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MicroRNA regulation of the bovine mammary immune response to a mastitis pathogen



A thesis submitted to the University of Dublin for the degree of

Doctor of Philosophy 2014

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Summary

Bovine mastitis is an inflammation-driven disease of the mammary gland that is associated with €2 billion in yearly losses to the European dairy industry. *Streptococcus uberis* is now ranked amongst the most prevalent mastitis-causing pathogens throughout the EU and in North America. Mastitis develops when bacteria on entering the mammary gland, encounter mammary epithelial cells and resident monocytes, triggering local and systemic inflammation. The innate response clears bacteria, but often results in significant tissue damage to the gland. Little is known, however, about how the immune response is regulated or resolved. Here, we examined the hypothesis that microRNAs (miRNAs), short non-coding RNAs which post-transcriptionally regulate gene expression, are central regulators of the transcriptional networks induced in the host response to a bovine mastitis pathogen, *Streptococcus uberis*. We first used next generation sequencing (NGS) to profile expression of bovine miRNAs in primary mammary epithelial cells challenged *in vitro* with *S. uberis*. We discovered 21 potentially novel bovine miRNAs, 2 of which had 100% identity to known miRNAs in other species, ssc-miR-664-3p (pig) and hsa-miR-219-1 (human). Ten miRNAs were differentially regulated in response to the pathogen. This miRNA response to the *S. uberis* is markedly different, however, to lipopolysaccharide (LPS) -induced miRNA expression. Target prediction analysis of differentially expressed miRNAs found that genes enriched in immune signalling pathways were preferentially targeted. The results suggested that differentially expressed miRNAs identified in this study are likely central regulators of the innate immune response to Gram-positive bacteria. To confirm this hypothesis *in vivo*, milk and blood CD14+ monocytes were FACS isolated from Holstein-Friesians infected

with *S. uberis*. Both mRNA and miRNA were isolated and sequenced. Hierarchical clustering of genes from milk-isolated monocytes (MIMs) revealed that control and infected animals clearly separated at 36 and 48 hpi. More than 3,500 genes underwent transcriptional change during infection. Pathway analysis revealed that upregulated genes in MIMs were significantly enriched for roles in inflammatory and other innate immune pathways (TLR, NLR and RIG-I signalling). Down-regulated genes were significantly associated with metabolic pathways. Ninteen miRNAs were differentially expressed in response to infection in MIMs. Pathway analysis revealed that predicted targets of down-regulated miRNAs were highly enriched for roles in innate immunity (FDR < $3.4E^{-8}$), while up-regulated miRNAs preferentially targeted genes involved in metabolism. We concluded that during *S. uberis* infection miRNAs are key amplifiers of monocyte inflammatory response networks and repressors of metabolism. By mining our database of mRNA and miRNA expression data, and integrating miRNA target predictive software, we selected bta-miR-200a as a potential regulator of IL8. Bta-miR-200a expression inversely correlated (-0.28864) with IL8 expression and was predicted by miRanda v3.3a to have a preferential binding site on positions 190-196 in the 3' UTR of IL8. This interaction was validated by transfecting synthetic mimics of bta-miR-200a and testing for IL8 downregulation by qPCR. In conclusion, these findings strongly suggest that bovine miRNAs are central regulators of the innate immune signalling, and play a key role in regulating the links between metabolism and inflammation. We have discovered over 30 novel bovine miRNAs including two human homologs. Regulatory miRNAs identified here, may have potential practical applications in veterinary medicine and could aid in identifying pathogen specific biomarkers of infection or targets for novel vaccination strategies.

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Lawless, N., A. B. Foroushani, et al. (2013). "Next Generation Sequencing Reveals the Expression of a Unique miRNA Profile in Response to a Gram-Positive Bacterial Infection." *PLOS ONE* 8(3): e57543".

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Abbreviations:

Ago2	Argonaute protein 2
APP	Acute Phase Protein
ATCC	American Type Culture Collection
C5a	Complement 5a
CCL2	Chemokine (C-C motif) Ligand 2
CD14	Cluster of Differentiation 14
CD8A	Cluster of Differentiation 8a
CXCR4	Chemokine Receptor Type 4
eIF4E	Eukaryotic Translation Initiation Factor 4E
ERAP1	Endoplasmic Reticulum Aminopeptidase 1
FACS	Fluorescence-Activated Cell Sorting
FBS	Fetal Bovine Serum
HITS-CLIP	High-Throughput Sequencing of RNA Isolated by Cross-Linking Immuno-Precipitation
IFN	Interferon
IL	Interleukin 1β, 6, 8, 10, 15
IRAK1	Interleukin-1 Receptor-Associated Kinase 1
IRES	Internal Ribosome Entry Site
IRF1	Interferon Regulatory Factor 1
LBP	Lipopolysaccharide Binding Protein
lincRNA	Large Intergenic Noncoding RNA
lncRNA	Long Non-Coding RNA
LPS	Lipopolysaccharide

LTA	Lipoteichoic acid
MyD88	Myeloid Differentiation Primary Response Gene 88
ncRNA	Non-coding RNA
NETS	Neutrophils Extracellular Traps
NFκB	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells
NFAT1	Nuclear Factor of Activated T-Cells
NGS	Next Generation Sequencing
ORF	Open Reading Frame
PAMPs	Pathogen-Associated Molecular Patterns
piRNAs	Piwi-Interacting RNAs
PPARgamma	Peroxisome Proliferator-Activated Receptor Gamma
PRR	Pattern Recognition Receptor
REL	Proto-Oncogene c-Rel
RIN	RNA Integrity Number
RISC	RNA-Induced Silencing Complex
SAA	Serum Amyloid A
SHIP1	SH2-Containing Inositol 5'-Phosphatase 1
siRNA	Small Interfering RNA
snoRNA	Small Nucleolar RNA
STAT	Signal Transducer and Activator of Transcription
SUAM	*Streptococcus uberis* Adhesion Molecule
TGFI-R2	Transforming Growth Factor Beta Receptor 2
TNF	Tumour Necrosis Factor
TP63	Tumour Protein p63
TPM	Tags Per Million

TRAF	TNF Receptor-Associated Factor
TRBP	TAR RNA Binding Protein
UCB	Umbilical Cord Blood
UTR	Untranslated Region
DGCR8	DiGeorge Syndrome Critical Region Gene 8

CHAPTER 1: Introduction

1.1 Bovine mastitis

Bovine mastitis is an inflammatory condition of the bovine mammary gland induced by the innate immune system responding to invasive bacteria which have overcome the anatomical barrier of the teat canal (Wellnitz and Bruckmaier 2012). A number of factors contribute to the complex aetiology of mastitis, including the pathogen, the host, and the environment. The innate immune response mediates clearance of bacterial pathogens from the mammary gland and is a key determinant of the outcome of an intra-mammary infection. A host of innate immune mediators including local immune cells, recruited immune cells and epithelial cells, all play important combinatorial roles in clearing infection through the production of cytokines and other inflammatory mediators (Zhong, Tien et al. 2006, Yang, Zerbe et al. 2008, Swanson, Stelwagen et al. 2009).

Intensive selection for milk production traits has resulted in a decline in the udder health of a number of dairy breeds, including the Holstein-Friesian, and has been negatively correlated with a high incidence of mastitis (Hansen, Lund et al. 2002). Worldwide, mastitis is estimated to cost the dairy industry over €2.5 billion per annum. Recent reports have determined that the cost per animal averages between $80 and $120 per animal each year in the U.S. (Henk Hogeveen 2011) and €72 per animal in Ireland (Berry and Amer. 2005). Despite widespread implementation of control strategies, financial losses incurred due to mastitis include increased veterinary treatment costs, withdrawal periods, milk loss, sub-fertility, infertility, and animal welfare issue. This re-occurring financial loss, combined with high incidence rates, has begun to seriously limit the competitiveness of the dairy sector, making mastitis an economic priority (Denis, Parlane et al. 2006). Furthermore, existing control

methods, primarily antibiotic treatment, are becoming increasingly ineffective, ethically undesirable, and unpopular both within the scientific community and by the increasingly well-informed consumer.

1.1.2 Mastitis causing pathogens

Mastitis or inflammation of the mammary gland is typically described in bovine as clinical or sub-clinical. Clinical mastitis is associated with an inflammatory reaction to bacterial infection, resulting in swelling, heat, elevated somatic cell count (cell population in milk consisting of mixed white blood cells), severe tissue damage, and sometimes death. Sub-clinical mastitis is often associated with little or no swelling, and a small sustained increase in somatic cell count. The causal pathogenic agents of mastitis are wide-ranging. Bacteria, yeast, fungi and algae are all known to be capable of causing mastitis (Bannerman, Paape et al. 2004, Petzl, Zerbe et al. 2008, Wellnitz and Bruckmaier 2012). The most common mastitis-causing pathogens in the bovine are bacteria from three groups, coliforms (*Escherichia coli*), staphylococci (*Staphylococcus aureus*), and streptococci (*Streptococcus uberis*) (Oviedo-Boyso, Valdez-Alarcon et al. 2007, Wellnitz and Bruckmaier 2012). Clinical mastitis is usually caused by a virulent bacterial strain such as *E. coli* (Porcherie, Cunha et al. 2012), but many commensally residing bacteria can be mastitis-causing. Sub-clinical mastitis/chronic mastitis, is more difficult to describe in clinical terms as symptoms are often mild or absent. The mammary gland can have a latent infection or the bacteria may subvert the innate response and remain unrecognised. In many cases bacterial colonies will be very difficult to clear, and as a consequence a small elevation in the somatic cell count may persist (Tassi, McNeilly et al. 2013).

S. uberis has emerged as a major bacterial mastitis-causing pathogen in rural regions world-wide, including Ireland (Barrett, Healy et al. 2005), New Zealand (Petrovski, Williamson et al. 2011), North America (Luther, Almeida et al. 2008, Olde Riekerink, Barkema et al. 2008) and the United Kingdom. Originally seen as an environmental bacterium, being ubiquitous throughout the dairy environment (Zadoks, Tikofsky et al. 2005), *S. uberis* has also been described as a contagious bacterial species transmitting from animal to animal (Zadoks, Allore et al. 2001). Natural reservoirs include the skin, GI tract, gut, and animal bedding. Outbreaks of mastitis have been associated with multiple strains of *S. uberis* and several of these strains have subsequently been isolated from clinical cases and used in bacterial challenge studies. Examples include *S. uberis* 233 (Swanson, Stelwagen et al. 2009) *S. uberis* 0140J (Denis, Parlane et al. 2006) and *S.uberis* UT888 (Chen, Dego et al. 2011). The complete genome of *S. uberis* 0140J was sequenced by Ward et al in 2009, with the aim of elucidating what makes this strain so well-adapted to invasion of the mammary gland (Ward, Holden et al. 2009). Strain 0140J has also been used in at least eight independent mastitis studies dating back to 1988, making it one of the most thoroughly characterised mastitis-causing *S. uberis* strains (Hill 1988, Hill, Finch et al. 1994, Lang, Lefebure et al. 2009, Leigh, Egan et al. 2010). For this reason, *S. uberis* 0140J was chosen as the pathogen for the *in vitro* challenge study in this thesis. A related strain (0140), originally isolated by Dr A.J. Bramley in 1979 (Bramley, King et al. 1979) was used in the *in vivo* challenge in collaboration with US Department of Agriculture (USDA) National Animal Disease Center (NADC). *S. uberis* 0140 was chosen because of studies carried out by our collaborators (Nelson, Reinhardt et al. , Bannerman, Paape et al. 2004, Harp, Waters et al. 2006), their

familiarity with the infection kinetics of this strain and prior experience with a successful mastitis-causing infection in the trial herd.

1.1.3 Bovine mammary gland immunobiology

A diverse array of physical, cellular, and molecular factors combine to perform the actions of the innate and adaptive immune system in the mammary gland (Bannerman, Paape et al. 2004, Aitken, Corl et al. 2011). Immune protection against bacterial invasion begins in the teat cistern, which is the point of entry for bacteria to the mammary gland. The teat cistern is lined by keratin, often referred to as a 'keratin plug' and is tightly closed by a sphincter muscle outside times of milking. Together, the sphincter muscle and keratin plug provide a physical barrier that prevents bacteria entering the mammary gland. During periods of lactation, the muscle is relaxed and the keratin plug is lost, weakening the barrier and reducing keratin bacteriostatic ability (Aitken, Corl et al. 2011). Located immediately above the teat cistern is the gland cistern. This region is home to a diverse array of soluble antibacterial factors, such as lactoferrin and complement proteins that act against bacteria which have penetrated the teat canal. Invasive bacteria which have succeeded in entering the main cistern are recognised by epithelial and milk resident cells, which then initiate the host innate response (Mitterhuemer, Petzl et al. 2010).

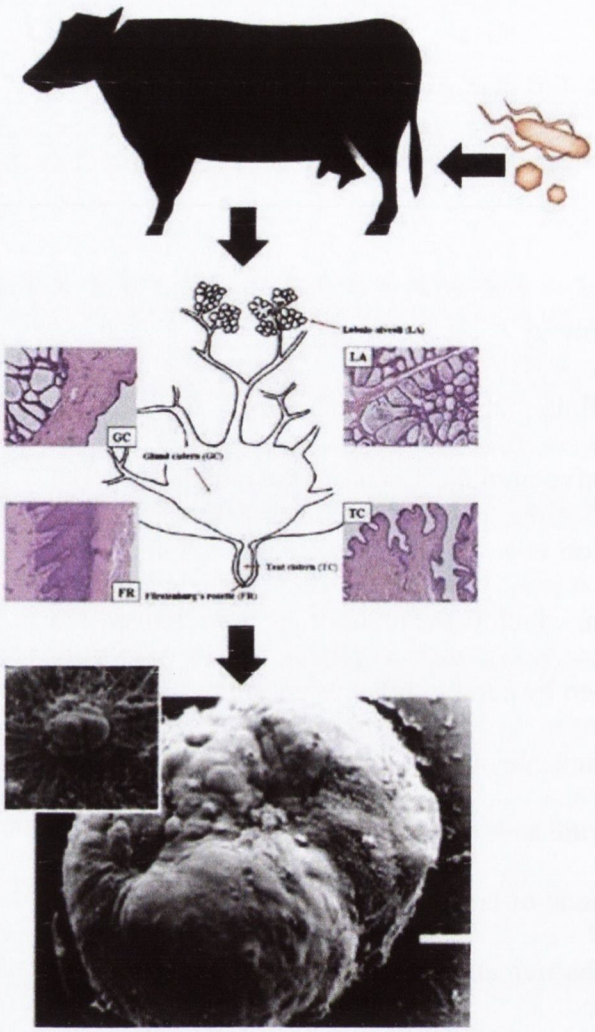


Figure 1.1 Bacterial infection of mammary gland. Disease causing bacteria come from multiple sources, including environment, self, and animals in the herd. Bacteria gain entry to the mammary gland through teat cistern, and enter into the gland cistern where they rapidly multiply in an attempt to establish an infection. Epithelial cells line the gland and milk resident monocytes acting in a sentinel capacity mobilise the innate immune response upon bacterial recognition. Figure taken and adapted from avanticell.com and (Rinaldi, Li et al. 2010).

1.1.4 Epithelial cells in mammary gland immunobiology

Among the first cells to come in contact with invading pathogens are mammary epithelial cells. Often referred to as sentinel cells, epithelial cells line the alveoli, teat canal, and gland cistern. Epithelial cells directly contribute to mammary gland immune surveillance by their expression of different combinations of cell-surface and endosomal pattern-recognition receptors (PRRs) (Swamy, Jamora et al. 2010). Invading bacteria often present specific surface patterns referred to as pathogen-associated molecular patterns (PAMPs). Examples of PAMPs include lipopolysaccharide (LPS) and lipoteichoic acid (LTA). PAMPS are recognised by PRRs, for instance Toll-like receptor 2 (TLR2) recognises LTA and peptidoglycan from Gram-positive bacteria (Moyes, Drackley et al. 2009). Initiated through either activated PRRs or proinflammatory cytokines, mammary epithelial cells activate pro-inflammatory intracellular signalling cascades (often through nuclear factor kappa light chain enhancer of activated B cells (NFκB) dependent signalling) (Rinaldi, Li et al. 2010). Multiple immune mediators are then released, including tumour necrosis factor α (TNF) and interleukin 8 (IL8), a chemotactic factor responsible for neutrophil recruitment, and activation (McClenahan, Krueger et al. 2006). Local production of acute phase proteins such as serum amyloid A3 (SAA3) and haptoglobin (Hp) has also been described (Whelehan, Meade et al. 2011). Together these factors initiate local physiological changes in vascular permeability, cell differentiation, and apoptosis. These and other cytokines are detected by milk somatic cells, including monocytes, which amplify inflammatory signals provoking acute phase protein (APP) production which acts systemically to suppress the spread of bacteria locally (Mitterhuemer, Petzl et al. 2010, Loor, Moyes et al. 2011).

1.1.5 Monocytes in mammary gland immunobiology

Milk somatic cells populations include neutrophils, macrophages, and monocytes as well as epithelial cells. The relative proportions of these populations change in response to bacterial recognition (Leitner, Shoshani et al. 2000). Monocytes typically make up between 10-30% of milk somatic cells; however, these data vary between studies. Monocytes have a similar surveillance role as epithelial cells; in addition to their effecter function. In response to bacterial infection, monocytes are released from the bone marrow into the circulation and travel along chemokine ligand-mediated cell migratory pathways to the mammary gland. There they differentiate into macrophage and dendritic cell populations, often increasing more than 10-fold (Bannerman, Chockalingam et al. 2005, Shi and Pamer 2011, Dong, Zhao et al. 2013). Upon activation of inflammation and induction of inflammatory mediators, neutrophils become the dominant cell type within the milk. Neutrophils make up the majority of immune cells in an infected gland during an infection and are tasked with directly clearing invasive bacteria via phagocytosis or neutrophil extracellular traps (NETS) (Lippolis, Reinhardt et al. 2006, Reinhardt, Sacco et al. 2013). Once recruited to the site of infection, monocytes and neutrophils orchestrate antimicrobial activity to counter bacterial spread and resolve the infection (Serbina, Jia et al. 2008, Dong, Zhao et al. 2013).

1.1.6 Profiling the innate immune response to *S. uberis* derived mastitis

Several studies examining the bovine mammary gland innate immune response to *S. uberis* infection have been carried out. qPCR and microarrays were the principle technologies. Microarray studies revealed large numbers of genes differentially expressed in response to an *S. uberis* (strain 0140J & 223/262 respectively) infection, the majority of which were immune related (Moyes, Drackley et al. 2009, Swanson, Stelwagen et al. 2009). Swanson *et al* described the differential expression of over 2,000 genes in a study of 5 Friesian heifers infected with *S. uberis* strain 223/262. In this study, the gene expression profiles of mammary alveolar tissue were examined at 60 and 132 hours post-infection, and a marked up-regulation of SAA3, Hp and lipopolysaccharide binding protein (LBP) was reported. A range of inflammatory cytokines, including IL1β, TNFα, IL8 and interferonγ (IFNγ) were also up-regulated. Up-regulation of the anti-inflammatory cytokine, IL10, was also detected. These findings were confirmed by Moyes *et al* in a study also using microarray technology which examined the response to *S. uberis* strain 0140J in the mammary tissue of 10 Holsteins 20 hours after infection. Again innate immune related genes featured among the top differentially expressed genes, including TNF, signal transducer and activator of transcription-3 (STAT3), Cluster of Differentiation 14 (CD14), IL1β, IL8, and IL6. A number of the genes found to undergo differential regulation were later confirmed in another study also using microarray and qPCR by De Greeff et al. However, only 239 genes were differentially expressed in this study. A number of innate immune genes including TLR2, lipopolysaccharide binding protein (LPB), chemokine receptor type 4 (CXCR4), CD14 and chemokine (C-C motif) ligand 2 (CCL2) were detected. However, the lower numbers of differentially expressed genes detected in this study when compared to the above, highlights

differing pathogenicities between bacterial strains within a species, and the complexity of mastitis (de Greeff, Zadoks et al. 2013).

Although we have an understanding of which inflammatory genes are active during mastitis, how the immune genes act as part of inflammatory networks remains undefined. Neither do we know fully how temporal changes affect inflammatory networks nor how they are switched off in specific cell populations once infection is resolved.

1.2 Non-coding RNA species

It is now becoming evident that pervasive expression of non-coding RNAs has a key role in regulating innate and adaptive immunity and many other cellular processes. The expression of non-coding RNAs in the mammalian genome and their roles in the regulation of many biological processes is now an established paradigm.

Critically, the emergence of innovative, high-throughput approaches has shown that several classes of RNAs are regulators of immune function and their deregulation, manipulation or circumvention are associated with disease progression (Esteller 2011, Trobaugh, Gardner et al. 2014). Regulatory classes of RNAs have been separated into a number of categories based on several criteria, including length and function. For example miRNAs and long noncoding RNAs (lncRNAs) are both non-coding, linked to immune regulation, but vary in length (Figure 1.2). There is a lack of consensus as to how different classes of non-coding RNAs regulate the transcriptome. The precise immune regulatory function of non-coding RNAs is increasingly under examination. Such classes of RNAs include lncRNAs found throughout the mammalian genome. They play a role in epigenetic regulation, directly interacting with ribonucleoproteins, and effecting immune cell differentiation and activation (Collier, Collins et al. 2012, Carpenter, Aiello et al. 2013, Fitzgerald and Caffrey 2014). Groups of shorter RNAs include piwi-interacting RNAs (piRNAs), which play a critical role in transposon regulation and protection against the activity of parasitic nucleic acids (Aravin, Hannon et al. 2007). Small nucleolar RNA (snoRNAs) dysregulation has also been associated with various cancers including chronic lymphocytic leukaemia, (Ronchetti, Mosca et al. 2013). siRNAs are believed to have originally evolved as a primitive immune response to viruses (Obbard, Gordon et al. 2009, Fabozzi, Nabel et al. 2011).

However, the puzzle of how one class of short non-coding RNAs, microRNAs (miRNAs), post-transcriptionally regulates gene expression has generated more interest in immunology than any other.

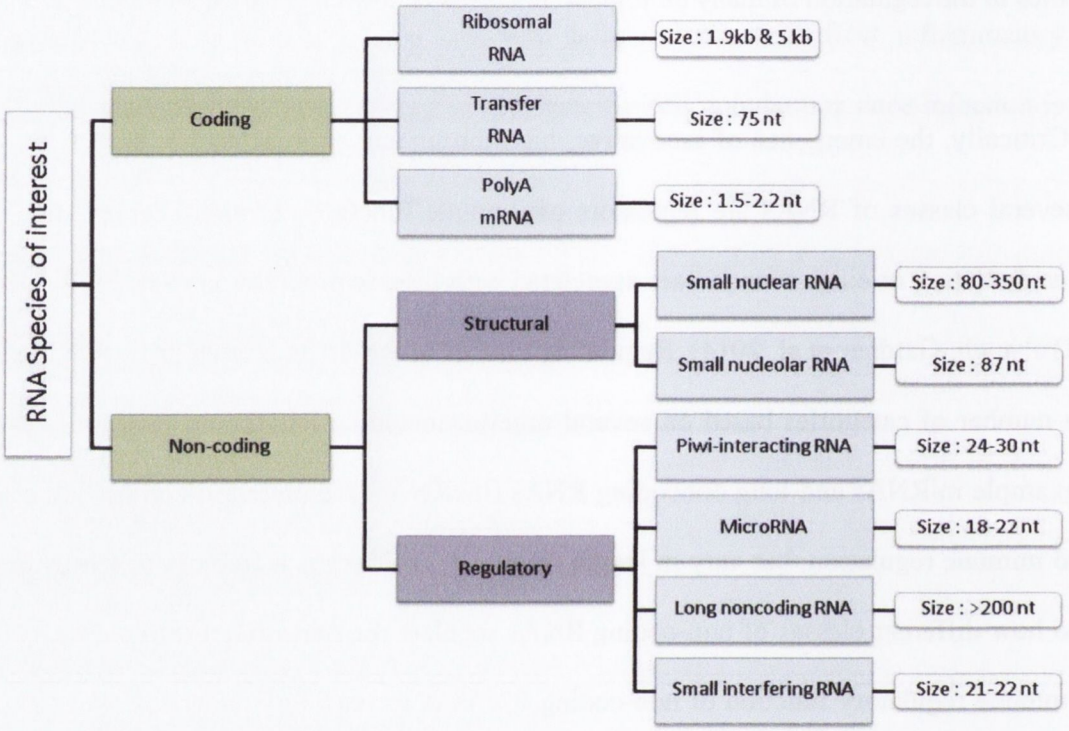


Figure 1.2 Coding and Non-coding RNA populations. RNAs include coding and non-coding categories; within these categories, RNAs are further divided based on proposed function and size.

1.2.1 MicroRNAs - important regulators of innate immunity

MicroRNAs, are short, non-coding RNAs, which post-transcriptionally regulate gene expression (Barral, Sarkar et al. 2009). Since their initial discovery in 1993 (Lee, Feinbaum et al. 1993), we have gained insight into the prolificacy of miRNAs. The latest miRBase database (release 20) contains 24,521 microRNA loci, processed to produce 30,424 mature microRNA products from 206 species (Kozomara and Griffiths-Jones 2014). A number of specific miRNAs have been experimentally validated as having a key role in the initiation, resolution, and regulation, of innate and adaptive immunity in human and mouse.

The immune response can be initiated through both up- and down-regulation of miRNAs, as they either repress or release genes. The prototypic mechanism of miRNA induction is miRNA-155, repressing Src homology-2 domain-containing inositol 5-phosphatase 1 (SHIP1) in mouse macrophages in response to LPS stimulation (O'Connell, Chaudhuri et al. 2009, Krol, Loedige et al. 2010). The same miRNA, targets TGF-beta activated kinase 1/MAP3K7 binding protein 2 (TAB2), modulating the interleukin-1 signalling pathway in human monocyte-derived dendritic cells again in response to LPS (Ceppi, Pereira et al. 2009). Often miRNAs are negatively regulated in response to a stimuli or pathogen, releasing inflammatory genes. Reduced expression of miR-149 in response to LPS or BCG releases myeloid differentiation primary response gene 88 (MyD88) driving inflammation in macrophage RAW264.7 cells (Xu, Zhang et al. 2014).

Sustained expression of miRNAs in a cell type or cell development stage has been shown to have a functional role in the survival and differentiation of cells. Regulation

of mature B cell production is controlled through miR-150 targeting of the transcription factor c-Myb (Xiao, Calado et al. 2007), miRNA-155 has a role in the normal immune function of CD4+ T cells (Rodriguez, Vigorito et al. 2007), and of note miRNA-146a targeting of IRAK1 (previously described), partially controls NF_KB expression in human plasmacytoid dendritic cells, controlling their maturation (Karrich, Jachimowski et al. 2013).

Several well-known miRNAs have been associated with resolving inflammation. Mmu-miR-146a, is induced during vesicular stomatitis virus (VSV) infection. Its role is to negatively regulate TNF receptor associated factor (TRAF6) and IRAK1 to suppress the RIG-I pathway (Hou, Wang et al. 2009). An extensively studied miRNA, miR-21, has been shown to reduce the expression of several inflammatory genes, most recently directly targeting phosphatase and tensin homolog (PTEN) and programmed cell death protein 4 (PDCD4) to resolve wound inflammation (Das, Ganesh et al. 2014).

Other interesting features of miRNA regulation include the fine tuning of toll-like receptor signalling (McCoy, Sheedy et al. 2010, O'Neill, Sheedy et al. 2011, Li and Shi 2013). In addition, regulation of human neutrophil senescence and partial control of myloid cell lung recruitment is under control of miR-223 via miRNA targeting of chemo-attractants during TB infection (Ward, Heath et al. 2011, Dorhoi, Iannaccone et al. 2013).

1.2.2 MicroRNA biogenesis

The precise mechanism behind miRNA biogenesis is a topic that has become increasingly well understood over the last decade (Hannon, Rivas et al. 2006). MicroRNAs are transcribed from poly cistronic transcription units in both non-coding and protein-coding loci. In the latter, miRNAs are found in both exonic and intronic regions, although the majority are found in intronic regions. These account for 40% of all miRNAs (Kim, Han et al. 2009). MicroRNAs are initially transcribed as long precursors called pri-miRNAs, which go through a series of processing steps before being exported from the nucleus. Pri-miRNAs are recognised and cleaved by the enzymes Drosha (ribonuclease 3) and DiGeorge syndrome critical region gene 8 (DGCR8) for processing to ~70nt hairpins (Bi, Liu et al. 2009, Heale, Keegan et al.). miRNAs are then exported from the nucleus by exportin 5 (RanGTP-dependent dsRNA-binding protein) to the cytoplasm.

On reaching the cytoplasm, the now precursor miRNAs (pre-miRNAs) are further processed into ~22nt double-stranded miRNA duplexes by Dicer (cytoplasmic RNaseIII enzyme) (Pedersen, Cheng et al. 2007). One strand of this miRNA duplex is termed the "guide strand" and the duplex is unwound to allow the guide strand to be incorporated/packaged into the RNA induced silencing complex (RISC/miRISC), a protein complex formed by Dicer, TRBP (dsRNA-binding domain protein) and Argonaute protein 2 (Ago2). The second strand, termed the "passenger strand" originally thought to be degraded, is now hypothesised to be functional in some cases (Griffiths-Jones, Hui et al. 2011, Yang, Phillips et al. 2011). The mature miRNA, functions to guide the RISC complex to its specific mRNA target (Bi, Liu et al. 2009, Heale, Keegan et al.).

1.2.3 Mechanism of microRNA immune target regulation

MicroRNAs act as post-transcriptional regulators of gene expression, altering mRNA stability or translation efficiency by hybridizing to 3' untranslated regions (UTRs) of certain subsets of mRNAs (collectively as many as 60% of all transcripts) (Barral, Sarkar et al. 2009). In animals, very few miRNAs (30-45%) regulate their targets by forming fully complementary duplexes, with the majority binding imperfectly to homologous mRNA targets. Plant miRNA-mRNA duplexes are, as a general rule, are more highly complementary (Yekta, Shih et al. 2004, Kong, Cannell et al. 2008, Ghildiyal and Zamore 2009). The primary region of pairing termed the "seed region" or "motif" is the pairing between nucleotides 2 and 7 at the 5' end of the miRNA. An imperfect pairing at the 5' end of the miRNA can also be compensated by extensive 3' end interactions and complimentary centred sites, where the middle region of the miRNA makes the most proximate pairing with a target sequence (Pasquinelli 2012, Martin, Wani et al. 2014).

Additional factors that influence target site selection include the location of the target site within an mRNA, their location in 3' UTRs, AU richness and location in RNA unstructured areas. Conserved seed pairings in unstructured and AU-rich regions can be good indicators of potential miRNA sites (Bartel 2009). Target sites tend not to be located beside sequences immediately after the translational stop codon that would fall into the ribosome shadow, a 15 nt region down from the stop codon bound by the ribosome before it dissociates from the mRNA.

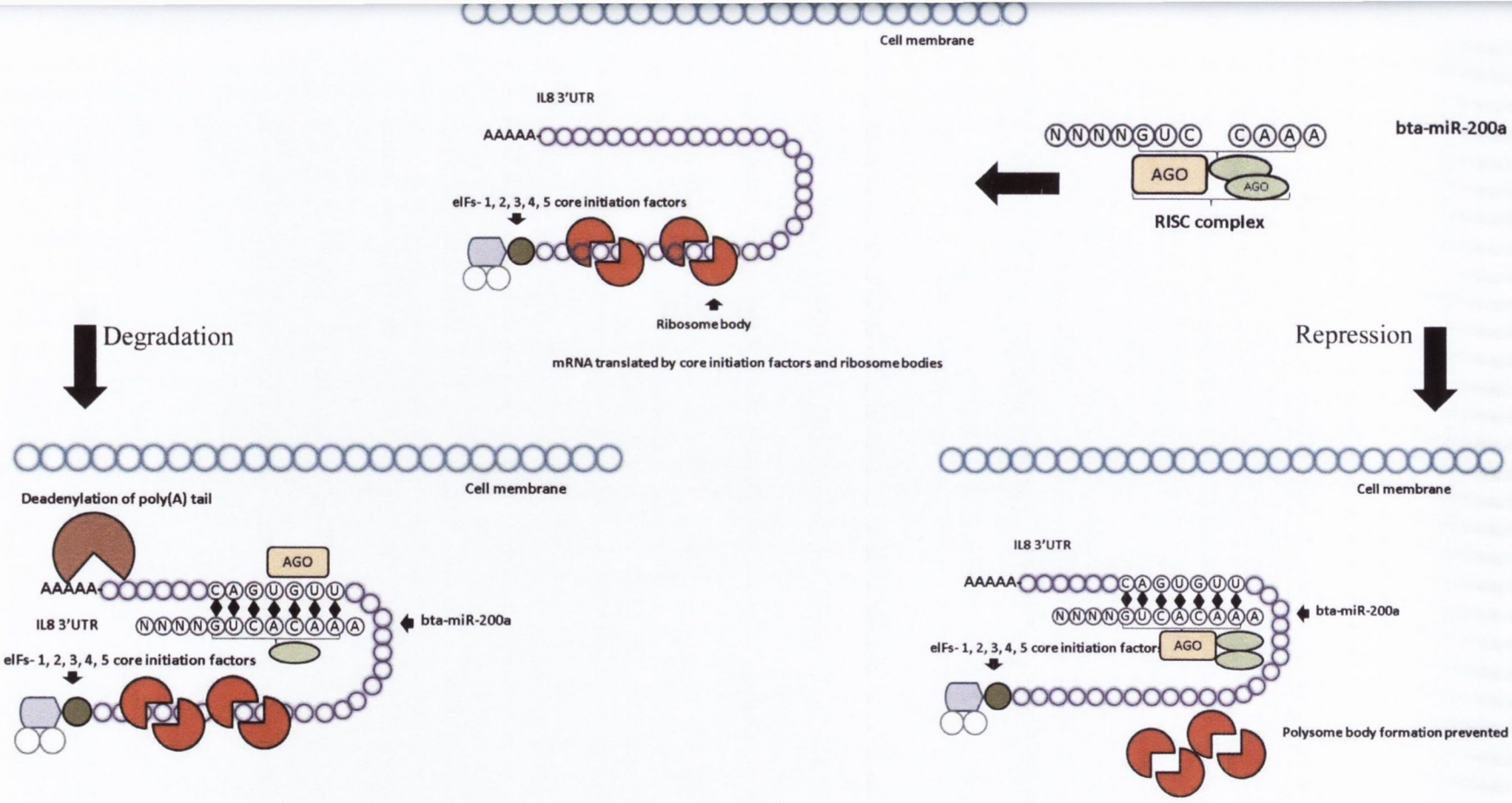


Figure 1.3 miRNA targeting of mRNA. Typically mature miRNA is packaged in RNA induced silencing complex (RISC) before guiding complex to the 3' UTR of target gene. The "seed region" will form a pairing with the target region, which is either directly degraded or the ribosome body drops off, partially inhibiting Eukaryotic translation initiation factors -1 -2 -3 -4 -5 and preventing translation.

1.2.4 MicroRNA degradation and translational repression

The mechanisms miRNAs use to regulate target gene expression are still unclear, and a topic of much debate. However, two major modes of action are generally considered: target degradation and translational repression. Several studies have suggested the basic concept that when specific miRNAs in cultured cells are increased, mRNA transcripts containing matching binding sites for those miRNAs are decreased (Lim, Lau et al. 2005, Hendrickson, Hogan et al. 2009). This has been attributed to mRNA destabilization, a method by which miRNA initiates deadenylation of the poly(A) tail. mRNA destabilisation is now considered the primary method of mammalian miRNA action (Huntzinger and Izaurralde 2011). The poly(A) tail is susceptible to a number of different deadenylation enzymes. Susceptibility to degradation can depend on a combination of local conditions. An example of deadenylation is the regulatory action of miR-430 during the onset of zygotic transcription in zebrafish embryogenesis where several hundred mRNAs are degraded (Giraldez, Mishima et al. 2006, Bazzini, Lee et al. 2012).

Translational repression, previously described as a principal method of miRNA regulation, is now considered to be secondary to mRNA degradation in mammals (Huntzinger and Izaurralde 2011). Briefly, the idea of translational repression centres on stopping the formation of a polysome body or preventing the completion of the polyribosome action, thus preventing protein formation. This action can be stopped/repressed at several stages (Kong, Cannell et al. 2008, Huntzinger and Izaurralde 2011, Pasquinelli 2012). Recent findings suggest that the mRNA cap structure, or the cap binding complex eIF4E (Eukaryotic translation initiation factor 4E), is interfered with. eIF4E is the target 5'cap binding protein, generally controlled

by a family of proteins called 4E-binding proteins. eIF4E has been previously described as a target for translational control due to its importance in forming circular mRNAs which protect against degradation (Yanagiya, Suyama et al. 2012). Other examples of translational repression include "ribosomal drop off", a process in which miRNAs cause ribosomes to exit prematurely from their bound mRNA. This may be caused by the recruitment of termination factors or terminated directly by miRNAs (Petersen, Bordeleau et al. 2006). The presence of an internal ribosome entry site (IRES) as an alternative miRNA translational target has also been discussed in the "ribosomal drop off" process (Petersen, Bordeleau et al. 2006). IRESs facilitate cap-independent mechanisms of translation, and provide an alternative repression pathway, although the principle remains largely the same (Humphreys, Westman et al. 2005). A comprehensive picture of translational repression has yet to be explained in full detail (Hannon, Rivas et al. 2006, Pasquinelli 2012).

1.2.5 MicroRNAs in bovine immunity

Identification of miRNAs in bovine immunity or their regulatory roles have been poorly explored to date. qPCR has demonstrated that bovine miRNAs are expressed in a wide range of tissues, including immune-related ones (Coutinho, Matukumalli et al. 2007, Jin, Grant et al. 2009, Xu, Zhang et al. 2009), but few studies have investigated how the expression of bovine miRNAs is altered in response to infection and only three of these have globally profiled the miRNA response to local infection. A recent study described differential expression of five inflammatory related miRNAs (miR-9, miR-125b, miR-155, miR-146a and miR-223) in response to *E. coli* LPS and *S. aureus* enterotoxin B stimulation of bovine monocytes (Dilda, Gioia et al. 2011). In another study, Hou et al identified three up-regulated (miR-296,

miR-2430, and miR-671) and one down-regulated miRNA (miR-2318) in the mammary gland tissue of cattle with mastitis (Hou, Huang et al. 2011). Naaem et al, applied qRT-PCR to a panel of miRNAs (miR-10a, -15b, -16a, -17, -21, -31, -145, -146a, -146b, -155, -181a, -205, -221, and -223) to study differentially expressed mRNA targets previously identified by microarray during intra-mammary *S. uberis* infection (Naeem, Zhong et al. 2012). A study by Li et al examined miRNA differential expression in bovine peripheral blood in mastitic cattle, reporting the presence of immune related miRNAs (Li, Wang et al. 2013). The most recent study applied high-throughput sequencing, comparing miRNA expression after MAC-T cell stimulation with heat inactivated *S. aureus* or *E. coli*, the first study of its kind in a bovine model (Jin, Ibeagha-Awemu et al. 2014). These studies have strongly suggested roles for individual miRNAs in regulating bovine immunity. However, according to miRBase there are over 1,300 annotated bovine miRNAs. Studies which adopt genome-wide approaches are required to gain greater insight into the role of bovine miRNAs in immunity and infection.

1.2.6 MicroRNA as biomarkers of infection

As previously mentioned, the economic loss resulting from mastitis is estimated to cost the dairy industry €2 billion per annum in the E.U., yet a biomarker for early detection of mastitis is not commercially available. MicroRNAs exhibit many properties that have made them of significant interest as non-invasive biomarkers. They are abundantly expressed, in a stable form, in a range of extracellular fluids including plasma, milk, urine, saliva, and even semen. They are easily measured, and in many cases exhibit temporal and spatial specificity (Chen, Gao et al. 2010, Hata, Murakami et al. 2010, Kosaka, Izumi et al. 2010, Kusuda,

Cadetti et al. 2011). They have high information content - small numbers of miRNA panels can serve as accurate biomarkers or signatures of a disease condition. The use of miRNAs as a clinical biomarker is most advanced in cancer research in the human. In 2009 Prometheus laboratories released a panel of miRNA biomarkers that they claimed accurately identified 25 different tumour types (Ajit 2012). This year, exosomal-miRNA identification chips using microfluid chip based technology will enter the marketplace theoretically allowing quicker diagnosis of cancerous states (Kanwar, Dunlay et al. 2014).

The stability of miRNAs in extracellular fluids appears to be largely due to packaging in microvesicles or exosomes. The role that these circulating miRNAs might have is far from certain, although their extracellular abundance has raised many fundamental questions, as to whether miRNAs are actively or passively secreted (Kosaka, Izumi et al. 2010, Turchinovich, Weiz et al. 2011). Current hypotheses include cytokine-like cell-to-cell communication, activators of immunological/biological functions, or even a role in transferring immunity or initiating developmental change from parent to child. Immunologically related molecules have long been known to circulate in milk. Antibodies, complement, and immune cells are known to enhance offspring immunity, inhibit bacterial growth, and stimulate immune function in breast milk. Panels of miRNAs have been found in both human breast milk and bovine milk (Chen, Gao et al. 2010, Kosaka, Izumi et al. 2010). In human breast milk miRNAs have been found circulating in exosomes. Exosome-derived miRNAs were shown to be highly stable and resistant to treatment with RNase digestion, freeze thaw and low pH (Kosaka, Izumi et al. 2010, Zhou, Li et al. 2012). Similarly in bovine milk, miRNAs were shown to be stably expressed in micro-vesicles; examples included bta-

miR-223 and bta-miR-125b, both of which have been linked with immune regulation (Hata, Murakami et al. 2010). This observation was also confirmed by Chen et al who examined miRNAs in milk and milk-derived products during different periods of lactation. MicroRNA expression levels were observed to change throughout lactation periods and to be present in milk products as well as in raw milk (Chen, Gao et al. 2010) an important factor if milk miRNAs are to be used as a biomarker.

1.3 Profiling miRNA expression

Methods for measuring or profiling miRNA expression have moved forward in tandem with our burgeoning knowledge on the subject. At present, three proven techniques exist for analysing miRNAs, qPCR, microarray hybridisation, and Next Generation Sequencing (NGS), however, new technologies are constantly evolving (Git, Dvinge et al. 2010, Zhang, Lee et al. 2013, Ueno and Funatsu 2014). Which technology is applied to any particular study depends on the question we wish to answer. For example we may wish to examine the relevant abundance of miRNAs – as either a group of several miRNAs with a specific function or a comprehensive profiling of all miRNAs. In order to make a fully informed choice about which technology best suits our study we must examine the general concepts of each technology along with the pros and cons of each. (Ragan, Zuker et al. 2011).

1.3.1 Quantitative reverse transcription PCR

Quantitative reverse transcription PCR-based techniques have been a cornerstone of transcriptome biology for many years. PCR amplifies target pieces of DNA or cDNA and simultaneously quantities them in real time. The natural progression to miRNA based qPCR has enabled absolute quantification of individual miRNAs. This technology is now commercially available from a range of life science companies. The method is easily incorporated into existing laboratories, and can provide an accurate quantitative measurement of expression of specific miRNAs (Ach, Wang et al. 2008). Several variations of this technology exist, including the addition of a poly (A) tail or a stem loop as a universal primer-binding site, TaqMan polymerase which increases accuracy, and enlarged PCR plates which increase

throughput (Pritchard, Cheng et al. 2012). Digital PCR (dPCR), relies on the basic PCR methodology to amplify the gene of interest, however, dPCR partitions the sample of interest, allowing a more accurate estimation and can provide accurate RNA quantification (Sanders, Mason et al. 2013). PCR-based techniques are not without their limitations. The structure and short length of miRNAs make them among the more difficult RNA transcripts to measure. As mentioned above, there is no universal binding site that naturally occurs on miRNAs so one must be added, for example the addition of a poly (A) tail, which additionally helps select for miRNAs in a small RNA population which contains many small RNA species (Jensen, Lamy et al. 2011, Pritchard, Cheng et al. 2012). Other issues include the unintended detection of circulating miRNAs in their unprocessed form and the difficulty in distinguishing the many isomer types of a given miRNA (isomiRs) which can have different binding targets affecting down-stream analysis (Chiang, Schoenfeld et al. 2010, Pritchard, Cheng et al. 2012). More practical considerations include cost, which is in comparison to other technologies is relatively low, and the preparation time taken, which is considered long, but depends on the respective pipeline of each lab (Git, Dvinge et al. 2010).

1.3.3 Microarray hybridisation

Microarray hybridisation like qPCR is well established in the market-place through companies including Agilent, Exiqon, and Ambion and has been extensively used for the parallel analysis of large numbers of miRNAs (Git, Dvinge et al. 2010). Current microarray technology allows a highly sensitive accurate measurement of differences in miRNA across states, pre-miRNA hairpins, and recently miRNA variants (3' modifications), in a given sample (Wyman, Knouf et al. 2011). Advocates

of this technology highlight that microarray technology is relatively high-throughput and will allow for a relatively global profiling of miRNAs in a sample. Arrays are regularly updated with the latest mirBase coverage, and provide a quick method of comparing the miRNA content between two states (Pritchard, Cheng et al. 2012). Disadvantages of microarray hybridisation techniques greatly depend on the experimental aim of the study. Basic issues include the amount of RNA, as many individual cell populations yield a low RNA amount, making it impossible to utilise several existing microarray platforms. For example, 1000ng of RNA is required for the Exiqon mercury LNA microRNA Array, v9.2. Fluorescent labelling of miRNAs can in some instances result in bias, and incorrect labelling of RNA species can occur which may cause background interference or false positives (Nelson, Wang et al. 2008, Pritchard, Cheng et al. 2012). Other more specific limitations of microarray platforms include their lack of accuracy in quantifying absolute miRNA content in a sample, and an inability to discover previously unknown miRNAs. Results across multiple platforms are not always the same due to different mirBase content and species coverage. Post-transcriptional modifications of miRNAs affect the function of many miRNAs and these are generally not readily detectable.

1.3.4 Next Generation Sequencing

Next generation sequencing technology has become the established technology of choice for a variety of biological problems. Currently, NGS platforms are available from six companies, Illumina, Roche 454, Helicos Bioscience, Life Technologies, Pacific Biosciences and Nanopore. Each has its own specific set of protocols which distinguishes one from another. Advances in small miRNA discovery, DNA-DNA bound protein interactions, and mRNA transcriptome

profiling/discovery can be attributed to NGS. Much of our understanding of the role of non-coding RNA populations has coincided with the emergence of NGS technology. MicroRNAs, siRNAs, piRNAs, and other non-coding RNA populations are now known to play a role in nearly every cellular process, with many of these findings have come about through the innovative use of NGS (Hannon, Rivas et al. 2006, Ghildiyal and Zamore 2009, Guttman, Garber et al. 2010, Ebert and Sharp 2012). The main advantage of RNAseq is its ability to provide a global snapshot of a miRNA population leading to the discovery of both known and unknown miRNAs. Landmark publications using NGS to study immunology include the first description of the NK cell miRNA transcriptome (Fehniger, Wylie et al. 2010), and a definition of chromatin modifications, the transcriptome and the miRNAome of developing lymphocytes (Kuchen, Resch et al. 2010). The precise identification of miRNAs allows us to examine isomiR variants within miRNA families and post-transcriptional modifications (Chiang, Schoenfeld et al. 2010, Pritchard, Cheng et al. 2012). Additionally, RNAseq is not subject to the issues associated with melting temperatures (Git, Dvinge et al. 2010). Libraries can be prepared for discrete cell populations, as the RNA input required is low, for example, a single blastomere was sequenced in a method termed "single-cell mRNAseq" resulting in the discovery of 5,000 more expressed genes than had previously been seen in microarray analysis (Tang, Barbacioru et al. 2009).

The most recent examples of RNAseq technology being applied in bovine studies include an examination of small non-coding RNAs in retinal microvascular endothelial cells that regulate angiogenesis (Guduric-Fuchs, O'Connor et al. 2012) the effect of butyrate on alternative splicing in bovine gut epithelial cells (Wu, Li et

al. 2012), an examination of the transcriptome differences between *in vitro* and *in vivo* bovine embryo populations destined for in-vitro fertilisation (IVF) (Driver, Penagaricano et al. 2012), and a comparison of the effects of heat in-activated *S. aureus* or E. coli, on MAC-T cells *in vitro* (Jin, Ibeagha-Awemu et al. 2014). These studies are amongst the first to sequence miRNAs in any bovine tissue. However they lack both sequencing depth, and sufficient replicates, reducing the power of each experiment (Liu, Zhou et al. 2014).

When selecting a technical platform, it is important to consider the method used to sequence samples. Not all of the above platforms are suitable for the examination of miRNAs. The generation of reads can be described in a three step process, library generation (wet-lab), cluster generation (platform based), and sequencing (platform based). Illumina apply a method in which all clusters are sequenced simultaneously in a process called cyclic reversible termination (CRT) in which the cDNA templates are sequenced base by base in parallel using four fluorescently modified nucleotides. A single base is read in each cycle. Cycles range from 50-200; our studies are carried out with a 50-cycle sequencing kit because an average miRNA is 22-nt in length. RNAseq is a technology that is constantly evolving and is not without its limitations. Library preparation methods have been previously shown to introduce bias for some miRNA species during PCR enrichment steps. Absolute quantification of miRNA populations, in general align well to q-PCR, and are increasing in reliability (Tam, de Borja et al. 2014). The relatively high cost is another major factor for consideration. However, multiplexing has alleviated this issue to an extent, while new versions of sequencing machines allow for adequate coverage of multiplexed samples (Linsen, de Wit et al. 2009)

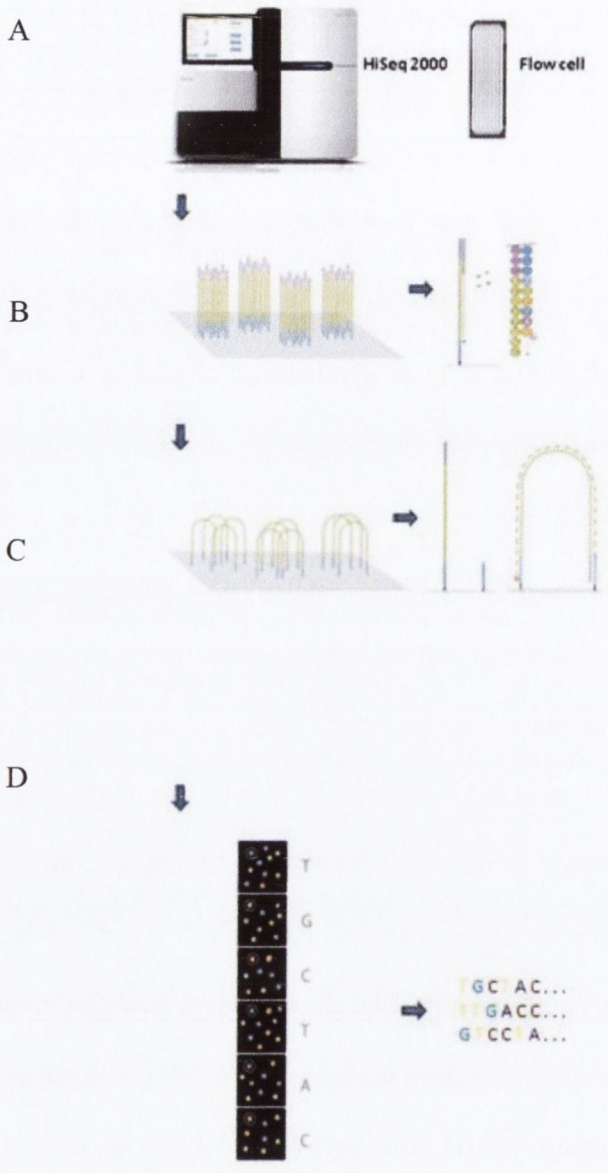


Figure 1.4 Illumina sequencing generation pipeline. (A) Multiplexed libraries are loaded onto a flow-cell. (B) Each library is then amplified. (C) All clusters are sequenced simultaneously and (D) imaged before reads are examined for quality control and analysis. Figure taken and adapted from www.Illumina.com.

1.4 Overall rationale underpinning thesis

Hypothesis

In this thesis we propose that microRNAs (miRNAs), short non-coding RNAs which post-transcriptionally regulate gene expression, are central regulators of the transcriptional networks induced in the host response to a bovine mastitis pathogen, *Streptococcus uberis*.

Overall Aims

The overall aims of this thesis are twofold.

1. To gain novel insight into how miRNAs regulate the host immune system.

2. To identify one or several miRNAs that could be developed as an early indicator (biomarker) of bovine mastitis, an inflammatory condition of the bovine mammary gland.

Importance of investigation

In this, study we have exploited recent advances in NGS technology to explore bovine immune cell regulatory networks operating during mastitis. In comparison to previous studies, we have sequenced un-pooled mRNA and miRNA libraries to a depth several orders of magnitude higher than previously used. We have investigated several cell populations including post-primary mammary epithelial cells which were challenged *in vitro*. We also investigated mRNA and miRNA from isolated CD14+ blood and milk monocytes in a large animal infection model. This model allowed us to explore the statistically significant temporal changes in both mRNA and miRNA expression in response to infection, the first of its type. Based on the sequencing data complimented with target prediction software we selected bta-miR-200a as a possible regulator of IL8 in a miRNA target validation study.

Specific aims:

1. To identify the miRNAs and the transcriptional networks that are differentially expressed over a time-course in an *in vitro S. uberis* challenge of bovine primary mammary epithelial cells, using a next-generation sequencing (NGS) approach.

2. To identify the miRNAs and the transcriptional networks that are differentially expressed over a time-course in blood and milk isolated CD14+ monocytes in an *in vivo S. uberis* challenge study.

3. To functionally validate the role of one of the differentially expressed miRNAs in regulating the host response to *S. uberis*.

CHAPTER 2: Material and methods

2.1 Materials

2.1.1 General materials

General laboratory chemicals were purchased from Sigma-Aldrich Ireland, with the exception of 8% TBE Urea Gel (1.00 mm x 10 well) (Invitrogen, CA, USA), Brain Heart Infusion Broth, Todd Hewitt Broth, Tryptic Soy Agar (BD & Company, Becton, Dickinson and Company, Le Point de Cloix, France), Collagen Type 1 Coated Flask (Greiner bio-one, Wemmel, Belgium), High Sensitivity DNA Kit, RNA 6000 Nano Kit, Small RNA Kit (Agilent, CO, USA), MacConkey Agar, (Thermo Fisher Scientific (Oxoid), (Hyclone), USA), mirVana™ RNA Isolation Kit (Ambion, TX, USA), RNA Clean and Concentrator (Zymogen, CA, USA), ScriptMinerTM Small RNAseq Library Preparation Kit (Epicentre, WI, USA), *Streptococcus uberis* 0140J (ATCC® Number BAA-854) (LGC Standards, Middlesex, UK), Trypan Blue Stain (VWR, USA), (Careforde, IL, USA).

2.1.2 Cell lines

Primary bovine mammary epithelial cells, which had been isolated from mammary parenchyma, were purchased from AvantiCell (Avanticell Science, Ayr, Scotland). Bovine macrophage (BoMac) cells were kindly gifted from Dr Cormac Whelehan (Trinity College Dublin, Ireland).

2.1.3 Cell culture reagents

Epithelial cell proliferation and growth medium (199/Ham's F12 (50:50) pH 7.4 containing 5% (v/v) horse serum, 5% (v/v) fetal bovine serum, 5 µg/mL bovine insulin, 1 µg/mL hydrocortisone, 3 µg/mL cortisol, 10 ng/mL

epidermal growth factor (EGF), 2 mM sodium acetate, 10 mM Hepes, U/mL penicillin/streptomycin and single strength Fungizone™) were purchased from AvantiCell (Avanticell Science, Ayr, Scotland). Roswell Park Memorial Institute (RPMI), trypsin-EDTA, and foetal calf serum was purchased from Biosciences Dublin. Pen-Strep was purchased from Thermo Fisher Scientific (Oxoid), (Hyclone), USA. Trypan blue was purchased from Sigma, USA, Lipofectamine was purchased from Invitrogen USA.

2.2 Cell culture

Primary bovine mammary epithelial cells were isolated from mammary parenchyma. The source animal was in her third trimester of first pregnancy, was aged between 26-30 months, and was negative for bovine viral diarrhoea and Bovine spongiform encephalopathy. Cells were plated (seed density of $1x10^6$) directly onto collagen coated plastic flasks. Media was initially replaced after 48 h. Cells were split twice (75 cm^2 and 175 cm^2) and were then seeded at a concentration of $1.8x10^5$ cells/well into collagen coated 6-well plates. Media was then changed after 24 h, and cells were inspected under microscopy for confluence. Cells were harvested by washing with Hanks Balanced Salt Solution (HBSS) pH 7.4 and treated with 4 mL of 0.25% trypsin for approximately 5 min at 37°C. An equal volume of medium to trypsin (1:1) neutralised trypsin.

Bovine macrophage (BoMac) cells were plated (seed density of $3x10^5$) directly onto collagen coated plastic flasks (Greiner-Bio-One GmbH, Frickenhausen, Germany) and immersed in RPMI medium containing 5% (v/v) fetal bovine serum 1 mL penicillin/streptomycin). Media was initially replaced after 48 h. Cells were split twice (75 cm^2 and 175 cm^2) and were then seeded at a concentration of $3x10^5$ cells/well into collagen coated 6-well plates. Media was then changed after 24 h, and cells were inspected under microscopy for confluence. Cells were harvested by washing with Hanks Balanced Salt Solution (HBSS) pH 7.4 and treated with 4 mL of 0.25% trypsin for approximately 5 min at 37°C. An equal volume of medium to trypsin (1:1) neutralised trypsin.

2.2.1 Bacterial strain preparation

Streptococcus uberis 0140J was purchased from the American Type Culture Collection (ATCC, Cat# BAA-854). *S. uberis* 0140J was first isolated in milk obtained from a clinical case of bovine mastitis in the United Kingdom in 1972. *S. uberis* pellet was initially rehydrated and propagated with brain-heart infusion (BHI) (BD & Company, Becton, Dickinson and Company, Le Point de Cloix, France), which was made up following manufacturer's instructions aseptically. Following rehydration of pellet, a tryptone soya agar (TSA) plate was inoculated, spread, and incubated at 37°C for 24 hours. When lawn growth was visible (24 h), Mc Conkey (BD & Company, Becton, Dickinson and Company, Le Point de Cloix, France) broth was made up as per manufacturer's instructions and loop of was used to inoculate 5 mL of Mc Conkey broth overnight in shaker at 37°C. For long term storage 1 mL of broth and 475 mL were added to 1.5 mL cryovial and mixed. Cryovial was then

stored at -80°C. For all further challenges, 100 µL of *S. uberis* mix was removed from cryovial and added to 5 mL THB broth, and put on shaker for 24 h at 37°C. Following visual examination for clouded appearance, serial dilutions of broth were made by withdrawing 500 µL from neat broth sample and adding to 4.5 mL of fresh broth, across eight tubes (10^{-8}). 100 µL of each 5 mL broth was then used to inoculate a TSA plate from which colony forming units were counted. From the same broth 1 mL was measured on a photometer (Eppendorf biophotometer, Hamburg, Germany) to determine the optical density, from which future counts and the subsequent multiplicity of infection (MOI) was calculated.

2.2.2 Lipoteichoic acid preparation

Lipoteichoic acid (LTA) (Sigma Ireland), a teichoic acid and component of gram positive bacteria was made up according to manufacturers' protocols. LTA pellet was initially rehydrated using nuclease free water to a concentration of 1 mg/mL and stored at -20°C. Dilutions were then made from this master stock as required in nuclease free water.

2.2.3 Infection of Cells with *S. uberis* 0140J

Bovine mammary epithelial cells were challenged with *S. uberis* 0140J at a MOI of 50, over a time course of 1, 2, 4, & 6h. Three replicates were infected at each time point and three replicate uninfected controls were also maintained for each time point. BoMac cells were challenged with *S. uberis* 0140J at a MOI of 50, over a time course of 6, 12, & 24 h & an MOI of 1 & 5 over a time course of 12 & 24 h. Three

replicates were infected at each time point and three replicate uninfected controls were also maintained for each time point.

2.2.4 Infection of cells with lipoteichoic acid

Bovine macrophage (BoMac) cells were cultured in 150 cm^2 flasks as previously described, until 95% confluence. The cells were then washed once in PBS before subsequent addition of fresh media with or without LTA at final concentration 10, 20 and 50 μg/mL. After incubation of the cells for 2, 4 and 6 h at each concentration of LTA, cells were washed with PBS before being harvested for subsequent RNA extraction.

2.2.5 MicroRNA mimics

MicroRNA mimics, small double stranded RNAs were purchased from Life Technologies. Bovine miRNA mimics are specific to miRBase version v20, and were prepared according to standard protocols (Life Technologies, Carlsbad, CA, USA). Mimics for the following miRNAs were designed as bta-miR-200a (miRBase accession: MIMAT0003822, assay ID: MC 11730). In addition two control mimics a positive mimic; miR-1 which negatively regulates Protein Tyrosine Kinase 9 (PTK9) and bta-miR-146a (miRBase accession: MIMAT0009236, assay ID: MC 14988). We also transfected a negative scramble mimic with no mRNA target. Briefly, mimics were initially resuspended at a concentration of 100 μM. Aliquots were taken from stock solution and diluted down to 10 μM, to give a final concentration of 10 pmol/μL. A total of 90 pmol was prepared for transfection per well (6-well plates) for bta-miR-200a, bta-miR-150, bta-miR-146a, miR-1 and negative scramble. Transfection complexes were prepared with lipofectamine in a reduced serum

medium (opti-MEM), and transfected into bovine macrophage cell lines for 24 h. All experiments were carried out in triplicate.

2.3 Live animal infection model study

2.3.1 Animals

10 female Holstein Friesians in the middle of their first lactation period, aged between 26-30 months, and 3-5 months post-partum, were selected for this study. The trial was conducted at the USDA National Animal Disease Centre (NADC) Ames, Iowa. All animals had a medical history that was free from mastitis. The National Animal Disease Centre's Animal Care and Use Committee approved all procedures used in this study.

2.3.2 Infection of Holstein Friesians with *S. uberis* 0140

Five animals were infected via the teat canal of the right front quarter with approximately 500 colony forming units (CFU) of a mastitis-causing pathogen, *Streptococcus uberis* 0140, in 10 mL of saline. Five control animals were inoculated with saline only. Milk and blood samples were obtained from each animal at 0, 12, 24, 36 and 48 h post-infection (or mock infection) (Figure 2.1). At each time point rectal temperature, total volume of milk, somatic cell count, bacterial counts, ambient temperature, humidity, and additional observations were recorded for each animal. Bacterial counts were determined from 5 mL milk samples collected aseptically from the infected quarter. Milk was serially diluted in sterile phosphate-buffered saline and spread on blood agar plates, then incubated for 24 h at 37°C. Following incubation,

plates were examined for bacterial growth and colony forming units (CFU) per mL were determined.

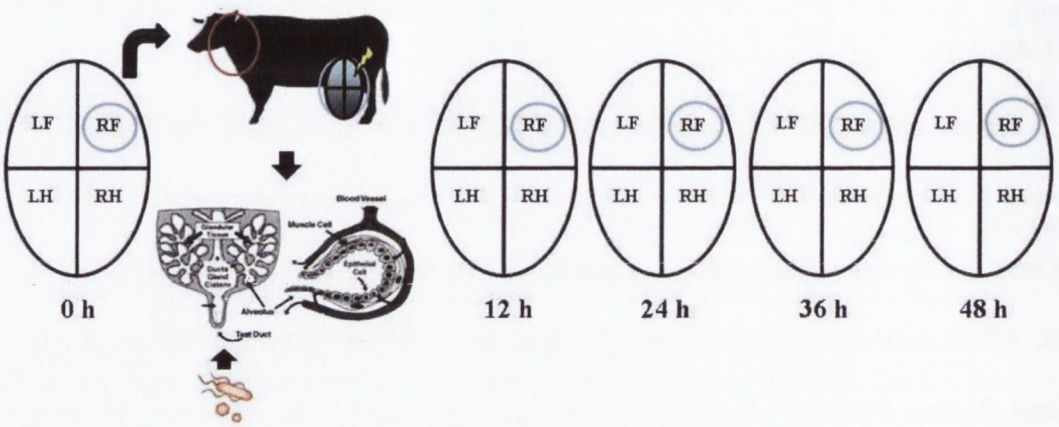


Figure 2.1 Schematic of *in vivo* infection protocol. Animals infected (n=5) with live *S. uberis* were inoculated in front right quarter only. In control animals (n=5) milk was also drawn from front right quarter only. Milk was drawn at 0 h, quarter was then infected, and milk was then drawn at 12, 24, 36, and 48 h.

2.3.3 Cell extraction from milk

Milk was collected using a sterilised quarter milker from infected and control animals at each time-point. The total volume of milk was noted. 5 mL of milk was isolated for milk bacteriology. The remaining milk was then diluted into Hanks balanced salt solution w/o Ca, Mg, Phenol red (HBSS) + 10 mM EDTA. The mixture was inverted several times, and transferred to a 1L centrifuge bottle and centrifuged in a fixed angle rotor at 10,000 x g for 30 mins. After centrifugation the supernatant was poured off and the pellets were resuspended in 150 mL of HBSS + 5 mM EDTA. The resuspended pellets were then transferred to fresh centrifuge tubes and spun at 2,500 x g for 30 mins. After the second spin, the supernatant was poured off and the pellet from each sample was resuspended in 20 mL of RPMI 1640 + 1 mM Sodium Pyruvate + 2 mM glutamine + 50 ug/mL gentamycin + 10% FBS (cRPMI). The 20 mL of cell suspension was divided into 2 x 10 mL aliquots in 15 mL conical centrifuge tubes. The cells were pelleted by centrifuge at 650 x g for 10 mins. After this spin the pellets were pooled into 4 mL of cRPMI and were labelled for cell sorting. The cells were counted by trypsin blue exclusion (Careforde, IL, USA) to determine the cell count.

2.3.4 Cell extraction from blood

At each time-point, animals were lead into a crush, and 2 x 60 cc syringes of blood were extracted by venipuncture and immediately placed on ice. The total volume of blood and total number of cells in blood were determined using a haemocytometer. Blood was spun for 20 mins at 1,200 x g. The buffy coat was observed between serum and red blood cell phase, and removed. Contaminating red blood cells were lysed by adding 1 volume lysis solution (10.6 mM Na2HPO4; 2.7

mM NaH2PO4) and inverting the tubes several times immediately followed by adding ½ volume restore solution (10.6 mM Na2HPO4; 2.7 mM NaH2PO4; 460 mM NaCl) and inverting the tubes. The buffy coat was then spun for 10 mins at 650 x g, and the red supernatant was poured off. Cells were re-suspended in red blood cell lysis solution and then restore solution again as above. Cell were spun for a further 5 mins at 650 x g, and re-suspended in 5-10 mL of media.

2.3.5 Isolation of CD14+ monocytes by flow cytometry

All flow cytometry was carried out by Bruce Pesch at the USDA-NADC Ames, Iowa. Milk and blood derived CD14+ monocytes were isolated by Fluorescence-activated cell sorting (FACS). Briefly, cells were labelled with monoclonal anti-bovine CD14 (Clone CAM36A, VMRD, Pullman, WA, USA) and a PE-conjugated anti-mouse IgG1 antibody (Southern Biotechnology, Birmingham, AL, USA). Labelled cells were separated based on fluorescence intensity using the BD FACS Aria Cell Sorting System (BD Biosciences, CA, USA). Cells with greater than 95% purity were isolated from the milk and peripheral blood of each animal (Appendix 1.1).

2.4 RNA extraction

RNA extraction and enrichment of different RNA species was carried out with two RNA extraction kits, the mirVana™ RNA isolation kit (Life Technologies) and MirPremier™ microRNA Isolation Kit (Sigma). The mirVana RNA isolation kit had the capacity to separate RNA populations by size, primarily greater or smaller than 200 nucleotides. RNA extraction procedures were largely the same, with the exception of the addition of miRNA homogenates and differing concentrations of ethanol used to trap RNA on glass-fibre filter for the mirVana™ RNA isolation kit.

2.4.1 Dual RNA and miRNA extraction

Total RNA and small RNA was extracted from 24 mammary epithelial cell sample using the mirVana™ RNA Isolation Kit (Life Technologies) according to the manufacturer's instructions. Cells were lysed using 500 µL lysis/binding solution directly on a culture plate. 50 µL of miRNA homogenate was added; solution was mixed by vortex and left on ice for 10 min. 500 µL of acid-phenol chloroform was added and the solution was mixed by vortexing for approximately 60 seconds. The solution was then centrifuged for 5 min at 10,000 x g at room temperature to separate the phases. The aqueous (upper) phase was removed and transferred to a separate tube. 1/3 volume of 100% ethanol was added to the aqueous phase and mixed by vortexing. Samples were passed through a filter cartridge (glass-fiber filter) by centrifuge for approximately 30 seconds at 10,000 x g. The filtrate was collected (residue on the filter contained RNA < 200 nt and was retained for later use) and 2/3 volume 100% ethanol was added and mixed by vortexing. Filtrate was passed through a second filter cartridge by centrifuge for ~30 seconds at 10,000 x g. The flow

through was discarded, and the filter was washed with 700 µL wash solution 1 and 500 µL wash solution 2 (twice). After discarding all flow through after each step, the filter was centrifuged for a further 1 min. 50 µL of pre-heated (95°C) nuclease free water was applied to the filter for 1 min, and the filter was centrifuged for 30 seconds. The purified RNA was stored at -80°C.

2.4.2 mRNA extraction

Total RNA samples were prepared independently for 50 blood-isolated CD14+ monocyte samples, 50 milk-isolated CD14+ monocyte samples and 50 bovine macrophage (BoMac) samples using the mirVana^{TM} miRNA Isolation Kit (Life Technologies, Carlsbad, CA, USA). For each sample, an initial volume of 600 µL of lysis/binding solution (provided with kit) was added to the cells and mixed by vortex to fully disrupt cells and form a homogenous lysate. One volume of acid-phenol: chloroform equal to the lysate volume was then added and the sample was mixed by vortex for 30-60 seconds. The sample was then centrifuged for 5 min at 10,000 x g at room temperature to separate the aqueous and organic phases. The aqueous (upper) phase was removed and transferred to a fresh tube. 1.25 volumes of 100% ethanol were added to the aqueous phase and mixed thoroughly by vortexing. The lysate/ethanol mixture was then pipetted onto the filter cartridge (provided with kit) and centrifuged at 10,000 x g for ~15 seconds to pass the mixture through the filter. The flow-through was discarded, and 700 µL wash solution 1 (working solution mixed with ethanol) added to the filter cartridge and centrifuged for ~5-10 seconds. Again, the flow-through from the collection tube was discarded, and the filter replaced in the cartridge as in the previous step. 500 µL wash solution 2/3 (working solution mixed with ethanol) was then added to the filter cartridge and centrifuged for

~5-10 seconds. This step was then repeated. After discarding the flow-through from the last wash, the filter was replaced in the cartridge in the same collection tube and the assembly was spun down for 1 min to remove residual fluid from the filter. The filter cartridge was transferred into a fresh collection tube (provided with kit.). 50 µL of pre-heated (95°C) nuclease free water was added to the centre of the filter, and centrifuged for ~20-30 seconds at max speed to recover the RNA. The purified RNA was stored at -80°C.

2.4.3 miRNA Extraction

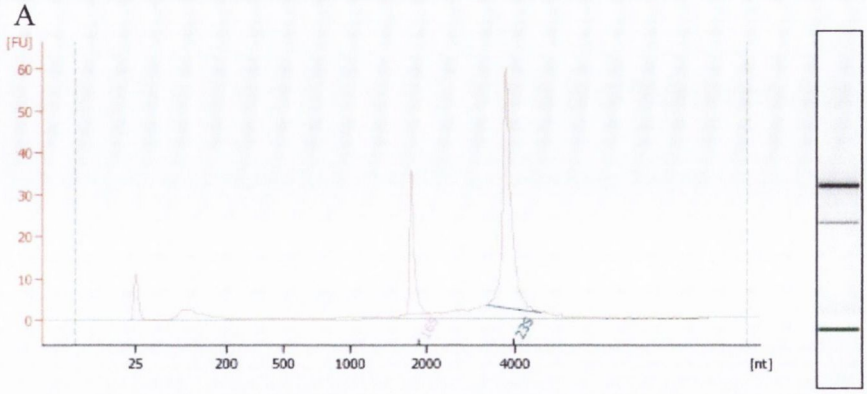
Small RNA samples were prepared independently for the same 50 blood-isolated CD14+ monocyte samples and 50 milk-isolated CD14+ monocyte samples. MicroRNA lysis buffer was made according to the standard Sigma-Aldrich protocol. Equal volumes of lysis buffer, and binding solution were made up with 10 mL of 2-mercaptoethanol per 1 mL. The cell pellet was shaken for 1-2 seconds to loosen cells. The Lysis Mix was added to the cell pellet and mixed by vortex immediately but gently and briefly (2-3 seconds) to disrupt the cell pellet. The sample was incubated at room temperature for 5 mins and mixed 2-3 times in between by gentle shaking. The sample was then centrifuged at maximum speed (16,000 x g) in a standard microcentrifuge for 5 mins to remove cellular debris, genomic DNA, and large RNA. The supernatant was transferred to a clean 2 mL collection tube. 1.1 volumes of 100% ethanol was then added to the clarified lysate for RNA binding, and mixed immediately and thoroughly by vortex or inversion. 700 mL of the mixture was pipetted into a binding column and centrifuged at 16,000 x g for 30 seconds. The flow through was decanted, and the step repeated. 700 mL of 100% ethanol was then added into the column and centrifuged at maximum speed (16,000 x g) for 30 seconds. The

binding column was then transferred into a fresh collection tube, into which 500 mL of the Ethanol-diluted Wash Solution 2 (provided by Sigma-Aldrich) was added into the column. The column was then centrifuged at maximum speed (14,000 –16,000 x g) for 30 seconds. The flow-through was discarded and the column returned to the Collection Tube. Another 500 mL of the Ethanol-diluted Wash Solution 2 was added into the column and centrifuged at maximum speed (16,000 x g) for 30 seconds. The flow-through was discarded the column returned to the collection tube. To dry the column, the column was centrifuged at maximum speed (16,000 x g) for 1 min to dry, and carefully removed from the centrifuge to avoid splashing the residual flow through liquid to the dried column. To elute RNA, the column was added to a new 2 mL collection tube and 30 mL of nuclease free water was added directly onto the centre of the filter inside the column and incubated at room temperature for 1 minute. The tube was then centrifuged at maximum speed (16,000 x g) for 1 minute to elute the RNA. Elute was collected, and the elution step was repeated once. The purified RNA was stored at -80°C.

2.4.4 RNA integrity and quantification

Total RNA was measured by the Agilent RNA 6000 Nano Kit using the 2100 Bioanalyzer (Agilent Technologies, USA). The integrity of each RNA sample was assessed before proceeding with sequencing (Figure 2.2). The Agilent Small RNA Kit (Agilent Technologies, USA) was used to quantify miRNA. All miRNA samples were above the required quantity for sequencing

A



Overall Results for sample 1 : **Sample 1**

RNA Area:	187.9	rRNA Ratio [23s / 16s]:	2.7
RNA Concentration:	119 ng/µl	RNA Integrity Number (RIN):	9.9 (B.02.08)

Fragment table for sample 1 : **Sample 1**

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
16S	1,748	2,109	29.9	15.9
23S	3,371	4,602	81.7	43.5

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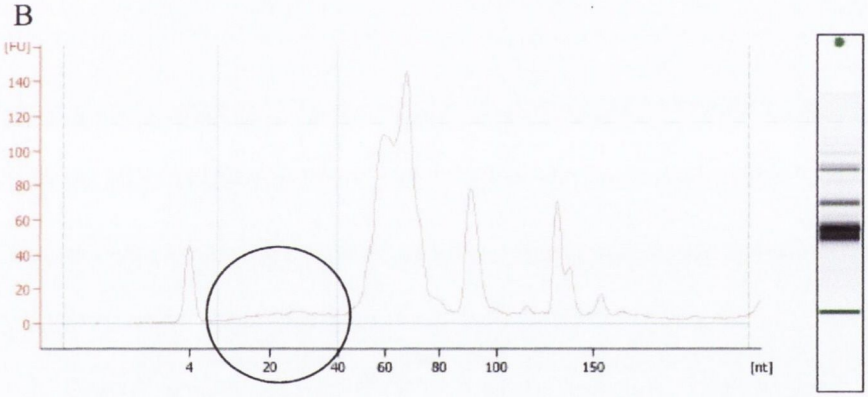
B



Overall Results for sample 4 : **Sample 4**

Small RNA Concentration [pg/µl]:	4,243.5	Result Flagging Color:	
miRNA Concentration [pg/µl]:	622.6	Result Flagging Label:	15 % miRNA; Concentration: 622.60 pg/µl
miRNA / Small RNA Ratio [%]:	15		

Region table for sample 4 : **Sample 4**

Name	From [nt]	To [nt]	Average Size [nt]	Size distribution in CV [%]	Conc. [pg/µl]	% of Total	Color
Small RNA	0	231	80	52.7	4,243.5	100	
miRNA	10	40	25	32.3	622.6	15	

Figure 2.2 (A) Selected example of total RNA and (B) miRNA samples showing optimum integrity. RNA extracted from primary mammary epithelial cells was assessed using micro-capillary electrophoresis (Agilent Bioanalyzer). Reference ladder visible at (A) 25 nt and (B) 4 nt respectively. (A) RNA degradation is estimated as the area under the 18S and 28S peaks at 2000 nt and 4000 nt. In (B) miRNA quantity is in 10-40 nt region. [FU] = fluorescent units and [nt] = nucleotides.

2.5 Quantitative real-time PCR (qPCR)

One microgram of total RNA from each sample was reverse transcribed into cDNA using a high capacity cDNA reverse transcription kit purchased from Applied Biosystems according to manufacturer's instructions (Life Technologies, CA, USA). The cDNA was quantified using a ND-1000 Nanodrop spectrophotometer, and then diluted to a working concentration of 50 ng per 20 uL reaction. Gene specific primers were designed using NCBI Primer BLAST software (Table 2.2).Primers were designed to span introns where possible to minimise the potential of amplifying genomic DNA. Primers were commercially synthesised by Sigma-Aldrich. Quantitative real time PCR was carried out using Fast SYBR Green Master Mix (Invitrogen, USA) and the MX3000P® quantitative PCR system (Stratagene Corp., CA, USA). PCR conditions used (shown in Table 2.1) were those outlined in the Fast SYBR Green Master Mix instructions. Amplicon dissociation was as follows; 95°C for 1 min, 55°C for 30 s and 95°C for 30 s. Each reaction was carried out in duplicate in a final volume of 20 µL, with 10 µL of Fast SYBR Green Master Mix, 8 µL H_2O, 1 µL (50 ng/µL) cDNA, and 1 µL of primer (combination of 5 µM forward primer and 5 µM reverse primer).

Table 2.1 Fast SYBR green Master Mix PCR conditions.

Temperature:	Time:	Cycles:
95°C	20 s	1
95°C	3 s	40
60°C	30 s	40

2.5.1 Primer optimisation

Lyophilised primers were diluted to a working stock concentration of 100 µM according to manufacturer's instructions. 12.5 µL forward and 12.5 µL reverse were combined then added to 225 µL nuclease free water to dilute primers to a working concentration of 5 µM per primer. From this 1 µL was added to each 20 µL reaction to give a final reaction concentration of 250 nM forward primer and 250 nM reverse primer. For primer optimisation, BoMac cDNA (control samples x 3 technical replicates) was used at a concentration of 50 ng per 20 µL reaction. For testing the full range of primer efficiency, serial dilutions were made at 750 nM, 500 nM, 250 nM, and 125 nM and run in duplicate. Dissociation curves and Cq data were examined for fluorescence and to ensure the primers were specific to reference genes and primer dimer amplification was absent. Once optimal primer concentrations were selected, qPCR was performed on serial dilutions of cDNA (100 ng, 50 ng, 25 ng, 12.5 ng, 6.25 ng, 3.125 ng, and 1.56 ng), to determine optimum cDNA input. PCR efficiency was calculated from the corresponding Cq values. Primers were used if the PCR efficiency ranged from 90-110%.

2.5.2 Selection of a stably expressed reference gene

To minimise inaccurate gene expression data between samples, primers were designed for four reference genes Peptidylprolyl Isomerase A (Cyclophilin A) (PPIA), Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), H3 Histone, Family 3A (H3F3A), and mitochondrial ribosomal protein S6 (MRPS6). The stability of each reference gene was determined using geNorm software (version 3.4 Microsoft Excel add-in software package.

2.5.3 Data analysis

For differential expression analysis of Data analysis was carried out using the $2^{-\Delta\Delta CT}$ method. Ct values were initially inputted into the formula $2^{\wedge-(ct)}$. A ratio of gene of interest relative to housekeeping gene was generating by normalising the gene of interest to a stable internal housekeeping control gene, and confirmed using Genex software. Statistical analysis on relative gene expression values were carried out using the non-parametric Mann-Whitney U test as implemented in version 5.01 of Graphpad Prism Software, San Diego, CA). Genes were considered to be significantly differentially expressed between treatment groups if comparison of the relative gene expression values from samples in those groups resulted in P values of <0.05.

Table 2.2 Primers sequences specific to genes used for qPCR.

Gene symbol	Accession number	Forward primers (5'-3)	Reverse Primer Sequence 3'-5'	Amplicon size (bp)	Primer concentration (nM)
PPIA	NM_178320.2	CCACCGTGTTCTTCGACAT	CTGTGAAAGCAGGAACCTTT	154	250
H3F3A	NM_001014389.2	CATGGCTCGTACAAAGCAGA	ACCAGGCCTGTAACGATGAG	136	250
GAPDH	NM_001034034.2	CTCCCAACGTGTCTGTTGTG	TGAGCTTGACAAAGTGGTCG	222	250
MRPS6	NM_001040584.2	CGCACGCTTCCCTATAAGAT	TTCACTTCCTGGGTCAGAGG	176	250
IL8	NM_173925.2	TTGTGAAGAGAGCTGAGAAGCAA	CCAGACCCACACAGAACATGAG	150	250
NOD2	NM_001002889.1	CGCCAAAGGACTTGCAAGA	CCTCGGAGCCAGACTTCCA	135	250
PTK9	NM_001025320.2	ACCCCTGTTGGAGGACAAAC	ACGTCAGTCTGTACCTCATTAATTT	324	500
IRAK1	NM_001040555.1	CCAATGACCCAGGTGTACCA	TAGGAGTTCTCTTGCGGGGA	113	500

2.6 High throughput sequencing

2.6.1 Small RNA library preparation (ScriptMiner™ v1)

Twenty-four indexed bovine mammary epithelial cell miRNA libraries were prepared using the ScriptMiner™ Small RNAseq Library Preparation Kit (Epicentre, USA). Procedures were performed according to the manufacturer's protocol. A 3'-tagging sequence was added to the 3'- end of the RNA followed by treatment with a degradase enzyme to reduce excess 3' adaptor oligo. A tagging sequence was then added to the 5' end of the RNA. The di-tagged RNA was purified using the Zymogen RNA Clean and Concentrator (Zymogen, USA). The di-tagged RNA was then reverse transcribed into cDNA, and the remaining RNA was removed using RNase. The PCR step used by the ScriptMiner™ kit, is a two stage process, firstly an analytical PCR step was carried out to optimise the number of cycles necessary for amplification. Once this was determined, the libraries were amplified and adaptors were added. Size fractionation of the miRNA libraries to separate them from adapter dimers was achieved by electrophoresis on an 8% TBE polyacrylamide gel (Life Technologies, USA) (1.00 mm x 10 well). The libraries were then purified from the gel and the Agilent High Sensitivity DNA Kit (Agilent Technologies, USA) was used to quantify the molarity and size of finished miRNA-seq libraries. The samples were sequenced on an Illumina HiSeq 2000. The sequencing service was provided by the Norwegian Sequencing Centre (www.sequencing.uio.no), a national technology platform hosted by the University of Oslo.

2.6.2 miRNA sample preparation kit (Illumina™ TruSeq v2 50 cycles)

One-hundred indexed CD14+ monocyte miRNA libraries were prepared using the TruSeq v2 Library Preparation Kit (Illumina, USA). The 3' and 5' adaptors were sequentially ligated to each sample. cDNA was made from each sample, ligated with both adaptors via reverse transcriptase and enriched by PCR. The amplified cDNA product was purified through a 6% Novex TBE PAGE gel. Samples were re-eluted in 10 mM Tris-HCL, pH 8.5, and pooled based using Illumina recommended multiplexing protocols. The completed libraries were validated on an Agilent 2100 bioanalyser using an Agilent DNA high sensitivity chip (Agilent, USA). The samples were sequenced on an Illumina HiSeq 2000. The sequencing service was provided by the Iowa State Sequencing Centre, DNA Facility, Iowa State University, Ames, Iowa 50011, USA.

2.6.3 mRNA sample preparation Kit (Illumina™ TruSeq v2 50 cycles)

One-hundred indexed CD14+ monocyte mRNA Illumina sequencing libraries were prepared using the Illumina Truseq RNA sample prep kit version 2 (Illumina, US) according to the manufacturer's instructions. Briefly, two rounds of selection using magnetic oligo dT beads were used to purify the poly-A containing mRNA. Once purified, mRNA was fragmented and primed with random hexamers into first strand cDNA using reverse transcriptase/random primers. The RNA template was then removed and double stranded cDNA was generated using DNA polymerase I and RNase H. 3' and 5' overhangs of the cDNA fragments were repaired, and the 3' ends were adenylated with a single 'A' before ligating the adaptors. Samples were then purified and the products with adaptors were selectively enriched by 14 cycles of PCR. The completed libraries were validated on an Agilent 2100 bioanalyser using an Agilent DNA1000 chip (Agilent, Colorado Springs, CO, USA. The libraries were sequenced on an Illumina HiSeq 2000. The sequencing service was provided by the Iowa State Sequencing Centre, DNA Facility, Iowa State University, Ames, Iowa 50011, USA.

2.6.4 Blocking and randomisation

All library sets were randomised to avoid confounding flow cell/lane effects (Auer and Doerge 2010). The following randomisation and blocking was applied. Mammary epithelial cell miRNA libraries were randomised across three lanes of a flowcell, with eight indexed samples on each lane. Libraries were sequenced on an Illumina HiSeq 2000 by the Norwegian Sequencing Centre with TruSeq v2 reagents. FASTq files were produced using the CASAVA pipeline v1.8.2. 50 blood-isolated and 50 milk-isolated CD14+ monocyte mRNA samples (n =100) were randomised

across four flow cells (i.e. 3 or 4 samples multiplexed per lane). 50 blood-isolated and 50 milk-isolated CD14+ monocyte miRNA samples (n =100) were randomised across three flow cells (i.e. 7 or 8 samples multiplexed per lane). Libraries were sequenced on an Illumina HiSeq 2000 by the Iowa State Sequencing Centre with TruSeq v2 reagents. Fastq files were produced using the CASAVA 1.8 pipeline. The barcode compatibility charts provided with the TruSeq RNA/miRNA sample preparation kit were adhered to when pooling libraries.

2.6.5 Cluster generation

Cluster generation is the process in which single cDNA molecules are isothermally amplified on an 8-channel flow cell. Each flow cell has a dense lawn of oligonucleotides grafted to its surface. The role of the oligonucleotides is to bind to the adaptors that are ligated to the library fragments. Single DNA molecules hybridise to the lawn of oligonucleotides and the bound fragments are extended to create copies which are covalently bound to the flow cell surface. This produces randomly distributed clonally amplified clusters (the ratio of the primers to the template on the support defines the surface density of the amplified clusters) (Metzker 2010). Each library fragment is clonally amplified through a series of extensions and isothermal bridge amplifications resulting in millions of spatially separated unique clusters. The reverse strands are cleaved and washed away. Ends are blocked and a sequencing primer is hybridised to the DNA templates. After this step the libraries are ready for sequencing.

2.6.6 Sequencing and imaging

Upon the successful completion of the cluster generation step, each lane of the flow cell will be imbedded with tens of millions of unique clusters. Each cluster will contain multiple copies of the same strand of cDNA. All clusters are sequenced simultaneously in a process called cyclic reversible termination (CRT) in which the cDNA templates are sequenced base by base in parallel using four fluorescently modified reversibly terminated nucleotides. All four bases compete to bind to the template. This ensures accurate calling of each base. After each round of synthesis a single nucleotide is incorporated to the primed templates in each cluster; following incorporation, the remaining nucleotides are washed away. The clusters are excited by a laser emitting a colour that identifies the newly incorporated nucleotide; the four colours are detected by total internal reflection fluorescence (TIRF) imaging using two lasers. The colour emitted by the cluster is picked up by a camera (imaging) and recorded in real time. The fluorescent label and blocking/inhibiting group (3'-blocked terminators containing a cleavable group attached to the 3'-oxygen of the 2'-deoxyribose sugar (Metzker 2010) are then cleaved and followed by another washing step, allowing for the addition of the next base. A single base is read in each cycle. Cycles range from 25-200; this study was carried out with a 50-cycle sequencing kit.

2.7 Mammary epithelial cell high throughput small RNA sequencing analysis

2.7.1 Mammary epithelial cell small RNAseq analysis

Preliminary quality control analyses of the 24 mammary epithelial cell fastq files were carried out with FASTQC software v0.10.0. Cutadapt v1.1 (code.google.com/p/cutadapt/) was then used to trim 3' adaptor sequences. Reads which were shorter than 18 nucleotides after trimming were discarded. Trimmed reads were then further filtered using the fastq quality filter (http://hannonlab.cshl.edu/fastx_toolkit/) v0.0.13. Reads where at least 50% of the bases had a Phred score < 20 were removed (Cock, Fields et al. 2010). Finally, reads passing all the above filters were also trimmed at their ends to remove low quality bases (Phred score < 20). Reads which successfully passed filtering were aligned to the bovine genome (UMD3.1) using novoalign version 2.07.11 (http://www.novocraft.com). HTSeq version 0.5.3p3 (http://www-huber.embl.de/users/anders/HTSeq/doc/overview.html) using the union model was used to assign uniquely aligned reads to Ensembl (v66) bovine gene and miRNA annotation (separately).

2.7.2 Mammary epithelial cell small RNAseq differential expression analysis

Prior to assessing differential expression, count data were first normalised across libraries using either the trimmed mean of M-values (TMM) normalisation method (Robinson, McCarthy et al. 2010) or upper-quantile normalisation (Bullard, Purdom et al. 2010). Differential expression analysis of miRNAseq data has been shown to be sensitive to the normalisation approach implemented (Garmire and Subramaniam 2012). To address this issue, we identified differentially expressed

miRNAs in two alternatively normalised datasets; TMM-normalised, upper-quantile normalised and no normalisation. Only miRNAs which were identified as differentially expressed across all three datasets were considered further i.e. the differential expression of these miRNAs was robust to the normalisation procedure. As an aside, we found that the two different normalisation approaches resulted in very similar miRNAs being detected as differentially expressed.

The R (version 2.14.1) Bioconductor package EdgeR (v2.4.6) (Robinson, McCarthy et al. 2010), which uses an negative binomial distribution model to account for both biological and technical variability was applied to identify statistically significant differentially expressed miRNAs. Only miRNAs that had at least 10 count per million in at least 3 samples were analysed for evidence of differential gene expression. The analysis was undertaken using moderated tagwise dispersions. Differentially expressed miRNAs were defined as having a Benjamini and Hochberg (Benjamini and Hochberg 1995) corrected P value of < 0.05.

2.7.3 Mammary epithelial cell small RNAseq novel miRNA discovery

In addition to profiling the expression of known miRNAs, miRNAseq data can also be used to identify the expression of potentially novel miRNAs. To do this, miRNAseq data from this study was analysed using the software package miRDeep2 v0.0.5 (Mackowiak 2011). The mirDeep2 algorithm mines high-throughput sequencing data for the presence of multiple sequenced RNAs corresponding to predicted miRNA hairpin structures in the genome. It then uses Bayesian statistics to score the fit of sequenced RNAs to the biological model of miRNA biogenesis. MiRDeep2 predicted a large number of potentially novel miRNAs from our

miRNAseq data. We further parsed this data using a number of different parameters to identify those novel miRNAs that have the highest likelihood of being true positives. Specifically, we identified those predictions where both the mature and star stands were expressed with a minimum of 5 reads each; where mirDeep2 predicted that the miRNA had > 90% probability of being a true positive; where the hairpin structure had a significant Randfold p-value and where the novel miRNA was independently predicted in two or more different miRNAseq samples.

Customised Perl scripts were also written in house to examine the mirDeep2 output for the presence of miRNA isomiRs. These scripts were used to identify isomiRs that were expressed at a level of at least 100 reads and to identify cases where the expression of the isomiR was higher than the expression of the miRBase consensus mature sequence. Furthermore, we identified whether isomiRs were modified at the 5' or 3' ends (first and last 5 nucleotides).

2.7.4 Mammary epithelial cell small RNAseq miRNA target prediction

Target genes that are potentially regulated by differentially expressed miRNAs were predicted using the consensus of two computational approaches, miRanda v3.3a (Betel, Wilson et al. 2008) and TargetScan v6.2 (Lewis, Burge et al. 2005, Grimson, Farh et al. 2007, Friedman, Farh et al. 2009). Given the high false positive rates for miRNA target prediction, we identified only those potential target genes that were predicted by both methods. More specifically, we first established a broad pool of potential targets by applying miRanda to bovine mature miRNA (miRBase v18) and cDNA sequences (UMD3.1, Ensembl v66) under default threshold settings. This resulted in the prediction of thousands of possible target genes per differentially expressed miRNA. To narrow down this pool of potential targets, we used TargetScan to independently identify conserved targets with a PCT-score above 0.9 and/or non-conserved targets with a context+ score above 90%. Target genes that were not corroborated by one of the two methods (in addition to Targetscan) were discarded. Pathway analysis of predicted gene targets was undertaken using the SIGORA R package (Foroushani, Brinkman, and Lynn et al, submitted) with KEGG pathway annotations (Kanehisa, Goto et al. 2012). Genes that have an annotated role in innate immunity were identified using www.innatedb.com (Lynn, Winsor et al. 2008), a curated database of innate immunity genes, pathways and molecular interactions.

2.8 Milk and blood isolated CD14+ high throughput mRNA sequencing analysis

2.8.1 Milk and blood isolated CD14+ mRNAseq analysis

100 fastq files were generated containing the sequencing data for each of the 100 mRNAseq libraries. The quality and number of the reads for each sample were then assessed using FASTQC v0.10.0. Each sample was then put through a number of quality control filters. Firstly, reads were filtered using the fastq Illumina filter v0.1 (http://cancan.cshl.edu/labmembers/gordon/fastq_illumina_filter/), which removes reads from the FASTQq files which were flagged as not passing the Illumina CASAVA pipeline filters. Cutadapt v1.2 (http://code.google.com/p/cutadapt/) was used to trim the adaptors from reads where necessary. The remaining reads were then further filtered using the FASTQ quality filter package (http://hannonlab.cshl.edu/fastx_toolkit/) v0.0.13.2. Reads were at least 70% of the bases had a Phred score < 20 were removed. Reads passing all the above filters were also trimmed at their ends to remove low quality bases (Phred score < 20). Reads which were < 20 nt after trimming were discarded. Reads which passed all quality control steps were then aligned to the bovine genome (UMD3.1 assembly (Zimin, Delcher et al. 2009)) using TopHat v 2.0.8 (Trapnell, Pachter et al. 2009) allowing 1 mismatch. Reads that did not uniquely align to the genome were discarded. HTSeq-count version 0.5.3p3 (http://wwwhuber.embl.de/users/anders/HTSeq/doc/overview.html) using the union model was used to assign uniquely aligned reads to Ensembl (v69) annotated bovine genes.

2.8.2 Differential gene expression analysis

Data were normalised across libraries using the trimmed mean of M-values (TMM) normalisation method (Bullard, Purdom et al. 2010). The R (version 2.15.2) Bioconductor package EdgeR (v2.4.6) (Robinson, McCarthy et al. 2010), which uses a negative binomial distribution model to account for both biological and technical variability was applied to identify statistically significant differentially expressed genes. Any samples that had < 5 million uniquely aligning reads were removed from further analysis. Only genes that had at least 1 count per million in at least 3 samples were analysed for evidence of differential gene expression. The analysis was undertaken using moderated tagwise dispersions. Differentially expressed genes were defined as having a fold change in gene expression > 1.5 and a Benjamini and Hochberg corrected FDR of < 0.05 (Benjamini and Hochberg 1995).

2.8.3 Hierarchical clustering

Hierarchical clustering of milk and blood mRNA normalised read counts were carried out in the R (version 2.15.2) hclust package. Heatmaps were generated using the R heatmap package.

2.8.4 Gene ontology and pathway analysis

The R (version 2.15.2) Bioconductor package GOseq (version 1.10.0) which corrects for gene length bias (Young, Wakefield et al. 2010) was used to identify over-represented pathways using pathway annotation imported from the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa and Goto 2000) database. KEGG disease pathways were excluded to focus the analysis on primary signalling

pathways. Pathways were considered significantly over-represented with an FDR < 0.05. Pathway analysis was undertaken using Ensembl predicted human 1:1 orthologs of the bovine differentially expressed genes.

Additionally, we manually generated two pathway annotations that were of interest but not annotated in detail in KEGG; the "inflammasome" and "interferon" pathways. Gene IDs for these pathways were sourced from SA biosciences (Qiagen) RT² Profiler™ PCR Array Human Interferon and Receptors (PAHS-064A), and RT² Profiler™ PCR Array Human Inflammasome (PAHS-097A) annotations. The interferon pathway consisted of 84 genes (Appendix 2) whose expression is controlled by or involved in cell signalling mediated by interferon ligands and receptors, while the inflammasome pathway consisted of 95 key genes (Appendix 2) involved in the function of inflammasomes, protein complex's involved in innate immunity, as well as general NOD-like receptor (NLR) signalling.

2.8.5 Network analysis methods

To generate molecular interaction networks, the human 1:1 orthologs of bovine genes that were differentially expressed in at least one of the four time-points, in milk isolated monocytes (MIMs) from *S. uberis* infected animals, were uploaded to InnateDB (www.innatedb.com) (Lynn, Winsor et al. 2008). InnateDB is one of the most comprehensive databases of all human and mouse experimentally-supported molecular interactions (>300,000 interactions in July 2013) but also specifically includes annotation on more than 19,000 manually curated human and mouse innate immunity relevant interactions, many of which are

not present in any other database (Lynn, Chan et al. 2010). Networks were visualized using Cytoscape 2.8.2 (Shannon, Markiel et al. 2003).

The network was analyzed using the cytoHubba plugin (Abd El Naby, Hagos et al. 2011) for Cytoscape 2.8.2 (Shannon, Markiel et al. 2003) to identify network hubs and bottlenecks using default parameters. The jActiveModules plugin (Ideker, Ozier et al. 2002) in Cytoscape 2.8.2 (Shannon, Markiel et al. 2003) was also used to identify high-scoring differentially expressed sub-networks (Overlap Threshold = 0.3; Search depth = 3; Number of modules = 5; "Regional Scoring" and "Adjust score for size" both enabled). The InnateDB pathway analysis tool was used to identify over-represented pathways among module genes.

2.9 Milk and blood isolated CD14+ high throughput miRNA sequencing analysis

2.9.1 Milk and blood isolated CD14+ miRNAseq analysis

Preliminary quality control analysis of the 100 miRNAseq fastq files was again carried out with FASTQC software v0.10.0 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Cutadapt v1.2 (code.google.com/p/cutadapt/) was then used to trim 3' adaptor sequences. Reads which were shorter than 18 nucleotides after trimming were discarded. Trimmed reads were then further filtered using the fastq quality filter (http://hannonlab.cshl.edu/fastx_toolkit/) v0.0.13.2. Reads where at least 70% of the bases had a Phred score < 20 were removed (Cock, Fields et al. 2010). Finally, reads passing all the above filters were also trimmed at their ends to remove low quality bases (Phred score < 20). Reads which successfully passed filtering were aligned to the bovine genome (UMD3.1) using novoalign v2.08.03 in miRNA mode (http://www.novocraft.com) allowing 1 mismatch. Non-uniquely aligning reads were discarded. HTSeq version 0.5.3p3 using the union model was used to assign uniquely aligned reads to miRBase v 19 miRNA annotation (Kozomara and Griffiths-Jones 2011).

2.9.2 Milk and blood isolated CD14+ miRNA differential expression analysis

Prior to assessing differential expression, miRNAseq count data were first normalised across libraries using either the trimmed mean of M-values (TMM) normalisation method (Robinson, McCarthy et al. 2010) or upper-quantile normalisation (Bullard, Purdom et al. 2010). Differential expression analysis of miRNAseq data has been shown to be sensitive to the normalisation approach

implemented (Garmire and Subramaniam 2012). To address this issue, we identified differentially expressed miRNAs in two alternatively normalised datasets; TMM-normalised (Robinson, McCarthy et al. 2010), upper-quantile normalised and with no normalisation. Only miRNAs which were identified as differentially expressed across all three datasets were considered further i.e. the differential expression of these miRNAs was robust to the normalisation procedure (Lawless, Foroushani et al. 2013). Any samples that had < 2 million uniquely aligning reads were removed from further analysis. The R (version 2.15.2) Bioconductor package EdgeR (v2.4.6) (Robinson, McCarthy et al. 2010) was applied to identify statistically significant differentially expressed miRNAs. The analysis was undertaken using moderated tagwise dispersions. Differentially expressed miRNAs were defined as having a Benjamini and Hochberg corrected P value of < 0.05 (Benjamini and Hochberg 1995).

2.9.3 Co-expression and target analysis of milk and blood isolated CD14+ miRNA and mRNA data

To identify mRNAs that were potentially regulated by differentially expressed (DE) miRNAs in MIMs, we first sought to calculate Pearson correlations, using the Apache commons Java statistics library, between all DE miRNA expression in reads per million (rpm) and all mRNA expression (TMM normalised read counts) over the time-course. A correlation matrix was constructed consisting of 26 (DE miRNAs) x 24,616 (All mRNAs) correlation coefficients. The resulting correlation matrix was then filtered to remove both non-significant correlations (critical value for Pearson's correlation for this matrix is r = -0.3116) and those inverse correlations that were not supported by miRanda predicted miRNA-target pairs (miRanda v3.3a). The mRNAs predicted to be targeted (i.e. had a significant anti-correlation relationship in the

expression of the mRNA and the miRNA, plus a predicted seed target) by either up- or down-regulated miRNAs were then selected for pathway analysis. Two-dimensional cluster analysis and visualization, using R version 2.15.3 *hclust* and *heatmap.plus* packages, was then applied to the filtered correlation matrix.

2.9.4 Milk and blood isolated CD14+ miRNA pathway analysis of predicted miRNA target genes

Target genes of differentially expressed miRNAs were submitted to InnateDB (Lynn, Winsor et al. 2008) for pathway analysis. Genes were submitted in two groups; those that were targets of up-regulated miRNAs, and those that were targets of down-regulated miRNAs. Significant pathways were calculated based on hypergeometric analysis, pathways of interest were defined as having a Benjamini and Hochberg corrected P value of < 0.05 (Benjamini and Hochberg 1995).

2.9.5 Milk and blood isolated CD14+ novel miRNA discovery

Using the software package miRDeep2 v0.0.5 (Mackowiak 2011) we examined whether milk/blood monocytes encoded for miRNAs not yet annotated in the bovine genome. We further parsed the data using a number of different parameters to identify those novel miRNAs that have the highest likelihood of being true positives as described previously (Lawless, Foroushani et al. 2013). Specifically, we identified those predictions where both the mature and star stands were expressed with a minimum of 5 reads each; where miRDeep2 predicted that the miRNA had > 90% probability of being a true positive; where the hairpin structure had a significant

Randfold p-value and where the novel miRNA was independently predicted in two or more different miRNAseq samples.

2.9.6 NCBI Gene Expression Omnibus (GEO) database identifiers

All datasets have been submitted to the NCBI Gene Expression Omnibus (GEO) database (Barrett, Troup et al. 2011). Bovine mammary epithelial cell miRNAseq fastq files experiment series accession number GSE41278. Milk and blood isolated CD14+ miRNAseq and mRNAseq fastq files experiment series accession number GSE51858.

Chapter 3: Next generation sequencing reveals the expression of a unique miRNA profile in response to a Gram-positive bacterial infection.

3.1 Introduction

MicroRNAs (miRNAs) are an abundant class of highly conserved, small (19-24nt long), non-coding, double-stranded RNA molecules. They act as post-transcriptional regulators of gene expression, altering mRNA stability and translation efficiency by hybridizing to the 3' untranslated regions (UTRs) of certain subsets of mRNAs (collectively as many as 60% of all mRNA transcripts) (Barral, Sarkar et al. 2009). Since their initial discovery in Caenorhabditis elegans in 1993 (Lee, Feinbaum et al. 1993), researchers have gained much insight into the prevalence of miRNAs in other species.

MicroRNAs have been shown to play key roles in the regulation of innate and adaptive immunity in humans and mice (O'Connell, Rao et al. 2010). miR-146a, for example, regulates the innate immune response to bacterial infection, targeting TNF receptor-associated factor 6 (TRAF6) and interleukin-1 receptor-associated kinase 1 (IRAK1) (Williams, Perry et al. 2008), while miR-150 regulates the production of mature B cells (Xiao, Calado et al. 2007). Studies elucidating the regulatory roles of miRNAs in bovine infection and immunity, however, are more limited. Bovine miRNAs have been shown to be expressed in a wide range of tissues, including immune-related ones (Coutinho, Matukumalli et al. 2007, Jin, Grant et al. 2009, Xu, Zhang et al. 2009), but only a handful of studies have investigated how the expression of bovine miRNAs are altered in response to infection. A recent qPCR study, for example, highlighted the differential expression of five inflammation related miRNAs (miR-9, miR-125b, miR-155, miR-146a and miR-223) in response to *E. coli* lipopolysaccharide (LPS) and *S. aureus* enterotoxin B stimulation of bovine monocytes (Dilda, Gioia et al. 2011). Two other studies have used a similar approach

to identify several miRNAs that were differentially expressed in the mammary gland tissue of cattle with mastitis (Hou, Huang et al. 2011, Naeem, Zhong et al. 2012). These and other studies suggest roles for individual miRNAs in regulating bovine immunity, however, according to Ensembl v66 (Hubbard, Aken et al. 2009, Flicek, Amode et al. 2012) there are over 1,300 annotated miRNAs in the bovine genome. Therefore, studies which adopt genome-wide approaches are required to gain greater insight into the repertoire of bovine miRNAs involved in immunity and infection.

Although microarray technologies to profile miRNA expression have been around for some time (Kim, Han et al. 2009), next generation sequencing (NGS) based technologies are revolutionising the field and provide the opportunity to profile the expression of known miRNAs with discriminating resolution and accuracy, and also to identify novel miRNAs (Buermans, Ariyurek et al. 2010). Furthermore, these technologies allow one to differentiate between the expression of alternative mature miRNAs from the same precursor and to identify the differential expression of miRNA isomiRs (Lee, Zhang et al. 2010). To date, a limited number of studies have applied these approaches to profile miRNAs in different bovine tissues (Chen, Gao et al. 2010, Huang, Ju et al. 2011, Guduric-Fuchs, O'Connor et al. 2012) and only one study has used an NGS approach to investigate the expression of bovine miRNAs in response to infection (Glazov, Kongsuwan et al. 2009).

In this study an NGS approach was implemented to profile the expression of bovine miRNAs at multiple time-points in primary mammary epithelial cells infected *in vitro* with *Streptococcus uberis*, a causative agent of bovine mastitis. This inflammatory disease of the mammary gland has significant economic impact on the global dairy

industry (Henk Hogeveen 2011) (Berry and Amer. 2005). To the best of our knowledge, this study represents the most comprehensive *in vitro* NGS study to date that profiles the host miRNA response to infection, in bovine. In comparison to previous studies, we have sequenced un-pooled miRNA libraries to a previously unprecedented sequencing depth from multiple replicates and controls across multiple time-points, allowing us to explore the statistically significant temporal changes in miRNA expression in response to infection.

3.2 Hypothesis

We propose that microRNAs (miRNAs), short non-coding RNAs which post-transcriptionally regulate gene expression, are central regulators of the transcriptional networks induced in the bovine mammary epithelial cell response to a bovine mastitis pathogen, *Streptococcus uberis*.

3.3 Specific aims

1. Identify the miRNAs that are differentially expressed over a time-course in an *in vitro S. uberis* challenge of bovine primary mammary epithelial cells, using a high throughput sequencing approach.

2. Identify the transcriptional networks that are targets of differentially expressed miRNAs in bovine primary mammary epithelial cells over the same time course.

3.4 Results

3.4.1 Isolation of small and total RNA from bovine mammary epithelial cells

Small RNA and total was isolated for NGS sequencing from *S. uberis* infected primary bovine mammary epithelial cells at 1, 2, 4 and 6 hours post-infection (hpi) (n = 3 infected and n = 3 controls at each time-point). Total RNA and small RNA were examined for quantity and integrity in each of the 24 samples (Table 3.1). Total RNA was assessed to be of high quality based on both Bioanalyzer and 28S/18S analysis. RNA integrity numbers (RIN numbers) for total RNA were >8 for each sample. The concentration of miRNA in each sample was also assessed (Table 3.1). Sufficient quantities were present to proceed with miRNAseq library preparation and qPCR.

Table 3.1 RNA integrity, quantity, 28S/18S ratio, and miRNA quantity for each of the 24 samples.

Sample Name	RNA Integrity Value (RIN)	RNA concentration ng/µl	rRNA Ratio (28S/18S)	miRNA concentration pg/µl
1 hour control (r1)	10	442	2.2	261.3
1 hour control (r2)	10	382	2.1	318.4
1 hour control (r3)	10	436	2.2	233.7
1 hour infected (r1)	10	488	2.2	415.6
1 hour infected (r2)	10	398	2.2	308.6
1 hour infected (r3)	10	469	2.1	442.1
2 hour control (r1)	10	331	2.3	958.3
2 hour control (r2)	10	288	1.9	628.2
2 hour control (r3)	10	248	2.2	630.2
2 hour infected (r1)	10	391	2.1	525.1
2 hour infected (r2)	10	437	2.2	382.7
2 hour infected (r3)	10	395	2.1	699.5
4 hour control (r1)	9.8	229	2	214.4
4 hour control (r2)	9.8	314	2	173.4
4 hour control (r3)	10	170	2	247.6
4 hour infected (r1)	9.7	272	1.9	622.6
4 hour infected (r2)	9.8	242	1.9	708
4 hour infected (r3)	9.9	302	2.1	814.4
6 hour control (r1)	9.8	284	2	696.7
6 hour control (r2)	9.8	259	2	662.2
6 hour control (r3)	9.7	240	2	566.7
6 hour infected (r1)	9.8	244	2.1	165.9
6 hour infected (r2)	9.6	226	1.9	173.9
6 hour infected (r3)	9.7	227	2	322.1

3.4.1 Validation of primers selected for stable reference gene and genes of interest

Primers were designed for each reference gene and gene of interest (GOI). The expression of three reference genes PPIA, GAPDH, H3F3A and two genes of interest TNF-α and IL8 were tested. Their RSq value, Y-value, and the efficiency of the line (Table 3.2) were all within required parameters. Reference genes were tested in geNorm in the primary mammary epithelial cell culture. All gene amplifications for bovine samples were normalised to GAPDH, H3F3A, and PPIA.

Table 3.2 Results of RSq, Y value, and efficiency values for primers used in this study

Gene name:	RSq:	Y value:	Efficiency:
GAPDH	0.996	-3.216	104.6%
H3F3A	0.994	-3.146	107.9%
PPIA	0.947	-3.403	96.6%
IL8	0.92	-3.223	104.3%
TNF-α	0.992	-3.31	102%

3.4.2 TNF-α and IL8 are induced in response to an *S. uberis* challenge in mammary epithelial cells

To determine whether this *in vitro* infection model was suitable for miRNA expression studies, primary mammary epithelial cells were challenged at 4 hours and 6 hours with live *S. uberis* (moi 50). The fold change in expression of IL8 and TNF-α were determined relative to internal control genes PPIA, H3F3A, and GAPDH, as was

the relative expression of both genes. The expression of both IL8 (3.2 fold @ 6 hpi, P < 0.05; figure 3.1A) and TNF-α (2.2 fold @ 4 HPI, not significant; 3.8 fold @ 6 hpi, P < 0.05; figure 3.1 B) increased relative to control samples at an MOI of 50.

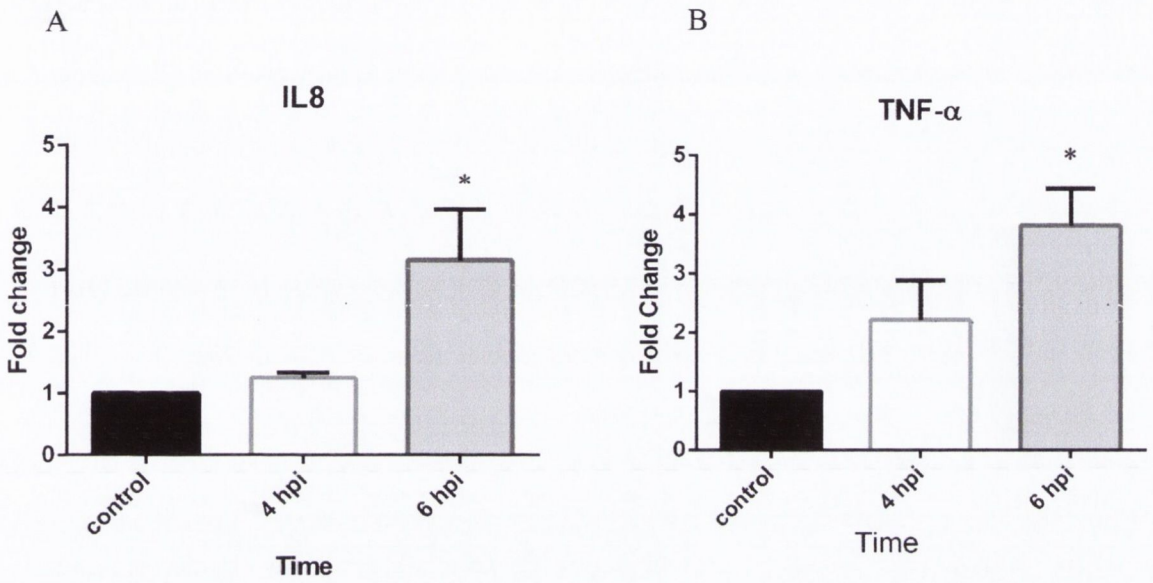


Figure 3.1 *S. uberis* stimulated A) IL8 and B) TNF-α expression in *ex vivo* primary mammary epithelial cells. Expression of IL8 and TNF-α were analysed using RT-qPCR (n= 3). Gene expression values were compared using a one-way ANOVA test (Kruskal-Wallis test), implemented in the GraphPad Prism software, version 5.0. P <0.05 was considered statistically significant (*).

3.4.2 High-throughput sequencing of small RNA libraries

Small RNA libraries were prepared from size selected RNA (<200 nucleotides). Libraries were prepared using the ScriptMiner™ protocol with indexing and each library was assessed for size and quality by micro-capillary electrophoresis (Agilent Bioanalyzer) on a high sensitivity DNA analysis chip (Appendix 3) before

cluster generation, sequencing and imaging on an Illumina Hiseq 2000. Samples were randomly multiplexed over 3 flowcell lanes (Table 3.3) for sequencing. Sequencing of small RNA libraries yielded more than 450 million raw sequence reads from mammary epithelial cells. Following a pipeline of adaptor removal, quality filtering and the removal of sequences that were too short, fastQC checks were run on each library to ensure reads were of sufficient quality to proceed with analysis (Appendix 4). More than 213 million reads were retained for further analysis (78,604,161 and 134,850,887 for control and infected replicates, respectively) (Table 3.4). These filtered reads were then aligned to the reference Bos taurus UMD 3.1 genome. Over 116 million reads aligned uniquely to the genome (Table 3.4). Reads that aligned to more than one position in the genome were discarded. Uniquely aligning reads were then assigned to known miRNAs using HTseq, based on Ensembl v66 (Hubbard, Aken et al. 2009, Flicek, Amode et al. 2012) annotation of the bovine genome. Correlation coefficients of each miRNA normalized read count dataset between samples at each time-point for control and infected technical replicates were carried out to examine the reproducibility of datasets. The average correlation between each time-point for both control and infected was > 0.98 (Appendix 5.1). Reads were further examined using expression density plots to examine the variation of aligned reads before and after normalisation (Appendix 5.2) Multidimension scaling (MDS) analysis was undertaken to assess the variation among samples based on miRNA expression at 1, 2, 4 and 6 hours post-infection (Appendix 6). A small separation can be seen in both the first dimension (MDS; Appendix 6) between control and infected samples at 4 h and 6 hours post infection respectively.

Table 3.3 Summary barcodes sequences and flow cell identification for each mammary epithelial cell miRNAseq library

Sample	Barcode	Flow Cell ID	Lane Number	Sequencing Chemistry
1 hrs control	GGCTAC	67	6	TruSeq V3 reagents
1 hrs control	CGATGT	67	7	TruSeq V3 reagents
1 hrs control	TTAGGC	67	4	TruSeq V3 reagents
1 hrs infected	ACTTGA	67	6	TruSeq V3 reagents
1 hrs infected	GATCAG	67	7	TruSeq V3 reagents
1 hrs infected	TAGCTT	67	1	TruSeq V3 reagents
2 hrs control	CAGATC	67	7	TruSeq V3 reagents
2 hrs control	ACTTGA	67	4	TruSeq V3 reagents
2 hrs control	GATCAG	67	4	TruSeq V3 reagents
2 hrs infected	TGACCA	67	4	TruSeq V3 reagents
2 hrs infected	ACAGTG	67	6	TruSeq V3 reagents
2 hrs infected	GCCAAT	67	7	TruSeq V3 reagents
4 hrs control	TTAGGC	67	7	TruSeq V3 reagents
4 hrs control	TGACCA	67	6	TruSeq V3 reagents
4 hrs control	ACAGTG	67	4	TruSeq V3 reagents
4 hrs infected	TAGCTT	67	7	TruSeq V3 reagents
4 hrs infected	GGCTAC	67	4	TruSeq V3 reagents
4 hrs infected	CGATGT	67	6	TruSeq V3 reagents
6 hrs control	GATCAG	67	6	TruSeq V3 reagents
6 hrs control	TAGCTT	67	6	TruSeq V3 reagents
6 hrs control	GGCTAC	67	7	TruSeq V3 reagents
6 hrs infected	GCCAAT	67	1	TruSeq V3 reagents
6 hrs infected	CAGATC	67	1	TruSeq V3 reagents

Table 3.4 Summary read statistics for each mammary epithelial cell miRNAseq library

Sample	Raw Reads	Reads Trimmed by Cutadapt	Reads too short to align	Reads After fastx suite QC	Reads Novoalign Uniquely Aligned to UMD3.1
1 hrs control	13,416,237	13,209,202	9,156,633	4,040,468	1,665,740*
1 hrs control	14,190,052	13,532,487	4,842,877	8,674,963	4,965,027
1 hrs control	16,743,831	15,333,780	10,199,375	5,073,997	2,552,886
1 hrs infected	14,271,728	13,633,096	4,425,619	9,185,280	5,207,454
1 hrs infected	13,794,847	13,413,074	6,095,463	7,302,460	3,960,033
1 hrs infected	28,960,252	27,630,244	6,983,901	20,636,491	14,453,054
2 hrs control	28,322,206	27,529,314	17,071,484	10,435,487	4,384,975
2 hrs control	30,086,589	27,929,416	19,896,884	7,925,919	2,743,404
2 hrs control	13,938,395	12,846,710	9,938,550	2,857,745	752,590*
2 hrs infected	16,524,815	14,475,572	7,806,196	6,599,817	3,569,339
2 hrs infected	17,085,890	16,576,231	9,666,656	6,891,188	3,901,877
2 hrs infected	18,152,687	17,060,124	11,183,176	5,864,231	3,082,575
4 hrs control	15,273,961	14,772,247	7,841,977	6,915,758	3,787,608
4 hrs control	13,543,447	12,998,729	6,557,510	6,424,760	3,600,583
4 hrs control	17,329,021	15,712,417	9,994,471	5,647,588	2,996,807
4 hrs infected	15,351,189	14,833,193	7,864,890	6,954,280	3,946,736
4 hrs infected	15,887,867	14,228,044	7,810,805	6,341,169	3,590,914
4 hrs infected	15,968,339	15,467,085	7,485,946	7,959,984	4,259,673
6 hrs control	17,494,397	16,956,074	8,611,706	8,319,808	4,136,993
6 hrs control	13,965,218	13,506,854	7,176,006	6,312,822	3,006,534
6 hrs control	13,610,030	13,355,930	7,791,526	5,553,854	2,553,380
6 hrs infected	28,378,850	27,086,872	10,525,013	16,552,189	9,283,191
6 hrs infected	26,195,614	25,317,605	10,854,398	14,455,131	7,790,276
6 hrs infected	32,437,670	30,214,603	4,317,752	25,884,920	16,061,953

* Dropped from further analysis

3.4.3 Repertoire of RNA species in small RNA libraries

The proportion of reads (averaged across 24 samples) uniquely aligning to different RNA biotypes demonstrates that miRNAs are the dominant ncRNA species sequenced in our small RNA libraries (Figure 3.2). The vast majority (>90%) of reads that align uniquely to known ncRNAs align to known miRNAs. There was no significant difference in the proportion of reads aligning to different RNA biotypes in the infected and control samples. The majority of the remaining reads primarily mapped to snoRNAs (Figure 3.2) (Guduric-Fuchs, O'Connor et al. 2012). Although the vast majority of reads align to known bovine ncRNAs, a low density of reads can be observed along each chromosome. These possibly represent mRNA degradation products.

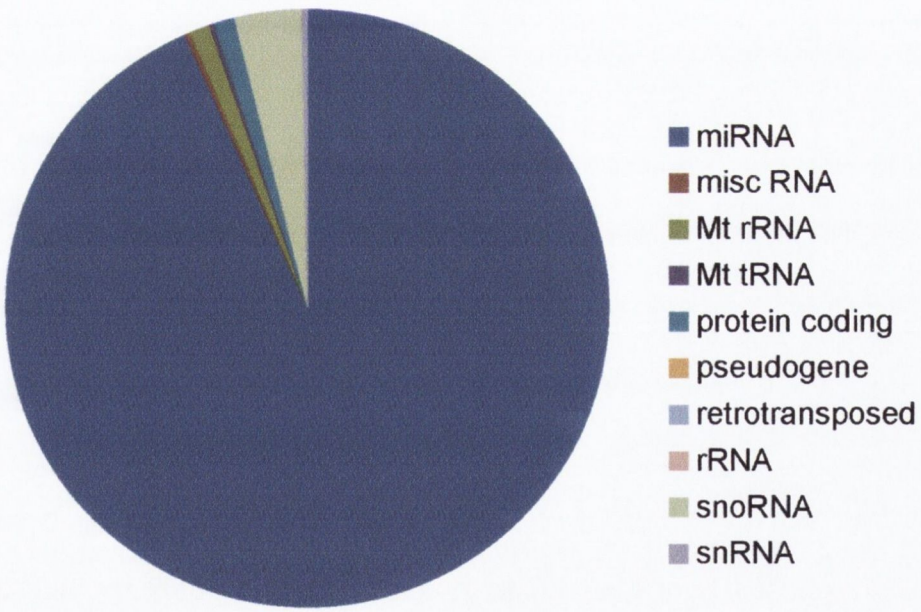


Figure 3.2 The proportion of reads aligning uniquely to bovine ncRNAs (averaged across 24 samples). The vast majority of reads aligned to known miRNAs.

3.4.4 The expression of miRNAs in primary bovine mammary epithelial cells

To characterise the bovine mammary epithelial cell microRNome, miRNAs that were expressed at an appreciable level (based on mapped read counts in tags per million sequenced (tpm)) were identified. 247 miRNAs had a count of greater than 1 tpm (Appendix 7). Of these, 114 miRNAs were expressed at a level >100 tpm (Appendix 7). To determine whether these miRNAs were expressed from related genomic regions, all miRNAs with >100 tpm were examined for genomic clustering (Figure 3.3). There was no evidence of a substantial genomic bias from which these miRNAs were encoded. The top 10 highly expressed miRNAs, which accounted for >80% of all aligned reads were evolutionarily conserved across multiple species. These miRNAs represent seven different miRNA families; miR-let-7 (bta-let-7b & bta-miR-3596), miR-21 (bta-miR-21), miR-27 (bta-miR-27a & bta-miR-27b), miR-28 (bta-miR-151), miR-184 (bta-miR-184), miR-200 (bta-miR-200a & bta-miR-200b), and miR-205 (bta-miR-205) (Appendix 7). Many of these miRNAs have been shown to have pleiotropic roles in other species (Table 3.5). miR-21 and miR-205, have been shown to have role in cancer, regulating tumour suppressor genes such as VEGF-A and TGFI-R2 (Wu, Zhu et al. 2009, Yang, Schlehe et al. 2010, Yu, Kanwar et al. 2012, Yue, Wang et al. 2012).

Of particular interest to this study is the fact that several of the most highly expressed miRNAs in BMEs have been shown to have a role in immunity. miR-27b, for example, has been shown to negatively regulate the mRNA stability of peroxisome proliferator-activated receptor gamma (PPARgamma), a transcriptional regulator of the inflammatory response (Jennewein, von Knethen et al. 2010). Interestingly, miR-27b has also been found to be degraded by a viral transcript in lytic murine

cytomegalovirus (MCMV) infection, further highlighting its role in immunity (Marcinowski, Tanguy et al. 2012). miR-21 has also recently been shown to be the most highly expressed miRNA in Mycobacterium leprae infected monocytes and to negatively regulate the Vitamin D-dependent antimicrobial pathway (Liu, Wheelwright et al. 2012). Evidence from previous studies also suggests that highly expressed miRNAs in BMEs may also regulate each other. miR-184, for example, has been demonstrated to antagonise miR-205 to maintain SHIP2 levels in epithelia (Yu, Ryan et al. 2008) (Table 3.5).

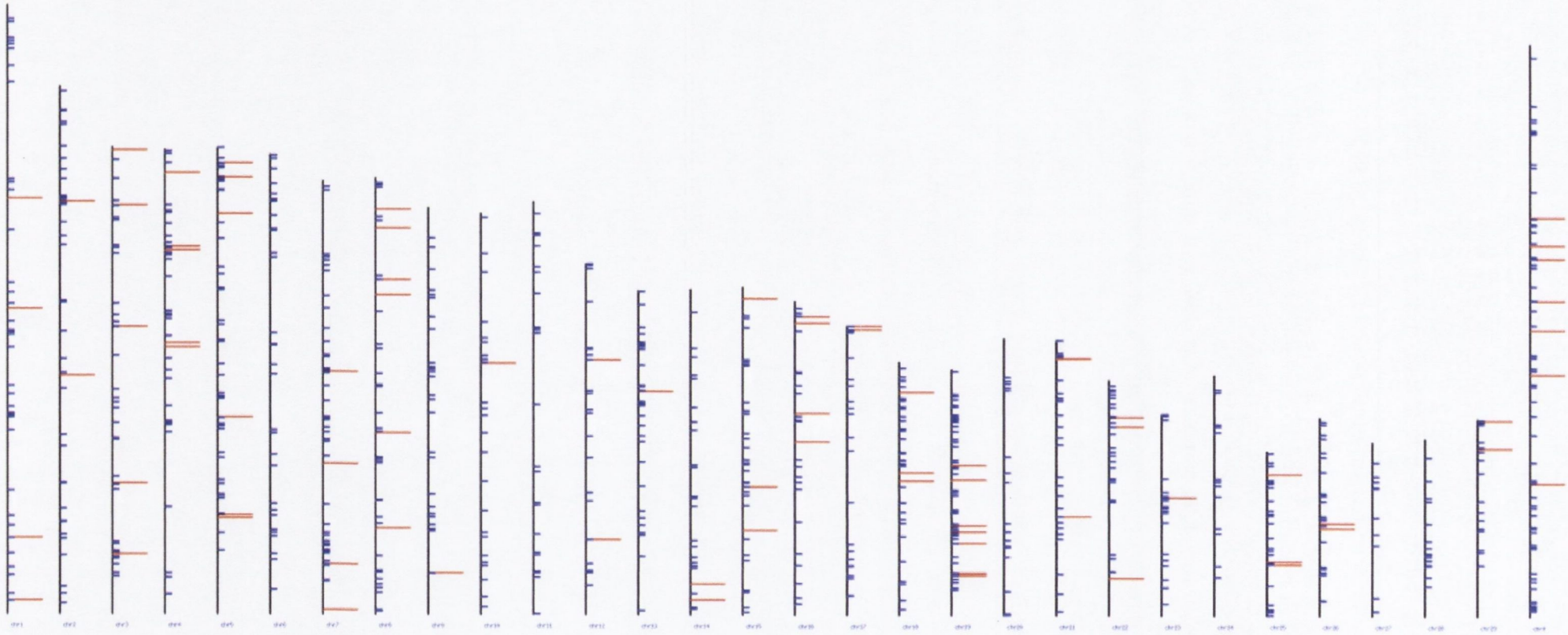


Figure 3.3 The genomic position of bovine mammary epithelial cell expressed miRNAs with >100 tpm (red). The position of other annotated bovine miRNAs is shown (blue) across the bovine genome in the following order left to right (chr1, chr2, chr3, chr4, chr5, chr6, chr7, chr8, chr9, chr10, chr11, chr12, chr13, chr14, chr15, chr16, chr17, chr18, chr19, chr20, chr21, chr22, chr23, chr24, chr25, chr26, chr27, chr28, chr29, chrX)

Table 3.5 Highly expressed miRNAs in bovine mammary epithelial cells have been shown to have pleiotropic functions in other species

miRNA	Species	Tissue	Target	Function	Reference
miR-21	Human	Monocytes	CAMP/ DEFB4A	Immune	(Liu, Wheelwright et al. 2012)
miR-21	Human	Colon Cancer Cell	TGFI-R2	Cancer	(Yu, Kanwar et al. 2012)
miR-184	Human	HeLa/HEK	SHIP2	Immune	(Yu, Ryan et al. 2008)
miR-205	Human	MCF-7, MDA-MB-231, MDA-MB-453 and MDA-MB-468 cells	VEGF-A	Cancer	(Wu, Zhu et al. 2009) (Yue, Wang et al. 2012)
miR-27b	Human	Monocytes	PPAR Gamma	Immune	(Jennewein, von Knethen et al. 2010)

3.4.5 Multiple miRNAs are differentially expressed in response to *S. uberis* infection

Once constitutively expressed miRNAs were characterised in unchallenged bovine mammary epithelial cells, the EdgeR statistical package was used (Robinson, McCarthy et al. 2010) to determine which miRNAs were significantly differentially expressed in response to *S. uberis* infection at 1, 2, 4 and 6 hpi. It has been suggested that differential expression analysis of miRNAseq is sensitive to the normalisation approach implemented (Garmire and Subramaniam 2012). To address this issue, differentially expressed miRNAs in two alternatively normalised datasets; TMM-normalised (Bullard, Purdom et al. 2010), and upper-quantile normalised were identified. Only miRNAs which were identified as differentially expressed across both datasets were considered as significantly differentially expressed i.e. the differential expression of these miRNAs was robust to the normalisation procedure. It was found that the two different normalisation approaches actually resulted in very similar miRNAs being detected as differentially expressed. To confirm miRNAs identified as being differentially expressed and remove results which may have been generated as artefacts of pairwise statistical comparison each miRNA that was identified by EdgeR (Robinson, McCarthy et al. 2010) as being differentially expressed was then plotted for each sample across the full time course as normalised tag counts. By examining miRNAs at both specific time-points and across full datasets, it is clear that the strategy of pairwise analysis can generate spurious signals of gene expression which are likely false positives. In addition any miRNAs under 10 tags per million were removed to further reduce the false positive results. In total ten miRNAs were considered to be differentially expressed when examined across all time-points (Appendix 8 A bta-miR-708, B) bta-let-7b, C) bta-miR-130a, D) bta-let-d,

E) bta-miR-185, F) bta-miR-200c, G) bta-miR-210, H) bta-let-7e, I) bta-miR-652, J) and bta-miR-100.).

No miRNAs were identified as differentially expressed at 1 hpi. At 2 hpi, 2 miRNAs, bta-miR-29e, and bta-mir-708, were found to be upregulated (Table 3.6). However when examined across all time points bta-miR-29e was found not to be upregulated and removed from further analysis. At 4 hpi, bta-miR-98 was up-regulated based on edgeR results however it was discounted as the expression values 4 hpi were lower than any other time point with the exception of control samples at 4 hpi, thus generating the spurious result. Bta-let-7b was upregulated at both 4 and 6 hpi, in both the edgeR data, however, at 4 hpi the upregulation appears marginal (Appendix 8 B). At 6 hpi, 11 miRNAs were found to be upregulated however three more bta-miR-185, bta-miR-24-2, and bta-miR-494, were removed when examined across all time-points. In particular it was evident bta-miR-494 did not undergo differential expression. In contrast, bta-miR-200c (Appendix 8 F) was evenly expressed across all samples and underwent a subtle increase in expression at 6 hpi.

Seven different miRNAs were identified as down-regulated in response to the *S. uberis* challenge (Table 3.6). No miRNAs were down-regulated at 1 or 2 hpi. At 4 hpi, bta-miR-29b-2, bta-miR-193a, and bta-miR-130a were down-regulated. When compared across all samples both bta-miR-193a and bta-miR-29b-2 were discounted from further analysis. At 6 hpi, bta-miR-29b-2, bta-miR-29c, bta-miR-29e, bta-miR-100, and bta-miR-130a were down-regulated (Table 3.6). Bta-miR-29e, bta-miR-29c, and bta-miR-29b-2 were removed based on expression values.

These results indicate that there are rapid temporal changes in the expression of miRNAs in response to a Gram-positive infection, with different miRNA repertoires being identified as differentially expressed at time-points that are 2 hours apart.

Table 3.6 Fold changes and false discovery rates of differentially expressed miRNAs at 2, 4, 6, and 8 hours post-infection in mammary epithelial cell

Hours Post Infection (hpi)	miRNA Name	Fold Change (FC)	False Discovery Rate	Considered
2	bta-miR-29e	1.8	0.00254	No
2	bta-mir-708	2.1	0.000368	Yes
4	bta-let-7b	1.34	0.001	Yes
4	bta-mir-98	1.25	0.025	No
4	bta-mir-193a	-1.35	0.001	No
4	bta-mir-29b-2	-1.41	0.008	No
4	bta-mir-130a	-1.28	0.008	Yes
6	bta-let-7d	1.37	0.00052	Yes
6	bta-mir-128-2	1.28	0.014	No
6	bta-mir-185	1.26	0.014	Yes
6	bta-let-7b	1.26	0.021	Yes
6	bta-mir-200c	1.22	0.021	Yes
6	bta-mir-24-2	1.22	0.021	No
6	bta-mir-210	1.23	0.028	Yes
6	bta-mir-128-1	1.25	0.042	No
6	bta-let-7e	1.22	0.047	Yes
6	bta-mir-652	1.26	0.072	Yes
6	bta-mir-494	1.66	0.078	No
6	bta-mir-29e	-14.61	0.00002	No
6	bta-mir-29b-2	-1.62	0.000052	No
6	bta-mir-100	-1.41	0.000011	Yes
6	bta-mir-130a	-1.37	0.000012	Yes
6	bta-mir-29c	-1.40	0.003	No

3.4.6 The miRNA response to the Gram-positive *S. uberis* is markedly different to the LPS miRNA response

To date, many immune-relevant miRNAs have been identified as part of the host response to lipopolysaccharide (LPS) stimulation (O'Neill, Sheedy et al. 2011, Qi, Qiao et al. 2012), which is frequently used to mimic a Gram-negative bacterial infection. A literature survey identified over 145 miRNAs that have been shown to be differentially expressed in response to LPS across multiple different species and tissues (Appendix 9). Of the ten unique miRNAs that we identified as being differentially expressed in response to the Gram-positive *S. uberis*, only 6 (bta-let-7d, bta-let-7b, bta-miR-100, bta-mir-130a, bta-miR-210, bta-miR-652) have also been reported to be differentially expressed in response to LPS in other species. Furthermore, two of this group (bta-miR-100, bta-miR-210,) show an inverse response to *S. uberis* infection in comparison to LPS. Most notably, bta-miR-100 which was previously identified as upregulated in mouse lung 6h post-stimulation with LPS, showed the inverse response at the same time-point in response to *S. uberis* infection (Moschos, Williams et al. 2007, Hsieh, Rau et al. 2012). This would suggest that the miRNA response to Gram-positive bacteria may be markedly different to Gram-negative.

3.4.7 Predicted targets of down-regulated miRNAs are enriched for genes with a role in innate immunity

Target genes that are potentially regulated by differentially expressed miRNAs in response to *S. uberis* infection at 2, 4 and 6hpi were predicted using two computational approaches, miRanda (Enright, John et al. 2003, Betel, Wilson et al. 2008) and TargetScan (Lewis, Burge et al. 2005, Grimson, Farh et al. 2007, Friedman, Farh et al. 2009). Given the high false positive rates for miRNA target prediction, those potential target genes that were predicted by both methods were identified (Table 3.3). Target genes that were not corroborated by one of the two methods were discarded. In total 1,412 unique genes were predicted to be targeted by differentially expressed miRNAs (Appendix 10). This resulted in 1412 miRNA-target interactions; 64 of these were targeted by down-regulated miRNAs; 1,353 were targeted by up-regulated miRNAs. Because of the difficulties in accurately predicting miRNA targets, it is more appropriate to examine whether broad functional categories of genes are statistically over-represented among predicted target genes, rather than focusing on individual gene predictions. Statistical analysis (Hypergeometric test) revealed that the predicted target genes of down-regulated miRNAs at 6 hpi were significantly enriched ($P = 0.1$) in genes annotated by www.innatedb.com (Lynn, Winsor et al. 2008) as having a role in innate immunity (Table 3.7). The predicted target genes of up-regulated miRNAs were not enriched for the same innate immune pathways suggesting that up and down-regulated miRNAs target different processes in response to *S. uberis* infection.

Pathway analysis of the predicted gene targets of up-regulated miRNAs (at 4 and 6 (not significant) hpi), revealed that pathways which have been previously implicated

in mastitis are statistically enriched among the predicted gene targets of up-regulated miRNAs (Table 3.7). These pathways include MAPK signalling and Cytokine-Cytokine Receptor Signalling. The MAPK signalling pathway, for instance, has been identified as one of the top canonical pathways highlighted in a microarray study examining the bovine mammary tissue response to mastitis, 20 hpi with *S. uberis* (Moyes, Drackley et al. 2009). The majority of the pathways that were identified as statistically enriched among the predicted gene targets of up-regulated miRNAs were also highlighted as significant in this previous microarray study. Taken together, these analyses strongly suggest that miRNAs that are differentially expressed during infection of BMEs with *S. uberis* are key regulators of the host response to this pathogen.

Table 3.7 miRNA target predictions by miRanda and TargetScan and their intersect.

Name	MiRanda Targets (default)	TargetScan = (PCT>0.9)	TargetScan = (Quantile>0.9)	Number of Intersecting Targets
bta-let-7b	6377	576	58	311
bta-let-7d	5637	576	53	290
bta-let-7e	5447	576	60	280
bta-mir-185	5696	0	338	177
bta-mir-200c	3362	74	201	123
bta-mir-708	6414	0	179	84
bta-mir-210	4012	0	206	83
bta-mir-130a	2630	87	76	63
bta-mir-100	946	0	13	1
bta-mir-652	0	0	0	0

Table 3.8 Pathway analysis of the predicted target genes of up-regulated miRNAs 4 and 6 hours post-infection and down regulated miRNAs 6 hours post infection. FDR = False discovery Rate (corrected P-value < 0.1), hpi = hours post infection.

KEGG Pathway	FDR (up) 4 hpi	FDR (up) 6 hpi	FDR (down) 6 hpi
MAPK signaling pathway	0.050226133	ns	ns
Cytokine-cytokine receptor interaction	0.090165729	ns	ns
Calcium signaling pathway	0.098070917	ns	ns
Lysosome	ns	ns	0.086343134
Cell adhesion molecules (CAMs)	ns	ns	0.092614978
Bacterial invasion of epithelial cells	ns	ns	0.099443157

3.4.8 MicroRNA isomiRs

MicroRNA isomiRs are heterogeneous variants of canonical miRNA species, which are, increasingly, being suggested to be of functional importance (Cloonan, Wani et al. 2011). It has been suggested that these miRNA variants can be cell type specific, have functional differences, and vary in their response to biological stimuli (Neilsen, Goodall et al. 2012). Evidence suggests that although isomiRs show similar expression patterns to their equivalent canonical miRNA, their targets can vary (Peng, Cheng et al. 2012). Deletions at both the 5' and 3' end of isomiRs may change the specificity of the seed binding region effecting miRNA function.

It was found that the expression of isomiRs was common for the majority of BME expressed miRNAs (Table 3.8). 100 known miRNAs were found to have at least one isomiR expressed at a level of >100 reads, and more than 1,000 different isomiRs were identified. Notably, in 40% of cases at least one isomiR was more highly expressed than the miRbase consensus sequence, suggesting that the isomiR should in fact be annotated as the consensus. On further examination, it was found that isomiR 'nibbling' was 1.4 more times likely than post-transcriptional additions and isomiR editing was 2.3 times more likely to be 3' modified than 5' modified, agreeing with current literature (Neilsen, Goodall et al. 2012). That said, almost 40% of isomiRs were 5' modified, potentially impacting on which targets they regulate, though the majority of 5' modified isomiRs were expressed at low levels. Further work is required to determine whether isomiRs have a functional role in response to infection.

3.4.9 Novel miRNA Discovery

In addition to profiling the expression of known miRNAs, miRNAseq data can also be used to identify the expression of potentially novel miRNAs. To do this, miRNAseq data from this study was analysed using the software package, miRDeep2 (Mackowiak 2011). Twenty-one high confidence, putatively novel, bovine miRNAs that were found to be independently predicted in multiple BME miRNAseq datasets (Table 3.10). Homology searching of the miRBase database (v 19) (Kozomara and Griffiths-Jones 2011) using BLAST (Altschul, Gish et al. 1990) identified that 2 of the novel miRNAs had 100% identity to known miRNAs in other species, ssc-miR-664-3p (pig) and hsa-miR-219-1 (human). Additionally 5 of the novel bovine miRNAs had significant homology with the bta-mir-2285 family. The bta-mir-2285 family has over 40 members spanning the entire bovine genome, (Guduric-Fuchs, O'Connor et al. 2012). Two additional novel miRNAs showed homology to the bta-mir-2284 family. The remaining miRNAs did not show significant homology to other known miRNAs in other species. However, given the very high read counts observed for several of these predicted miRNAs, and the fact that they were independently predicted in multiple different samples would suggest that many of these predictions represent true novel bovine miRNAs

Table 3.9 Analysis of isomiR heterogeneity across 24 miRNAseq samples. Only isomiRs present at > 100 are shown

Sample	miRs with isomiRs*	isomiRs	longer than consensus	Shorter than consensus	5' modified	3' modified	IsomiR > than consensus
1 hour control (r2)	108	1159	354	520	437	948	42
1 hour control (r3)	95	818	242	372	260	694	36
1 hour infected (r1)	103	1148	355	514	442	936	39
1 hour infected (r2)	94	810	233	378	269	682	34
1 hour infected (r3)	52	341	111	167	97	309	21
2 hour control (r1)	104	1114	341	515	426	922	41
2 hour control (r2)	94	999	307	460	390	828	40
2 hour infected (r1)	98	1075	321	513	437	881	37
2 hour infected (r2)	91	870	259	418	346	713	40
2 hour infected (r3)	91	829	249	366	297	676	37
4 hour control (r1)	114	1165	362	526	478	945	41
4 hour control (r2)	101	1086	310	482	411	879	39
4 hour control (r3)	141	1629	572	651	549	1341	59
4 hour infected (r1)	98	1048	314	446	343	867	37
4 hour infected (r2)	100	1008	313	467	410	824	32
4 hour infected (r3)	91	899	269	406	338	738	43
6 hour control (r1)	96	1056	321	495	419	881	40
6 hour control (r2)	97	1002	307	459	349	851	39
6 hour control (r3)	102	1062	330	504	441	881	38
6 hour infected (r1)	120	1245	448	533	397	1070	51
6 hour infected (r2)	119	1165	408	510	371	998	52
6 hour infected (r3)	137	1796	633	723	597	1500	63

Table 3.10 Putative novel bovine miRNAs discovered through miRDeep2 analysis of miRNAseq data from 24 bovine BMEs.

Name	Mature Sequence Best miRBase BLAST Hit (e-value < 1)	Samples miRNA is Predicted	Mature Tag Count	Predicted Mature Sequence
bta-mir-6537	N/A	7	272,924	Gugggacgcgugcguuuu
bta-mir-6538	N/A	22	22,094	auagccaguuggggaagaaugc
bta-mir-6539	N/A	20	9,687	acgcaauucuucaaaaucuuagc
bta-mir-6540	N/A	16	2,840	aaaaacuggcagcuucauguaa
bta-mir-2285i-1	bta-miR-2285i	13	2,241	aaaacuggaacgaacuuuugggc
bta-mir-2285f-3	bta-miR-2285f	18	2,202	aaaaccugaaugaacuucuugg
bta-mir-2284z-8	bta-miR-2284z	15	2,013	uaaaaguuugguuggguuuuu
bta-mir-664b	ssc-miR-664-3p	20	1,736	uauucauuuaucucccagccuac
bta-mir-6541	N/A	8	1,501	uggagcggcugcacagagcgu
bta-mir-2285c-1	bta-miR-2285c	14	694	aaaaccugaagagacuuuuugg
bta-mir-6542	N/A	13	693	ugcuaccuagucugagugaguga
bta-mir-6544	N/A	18	332	uggugcucccuggagcugagc
bta-mir-6516	gga-miR-6516-5p	7	242	uuugcaguaacaggugugaac
bta-mir-219-1	hsa-miR-219-1-3p	3	173	agaguugagucuggacgucccg
bta-mir-6545	N/A	3	121	auggacugucaccugaggagc
bta-mir-2285m-6	bta-miR-2285m	2	107	aaaacccaaaugaacuuuuugg
bta-mir-2284b-1	bta-miR-2284b	5	86	aaauguucgcuuggcuuuuucc
bta-mir-2285f-4	bta-miR-2285f	2	64	agaaaguucauuuagguuuuuc
bta-mir-6546	N/A	4	52	cuuccucuuccgguuggcaga
bta-mir-6547	N/A	3	29	auucuccauuggauauaauagu
bta-mir-6643	gga-miR-6643-5p	2	21	cagggagggcaggggaggg

3.5 Discussion

In this study, a next generation sequencing approach was used to profile the expression of bovine miRNAs at multiple time-points in primary bovine mammary epithelial cells (BMEs) infected *in vitro* with *S. uberis*, a causative agent of bovine mastitis. In comparison to previous NGS studies investigating the host miRNA response to infection, un-pooled miRNA libraries were sequenced to a previously unprecedented sequencing depth from multiple replicates and controls across multiple time-points. This has allowed us to explore statistically significant temporal changes in miRNA expression in response to infection. Analysing over 450 million sequencing reads, we found that approximately 20% of known bovine miRNAs are expressed in BMEs. A similar diversity of miRNA expression has also been recently reported in other tissues, including bovine retinal microvascular endothelial cells (RMECs) (Guduric-Fuchs, O'Connor et al. 2012) and in testicular and ovarian tissues (Huang, Ju et al. 2011). As has also been reported in other studies, there is a significant dynamic range in the expression of known miRNAs in BMEs. A few miRNAs are expressed at very high levels, with the majority being expressed at low levels. The top 10 most highly expressed miRNAs account for >80% of all aligned reads and are highly conserved across species. Whether or not the other more lowly expressed miRNAs play a significant biological role remains an open question.

It was also found that the expression of isomiRs was common for many of the BME expressed miRNAs. Most significantly, in 40% of cases, at least one isomiR was more highly expressed than the miRbase consensus sequence, indicating that studies such as ours can also be used to improve miRNA annotation. In particular, changes to the 5'

of the consensus sequence could lead to dramatically different target genes being computationally predicted. A large number of 5' isomiRs were identified although they were mostly expressed at low levels.

In addition to profiling known miRNAs, sequencing data was also analysed to identify potentially novel bovine miRNAs. Twenty-one high-confidence, putatively novel, bovine miRNAs were identified independently across multiple different samples. The mature sequences of two of the novel miRNAs were 100% identical to known miRNAs in other species. Seven of the other predicted miRNAs exhibited significant homology to two bovine miRNA families, bta-mir-2284 and bta-mir-2285.

Few studies have previously investigated temporal changes in global miRNA expression using an NGS approach (Cui, Guo et al. 2010), although several have used microarray technology (Wang, Weber et al. 2008, Li, Branham et al. 2010, Mukhopadhyay, Brock et al. 2010, Reinsbach, Nazarov et al. 2012). Thus far, many immune-relevant miRNAs have been identified as part of the host response to LPS stimulation (O'Neill, Sheedy et al. 2011, Qi, Qiao et al. 2012). A literature survey identified more than 145 miRNAs across multiple different species and tissues that have been shown to be LPS responsive. In this study, the miRNA response to the Gram-positive *S. uberis* was markedly different to the reported LPS miRNA response. Over the 6 hour time-course, we identified 10 known bovine miRNAs as significantly differentially expressed in response to the *S. uberis* challenge. Only 6 of these miRNAs have also been reported to be differentially expressed in response to LPS. For those in common, an inverse pattern of expression was observed in 2 of the 6 cases suggesting that the miRNA response to Gram-positive bacteria may be

markedly different to Gram-negative. Further global studies of the miRNA response to Gram-positive and Gram-negative bacteria in the same tissue at the same time-points will be required to confirm this.

It is notable that we found most miRNAs were differentially expressed at different time-points post-infection, suggesting that miRNAs exhibit rapid dynamics in their temporal expression. It is also notable that the majority of miRNAs that we report as being differentially expressed exhibit relatively subtle changes in gene expression in response to infection. This subtle change in expression is in line with existing literature and strengthens the hypothesis that miRNAs are fine-tuners of gene expression (Bartel 2009, O'Neill, Sheedy et al. 2011). For example, miR-let-7d and miR-652 demonstrated similar levels of differential expression 6 hours post LTA stimulation in mouse tissues (Hsieh, Rau et al. 2012).

Computational analysis revealed that the predicted target genes of *S. uberis* down-regulated miRNAs were statistically enriched for roles in innate immunity. This would suggest that these miRNAs may significantly regulate the sentinel capacity of mammary epithelial cells to mobilise the innate immune system (Swamy, Jamora et al. 2010). Pathway analysis of the predicted targets of up-regulated miRNAs has also identified the statistical over-representation of several pathways previously implicated in the host response to mastitis, such as the MAPK, and cytokine signalling pathways. Furthermore, several of the differentially expressed miRNAs have been shown to have roles in the immune systems of other species. For example, bta-let-7 miRNAs were up-regulated at both 4 and 6 hours post-infection with *S. uberis*. The let-7 family has been extensively described in the literature for having a role in immunity. The

down-regulation of let-7 family members, for example, was shown to promote expression of IL10 and IL6 in HeLa cells infected with Salmonella enterica serovar Typhimurium (Schulte, Eulalio et al. 2011). The observed up-regulation of let-7 miRNAs in our study may lead to the repression of anti-inflammatory cytokines to promote innate immunity.

In addition, the down-regulation of miRNAs bta-miR-130a, has a known role in immunity and infection in other species. LPS induced TNF-α expression in neonatal and adult monocytes has been shown to be greatly suppressed by the induction of miR-130a (Huang, Yu et al. 2012). Taken together, the evidence strongly suggests that the differentially expressed miRNAs identified in this study are likely central regulators of the innate immune response to *S. uberis* and thus represent potential therapeutic targets or novel biomarkers of infection and inflammation.

Chapter 4: MicroRNAs are amplifiers of bovine monocyte inflammatory networks and repressors of metabolism.

4.1 Introduction

Mastitis develops as bacteria entering the udder via the teat canal stimulate a pathological form of inflammation. Bacteria encounter epithelial cells lining the mammary gland stimulating a local inflammatory response which facilitates their transport across the epithelial barrier where they are detected by resident immune cells, such as monocytes. Both cell types constitutively express surface pathogen recognition molecules such as Toll-like Receptors (TLRs) enabling them to function in a sentinel capacity. Invasive *S. uberis* triggers TLR2 and 4 mobilising local and systemic inflammatory mediators (Bannerman, Paape et al. 2004, Moyes, Drackley et al. 2009). Typically, chemokines, interleukins (ILs) and tumour necrosis factor α (TNF) initiate local physiological changes in vascular permeability, cell differentiation, and apoptosis. Concurrently, systemic innate immune changes provoke acute phase protein (APP) production which is distributed systemically to suppress the spread of bacteria locally (Mitterhuemer, Petzl et al. 2010). During this phase, immune cells are recruited to the point of infection (Rinaldi, Li et al. 2010). Monocytes are released from the bone marrow into the circulatory system and eventually reach the mammary gland via chemokine ligand-mediated cell migration. There they differentiate into macrophage and dendritic cell populations (Shi and Pamer 2011, Dong, Zhao et al. 2013). Neutrophils make up the majority of immune cells in an infected gland during an infection. Neutrophils are tasked with directly clearing invasive bacteria via phagocytosis or neutrophil extracellular traps (NETS) and subsequently aid in resolution of inflammation (Lippolis, Reinhardt et al. 2006, Reinhardt, Sacco et al. 2013). Once recruited to the site of infection, monocytes and neutrophils orchestrate antimicrobial activity to control bacterial spread and resolve the infection (Serbina, Jia et al. 2008, Dong, Zhao et al. 2013). Immune cells and

other somatic cells can be detected in the milk of infected animals and the counts of the number of such somatic cells per mL, called the somatic cell count, is an indicator of mastitis (Jones and Bailey 2009).

The local immune response in mammary tissues has been examined by several approaches both *in vivo* and *in vitro*. Candidate gene based approaches and microarray technology have determined that over 2,000 genes spanning immunity, metabolism, & tissue remodelling are active during mastitis (Moyes, Drackley et al. 2009, Swanson, Stelwagen et al. 2009, Mitterhuemer, Petzl et al. 2010). Modest data are, however, available examining transcriptional activity in either milk or blood monocytes from infected animals (Prgomet, Sarikaya et al. 2005) and little is known regarding the role microRNAs play in regulating these responses.

MicroRNAs (miRNAs) are small, non-coding RNAs, which play a key role in the regulation of innate and adaptive immunity as post-transcriptional regulators of gene expression (O'Connell, Rao et al. 2010). They have been shown to regulate immune function in several cell types. Neutrophil senescence, for example, is regulated by a discrete miRNA repertoire (Ward, Heath et al. 2011). Naïve mouse B cells are indirectly regulated by miR-155 via histone deacetylase 4 repression, while naive CD4+ T cell differentiation and function is regulated by global changes in miRNAs (Sandhu, Volinia et al. 2012, Bronevetsky, Villarino et al. 2013). In a recent study, we concluded that miRNAs likely play a key role in regulating the innate immune response in mammary epithelial cells to a bovine mastitis pathogen *in vitro* (chapter 3).

Although miRNA expression is abundant in numerous bovine tissues, genome-wide studies elucidating the regulatory roles of miRNAs in bovine immunity are limited (Coutinho, Matukumalli et al. 2007, Jin, Grant et al. 2009, Xu, Zhang et al. 2009). Furthermore, no bovine studies to date have applied next generation sequencing (NGS) to examine global miRNA expression in immunity and infection *in vivo*. In this study, an NGS approach is reported, to profile the expression of bovine miRNAs & mRNAs at multiple time-points in milk and blood isolated CD14+ monocyte cells isolated from Holstein Friesians infected *in vivo* with *Streptococcus uberis* a causative agent of bovine mastitis.

4.2 Hypothesis

The hypothesis of this thesis is that microRNAs (miRNAs), short non-coding RNAs which post-transcriptionally regulate gene expression, are central regulators of the transcriptional networks induced in the host response to a bovine mastitis pathogen, *Streptococcus uberis*. This hypothesis will be addressed in this thesis via two major objectives.

4.3 Specific aims

1. Identify the miRNAs and the transcriptional networks that are differentially expressed over a time-course in blood and milk isolated CD14+ monocytes in an *in vivo S. uberis* challenge study.
2. Confirm the observations made in chapter 3, that miRNAs carry out a regulatory role in bovine immunity.

4.4 Results

4.4.1 The kinetics of *S. uberis* infection *in vivo*

To investigate the host monocyte transcriptional and post-transcriptional response to a mastitis-causing pathogen *in vivo*, five Holstein Friesian animals were infected via the teat canal with approximately 500 CFU of *Streptococcus uberis* 0140, in 10 mL of saline. Five control animals were inoculated with saline only. Blood and milk samples were taken at 0, 12, 24, 36 and 48 hours post infection (hpi) and CD14+ monocytes were isolated by FACS (Figure 4.1). The infection was monitored using recorded milk bacterial counts (CFU/mL) and somatic cell counts (per mL) at each of the five time points for each animal (control & infected). On average, bacterial counts peaked in the infected animals at 24 hpi, whereas no change was observed in the uninfected controls. There was, however, significant heterogeneity among the CFU data for each infected animal, in terms of the magnitude and the timing of the response (Figure 4.2A and (Appendix 11). One infected animal (TI3 - which had the highest CFU/mL data) peaked at 24 hpi, two others (TI1 and TI4) peaked at 36 hpi and CFU data for one animal was still climbing at 48 hpi (TI5). Additionally, one infected animal was observed to have only a very modest increase in bacterial counts (TI2). Somatic cell count (SCC) data also confirmed the presence of the infection in the challenged animals and not in the controls. SCC was observed to increase at 24 hpi and by 36 hpi was, on average, >900,000 cells/mL in infected animals. In comparison, the average SCC in control animals at 36 hpi was <52,000 cells/mL (Figure 4.2B). An SCC reading >200,000 cells/mL is generally considered diagnostic of mastitis (Dufour and Dohoo 2013). Again there was heterogeneity in the SCC response in the infected animals. Interestingly, the infected animal that was observed

to have only a modest increase in CFU/mL (TI2) had a relatively robust SCC response.

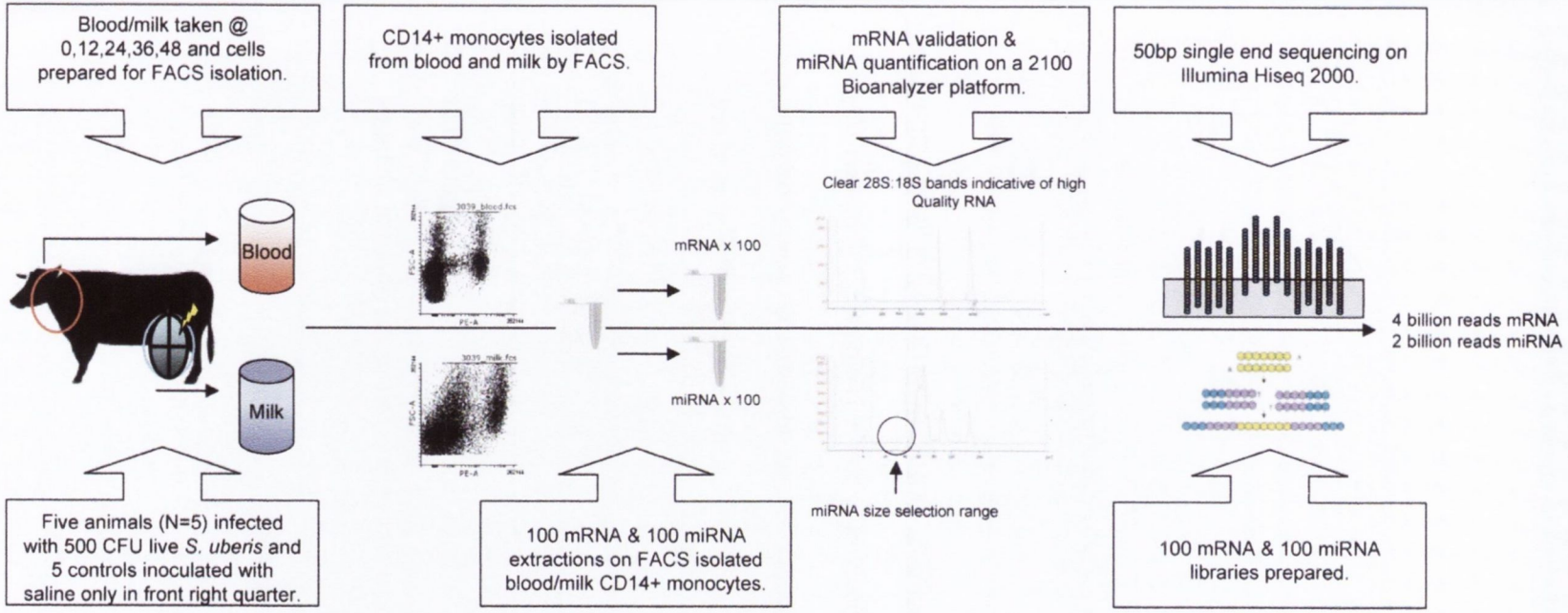


Figure 4.1 Overview of the experimental design. Five Holstein Friesian animals were infected via the teat canal of the right front quarter with approximately 500 CFU of a mastitis-causing pathogen, *Streptococcus uberis* 0140, in 10 mL of saline. Five control animals were inoculated with saline only. Milk and blood samples were obtained from each animal at 0, 12, 24, 36 and 48 hours post-infection (or mock infection). Milk and blood derived CD14+ monocytes were isolated by Fluorescence-activated cell sorting (FACS) from each sample. mRNA and miRNA were extracted and 200 Illumina-compatible libraries were prepared for sequencing on a HiSeq 2000 machine. More than 4 billion mRNA reads and > 2 billion miRNA reads were sequenced in total

4.4.2 Isolation of CD14+ monocytes by flow cytometry

The purity of milk and blood FACS-isolated CD14+ monocyte populations was estimated to be >95% FACS by the NADC FACs core facility. To assess this, tag counts were generated for six surface expression markers (Table 4.1, Appendix 1.2) in blood derived CD14+ monocyte and six surface expression markers (Table 4.1, Appendix 1.2) in milk derived CD14+ monocyte across all respective samples. We found that the average tag counts for all samples across each surface expression marker were within the expected range (<= 5%), however, a number of individual samples had an elevated level of contamination with other cell-types. Blood "CD14+ monocytes" were contaminated with B-cells and to a lesser extent T-cells at a low level across all samples. B-cell contamination was examined with a CD22 marker (B-lymphocyte cell adhesion molecule) (Appendix 1.2 E), and was elevated randomly across thirteen blood CD14+ monocyte samples. T-cell contamination was more restricted, with elevated peaks of T-cell surface receptor glycoprotein 4 (Appendix 1.2 C), and T-cell surface receptor glycoprotein CD3 gamma chain (Appendix 1.2 B) found in control animal 2 at 12 and 36 hours post infection compared to all other blood samples. There were no specific groups of animals at time-points that demonstrated elevated levels of contamination that may affect transcriptional changes contributed to *S. uberis* infection.

Milk CD14+ monocytes had a lower level of contamination overall, however, a number of individual samples were more heavily contaminated with epithelial cells than the 5% expected during FACs isolation. In particular infected animal 1 at 12, 24, 36, and 48 hours post infection demonstrated elevated levels of CD24 (angiotension I

converting enzyme (peptidyl-dipeptidase A) 3) (Appendix 1.2 J). Furthermore both control and infected animal at 36 hpi and 48 hpi were found to have elevated levels of STAT1 (Signal Transducers and Activators of Transcription) (Appendix 1.2 N) in comparison to samples at all other time points, however STAT1 expression is expected in CD14+ monocytes. Also control animal 4 at 36 hpi was found to have highly elevated levels of CK-8 (Keratin type II cytoskeleton 8) (Appendix 1.2 K) in comparison to all other samples.

In summary, this data indicates a low level of blood and milk CD14+ monocytes contamination across all cell populations as might be expected. Contamination of samples clustered in particular groups, may introduce spurious results in down-stream transcriptional and differential expression analysis. However, we have found that while a number of individual samples contained elevated contamination levels of non-monocytic cell types, no particular time-point/sub-group of samples were more heavily contaminated than another (Table 4.1). Representative dot plot flow cytometry profiles for both control and infected animals 1, 2, 3, 4, and 5 milk and blood isolated monocyte CD14+ subset populations show separation of CD14+ monocyte population in A), B), C), D), and E) (Appendix 1.1).

Table 4.1 Surface expression markers examined for non-specific cellular contamination of blood and milk derived CD14+ monocyte populations. Surface expression marker names, surface expression markers identifiers, related cell types, and average tag counts for cell surface expression markers are included.

Cell Type	Marker name	Surface Tag ID	Cell Type	Avg tag count
CD14+ blood monocytes	monocyte differentiation antigen CD14 precursor	CD14	CD14+ monocytes	480
CD14+ blood monocytes	B-lymphocyte cell adhesion molecule	CD22	B-cell	88
CD14+ blood monocytes	B-lymphocyte surface antigen	CD19	B-cell	25
CD14+ blood monocytes	T-cell surface glycoprotein CD4	CD4	T-cell	14
CD14+ blood monocytes	T-cell surface glycoprotein CD3 gamma chain	CD3	T-cell	10
CD14+ blood monocytes	Chemokine type 7 precursor	CCR7	T-cell central memory cell marker	6
CD14+ milk monocytes	monocyte differentiation antigen CD14 precursor	CD14	CD14+ monocytes	4328
CD14+ milk monocytes	Signal Transducers and Activators of Transcription	STAT1	T-helper cell	1245
CD14+ milk monocytes	aminopeptidase B	APB	Epithelial cells	32.8
CD14+ milk monocytes	angiotension I converting enzyme (peptidyl-dipeptidase A) 3 precursor	CD24	Epithelial cells	11.7
CD14+ milk monocytes	Keratin type II cytoskeleton 8	CK-8	Epithelial cells	7.8
CD14+ milk monocytes	T-cell surface glycoprotein CD4	CD4	T-cell	2.9

4.4.3 Profiling mRNA expression in blood and milk isolated CD14+ monocytes

A next generation sequencing approach was applied to monitor the transcriptional (mRNAseq) and post-transcriptional (miRNAseq) changes that occurred in both blood and milk isolated CD14+ monocytes in the infected and control animals (Figure 4.1). Sequencing of 100 mRNA Illumina libraries (i.e. 50 blood and 50 milk monocyte mRNA libraries) yielded >4 billion sequence reads which underwent a pipeline of quality control checks, including FastQC checks which were run on each library to ensure reads were of sufficient quality to proceed with analysis (Appendix 12 and 13). More than 3 billion reads of these mapped uniquely to the Bos taurus UMD 3.1 genome (Appendix 13). The average correlation coefficient of mRNA normalised read counts between samples at each time-point was 0.95 for control samples and 0.92 for infected samples (Appendix 14.1). The variation of aligned reads before and after normalisation from each dataset were examined using expression density plots (Appendix 14.2) Infected animal 2 samples, control animal 2, control animal 4 at 24 hpi infected, infected animal 3, and infected animal 4 at 24 hpi (all milk) were removed from differential expression analysis based on CFU and quality control data.

Variation among milk and blood mRNA expression samples (0, 12, 24, 36 and 48 hours post-infection) was assessed by multidimension scaling (MDS) analysis (Appendix 15.1-15.2, 15.5). In the first dimension in plots D and E control and infected milk mRNA samples clearly separate at 36 h and 48 hours post infection (Appendix 15.1). Hierarchical Clustering of normalised mRNA read counts from MIMs confirmed that the control and infected animals also separated at 36 and 48 hpi (Appendix 15.6) except for the one infected sample (TI2) which had very low

bacterial counts and likely did not developed a full infection (Figure 4.2C-F,). This sample was subsequently excluded from differential gene expression analysis. Hierarchical Clustering of the normalised mRNA read counts of genes that were differentially expressed in blood isolated monocytes (BIMs) revealed that only 3 of the infected animals (TI1, TI3 and TI4) separated from uninfected controls at 36 and 48 hpi (Figure 4.3, Appendix 15.7). These animals also had the highest SCC data at these time-points.

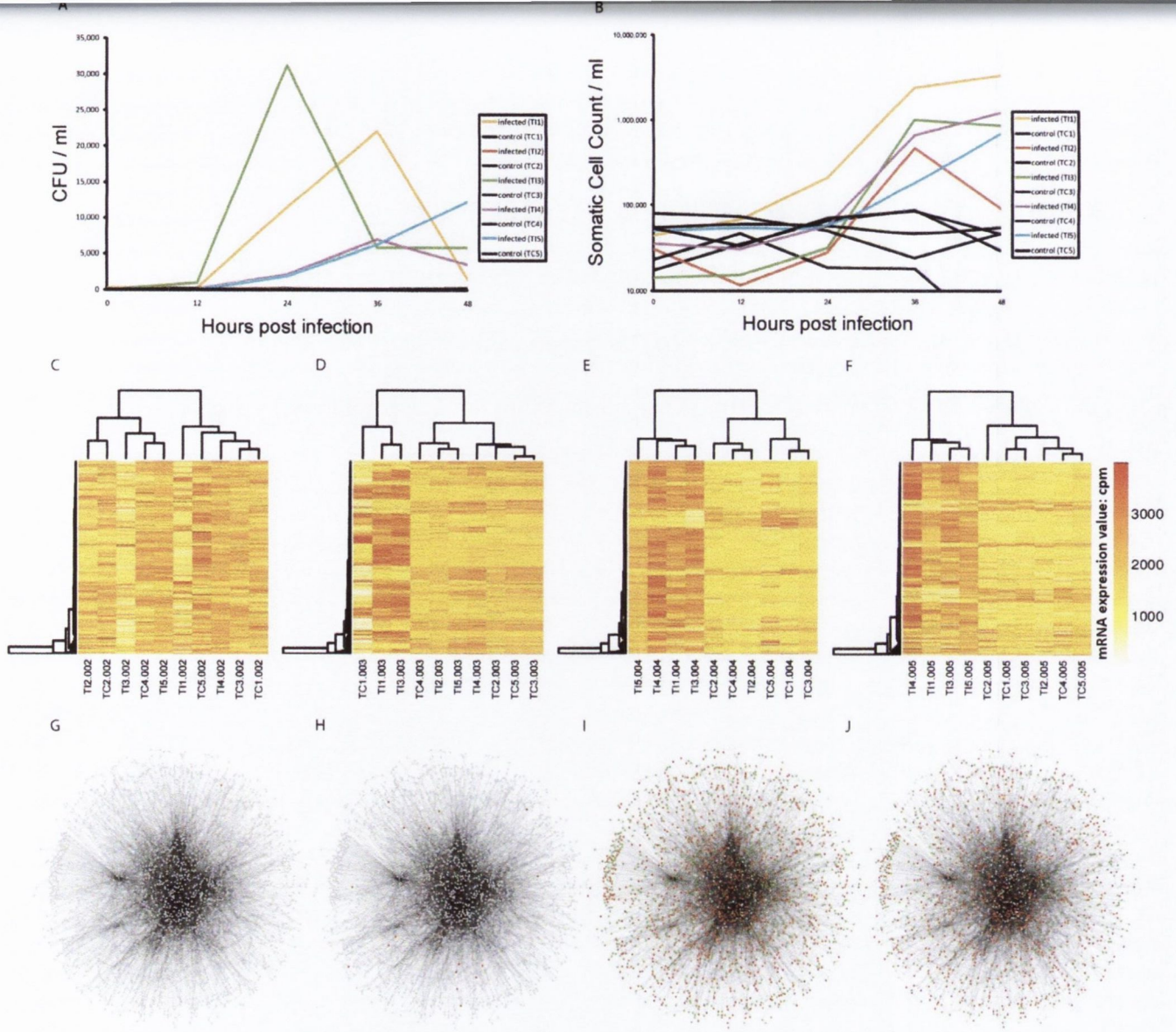
The EdgeR statistical package (Robinson, McCarthy et al. 2010) was utilised to determine which mRNAs were significantly differentially expressed in MIMs and BIMs in response to *S. uberis*. Additionally reference genes were plotted for all samples on normalised data sets as a quality control measure among samples (Appendix 16). Tyrosine 3-Monooxygenase (YWHAZ) (Appendix 16 A), Glyceraldehyde-3-phosphate (GAPDH) (Appendix 16 B), Phosphoglycerate kinase 1 (PGK1) (Appendix 16 C), Succinate dehydrogenase complex, subunit A (SDHA) (Appendix 16 D), and Hypoxanthine-guanine phosphoribosyltransferase (HRPT) (Appendix E), all exhibited a stable expression profile across all milk CD14+ samples.

A number of immunologically significant genes of interest including Chemokine (C-C motif) receptor 1 (CCR1), Interleukin 1, beta (IL1B), Interleukin 6 (IL6), Interleukin 8 (IL8), Toll like receptor 2 (TLR2), Toll like receptor 4 (TLR4), and Tumor necrosis factor alpha (TNFα) were examined for differential expression across milk CD14+ samples. The expression pattern of each of these immune genes was plotted across the

sample over the course of the infection model. As previously mentioned, each animal displayed a unique infection profile when examined over the 48 hour time course, and infections did not peak uniformly between animals, for example bacterial CFU counts steadily increased for infected animal 5 (TI5) up to 48 hpi, while infected animal 3 (TI3) peaked at 24hpi. Based on these observations, DE gene datasets were plotted to reflect the CFU heterogeneity between animals. Infected animal 1 (TI1) show a clear induction of both IL1B and IL6 (Appendix 17 B, G) at 24hpi; Infected animal 4 (TI4) shows a modest induction and infected animal 5 (TI5) and infected animal 2 (TI2) shows none at this time-point. TLR2 and TLR 4 expression (Appendix 17 D, E) in infected animal 1 (TI1) begin steadily climbing at 24 hpi. This matches the CFU data exactly where infected animal 1 had much higher CFUs than the others at 24hpi. By 36hpi the induction of IL1B and IL6 (Appendix 17 B, G) in infected animal 1 (TI1) starts to reduce; but infected animal 4 (TI4) shows maximal induction of IL1B (Appendix 17 B) (matching its CFU data). While TLR2 and TLR4 expression (Appendix 17 D, E) steadily rises in infected animal 1 (TI1), and sharply increases in infected animal 4 (TI4) and infected animal 5 (TI5) in line with CFU data. Infected animal 5 (TI5) shows the highest induction at 48hpi (again matching the CFU data). Infected animal 2 (TI2) did not show any evidence of infection based on the CFU data and this is reflected in the fact that IL6 and IL1B (Appendix 17 B, G) are not induced, while TLR2 and TLR 4 are mildly induced (Appendix 17 D, E).

In MIMs, there were 4, 36, 1774, and 1532 up-regulated genes at 12, 24, 36 and 48 hpi, respectively. The majority (1,254) of genes up-regulated at 48 hpi were also up-regulated at 36 hpi. Additionally, there were 5, 2, 1518 and 995 down-regulated genes in MIMs at those time-points. Of the 995 down-regulated genes at 48 hpi, 80% were

also down-regulated at 36 hpi. Overall, 2056 different genes were up-regulated and 1721 different genes were down-regulated for at least one time-point in MIMs in response to *S. uberis* infection. Traditionally, mastitis has been thought of as a local bacterial infection with a robust inflammatory response, which is largely reflected by the increased number of differentially expressed genes found in milk CD14+ monocytes compared to blood CD14+ monocytes. In BIMs, however, we observed a much more subtle but still quite significant response to *S. uberis* infection. Only ten genes were up-regulated in BIMs at 36 hpi but this increased to 83 genes by 48 hpi. Nine of the ten 36 hpi genes were also up-regulated at 48 hpi. Additionally, 3, 4, 26, and 39 genes were down-regulated in BIMs at 12, 24, 36 and 48 hpi, respectively.



infection was monitored using recorded milk bacterial counts (CFU/mL) and B) somatic cell counts (per mL) at each of the five time points (001 = 0 hpi, 002 = 12 hpi, 003 = 24 hpi, 004 = 36 hpi, 005 = 48 hpi.). for each for each of the five animals (control (TC1-5) & infected (TI1-5). Significant heterogeneity was observed in the CFU data among the infected animals. One infected animal (TI2) was observed to have only a very modest increase in bacterial counts. C-F) Hierarchical clustering of top 500 most variable probes in milk-isolated monocytes (MIMs) at 12, 24, 36 and 48 hpi, respectively, revealed that infected animals separated from control animals in their gene expression response at 36 and 48 hpi except for animal TI2. Heatmap is coded from dark red = $1x10^5$ to white $1x10^2$. G-J) InnateDB network analysis of genes that were differentially expressed at 12, 24, 36 and 48 hpi, respectively. The networks were visualised in Cytoscape.

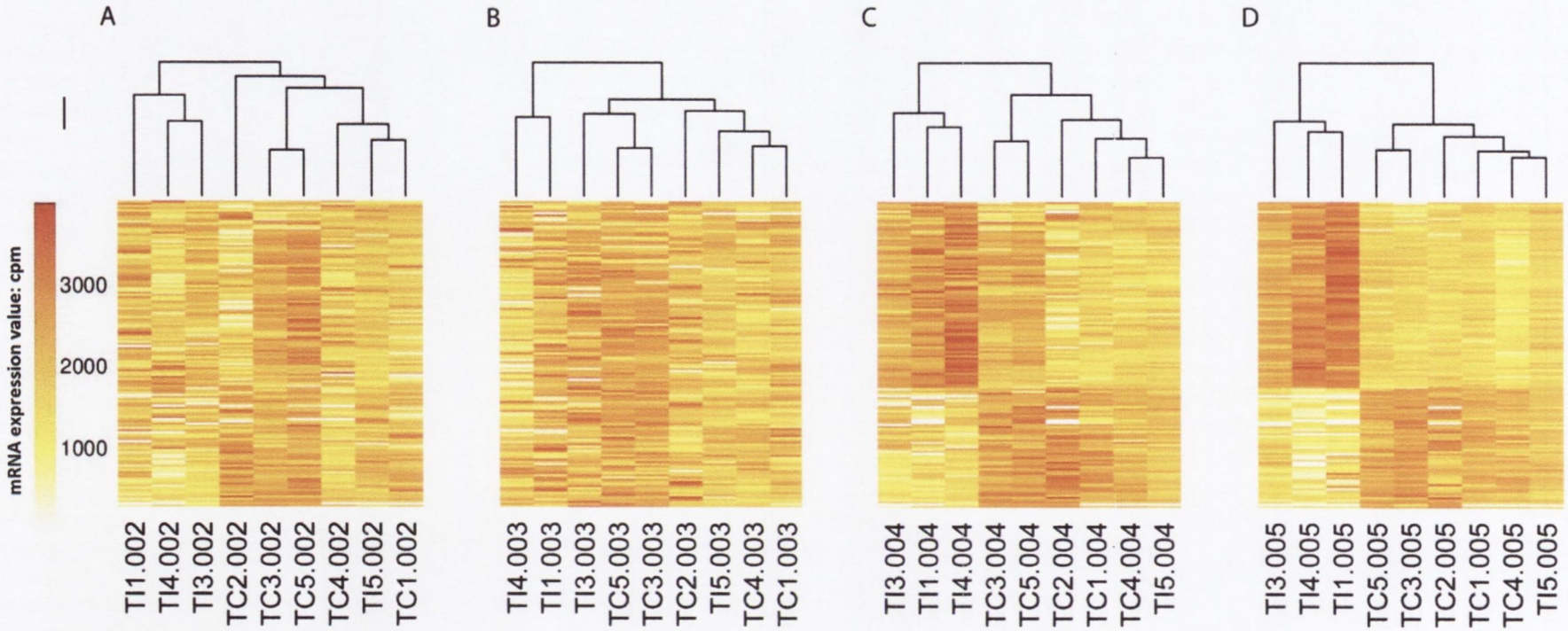
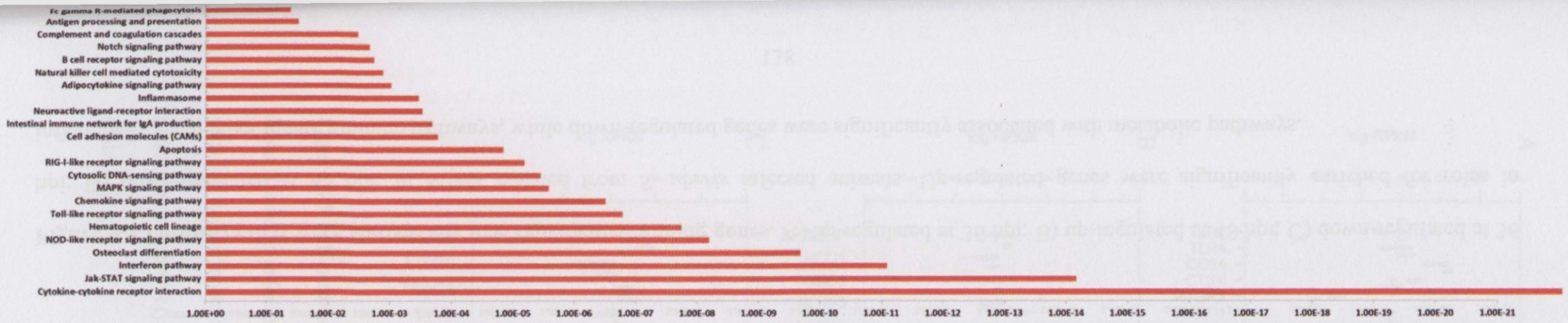


Figure 4.3 Heatmap clustering of all genes differentially expressed (tags per million) in blood isolated monocytes across infected and control animals at (A) 12 hours post infection, (B) 24 hours post infection, (C) 36 hours post infection, & (D) 48 hours post infection. The more red the colour the more highly expressed that gene is, R (V2.15.2) hclust package. Heatmap labelling: Five control animals (TC1-5) & five infected animals (TI1-5) at the following time-points 002 = 12 hpi, 003 = 24 hpi, 004 = 36 hpi, 005 = 48 hpi.).

4.4.4 Pathway analysis reveals the suppression of metabolic pathways and the up-regulation of inflammatory pathways in response to *S. uberis* infection

Pathway analysis of up- and down-regulated genes at each time-point was undertaken using GOseq (Young, Wakefield et al. 2010) with pathway annotation imported from the KEGG database (Kanehisa and Goto 2000) to identify which pathways were statistically overrepresented among DE genes in MIMs and BIMs. Two manually curated pathways (Interferon signalling pathway and the Inflammasome pathway) were also included (see Methods). No significant pathways were identified among either the BIM or MIM DE genes at 12 or 24 hpi. At 36 and 48 hpi, however, more than 20 different pathways were identified as being statistically overrepresented (Figure 4.4-4.5 and Table 4.2). In MIMs, down-regulated genes were predominantly associated with metabolic pathways (Figure 4.4), such as fatty acid and amino acid metabolism, the citric acid (TCA) cycle and glutathione metabolism, as well as DNA replication and repair pathways and the cell cycle. Down-regulated pathways were largely similar between 36 hpi and 48 hpi, though fewer pathways were significant at 48 hpi. Up-regulated genes, on the other hand, were primarily associated with well-known pattern recognition receptor (PRR) pathways (Figure 4.4) including the Toll-like receptor pathway (e.g. TLR2, TLR4, CD14, MYD88, TIRAP, and IRAK1 all up-regulated), the NOD-like receptor pathway (e.g. NOD1, NOD2, NLRP3 (NALP3), NLRC4 (IPAF), NAIP (NAIP5) up-regulated) and the RIG-I-like receptor pathway (e.g. DDX58 (RIG-I), IFIH1 (MDA5), CYLD, DHX58 (LGP2), DDX3X, TRIM25); interferon signalling and cytokine and chemokine signalling pathways. Up-regulated inflammatory cytokine and chemokine genes included the genes encoding TNF, IL1A, IL1B, IL6, IL8, IL12A and IL12B, IL17B and IL17C, IL18, IL23A, IL27, CCL3 (MIP1α) CCL4 (MIP1β), CCL5 (RANTES), CCL8 (MCP-

2) and CCL20 (MIP3A). The genes encoding TNF, IL1B, IL6, IL12 and CCL20 were more than 10 fold up-regulated at 36 hpi. All up-regulated pathways that were significant at 36 hpi were still significant at 48hpi, with 5 additional up-regulated pathways being significant only at 48 hpi. These pathways were primarily related to leukocyte migration and phagocytosis. In BIMs, only two pathways were statistically over-represented among 48 hpi up-regulated genes - Interferon signalling and Cytokine-cytokine receptor interaction. No pathways were significant at the other time-points. Among down-regulated genes, there were also few over-represented pathways. Those pathways that were significant were primarily related to the complement and focal adhesion pathways.


Fc gamma R-mediated phagocytosis
Antigen processing and presentation
Complement and coagulation cascades
Notch signaling pathway
B cell receptor signaling pathway
Natural killer cell mediated cytotoxicity
Adipocytokine signaling pathway
Inflammasome
Neuroactive ligand-receptor interaction
Intestinal immune network for IgA production
Cell adhesion molecules (CAMs)
Apoptosis
RIG-I-like receptor signaling pathway
Cytosolic DNA-sensing pathway
MAPK signaling pathway
Chemokine signaling pathway
Toll-like receptor signaling pathway
Hematopoietic cell lineage
NOD-like receptor signaling pathway
Osteoclast differentiation
Interferon pathway
Jak-STAT signaling pathway
Cytokine-cytokine receptor interaction
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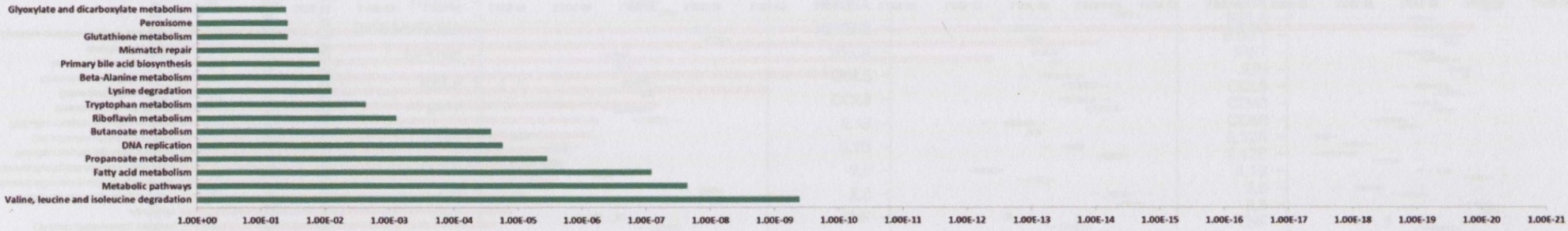
Glyoxylate and dicarboxylate metabolism
Peroxisome
Glutathione metabolism
Mismatch repair
Primary bile acid biosynthesis
Beta-Alanine metabolism
Lysine degradation
Tryptophan metabolism
Riboflavin metabolism
Butanoate metabolism
DNA replication
Propanoate metabolism
Fatty acid metabolism
Metabolic pathways
Valine, leucine and isoleucine degradation
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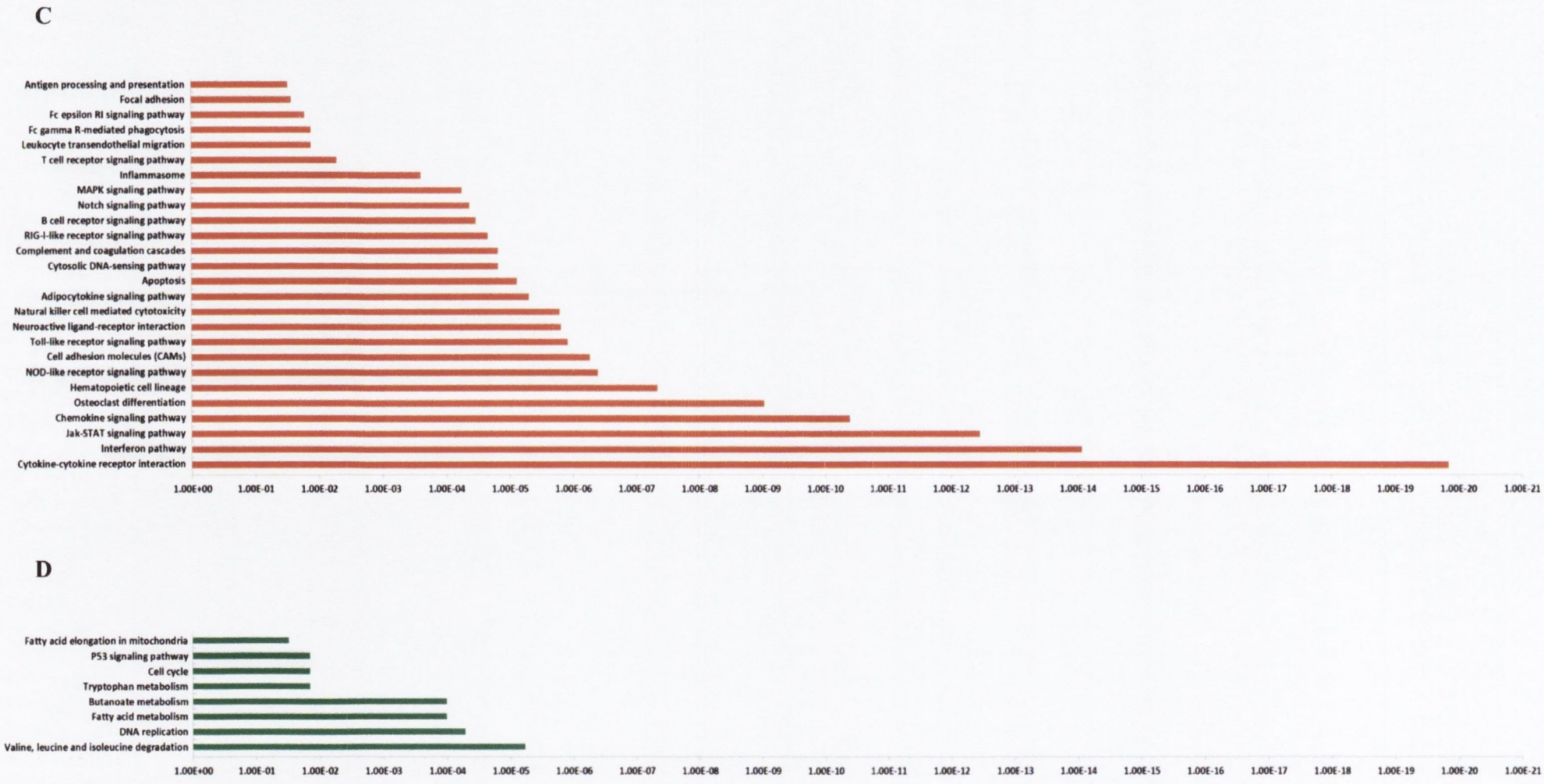


Figure 4.4 Pathways that were statistically overrepresented among genes. A) up-regulated at 36 hpi; B) up-regulated at 48 hpi; C) down-regulated at 36 hpi; D) down-regulated at 48 hpi; in MIMs isolated from *S. uberis* infected animals. Up-regulated genes were significantly enriched for roles in inflammatory and other innate immune pathways, while down-regulated genes were significantly associated with metabolic pathways.

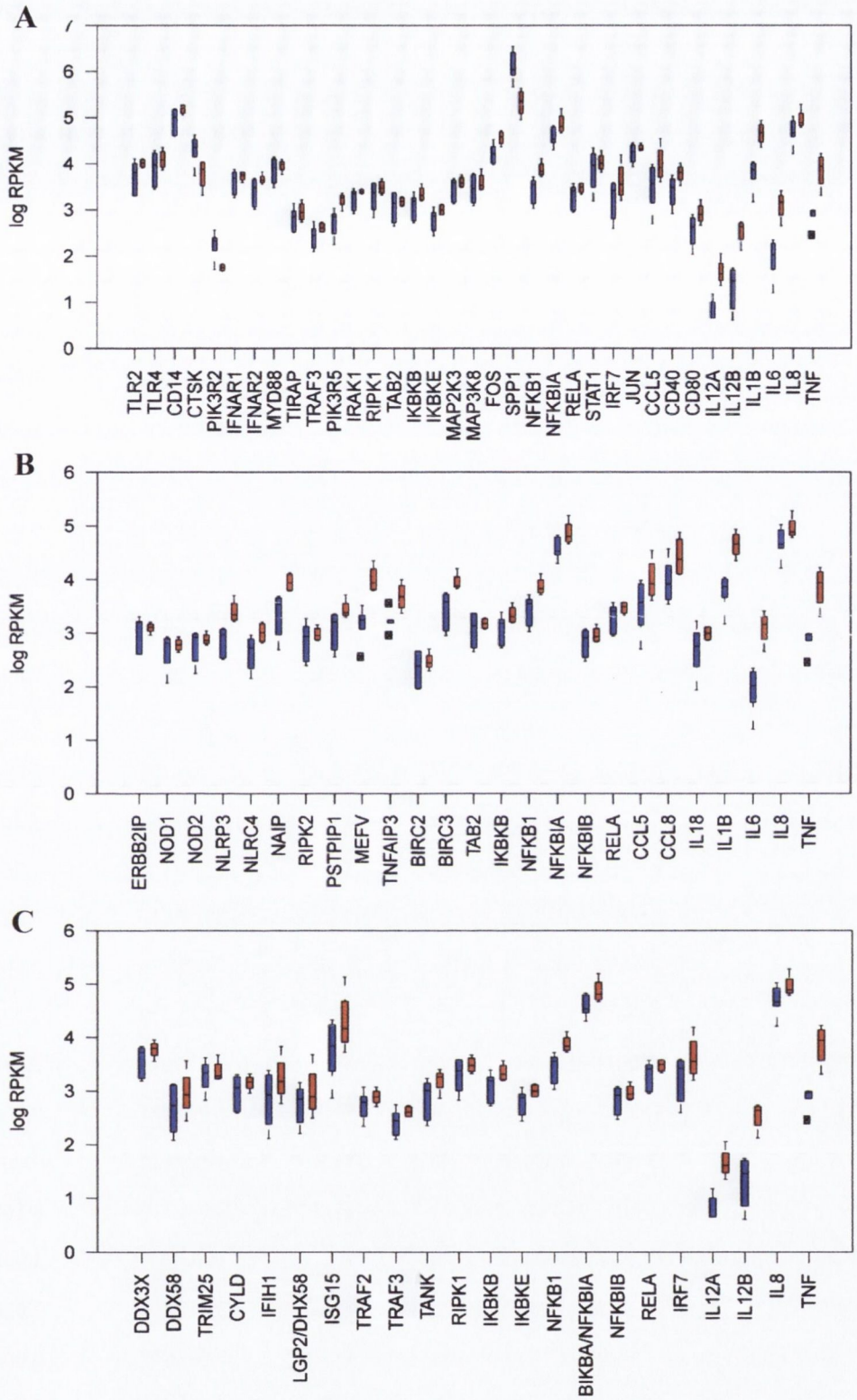


Figure 4.5 Differential gene expression in key innate immune pathways in MIMs from *S. uberis* infected animals. Reads Per Kilobase per Million (RPKM) values for each of the differentially expressed genes in the A) TLR, B) NLR and C) RIG-I pathways, respectively (Red = infected; Blue = Control). All counts converted to Log scale.

Table 4.2 Fold changes, P-values, and false discovery rate (FDR) of differentially expressed gene in TLR, NLR, and RIG-I pathways.

Time	Gene Symbol	Ensemble Bovine ID	Pathway	Fold Change	FDR
36	TLR2	ENSBTAG00000008008	TLR	2.3203	3.84E-07
36	TLR4	ENSBTAG00000006240	TLR	1.964966	0.000434051
36	CD14	ENSBTAG00000015032	TLR	2.3772	1.43E-06
36	CTSK	ENSBTAG00000021035	TLR	-2.21963	0.002331567
36	PIK3R2	ENSBTAG00000002350	TLR	-2.382877	0.00103899
36	IFNAR2	ENSBTAG00000015212	TLR	2.387939	1.22E-07
36	IFNAR1	ENSBTAG00000021819	TLR	1.796349	0.003417826
36	MYD88	ENSBTAG00000000563	TLR	1.669854	0.006881957
36	TIRAP	ENSBTAG00000021504	TLR	2.01164	0.003671467
36	TRAF3	ENSBTAG00000013475	TLR	2.086784	0.00093547
36	PIK3R5	ENSBTAG00000005978	TLR	3.287367	3.48E-17
36	IRAK1	ENSBTAG00000016085	TLR	1.850498	1.86E-05
36	RIPK1	ENSBTAG00000006378	TLR	2.05041	2.82E-05
36	TAB2	ENSBTAG00000013454	TLR	1.626723	0.002465213
36	IKBKB	ENSBTAG00000007599	TLR	2.208827	2.52E-08
36	IKBKE	ENSBTAG00000010408	TLR	2.250811	2.68E-05
36	MAP2K3	ENSBTAG00000010576	TLR	1.922774	0.000757148
36	MAP3K8	ENSBTAG00000011600	TLR	1.824822	0.005118556
36	FOS	ENSBTAG00000004322	TLR	3.043316	8.16E-07
36	SPP1	ENSBTAG00000005260	TLR	-4.638179	3.45E-09
36	NFKB1	ENSBTAG00000020270	TLR	3.812113	1.62E-12
36	BIKBA/NFKBIA	ENSBTAG00000016683	TLR	2.893679	0.000153566
36	RELA	ENSBTAG00000013895	TLR	2.031027	0.000134286
36	STAT1	ENSBTAG00000007867	TLR	1.638258	0.007678097
36	IRF7	ENSBTAG00000047680	TLR	4.026025	4.88E-07
36	JUN	ENSBTAG00000004037	TLR	1.814557	0.009897414
36	CCL5	ENSBTAG00000007191	TLR	4.423537	9.66E-08
36	CD40	ENSBTAG00000020736	TLR	3.161958	4.63E-10

36	IL12A	ENSBTAG00000015150	TLR	16.342053	2.88E-09
36	IL12B	ENSBTAG00000004741	TLR	12.174564	1.10E-19
36	IL1B	ENSBTAG00000001321	TLR	10.842898	7.30E-20
36	IL6	ENSBTAG00000014921	TLR	18.843583	9.70E-25
36	IL8	ENSBTAG00000019716	TLR	2.594864	0.000503626
36	TNF	ENSBTAG00000025471	TLR	17.460404	4.08E-20
36	ERBB2IP	ENSBTAG00000000415	NLR	1.561861	0.025108189
36	NOD1	ENSBTAG00000038235	NLR	1.703746	0.001790162
36	NOD2	ENSBTAG00000020936	NLR	1.787062	0.00039819
36	NLRP3	ENSBTAG00000001273	NLR	3.059285	1.98E-11
36	NLRC4	ENSBTAG00000008223	NLR	2.507742	7.99E-09
36	NAIP	ENSBTAG00000003326	NLR	4.86534	6.09E-17
36	RIPK2	ENSBTAG00000015271	NLR	1.581044	0.002998724
36	PSTPIP1	ENSBTAG00000021144	NLR	3.175166	5.18E-10
36	MEFV	ENSBTAG00000019123	NLR	6.340765	6.22E-14
36	TNFAIP3	ENSBTAG00000000436	NLR	4.370505	2.98E-06
36	BIRC2	ENSBTAG00000035735	NLR	1.723126	0.008541528
36	BIRC3	ENSBTAG00000024918	NLR	3.537959	6.74E-13
36	TAB2	ENSBTAG00000013454	NLR	1.626723	0.002465213
36	DDX3X	ENSBTAG00000035907	RIG-I	2.134491	2.68E-05
36	DDX58	ENSBTAG00000003366	RIG-I	3.012793	0.000149917
36	TRIM25	ENSBTAG00000009948	RIG-I	1.677935	0.021099029
36	CYLD	ENSBTAG00000006291	RIG-I	1.894857	0.000125499
36	IFIH1	ENSBTAG00000008142	RIG-I	3.383578	3.65E-05
36	LGP2/DHX58	ENSBTAG00000046580	RIG-I	3.368594	0.000191613
36	ISG15	ENSBTAG00000014707	RIG-I	6.488277	2.47E-06
36	TRAF2	ENSBTAG00000010497	RIG-I	1.911237	0.000833063
36	TRAF3	ENSBTAG00000013475	RIG-I	2.086784	0.00093547
36	TANK	ENSBTAG00000006654	RIG-I	2.765838	3.00E-09
36	RIPK1	ENSBTAG00000006378	RIG-I	2.05041	2.82E-05
36	IKBKB	ENSBTAG00000007599	RIG-I	2.208827	2.52E-08
36	IKBKE	ENSBTAG00000010408	RIG-I	2.250811	2.68E-05

36	NFKB1	ENSBTAG00000020270	RIG-I	3.812113	1.62E-12
36	BIKBA/NFKBIA	ENSBTAG00000016683	RIG-I	2.893679	0.000153566
36	NFKBIB	ENSBTAG00000001778	RIG-I	1.861495	2.83E-05
36	RELA	ENSBTAG00000013895	RIG-I	2.031027	0.000134286
36	IRF7	ENSBTAG00000047680	RIG-I	4.026025	4.88E-07
36	IL12A	ENSBTAG00000015150	RIG-I	16.342053	2.88E-09
36	IL12B	ENSBTAG00000004741	RIG-I	12.174564	1.10E-19
36	IL8	ENSBTAG00000019716	RIG-I	2.594864	0.000503626
36	TNF	ENSBTAG00000025471	RIG-I	17.460404	4.08E-20

4.4.5 Network analysis of differentially expressed genes

InnateDB was used to generate a network of experimentally-supported molecular interactions that have been annotated to occur directly between the differentially expressed genes and their encoded products. Gene expression data from each of the four post-infection time-points was then overlaid on this network and the network was visualised using Cytoscape 2.8.2 (Shannon, Markiel et al. 2003) (Figure 4.2 G-J). The network consisted of 2,185 nodes (representing differentially expressed genes and their encoded products) and 10,786 edges (representing annotated molecular interactions) between them.

The network was then analysed using cytoHubba (Abd El Naby, Hagos et al. 2011) to identify network hubs and bottlenecks which may represent the key regulatory nodes in the networks. Using the "Degree" algorithm the top 20 hubs (i.e. genes/proteins that are highly connected to other DE genes) in each network were identified (Appendix 17). InnateDB Gene Ontology analysis revealed that the top 20 hubs were highly enriched for roles in innate immunity (FDR $< 1.7e^{-8}$) and transcriptional regulation (FDR $< 1.5e^{-7}$). Indeed, many of the top 20 hubs were well-known transcriptional regulators of innate immunity including JUN, NFKB1, RELA, STAT1, STAT3, EP300, and CREBBP. These transcriptional regulators were located in the most densely connected portion of the network and share many connections (Figure 4.7A). One interpretation of this is that transcriptional regulation by several of these transcription factors is required for differential gene expression in response to the infection.

Bottlenecks are network nodes that are the key connector proteins in a network and have many "shortest paths" going through them, similar to bridges or tunnels on a highway map (Yu, Kim et al. 2007). 17 of the top 20 hubs were also identified as network bottlenecks. Beta-actin (ACTB), the transcriptional regulator, FOS (AP1), and ISG15, were additionally identified in the top 20 bottlenecks. ISG15 has been shown to act as a negative regulator of both the NFκB and RIG-I signalling pathways (Minakawa, Sone et al. 2008, Arnaud, Dabo et al. 2011)

Identifying hubs solely based on their degree can identify nodes that, in general, have been annotated to have lots of interactions. This fails to address whether the number of connections in a particular network of interest is more or less than is expected (given the number of known interactions for that node in the database and the size of the network). To address this issue, we have developed the Contextual Hub Analysis Tool (CHAT) (Wiencko et al., unpublished), a network analysis tool which identifies nodes in a network that are more highly connected to contextually relevant nodes (in this case differentially expressed nodes) than is expected by chance (Appendix 18). Applying this method to the network identified that several of the hub nodes (e.g. KIAA0101), did not have more connections to differentially expressed genes than expected by chance. This means that these genes, although highly connected, are less likely to be functionally important in our network. On the other hand, all of the transcriptional regulators of innate immunity that were identified as hubs in the analysis above (JUN, NFKB1, RELA, STAT1, STAT3, EP300, CREBBP), were also identified by CHAT to be significantly more connected to 36 hpi differentially expressed genes than expected by chance. CHAT also identified several other known innate immunity transcriptional regulators in the top 20 "contextual" hubs, including

REL, IRF1, and IRF9. Indeed, 15 of the top 20 contextual hubs are annotated by InnateDB as having a role in innate immunity (FDR < $1.16E^{-12}$). Although the ranking of the top contextual hubs changed from 36 to 48 hpi, the most significant hubs remained the same.

The network was also analyzed using the jActiveModules plugin (Ideker, Ozier et al. 2002) in Cytoscape 2.8.2 (Shannon, Markiel et al. 2003) to identify high-scoring differentially expressed sub-networks. This type of analysis can aid in the identification of functionally relevant groups of differentially expressed genes that may be acting in concert. A single highly connected component (>10 nodes) was identified consisting of 278 nodes and 1,585 interactions. This module consisted of several of the transcriptional hubs (CREBBP, EGR1, EP300, NFKB1, RELA) identified in the analysis above and their interactors (Figure 4.7B). Pathway analysis of genes in the module revealed that the module was statistically enriched for many of the same pathways that were identified in the analysis of all up-regulated genes including Jak-STAT signalling, the TLR, NLR and RIG-I pathways, apoptosis, and chemokine and cytokine signalling (Table 4.3).

Table 4.3 Significantly over-represented KEGG pathways among genes identified in the jActiveModules module.

Pathway Name	Source	Pathway p-value (corrected)
Jak-STAT signaling pathway	KEGG	1.04827E-15
Pathways in cancer	KEGG	3.90075E-14
Osteoclast differentiation	KEGG	4.23223E-14
Hepatitis C	KEGG	3.3719E-11
Toll-like receptor signaling pathway	KEGG	6.6151E-11
Chemokine signaling pathway	KEGG	7.54021E-11
Small cell lung cancer	KEGG	1.39694E-10
Apoptosis	KEGG	1.58047E-10
Toxoplasmosis	KEGG	2.95289E-09
Chagas disease	KEGG	3.15434E-09
Leishmaniasis	KEGG	5.76039E-09
B cell receptor signaling pathway	KEGG	1.40509E-08
T cell receptor signaling pathway	KEGG	2.20881E-07
Adipocytokine signaling pathway	KEGG	2.32238E-07
Neurotrophin signaling pathway	KEGG	2.7838E-07
Shigellosis	KEGG	5.26551E-07
NOD-like receptor signaling pathway	KEGG	6.18456E-07
Fc gamma R-mediated phagocytosis	KEGG	1.32693E-06
Acute myeloid leukemia	KEGG	1.85282E-06
RIG-I-like receptor signaling pathway	KEGG	2.2777E-06
Cytokine-cytokine receptor interaction	KEGG	3.95084E-06

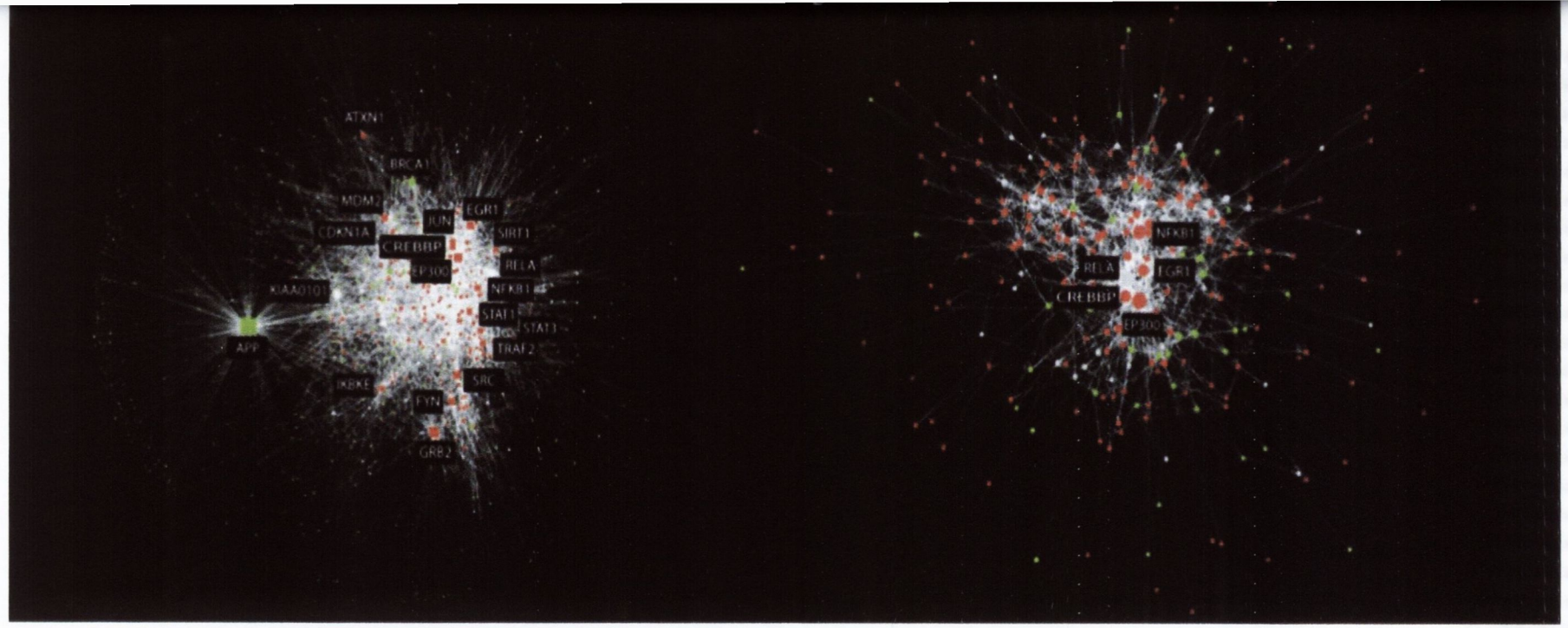


Figure 4.6 Network analyses of differentially expressed genes. InnateDB was used to generate a network of experimentally-supported molecular interactions that have been annotated to occur directly between the differentially expressed genes and their encoded products. A) The top 20 CytoHubba-predicted network hubs and their interactors. Overlain on the network is gene expression data from MIMs at 36 hpi (Red = up-regulated; Green = down-regulated). The top 20 hubs were highly enriched for roles in innate immunity. B) The high-scoring differentially expressed sub-network identified in the larger network using jActiveModules. This module was highly enriched for several innate immune relevant pathways.

4.4.6 Profiling miRNA expression in blood and milk isolated CD14+ monocytes

Sequencing of 100 miRNA Illumina libraries yielded over 1 billion reads for both the MIM and BIM samples. Following a pipeline of quality filtering and adaptor trimming, a total of 312 and 492 million reads from MIMs and BIMs, respectively, mapped uniquely to the Bos taurus UMD 3.1 genome (Appendix 12 and 13). Uniquely aligning reads were then assigned to known mRNAs/miRNAs using HTseq based on miRBase v19 annotation of the bovine genome to generate read counts per mature miRNA in each sample (Hubbard, Aken et al. 2009, Flicek, Amode et al. 2012). On average, 79% of MIM reads and 80% of BIM reads, uniquely mapped to known miRNAs (Figure 4.8). The average Pearson correlation at each time-point for both control and infected sample was > 0.92 among replicates (Appendix 19.1). One blood miRNA sample fell below the cut-off point of 0.85 (control animal 1 at 24 hpi) when correlated against the samples at 24 hpi, a second sample control animal 2 at 12 hpi also fell below 0.85 when correlated against control animal 1 at 12 hpi. No differentially expressed miRNAs were recorded at these time-points. Reads were further examined using expression density plots to examine the variation of aligned reads within samples before and after normalisation (Appendix 19.2). Milk and blood miRNA expression samples (12, 24, 36 and 48 hours post-infection) were assessed by multidimension scaling (MDS) analysis (Appendix 15.3-15.4) and hierarchical clustering (Appendix 15.8). In plots C and D control and infected milk miRNA samples separate at 36 and 48 hours post infection (Appendix 15.3, 15.4, and 15.6 C). Based on quality control and CFU data the following samples were removed from differential expression analysis, infected animal 2 all-time points infected animal 3 at 12h (milk), control animal 2 at 24h (milk), and control animal 4 at 24h (milk). RNAseq profiling revealed that the miRNAome of MIMs and BIMs were broadly

similar and exhibited a range of miRNA expression that has been observed in many other cell-types. 297 and 282 miRNAs were expressed at a threshold of >1 rpm in MIMs and BIMs, respectively. Of these, 136 and 116, respectively, were expressed at a level >100 rpm, a level of expression that has been shown to be associated with functional miRNAs (Mullokandov, Baccarini et al. 2012).

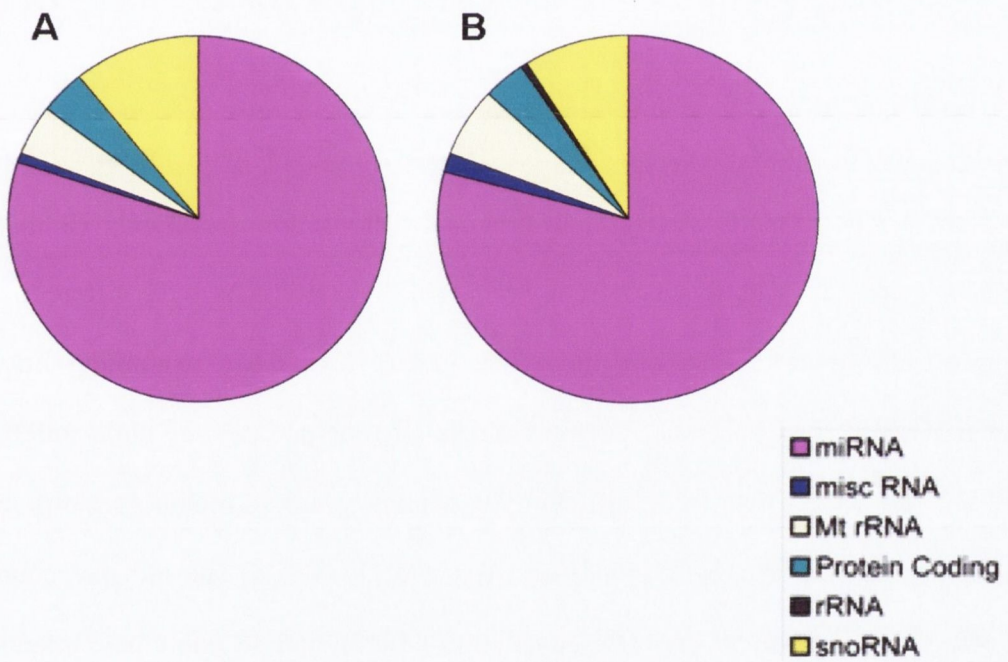


Figure 4.7 The proportion of reads aligning uniquely to bovine ncRNAs. A) Reads aligning to ncRNAs in milk isolated monocytes. B) Reads aligning to ncRNAs in blood isolated monocytes. The majority of reads align to known miRNAs.

4.4.7 Multiple miRNAs are differentially expressed in response to *S. uberis* infection

The EdgeR statistical package was utilised to determine which miRNAs were significantly differentially expressed in response to *S. uberis* infection at 12, 24, 36, and 48 hpi. To address any normalisation issues only those miRNAs that were robust to the normalisation procedure used in Chapter 3 were retained. Again all miRNAs identified by EdgeR (Robinson, McCarthy et al. 2010) as being differentially expressed were also plotted for each blood and milk sample as normalised tag counts. By examining miRNA expression patterns across blood and milk datasets we identified and removed false positive results (Appendix 20). Only miRNAs that had an average expression of >10 rpm across samples were included for further analysis. In MIMs, we identified that 26 unique miRNAs were differentially expressed, when compared against samples plotted at all time-points it was found that only 19 unique miRNAs were differentially expressed. Twelve of these miRNAs were differentially expressed across more than one time-point (Table 4.4), when examining EdgeR results. When compared against samples across all time-points, only eight miRNAs were left that were found to be differentially expressed across multiple time-points. Multidimension scaling (MDS) analysis (Appendix 15.3-15.4) showed that control and infected milk miRNA samples separated at both 36 and 48 hours post infection (Appendix 15.3 and 15.4).

Very few miRNAs were differentially expressed in blood monocytes. We found 3 in total based on EdgeR analyses, 1 at 24 hpi and 3 at 48 hpi (1 was differentially expressed at both time-points) (Table 4.4), however only one miRNA remained (bta-miR-146b), when each of the three were examined across all samples.

Table 4.4 Fold changes and false discovery rates of differentially expressed miRNAs at 12, 24, 36, and 48 hours post-infection in milk and blood isolated monocytes (Appendix 21).

Tissue	Hours post infection (hpi)	miR Name	Fold change	FDR	Considered
MIMs	12	bta-miR-615	16.53	0.003	No
MIMs	12	bta-miR-451	32.73	0.023	No
MIMs	12	bta-miR-486	1.77	0.051	No
MIMs	36	bta-miR-34a	-8.07	0.004	Yes
MIMs	36	bta-miR-200c	-7.12	0.022	No
MIMs	36	bta-miR-200b	-6.59	0.022	No
MIMs	36	bta-miR-182	-6.43	0.035	No
MIMs	36	bta-miR-125a	-5.86	0.001	Yes
MIMs	36	bta-miR-200a	-4.90	0.061	Yes
MIMs	36	bta-let-7e	-4.51	0.047	Yes
MIMs	36	bta-miR-760-3p	-4.38	0.031	Yes
MIMs	36	bta-miR-193a-5p	-4.13	0.099	No
MIMs	36	bta-miR-150	-4.05	0.015	Yes
MIMs	36	bta-miR-210	-4.03	0.000	Yes
MIMs	36	bta-miR-375	-3.88	0.022	Yes
MIMs	36	bta-miR-149-5p	-2.77	0.099	Yes
MIMs	36	bta-miR-30a-5p	-2.48	0.047	Yes
MIMs	36	bta-miR-142-5p	2.21	0.033	Yes
MIMs	36	bta-miR-363	2.23	0.050	Yes
MIMs	36	bta-miR-223	2.44	0.011	Yes
MIMs	36	bta-miR-338	2.51	0.018	Yes
MIMs	36	bta-miR-339a	2.82	0.016	Yes
MIMs	36	bta-miR-2898	2.98	0.004	Yes
MIMs	36	bta-miR-1291	3.07	0.033	Yes
MIMs	36	bta-miR-423-5p	5.73	0.015	Yes
MIMs	36	bta-miR-451	47.12	0.000	No
MIMs	48	bta-let-7e	-6.92	0.056	Yes
MIMs	48	bta-miR-200b	-6.06	0.056	No
MIMs	48	bta-miR-34a	-5.37	0.015	Yes
MIMs	48	bta-miR-149-5p	-4.01	0.056	Yes
MIMs	48	bta-miR-375	-3.76	0.007	Yes
MIMs	48	bta-miR-210	-3.39	0.073	Yes
MIMs	48	bta-miR-125a	-3.29	0.088	Yes
MIMs	48	bta-miR-150	-3.18	0.034	Yes
MIMs	48	bta-miR-99b	-2.9	0.056	Yes
MIMs	48	bta-miR-338	2.81	0.056	Yes
MIMs	48	bta-miR-486	12.79	0.007	No
MIMs	48	bta-miR-451	48	0.000	No
BIMs	24	bta-miR-146b	-23.26	0.024	Yes
BIMs	48	bta-miR-451	9.74	0.067	No
BIMs	48	bta-miR-146b	-3.52	0.067	Yes
BIMs	48	bta-miR-411a	-22.44	0.067	No

Many of the miRNAs identified as being differentially expressed have been shown to have a role in immunity in other species. miR-223, for example, which was up-regulated at 36 hpi in MIMs, has a multi-factorial role in neutrophils, regulating their proliferation, activation and granulopoiesis (Chen, Li et al. 2004, Fazi, Rosa et al. 2005). Interestingly, in RAW264.7 cells challenged with LPS, miR-223 has been reported to be down-regulated, allowing the up-regulation of signal transducer and activator of transcription 3 (STAT3), which promotes pro-inflammatory IL6 and IL1β transcription. In chapter 3 the differences in the miRNA response to LPS and *S. uberis*, a Gram-positive bacterium were discussed.

Other DE miRNAs in this study that have a demonstrated role in immunity and infection in other species includes let-7e, which was down-regulated in MIMs following *S. uberis* infection. Let-7e, has been shown to regulate Caspase 3 and 7 in human monocyte derived macrophages infected with Mycobacterium *avium hominissuis* (Sharbati, Lewin et al. 2011). Let-7e, as well as several other DE miRNAs in this study (bta-miR-200c, bta-miR-210, and bta-miR-193a) have also previously been identified as DE in bovine mammary epithelial cells stimulated with *S. uberis in vitro* (chapter 3). Another DE miRNA with a role in immune regulation is miR-150, which we observed to be down-regulated at both 36 and 48 hpi in MIMs. miR-150 targets MyD88 (up-regulated at both 36 and 48 hpi in MIMs), a key regulator of TLR signalling (Ghorpade, Holla et al. 2013). miR-150 has also been shown to target CXCR4 (Tano, Kim et al. 2011, Rolland-Turner, Goretti et al. 2013), which was up-regulated 2-fold at 36 and 48 hpi in MIMs. Finally, one of the miRNAs that was identified as down-regulated in BIMs, miR-146, has been shown to target

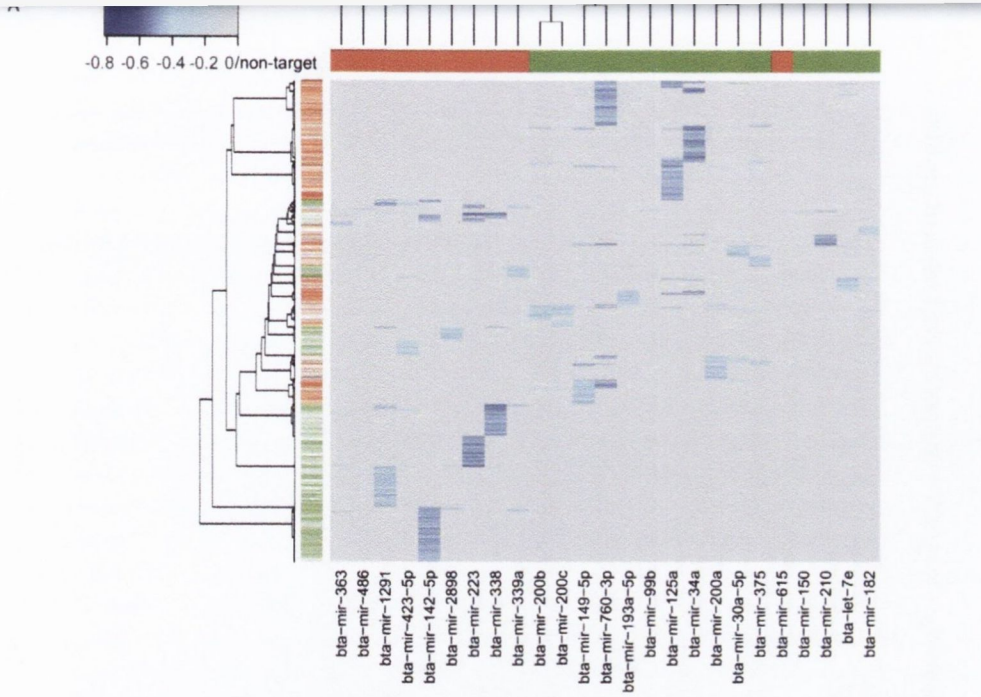
TNF receptor-associated factor 6 (TRAF6) and IL-1 receptor-associated kinase 1 (IRAK1) genes in THP-1 cells stimulated with LPS (Taganov, Boldin et al. 2006).

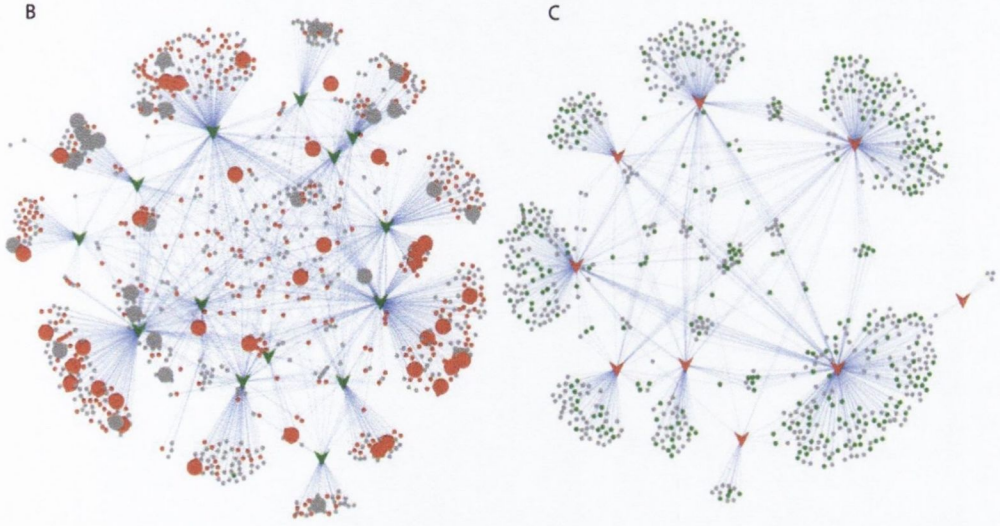
4.4.8 The predicted targets of down-regulated but not up-regulated miRNAs are highly enriched for roles in innate immunity

To identify the potential mRNA targets of differentially expressed miRNAs in MIMs isolated from infected animal mRNAs whose expression was significantly negatively correlated with miRNA expression was identified. These predictions were further refined by removing those miRNA-target predicted relationships that were not supported by a predicted seed region in the 3' UTR of the correlated mRNA (Figure 4.10. Analysis of the predicted target genes using InnateDB (www.innatedb.com) (Lynn, Winsor et al. 2008), revealed that the predicted targets of down-regulated but not up-regulated miRNAs were highly enriched for roles in innate immunity (FDR < $3.2E^{-8}$). More specifically, pathway analysis revealed that down-regulated miRNAs were predicted to preferentially target key pathogen recognition receptor (PRR) signalling pathways including the TLR, NLR and RIG-I signalling pathways (Table 4.5). Given that these pathways were also identified in the mRNAseq data as being among the top up-regulated pathways, this finding strongly suggests that miRNAs are key regulators of innate immune pathways which drive the host inflammatory response during mastitis. In contrast, the predicted targets of up-regulated miRNAs were enriched for roles in metabolism (FDR = 0.01). Pathway analysis of the mRNAseq data had already highlighted the down-regulation of metabolic pathways in response to *S. uberis* infection (see above). These results suggest that miRNAs may also be key regulators of the transcriptional suppression of metabolic pathways during mastitis.

Table 4.5 Significantly over-represented KEGG pathways among predicted miRNA targets.

Pathway Name	Source	Pathway p-value (corrected)
Toll-like receptor signaling pathway	KEGG	6.98584E-11
Osteoclast differentiation	KEGG	1.72904E-07
Apoptosis	KEGG	1.27797E-05
NOD-like receptor signaling pathway	KEGG	3.63936E-05
RIG-I-like receptor signaling pathway	KEGG	0.000592441
MAPK signaling pathway	KEGG	0.000610539
B cell receptor signaling pathway	KEGG	0.0009512
Steroid biosynthesis	KEGG	0.001777166
Neurotrophin signaling pathway	KEGG	0.002794434
Cytosolic DNA-sensing pathway	KEGG	0.004337574
MRNA surveillance pathway	KEGG	0.004414159
P53 signaling pathway	KEGG	0.00548788
Spliceosome	KEGG	0.006832377
Chemokine signaling pathway	KEGG	0.008376185
Phagosome	KEGG	0.011099319
Metabolic pathways	KEGG	0.009395686





cluster analysis and heatmap visualization of the miRNA/mRNA expression correlation data matrix after filtering by predicted miRNA-targets and correlation significance. The primary split in the upper hierarchical dendrogram largely aligns with the up-regulated miRNA (red) and down-regulated miRNA (green). miRNA/mRNA target expression correlations are coloured based on increasing significance (light blue to blue) with non-target/non-significant correlations masked (grey). B) A network representation of the predicted targets (circular nodes) of down-regulated miRNAs (green arrows). Red circles = up-regulated in MIM mRNA expression data at either 36 and/or 48hpi. Larger circular nodes represent those genes which have been annotated by InnateDB to have a role in innate immunity. C) A network representation of the predicted targets (circular nodes) of up-regulated miRNAs (red arrows). Green circles = down-regulated in MIM mRNA expression data at either 36 and/or 48hpi. Note that the predicted targets of down-regulated but not up-regulated miRNAs are highly enriched for roles in innate immunity.

4.4.9 Novel miRNA discovery

The miRNA sequencing data was also mined to determine if milk or blood monocytes expressed potentially novel miRNAs. In chapter 3 19 novel miRNAs were identified in bovine mammary epithelial cells using miRDeep2 (Lawless, Foroushani et al. 2013). Applying the same approach as this previous study a further 20 high-confidence putatively novel bovine miRNAs were identified that were independently predicted in multiple MIMs/BIMs miRNAseq data (Table 4.6).

Searching the miRBase database (v 20) using BLAST identified that 8 of the novel miRNAs had close homology to other miR-2284 family members. miRBase currently lists this miRNA family as having 102 members, yet virtually no data exists regarding what function such a large group of constitutively expressed miRNAs may have. Other novel miRNAs discovered in this study included a homolog of hsa-miR-3680-3p, a miRNA identified in human periphery blood (Vaz, Ahmad et al. 2010). The discovery of these miRNA further adds to the database of bovine miRNAs.

Table 4.6 Putative novel bovine miRNAs discovered through miRDeep2 analysis of miRNAseq data from 100 milk and blood isolated monocytes.

Cell type	Number of samples miRNA is predicted in	Mature Sequence Best miRBase BLAST Hit (e-value < 1)	Mature Tag Count	Predicted Mature Sequence
MIMs/BIMs	9	N/A	935	aaguuuaaagaagucugccuug
MIMs	6	N/A	25226	ccgagccugacagaucacaca
MIMs	10	N/A	355	ccccggcuuuuccucccccagg
MIMs	13	N/A	585	ccuccgccccgcgcucgccucgc
MIMs/BIMs	13	N/A	2528	agaacugaguccuuuggaaaaga
MIMs/BIMs	16	N/A	540	ucugcauccgaggacucaaccc
MIMs	5	N/A	175	uuguccuacuucucagcugucu
MIMs	8	N/A	589	cucccucggaacccggcugggacc
MIMs/BIMs	11	N/A	217	augugaaauuaccaguauuug
MIMs/BIMs	21	N/A	727	cacacucagauaaaguaggcacu
MIMs/BIMs	7	hsa-miR-3680-3p	1100	uugcaugacucugagaguaagu
MIMs/BIMs	8	bta-miR-7860	342	cacucugugcagccgcuccagg
MIMs/BIMs	55	bta-miR-2285t	39546	caaaaaguucguccagauuuuu
MIMs/BIMs	14	bta-miR-2285l	2444	aaaacccugaugaacuuuuuga
MIMs/BIMs	6	bta-miR-2285f	17481	aaaaccugaaugaacuuucugg
MIMs/BIMs	24	bta-miR-2285e	1539	aaacuugaacggacuuuuuggc
MIMs/BIMs	6	bta-miR-2285e	13046	aaaaccucaacgaacucuuugg
MIMs/BIMs	30	bta-miR-2284z	65924	agaaaaguuuguuuggguuuuu
MIMs/BIMs	21	bta-miR-2284z	845	agaaaguuuguuuggguuuuuc
MIMs/BIMs	16	bta-miR-2284d	1374	aaaaguacguuuggguuuuuca

4.5 Discussion

Infectious disease is a serious threat not only directly to human health but also to animal health, where it is associated with substantial annual economic losses, public confidence issues and food security concerns. Currently, there is a significant gap in the understanding of the molecular and genetic mechanisms which underpin susceptibility to infectious disease both in humans and in animals. An important reason for this is the fact that disease susceptibility is a multi-factorial complex phenotype, which is not the result of single genes acting in isolation but rather is due to perturbation at a network or systems level (Barabasi, Gulbahce et al. 2011). Such networks are regulated at multiple different levels (e.g. genetic, transcriptional, post-transcriptional) and as such a multi-omic integrative biology approach is needed to understand them.

Here, a next generation sequencing approach is reported, coupled with advanced network and pathway biology methods to simultaneously profile the mRNA and miRNA networks that are differentially regulated *in vivo* in blood and milk isolated CD14+ monocytes during infection with a bovine mastitis pathogen, *S. uberis*. Bovine mastitis is an inflammation-driven disease of the bovine mammary gland, which costs the global dairy industry billions of dollars per annum (Wells, Ott et al. 1998, Jones and Bailey 2009). Profiling genome-wide changes in mRNA expression in MIMs and BIMs using RNAseq, more than 3,500 genes were observed to be statistically altered in their expression in response to *S. uberis* challenge. Notably, this RNAseq approach identified approximately 1,000 more differentially expressed genes than had been previously reported in a microarray based analysis of RNA expression in mammary tissue of cattle infected with the same pathogen (Moyes, Drackley et al. 2009, Swanson, Stelwagen et al. 2009, Jensen, Gunther et al. 2013). As expected, given that mastitis is a

relatively localised inflammatory disease in the mammary gland, the majority of differentially expressed genes were identified in MIMs, which are recruited to the site of infection. This influx of immune cells (including monocytes) to the site of infection was observed in recorded SCC data only in the infected animals. A small but significant transcriptional response in BIMs to *S. uberis* infection was also observed, that was primarily associated with an interferon and cytokine signalling signature. Previous studies have also shown more systemic changes in gene expression in neighbouring uninfected mammary glands, the liver and in the blood (Blum, Dosogne et al. 2000, Jiang, Sorensen et al. 2008, Mitterhuemer, Petzl et al. 2010, Jensen, Gunther et al. 2013).

The predominant signature associated with up-regulated mRNAs in MIMs from *S. uberis* infected animals was the strong transcriptional activation of innate immune and inflammatory gene expression. In particular, a transcriptional activation of key pattern recognition pathways including the TLR, NLR and RIG-I pathways, which likely drive the observed pro-inflammatory response was noted. The involvement of TLR signalling (particularly TLR2 and TLR4) in the host response to mastitis is well documented (Mitterhuemer, Petzl et al. 2010, Buitenhuis, Rontved et al. 2011, Ma, Zhu et al. 2011, Whelehan, Meade et al. 2011, Porcherie, Cunha et al. 2012), however, less is known about the involvement of the NLR and RIG-I pathways (Moyes, Drackley et al. 2009). Interestingly, the RIG-I pathway is classically associated with viral RNA recognition, however, recent findings suggest that RIG-I can also recognise nucleic acids released by invasive bacteria and trigger IFNβ and inflammasome activation (Abdullah, Schlee et al. 2012). These findings concur well with the observed interferon and inflammasome activation transcriptional signatures observed in this study.

Several previous *in vitro* studies have strongly suggested roles for miRNAs in regulating bovine immunity (Dilda, Gioia et al. 2011, Lawless, Foroushani et al. 2013); however none of these have globally profiled the miRNA response to infection *in vivo*. In this study, a next generation sequencing approach was used to profile miRNA expression in MIMs and BIMs following *S. uberis* infection. 19 miRNAs were identified as DE in MIMs and one was identified in BIMs. Several of these have been previously described as targeting immune or inflammatory regulators in other species. Of particular interest is the finding that down-regulated but not up-regulated miRNAs in MIMs are predicted to preferentially target genes involved in innate immunity and inflammation. Furthermore, the TLR, NLR and RIG-I pathways discussed above were all preferentially predicted to be targeted. This strongly suggests that the transcriptional suppression of these miRNAs enables the activation and amplification of the pro-inflammatory response. Further supporting this conclusion is the fact that several of the DE miRNAs in our study have been validated to target genes in these pathways in other species. miR-149, for example, which was down-regulated in MIMs following *S. uberis* infection, has been shown to target mouse CD14 and IRAK1 (Chi, Zang et al. 2009), key signalling proteins in the TLR pathway. Both CD14 and IRAK1 are also predicted to be targets of bta-miR-149 in our study.

The other predominant transcriptional signature that were found in MIMs following *S. uberis* infection was the wide-spread repression of a number of metabolic processes (> 150 KEGG-annotated metabolism genes are down-regulated at 36 hpi). Interestingly, it was found that up-regulated miRNAs were predicted to preferentially target genes involved in metabolism, suggesting that miRNAs, which are up-regulated in response to *S. uberis* infection may contribute to the transcriptional suppression of metabolic pathways. This signature of metabolic gene transcriptional suppression may appear initially paradoxical in light of the

fact that production of inflammatory cytokines is expected to require substantial energy consumption. Indeed, it is now becoming widely appreciated that activated macrophages undergo the Warburg effect, switching their metabolism from oxidative phosphorylation to glycolysis (McGettrick and O'Neill 2013). This metabolic switch has recently been investigated using a combined metabolomics and microarray approach (Tannahill, Curtis et al. 2013) and has revealed the up-regulation of a number of genes involved in glycolysis in bone-marrow derived macrophages (BMDMs) challenged with lipopolysaccharide (LPS) (e.g. solute carrier family 2 (facilitated glucose transporter), member 1 (SLC2A1/GLUT1), hexokinase 3 (HK3), fructose-2,6-biphosphatase 3 (PFKFB3)) and the down-regulation of several key genes encoding enzymes in the TCA cycle (e.g. malate dehydrogenase 1 (MDH1) and isocitrate dehydrogenase 2 (IDH2)). The transcriptional data here are very consistent with the data presented in this paper (e.g. SLC2A1; HK2 and HK3 and PFKFB4 are all up-regulated and MDH1 and IDH1 are down-regulated in MIMs at 36 hpi) and suggests that although there is a broad signature of transcriptional suppression of metabolism, these cells are likely to be highly glycolytically active. Several other genes encoding enzymes in the TCA cycle (which was statistically over-represented among down-regulated genes) were also transcriptionally repressed in MIMs at 36 hpi including dihydrolipoamide dehydrogenase (DLD); fumarate hydratase (FH); IDH3B; pyruvate dehydrogenase beta (PDHB); MDH2; succinate dehydrogenase complex, subunit B (SDHB); succinate-CoA ligase, alpha subunit (SUCLG1). As has also recently been shown in BMDMs, LPS strongly increases levels of succinate, a TCA cycle intermediate (Tannahill, Curtis et al. 2013). Succinate acts as an inflammatory signal in macrophages inducing IL1B through the transcription factor HIF1α, both of which are transcriptionally activated in MIMs at 36 hpi (IL1B is 10 fold up-regulated). Another metabolic pathway that is significantly transcriptionally repressed in MIMs at 36 and 48 hpi is the KEGG Valine, leucine and isoleucine degradation pathway.

Valine, leucine and isoleucine are branch-chain amino acids which are converted into Acyl-CoA derivatives. These are converted either into acetyl-CoA or succinyl-CoA and enter the TCA cycle (Sears, Hsiao et al. 2009). Most of the other significantly down-regulated pathways, including Fatty acid metabolism; Propanoate metabolism, Butanoate metabolism, Tryptophan metabolism, beta-Alanine metabolism, Lysine degradation and Glyoxylate and dicarboxylate metabolism, also result in the production of acetyl-CoA and succinyl-CoA that enter the TCA cycle. A similar pattern of expression leading to the transcriptional down-regulation of the TCA cycle and alternative pathways involved in producing TCA cycle components has also been reported in other infection models (Chin, Monack et al. 2010). The transcriptional repression of these pathways is therefore also consistent with a switch in metabolism from oxidative phosphorylation to glycolysis during the pro-inflammatory response.

Another pathway that is significantly down-regulated is the KEGG primary bile acid biosynthesis pathway. Despite the potentially misleading name, this pathway primarily consists of the reactions involved in cholesterol metabolism. The down-regulation of genes involved in cholesterol metabolism has also been reported in monocytes isolated from HIV+ individuals (Feeney, McAuley et al. 2013) and a number of bacterial infections (Dushkin 2012). The accumulation of cholesterol in monocytes and macrophages leads to the formation of foam cells, which in humans are associated with the inflammatory disease, atherosclerosis (Ross 1999). The down-regulation of genes involved in cholesterol and fatty acid metabolism is likely driven in part by the transcriptional suppression of the PPAR γ transcription factor in MIMs at 36 hpi and the down-regulation of PPARα at 48 hpi, both of which are key transcriptional regulators of these pathways (Dushkin 2012). Interestingly, the PPARs also have a role in the regulation of inflammation, where their suppression is required to induce

inflammatory gene expression (Bensinger and Tontonoz 2008). Of further note is that one of the liver X receptors, LXRβ (NR1H2), which together with the PPARs is a key regulator of inflammation and lipid metabolism (Bensinger and Tontonoz 2008), is up-regulated in MIMs at 36 and 48 hpi. LXRs are also known to antagonise inflammatory gene expression, so it is somewhat surprising to find LXRβ to be up-regulated. This may reflect the fact that balance is needed to avoid excessive inflammation or that LXRs and PPARs do not completely overlap in the genes that they regulate.

An additional link between metabolism and inflammation that is currently under intensive investigation is the role of NAD+, sirtuins (SIRTs) and AMP-dependent protein kinase (AMPK) in suppressing inflammation (McGettrick and O'Neill 2013). The activation of TLR4, which along with TLR2 and TLR9 is transcriptionally up-regulated in MIMs at 36 hpi, has been shown to induce NAM phosphoribosyltransferase (NAMPT; up-regulated in MIMs at 36 hpi), which in turn activates SIRT1 (up-regulated in MIMs at 36hpi) via NAD+. SIRT1 limits inflammation by repressing RELA transcription factor activity, a key transcriptional hub identified in MIMs. SIRT1 also activates AMPK (up-regulated in MIMs at 36 hpi), a central regulator of energy metabolism. Activation of AMPK has been shown to decrease NF κB activity and TNFα production in macrophages stimulated with LPS, IL12 production in DCs; and HIF1α. This data suggests that at 36 hpi, the brakes are starting to be applied to limit the inflammatory response to *S. uberis* infection via SIRT1 and AMPK. The effect of this break is apparent at 48 hpi where the genes encoding TNFα, IL1B, IL12 and HIF1α are all down-regulated in comparison to 36hpi. The limiting of inflammation at this stage makes sense based on the bacterial count data, which shows that bacterial CFU/mL are dropping, suggesting that the infection is being resolved. Interestingly, AMPK activity also inhibits both the cholesterol and fatty acid metabolic pathways that, as discussed above, are down-

regulated in MIMs following *S. uberis* infection. This suppression of fatty acid metabolism has been shown to be beneficial to the host during a viral infection (Moser, Schieffer et al. 2012).

Finally, the statistically significant over-representation of down-regulated genes annotated in the KEGG glutathione metabolism pathway was observed. Glutathione has an important role in innate and adaptive immunity and has been shown to confer protection against microbial, viral and parasitic infections (Morris, Khurasany et al. 2013). Glutathione metabolism also plays an important role in macrophages in the detoxification of reactive oxygen species (ROS). The transcriptional suppression of this pathway in MIMs following *S. uberis* infection may be a consequence of the metabolic switch to glycolysis and may be detrimental to the host leading to an excess of oxidants in the cells, which could drive the inflammation and tissue damage that are characteristic of mastitis. Indeed, in patients with active tuberculosis, PBMC intracellular glutathione levels dropped by 70%, this was correlated with increased pro-inflammatory cytokines and enhanced bacterial growth (Guerra, Johal et al. 2012). Supplementation with glutathione has been demonstrated to lead to the control of mycobacterial growth (Venketaraman, Dayaram et al. 2003) and also appears to have beneficial effects in reducing inflammation in HIV+ patients (Morris, Guerra et al. 2012). This suggests that glutathione supplementation is a potential strategy to reduce the effects of inflammation in mastitis.

MicroRNAs also likely play a key role in regulating the links between inflammation and metabolism observed in this study. Human SIRT1, for example, which as discussed above limits inflammation, has been shown to be a target of miR-34a (Yamakuchi, Ferlito et al. 2008). The data presented here is consistent with this relationship also existing in MIMs,

where bta-miR-34a was found to be down-regulated at 36 and 48 hpi and SIRT1 up-regulated.

Aside from providing new insight into the regulatory role miRNAs play in *S. uberis* infection *in vivo*, this study also provides the ground-work for a number of potential practical applications in veterinary medicine. miRNAs, for example, exhibit many properties that have made them of significant interest as non-invasive biomarkers. miRNAs are abundantly and stably expressed in a range of accessible tissues including serum, milk, urine, saliva and semen where they can be readily measured (Chen, Gao et al. 2010, Hata, Murakami et al. 2010, Kosaka, Izumi et al. 2010). Importantly for a potential biomarker, miRNAs have a high information content, and the expression profile of small numbers of them have been shown to be diagnostic of disease (De Guire, Robitaille et al. 2013). The use of miRNAs as a clinical biomarker is most advanced in human cancer research. In 2009, Prometheus Laboratories released a miRNA biomarker to accurately identify 25 different tumour types (Ajit 2012), and miRNA biomarkers are now available for early cancer prognosis from two further companies Asuragen & Rosetta Genomics. Next generation sequencing based technologies, such as the approach used in this study, are empowering RNA expression profiling, including miRNAs, on a genome-wide scale with unprecedented resolution, accuracy and speed and at a relatively low cost (Schuster 2008). There is significant potential to develop these approaches as diagnostics of infection in animals and also in humans.

Chapter 5: MicroRNA regulation of IL8 during bovine mastitis.

5.1 Introduction

In chapter 4, an *in vivo* infection model in which more than 3,500 genes were identified as differentially expressed in response to *S. uberis* infection was described. Pathway analysis revealed that differentially expressed genes were enriched for a host of cellular processes including inflammatory and metabolic pathways. Twenty-six miRNAs were also identified as differentially expressed. Down-regulated miRNAs were predicted to preferentially target up-regulated mRNAs involved in innate immunity and inflammatory networks, while up-regulated miRNAs were predicted to preferentially target metabolic pathways. Downstream signalling from these inflammatory pathways mobilises a vast repertoire of cytokines, chemokines and effector molecules. One such cytokine, interleukin 8 (IL8), (upregulated 2.5 fold at both 36 and 48 hours post infection) is highly expressed during mastitis (Peli, Scagliarini et al. 2003, Lee, Bannerman et al. 2006). IL8 is a known chemotactic factor of neutrophils during infection. Neutrophils play a key role in clearing invasive bacteria and contribute to the substantial tissue damage associated with mastitis (Barber and Yang 1998, Stevens, Van Poucke et al. 2011). Better understanding of the posttranscriptional regulation of IL8 gene expression is therefore of significant interest.

From the studies in Chapter 4 a database of potential mRNA targets of miRNAs that are differentially expressed in inflammatory CD14+ monocytes during a mastitis infection was generated. These targets have been predicted through the careful integration of both computationally-predicted miRNA-mRNA targets and by correlating miRNA expression with mRNA expression in the same samples. Mining these data, bta-miR-200a was selected as a possible regulator of IL8. Previous analysis showed that during *S. uberis* infection of the bovine mammary gland, bta-miR-200a expression in CD14+ monocytes was inversely correlated (-0.28864) with IL8 expression (in the same samples). Additionally, bta-miR-200a

was predicted by the miRanda software to have a preferential binding site on positions 190-196 in the 3' UTR of IL8. Specifically, an exact seed match to positions 2-8 of the mature miRNA, in addition to position 8 (7mer-m8).

Bta-miR-200a is part of the miR-200 family, a family of miRNAs that has been studied in several species including bovine. Much evidence exists for miR-200 expression in human breast tissue. Historically known as a tumour suppressor, up-regulation of bta-miR-200a inhibits epithelial-mesenchymal transition (EMT), preventing cell migration and anchorage independent growth in the human breast (Eades, Yao et al. 2011). Further evidence of miR-200a expression in the mammary gland can be found in non-model organisms including the goat where miR-200a regulates mammary fat globule formation (Lin, Luo et al. 2013) and in bovine mammary epithelial cells, where it has been shown to regulate polarity and phenotype (Nagaoka, Zhang et al. 2013). Other studies have found miR-200a differentially expressed in response to several pathogens in various species. During influenza virus infection of murine lung tissue, mmu-miR200a was predicted to target and was inversely correlated with transcription factors STAT1/2, genes critical to interferon induction (Li, Chan et al. 2010). Recently in chicken, miR-200a was found to be differentially regulated during necrotic enteritis (Dinh, Hong et al. 2014). Finally miR-200b/c has been shown to target the MyD88 dependent pathway during TLR4 activation (Wendlandt, Graff et al. 2012). Together this literature indicates miR-200a expression is prominent in the mammary gland of several species and there is a precedence of miR-200a being active during infection.

This chapter will examine whether IL8 is upregulated *in vitro* during live *S. uberis* infection. A transformed cell line originally developed in 1995 (Stabel and Stabel 1995) was used for this study. It is the only bovine macrophage (BoMac) cell line available for bovine *in vitro*

infection studies. BoMac cells lack a number of cell surface markers including CD14 (Sager, Davis et al. 1999). Whether or not bta-miR-200a post-transcriptionally regulates IL8 by transfecting a synthetic mimic of bta-miR-200a into the cells will be examined. qPCR will be used to examine if IL8 is supressed in the presence of the bta-miR-200a mimic (but not in the presence of a scrambled negative control RNA). Using a live bacteria challenge for this transfection model could introduce an unfavourable layer of complexity and reduces specificity which could complicate the experiment. Therefore, it will be examined how lipoteichoic acid (LTA) effects IL8 expression in BoMac cells, with a view of using a simplified LTA infection model for transfections. To validate miRNA mimic transfections, a positive control miR mimic (miR-1) validated by Life Technologies to target Protein Tyrosine Kinase 9 (PTK9) was purchased. The miR-1 mimic is 100% homologous between human and bovine. A second validated miRNA, miR-146a known to target interleukin receptor associated kinase 1 (IRAK1) in human (Taganov, Boldin et al. 2006), was used as an "in house" positive control. Human and bovine miR-146a share a 100% homologous seed region and the human mature miRNA is two nucleotide longer than the bovine (84% homology).

5.2 Hypothesis

We propose that the microRNA (miRNA) bta-miR-200a, a short non-coding RNA molecule is a post-transcriptional regulator of interleukin 8 (IL8) during bovine mastitis.

5.3 Specific aims

1. Optimise qPCR for detection of bovine IL8 in bovine macrophage (BoMac) cell line.

2. Carry out a time course study of IL8 by challenging BoMac cells with live *S. uberis*.

3. Optimise an *in vitro* stimulation of gene regulation by miRNA mimics.

4. Measure the effect of a synthetic miRNA mimic (bta-miR-200a) on IL8 induction in a live *S. uberis* infection model.

5. Develop a more specific model of IL8 induction using LTA.

6. Measure the effect of a synthetic miRNA mimic (bta-miR-200a) on IL8 induction in a LTA stimulation model.

5.4 Results

5.4.1 Extraction and quantification of RNA from a bovine macrophages

BoMac cells were cultured *in vitro* in six-well plates as described in the methods section. Three technical replicates (n=3) alongside three controls (n=-3) were used for each stimulation/challenge. Twenty-seven LTA stimulated samples and twenty-one live *S. uberis* samples were lysed, and RNA was extracted for qPCR. RNA was examined for quantity and integrity in each sample. Total RNA was assessed to be of high quality on Bioanalyser. RIN numbers for total RNA were > 8 for each sample. Sufficient quantities were present to proceed with qPCR.

5.4.2 Validation of primers selected for stable reference gene and genes of interest

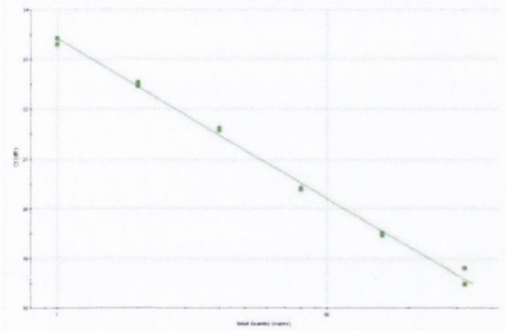
Primers were designed for each reference gene and gene of interest (GOI). The expression of four reference genes PPIA, GAPDH, H3F3A, MRSP6, and three genes of interest IL8, IRAK1, and PTK9 were tested. Their RSq value, Y-value, and the efficiency of the line (Table 5.1) were all within required parameters. Reference genes were tested in geNorm in the BoMac cell line. All gene amplifications for bovine samples were normalised to GAPDH and PPIA.

Table 5.1 Results of RSq, Y value, and efficiency values for primers used in this study

Gene name:	RSq:	Y value:	Efficiency:
GAPDH	0.996	-3.216	104.6%
H3F3A	0.994	-3.146	107.9%
PPIA	0.947	-3.403	96.6%
MRSP6	0.996	-3.111	109.6%
IRAK1	0.971	-3.29	101.3%
PTK9	0.996	-3.371	98%
IL8	0.92	-3.223	104.3%

Figure 5.1 Standard curves of optimised reference genes and GOI. A) GAPDH (slope of line -3.216), B) H3F3A (slope of line -3.146), C) PPIA (slope of line -3.403), D) MRSP6 (slope of line -3.111) E) IL8 (slope of line -3.223) F) PTK9 (slope of line -3.371), and G) IRAK1 (slope of line -3.29) generated on MXP3000P® quantitative PCR system.

A



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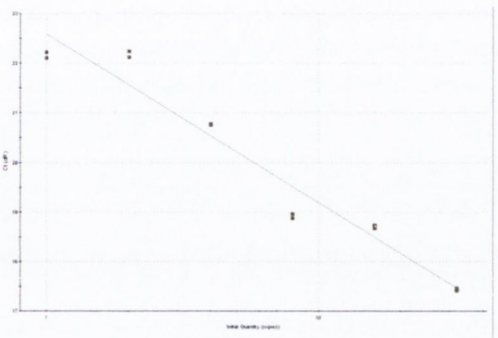

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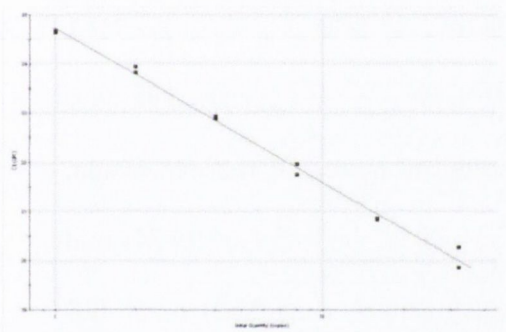

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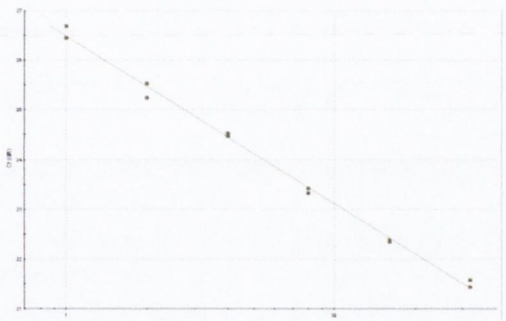

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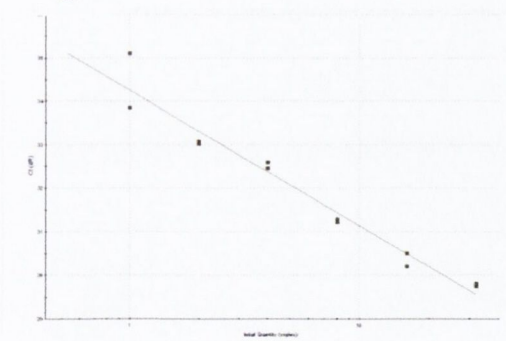

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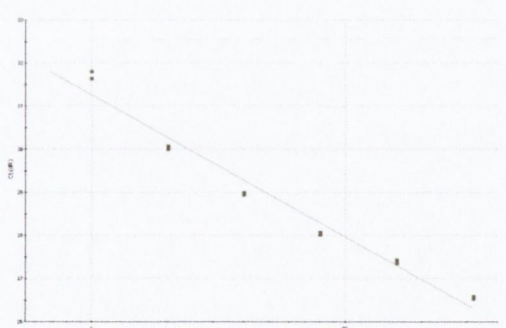

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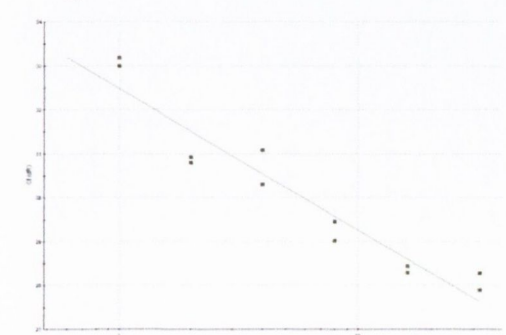

5.4.3 IL8 is transcriptionally induced in response to *S. uberis* challenge

To demonstrate how *S. uberis* infection of a bovine macrophage cell line caused differential expression of IL8 *in vitro*, BoMac cells (n=3) were challenged at 12 and 24 hpi see methods 2.2.3. Three multiplicities of infection 1, 5, and 50 were used to challenge cells. The fold change in expression of IL8 was determined relative to internal control genes PPIA and GAPDH, as was the relative expression of both genes.

IL8 expression increased relative to control samples at all-time points at an MOI of 50. Expression of IL8 peaked at 6 hpi (7.6 fold, $P < 0.05$; data not shown).It was observed that an MOI of 50 for a sustained time lead to cell death, and reduced RNA quantities. Therefore an MOI of 50 deemed unsuitable for future transfection studies. *S. uberis* challenges at an MOI of 1 and 5 also resulted in increased IL8 expression. At an MOI of 1, IL8 increased 4.6 (P-value = 0.011) and 3.2 (P-value = 0.0174) fold at 12 and 24 hpi, respectively, these results were confirmed at an MOI of 5: IL8 increased 5.8 (P-value = 0.0001) and 5.01 (P-value = 0.0002) fold at 12 and 24 hpi, respectively (Figure 5.2).

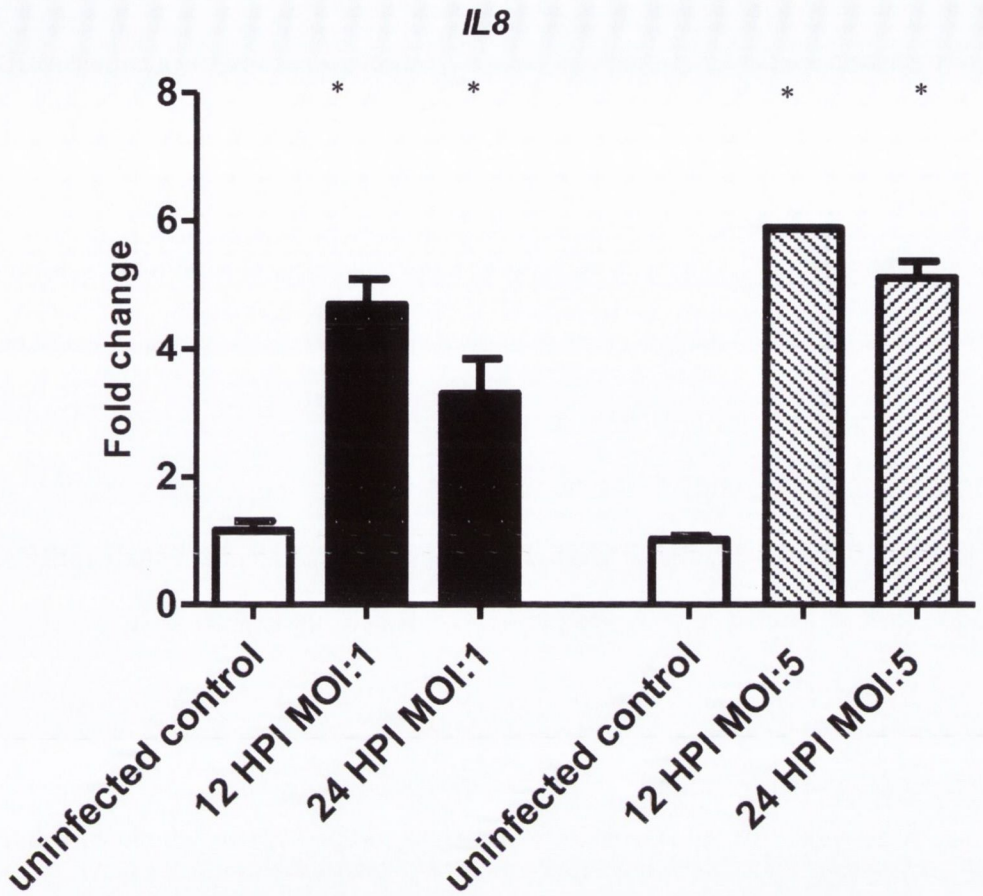

Figure 5.2 *S. uberis* stimulated IL8 expression in bovine macrophages. Expression of IL8 was analysed using qPCR. P values for comparison of uninfected and infected were generated using GraphPad Prism software, version 5.01 (P-value <0.05 deemed significant). Stimulation parameters were compared using one way ANOVA test (Kruskal-Wallis test). P <0.05=* was considered statistically significant. Mean fold changes are shown for *S. uberis* challenged BoMac cells at two multiplicities of infection 1 and 5 (n=3).

5.4.4 Mimic positive controls down regulate bovine mRNA targets

By parsing a dataset of potential miRNA targets (described in chapter 4) IL8 was found to be a potential target for bta-miR-200a. The miRanda software (miRanda v3.3a) predicted a pairing of IL8 target region and bta-miR-200a at position 190-196 on the 3' UTR of IL8. The miRNA also was inversely correlated (-0.28864) with IL8 mRNA expression in the dataset. To validate this predicted relationship, a miRNA mimic for bta-miR-200a (miRBase accession: MIMAT0003822, assay ID: MC 11730) was purchased from Life Technologies (miRBase v20). MiRNA mimics are small double stranded synthetic RNAs, which mimic the action of mature miRNAs within the cell. In addition, a scrambled negative control (mirVana™ miRNA Mimic Negative Control #1), and a positive control (mimic miR-1) which negatively regulates Protein Tyrosine Kinase 9 (PTK9) were also obtained. Bta-miR-146a (miRBase accession: MIMAT0009236, assay ID: MC 14988) which has been previously shown to negatively regulate interleukin receptor associated kinase 1 (IRAK1) in human was used as a further positive control (Williams, Perry et al. 2008). BoMac cells were incubated with each individual mimic~lipofectamine for 24 h at a mimic concentration of 90 pmol before challenge or RNA extraction.

The expression of PTK9, the target of the positive control (miR-1), and IRAK1, the target of bta-miR-146a were determined by qPCR. All samples were normalised to the reference genes PPIA and GAPDH. A small decrease in expression -1.3 fold; P-value = 0.0265; n=3 was detected in PTK9, when compared as a group against both the non-transfected control and the negative control, however it was not significant when compared against the non-transfected control alone (Fig 5.3). IRAK1 decreased by -1.5 fold; P-value = 0.0273; n =3 (Fig 5.4) when compared as a group against both

the non-transfected control and the negative control, however it was not significant when compared against the non-transfected control alone.

The expression of IL8 in BoMac cells transfected with mimic bta-mir-200a was compared against non-transfected control cells by qPCR. No significant reduction in IL8 expression was observed in mimic bta-mir-200a transfected cells (P-value = 0.858; n=3; not significant). BoMac cells were also transfected with a synthetic miRNA mimic for bta-miR-200a (for 24h) and were then challenged with *S. uberis* (MOI = 5). At 24 hpi, expression of IL8 was compared by qPCR to its expression in cells challenged under the same conditions but without the transfected mimic (5.4.3). Cells pre-incubated with mimic-200a showed a small reduction in IL8 expression (-1 fold; P-value = 0.003; n=3) compared to infected cells (Figure 5.5).

Other studies which have used miRNA mimics to reduce gene expression typically have transfected scrambled negative control (mirVana™ miRNA Mimic Negative Control #1), and the positive control (mimic miR-1) at a much higher concentration than that recommended in the manufacturer's instructions. For example, three studies have used 500 pmol (Barnes, Stephenson et al. 2012), 125 pmol per $1x10^7$ cells (Wang, Corrigan-Cummins et al. 2012), and 30 nM (Schaeffer, Hansen et al. 2012). Here, we have used the manufacturers recommendation's as a start point for which we can carry out further transfections studies.

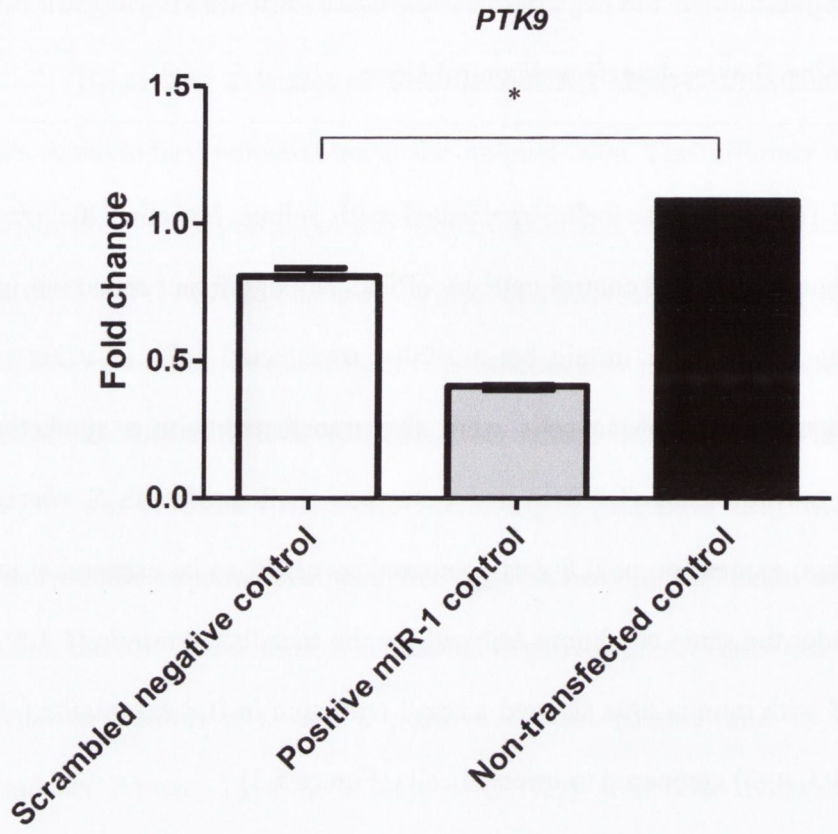

Figure 5.3 Effect of miR-1 on PTK9 gene expression in BoMac cells. Expression of PTK9 was analysed using qPCR. P values for comparison of uninfected and infected were generated using GraphPad Prism software, version 5.01 (P-value <0.05 deemed significant). Stimulation parameters were compared using one way ANOVA, the Kruskal Wallis test and the Mann-Whitney U test. P <0.05=* was considered statistically significant. All samples were normalised to reference genes PPIA/GAPDH. Mean fold changes are shown for BoMacs transfected with mimic miR-1, negative scramble mimic, and non-transfected controls (all n=6).

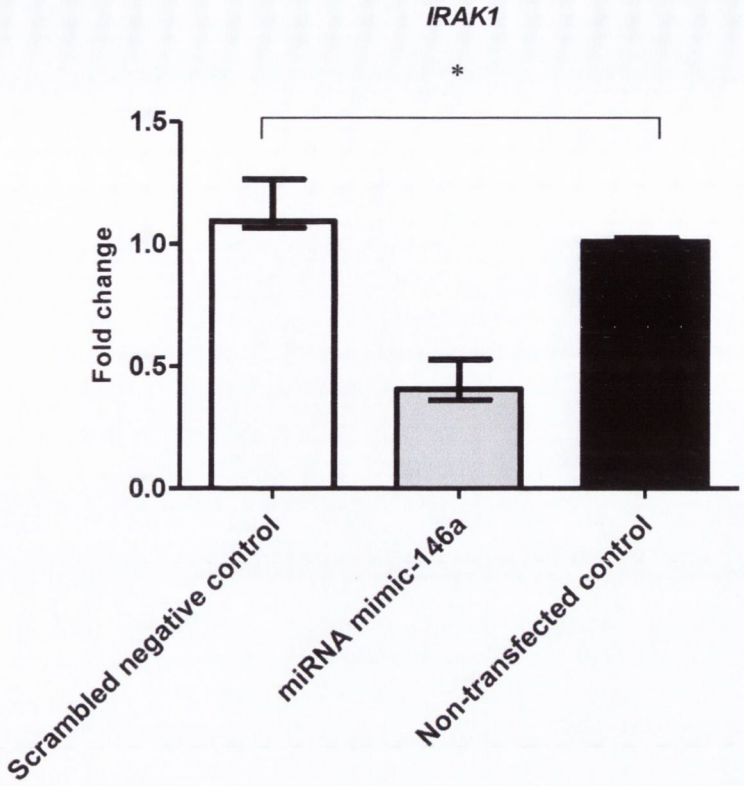

Figure 5.4 Effect of 146a on IRAK1 gene expression in BoMac cells. Expression of IRAK1 was analysed using qPCR. P values for comparison of uninfected and infected were generated using GraphPad Prism software, version 5.01 (P-value <0.05 deemed significant). Stimulation parameters were compared using one way ANOVA, the Kruskal Wallis test and the Mann-Whitney U test. P <0.05=* was considered statistically significant. All samples were normalised to reference genes PPIA/GAPDH. Mean fold changes are shown for BoMacs transfected with mimic miR-146a, negative scramble mimic, and non-transfected controls (all n=3).

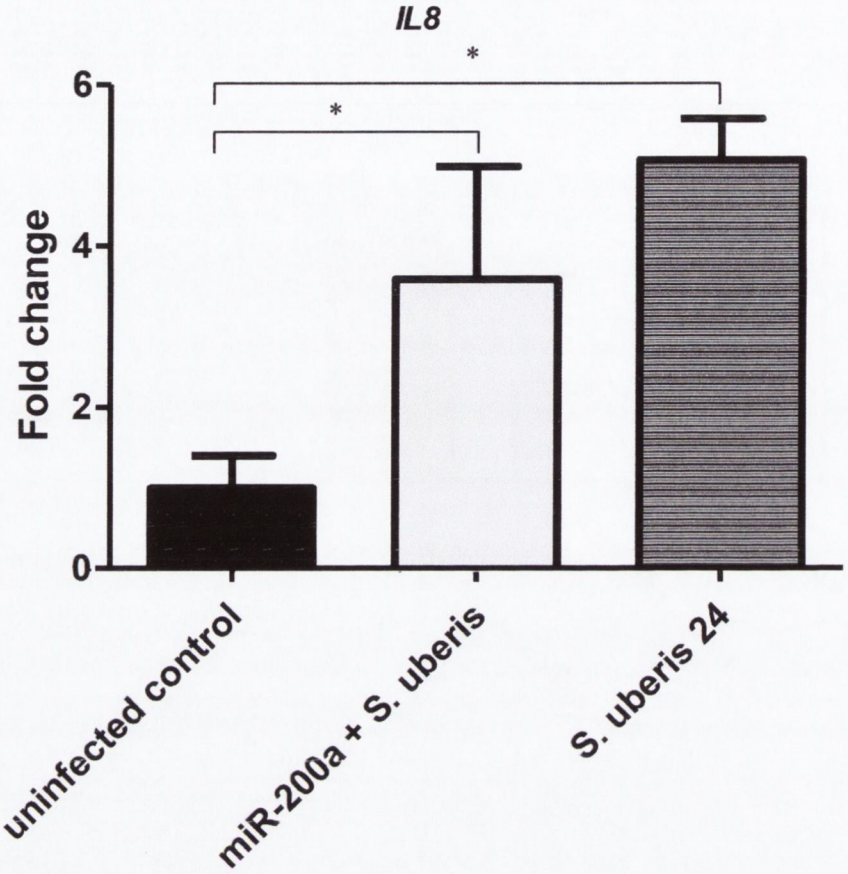

Figure 5.5 miR-200a mimic tends to inhibit IL8 gene expression in BoMac cells challenged with *S. uberis*. Expression of IL8 was analysed using qPCR. P values for comparison of uninfected and infected were generated using GraphPad Prism software, version 5.01 (P-value <0.05 deemed significant). Stimulation parameters were compared using one way ANOVA, the Kruskal Wallis test and the Mann-Whitney U test. P <0.05=* was considered statistically significant. All samples were normalised to reference genes PPIA/GAPDH. Mean fold changes are shown for *S. uberis* challenged at an MOI of 5 versus miR-200a transfected and *S. uberis* challenged cells (n=3).

5.4.5 IL8 is transcriptionally induced in response to LTA challenge in vitro

Lipoteichoic acid is a potent Gram positive bacterial cell wall molecule and known IL8 agonist, was used to stimulate IL8 expression in BoMac cells (Yu, Shi et al. 2010). Cells were challenged at 2, 4 and 6 hpi, with three concentrations of LTA 10 μg/mL, 20 μg/mL, and 50 μg/mL.

There was no expression change of IL8 in stimulated BoMac cells relative to un-stimulated control BoMac cells at 10 μg/mL. A small increase in expression at 20 μg/mL was observed. However, at 50 μg/mL, a fold change of 6 and 2.6 (P-value = 0.038) fold in IL8 expression at 4 and 6 h, respectively (Figure 5.6) was observed. Based on these results, it was determined that LTA at a dosage of 50 μg/mL is a suitable model for knock-down studies.

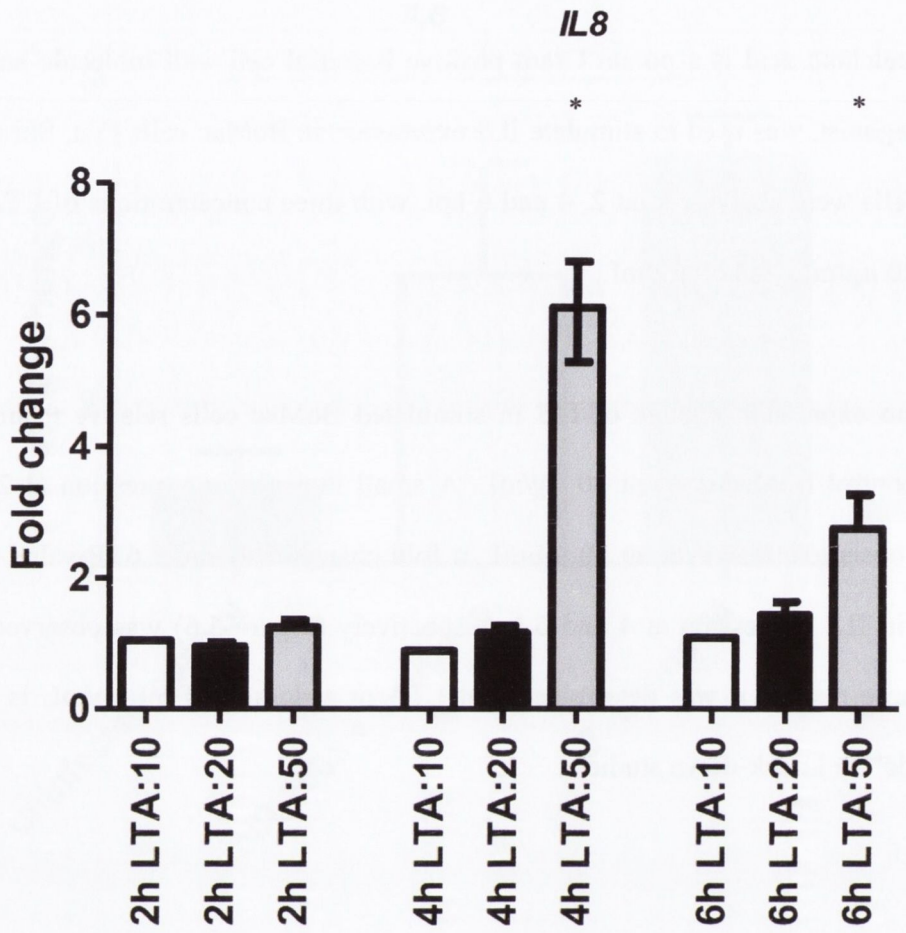

Figure 5.6 IL8 gene expression in BoMac cell line stimulated with LTA. Expression of IL8 was analysed using qPCR. All samples (stimulated and un-stimulated control) were normalised to reference gene PPIA/GAPDH. Mean fold changes are shown for LTA stimulated BoMacs at 10 μg/mL, 20 μg/mL, and 50 μg/mL at 2, 4, and 6 h (n=3). Data shown is the median (bars denote the upper and lower range) of n=3 technical replicates. Statistical analysis was carried out using a 1 way ANOVA (Kruskal-Wallis test). Statistical significance denoted as * = $P < 0.05$.

5.4.6 Mimic miR-200a reduces IL8 expression during LTA stimulation

The expression of IL8 in BoMac cells transfected with mimic bta-mir-200a then stimulated with LTA (50 µg/mL; @ 6 hpi) were compared against non-transfected stimulated (LTA 50 µg/mL; @ 6 hpi) BoMac cells, and negative scramble transfected then LTA (50 µg/mL; @ 6 hpi) BoMac cells, and control cells by qPCR. Synthetic miRNA mimic transfection quantities were increased to 130pmol for bta-miR-200a and negative scramble (for 24h). All samples were normalised to the reference genes PPIA and GAPDH.

Cells transfected with mimic bta-miR-200a and stimulated with LTA had a mean fold change of 2.35; (P-value = 0.0119; n=6). Cells stimulated with LTA only had a mean fold change of 4.2 fold (P-value = 0.015; n=6) and cells transfected with the negative scramble and stimulated with LTA had a fold change of 3.95 (P-value = 0.0126; n=6) when each was compared against control cells (Fig. 5.7). Overall IL8 expression in LTA stimulated BoMac cells is reduced by approximately 2 fold by bta-miR-200a mimic.

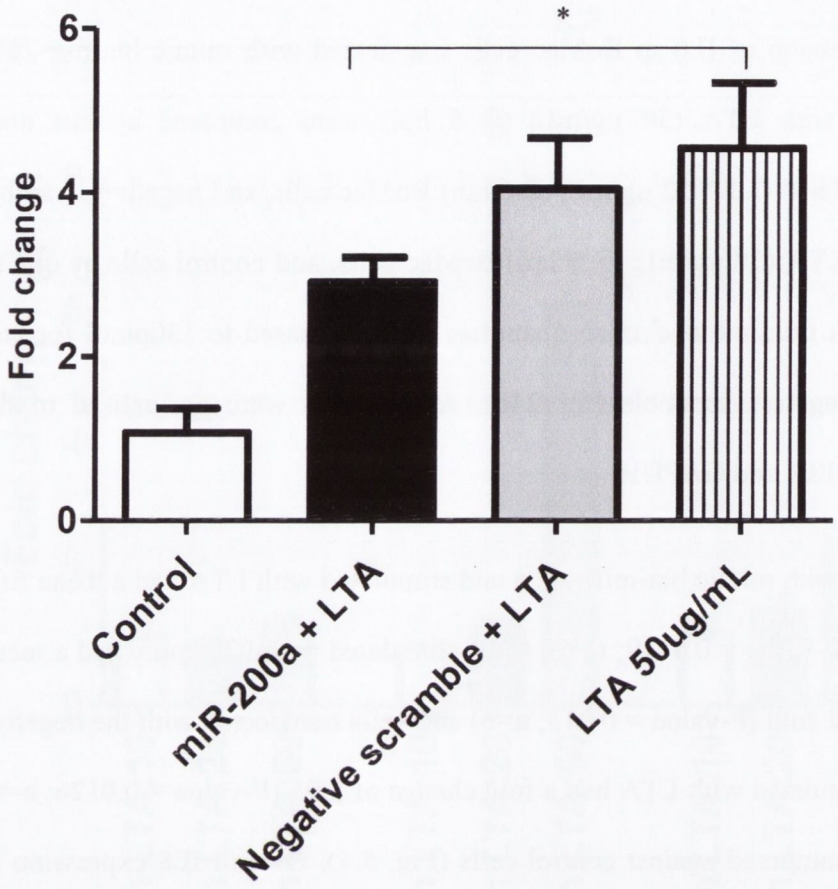

Figure 5.7 miR-200a mimic tends to inhibit IL8 gene expression in BoMac cells stimulated with LTA. Expression of IL8 was analysed using qPCR. P values for comparison of uninfected and infected were generated using GraphPad Prism software, version 5.01 (P-value <0.05 deemed significant). Stimulation parameters were compared using one way ANOVA, the Kruskal Wallis test and the Mann-Whitney U test. P <0.05=* was considered statistically significant. All samples were normalised to reference genes PPIA/GAPDH. Mean fold changes are shown for LTA stimulated 50 µg/mL versus miR-200a transfected and LTA stimulated 50 µg/mL (n=6).

5.5 Discussion

The growth in high-throughput technologies has been driven by increased sequencing capacity, the emergence of interpretative software (Breuer, Foroushani et al. 2013) and decreasing costs of sequencing and data warehousing. However, post-sequencing miRNA target validation has proven to be somewhat of a bottleneck. Many predictive miRNA software packages use the base pairing between the seed region of the miRNA and the 3' UTR of the target mRNA as a determining factor in predictive scoring (Zheng, Fu et al. 2013, Kozomara and Griffiths-Jones 2014). Due to natural genome redundancy, and imperfect genome annotation, particularly in the 3' UTR, there is still a need to further validate miRNA targets by both sequencing and functional validation (Witkos, Koscianska et al. 2011). In addition, miRNA target validation in non-model organism can provide a further challenge, due to the scarcity of suitable cell types.

Here a miRNA target validation study is reported, where miRNA and putative mRNA target selection was based on findings of an *in vivo* NGS study. The miRNA target of interest IL8, is expressed in several immune cell types including monocytes (Chapter 4), epithelial cells (Gunther, Liu et al. 2010), and neutrophils, three innate immune cell types highly relevant to bovine mastitis. IL8 is strongly associated with contributing to the inflammatory state which clears bacteria that lead to the mastitis, by recruiting neutrophils to the site of infection (Schroder, Mrowietz et al. 1987, Barber and Yang 1998). Here, IL8 transcriptional expression has been demonstrated to increase *in vitro* in a bovine macrophage cell line when challenged with either live *S. uberis* or stimulated with lipoteichoic acid. Challenging BoMac cells with a live pathogen at higher concentrations and longer time points decreased cell viability. In

addition live bacteria will induce a complex immune reaction in comparison to LTA, which may further inhibit mimic transfections.

It has been demonstrated that a positive control miRNA mimic, miR-1, and mimic miR-146a successfully reduced the transcriptional expression of target PTK9 and IRAK1, albeit modestly, as would be expected. Although miR-146a has been shown to target IRAK1 in human by luciferase assay, the gene has yet to be shown as a target of miR-146a in bovine. Bta-miR-146a has the highest possible seed match to the IRAK1 3'UTR (8mer). Preliminary experiments to determine whether bta-miR200a inhibits bovine IL8 expression are not yet conclusive, however initial findings suggest bta-miR-200a reduces the transcriptional expression of IL8.

Comparative mimic studies have transfected cells with up to 5 times more mimic than carried out here. By increasing the concentration of mimic on par with these studies it may be possible to more accurately assess bta-miR-200a suppression of IL8 expression. To validate the target region the 3' UTR target region will be cloned into plasmids, before co-transfection of both miRNA mimic and plasmid into BoMac cell, confirming miRNA targeting by luciferase assay.

Further mining of the dataset from Chapter 4 revealed a number of miRNA targets of interest. Bta-miR-150a expression in CD14+ monocytes was negatively correlated (-0.32593) with NOD2 expression and bta-miR-193a was negatively correlated with TLR2 (-0.3116). Bta-miR-150 was predicted by miRanda software (miRanda v3.3a) to have a preferential binding site on positions 32-38 on the 3' UTR of NOD2 and

bta-miR-193a was predicted to have a preferential binding site on positions 341-347on the 3' UTR of TLR2.

To conclude, IL8 has been shown to be up-regulated in a bovine macrophage cell line when challenged with live *S. uberis*. IL8 stimulation with LTA however, appears to be an improved model for transfection studies. Mimic positive controls resulted in a small reduction of mRNA targets of interest. Further work is required to confirm the initial finding that bta-miR-200a reduces transcriptional expression of IL8.

Chapter 6: Conclusions

6.1 Conclusions

Infectious disease in animals constitutes a significant threat to the global economy (Godfray, Beddington et al. 2010) and remains a major public health problem worldwide (Daszak, Cunningham et al. 2000, Jones, Patel et al. 2008). Pathogenic organisms have developed resistance to many front-line antibiotics (Fischbach and Walsh 2009), and biosecurity measures to prevent contagious transmission are not always effective (Barkema, Green et al. 2009). Costing the E.U. nearly €2 billion/annum, bovine mastitis is amongst the most economically significant animal diseases affecting agriculture (Wells, Ott et al. 1998, Rinaldi, Li et al. 2010). There remains a significant gap in our understanding of how inflammation is regulated during mastitis. In this thesis, I have applied a high-throughput approach to examine the early host immune response to a bovine mastitis pathogen (*S. uberis*) at the transcriptional and post-transcriptional levels *in vitro* in bovine mammary epithelial cells and *in vivo* in milk and blood isolated CD14+ monocytes. A particular focus was to investigate the role that miRNAs, small non-coding RNAs which post-transcriptionally regulate gene expression, play in shaping the host transcriptional networks in these cells in response to the infection.

In the first study (Chapter 3), temporal changes in miRNA expression were investigated *in vitro* in primary mammary epithelial cells challenged with live *S. uberis*. Epithelial cells actively drive inflammation during mastitis and are critical to the successful clearance of infection. An *in vivo* study of epithelial cells requires licencing and biopsy of the inner gland cistern. Instead, primary mammary epithelial cells were purchased commercially then grown and challenged *in vitro*. By sequencing unpooled miRNAseq libraries across multiple time-points 10 unique

miRNAs were found to be significantly differentially expressed in response to infection. Differential expression in the majority of miRNAs was observed to be subtle and varied across different time points. This was suggestive of a dynamic network of miRNAs responding to infection. Target prediction analysis of differentially expressed miRNAs found genes enriched in immune signalling pathways were preferentially targeted. It was concluded that the differentially expressed miRNAs identified in this study are central regulators of the innate immune response to *S. uberis*. When comparing the findings of chapter 3 against existing literature, it was also found that the miRNA response to the Gram-positive *S. uberis* was markedly different to that reported for the miRNA response to LPS stimulation. Less than half of the miRNAs described in Chapter 3 have previously been shown to be differently regulated in infection. Five of those had an inverse pattern of expression in LPS studies to the miRNA response to a Gram-positive bacterium. Based on this, it is suggested that the miRNA response to Gram-positive bacteria is markedly different to that seen in a Gram-negative response.

In the second study (Chapter 4), mRNA and miRNA were simultaneously profiled and networks differentially regulated *in vivo* in blood and milk isolated CD14+ monocytes during infection with the same bovine mastitis pathogen, *S. uberis* were examined. Monocytes maintain a constant vigil in the mammary gland and function in a surveillance capacity. Upon bacterial recognition, monocytes release a cascade of cytokines and chemoattractants, aiding in the resolution of mammary gland infection. As previously mentioned, examining mammary epithelial cells *in vivo* requires licencing. Furthermore, studying temporal change requires multiple biopsies of the mammary gland, making ethical approval problematic. Instead, temporal changes in

milk and blood CD14+ monocytes from multiple biological replicates (infected n=5 and control n=5) were investigated. This study represents the largest bovine *in vivo* NGS study of its kind. In MIMs, 2,056 and 1,721 genes were up- and down-regulated respectively across the five time points. A subtle but significant response to infection in BIMS was observed. Eighty three genes were up-regulated in BIMs by 48 hpi. Pathway analysis revealed that up-regulated genes in MIMs were significantly enriched for roles in inflammatory and other innate immune pathways (e.g. TLR, NLR and RIG-I signalling), while down-regulated genes were significantly associated with metabolic pathways. InnateDB network analysis of DE genes revealed that contextual hubs were highly enriched for roles in innate immunity (FDR < $1.16E^{-12}$). The majority of the top 20 contextual hubs were well known transcriptional regulators of innate immunity (e.g. CREBBP, EP300, IRF1, IRF9, JUN, NFKB1, REL, RELA, STAT1, STAT3). Genes up-regulated in BIMs showed a significant association with interferon and chemokine signalling. Ninteen miRNAs were DE in response to infection in milk monocytes, with 1 miRNAs found to be differentially expressed in BIMs. Pathway analysis revealed that predicted targets of down-regulated miRNAs were highly enriched for roles in innate immunity (FDR < $3.4E^{-8}$) in particular TLR signalling, while up-regulated miRNAs preferentially targeted genes involved in metabolism. It was concluded that during *S. uberis* infection miRNAs are key amplifiers of monocyte inflammatory response networks and repressors of metabolism.

Several studies have examined systemic changes in gene expression in neighbouring uninfected mammary glands, the liver and in the blood (Blum, Dosogne et al. 2000, Jiang, Sorensen et al. 2008, Mitterhuemer, Petzl et al. 2010, Jensen, Gunther et al.

2013). This is the first study to examine the role of BIMs in systemic changes during bovine mastitis. The gene set found to undergo differential expression here is consistent with existing literature. The transcriptional activation of key pattern recognition pathways including the TLR, NLR and RIG-I pathways was also described, which likely drive the observed pro-inflammatory response. Previous studies have shown the involvement of TLR signalling in the host response to mastitis is well documented (Mitterhuemer, Petzl et al. 2010, Buitenhuis, Rontved et al. 2011, Ma, Zhu et al. 2011, Whelehan, Meade et al. 2011, Porcherie, Cunha et al. 2012), however, less is known about the involvement of the NLR and RIG-I pathways (Moyes, Drackley et al. 2009). Classically these pathways are not associated with mastitis, however recent findings suggest that RIG-I can also recognise nucleic acids released by invasive bacteria and trigger IFN-β and inflammasome activation (Abdullah, Schlee et al. 2012). These findings concur well with the observed interferon and inflammasome activation transcriptional signatures observed in Chapter 4. Several of the DE miRNAs in our study have been validated to target genes in these pathways in other species. miR-149, for example, which was down-regulated in MIMs following *S. uberis* infection, has been shown to target mouse CD14 and IRAK1 (Chi, Zang et al. 2009), key signalling proteins in the TLR pathway. Both CD14 and IRAK1 are also predicted to be targets of bta-miR-149 in our study.

In the final study (Chapter 5), potential mRNA targets of miRNAs differentially expressed in inflammatory CD14+ monocytes were further investigated. IL8 was selected as a possible target of bta-miR-200a. Previous (Chapter 4) data showed that bovine mammary gland bta-miR-200a differential expression in CD14+ monocytes

was inversely correlated (-0.28864) with IL8 expression (in the same samples). Furthermore, the miRanda (v3.3) software predicted a preferential binding site for bta-miR-200a on positions 190-196 in the 3' UTR of IL8. Specifically, an exact seed match to positions 2-8 of the mature miRNA. To validate this predicted relationship, IL8 expression *in vitro* in a bovine macrophage cell line was examined. It was demonstrated that IL8 is differentially expressed in this cell line when challenged with a pathogen (*S. uberis*) or stimulated with an appropriate agonist (LTA). To validate the predicted miRNA~mRNA relationship a miRNA mimic for bta-miR-200a was purchased. IL8 expression in the presence of this miRNA mimic was examined. MiRNA mimics are small double stranded synthetic RNAs, which imitate the action of mature miRNAs within the cell. IL8 expression was observed to be reduced in the presence of the bta-miR-200a mimic, however, further optimisation of the experiment is required. Furthermore, IL8 represents just one potential target out of many predicted relationships. Further work will be required to characterise these.

Another added advantage of high-throughput sequencing is the potential to discover novel genes or non-coding RNAs. Using miRDeep2 a miRNA discovery software package, combined with strict manual parsing 21 novel miRNAs were identified in bovine mammary epithelial cells, and a further 20 novel bovine miRNAs in MIMs/BIMs. Amongst the most interesting aspect of these findings are the 13 novel miRNAs discovered across study 1 and 2 that share significant homology to miR-2284/2285 family members. Combined, these two families of miRNAs have over 100 members. Little data exist regarding the targets or function of any of these miRNAs. Neither have they been described in any other species. Another interesting finding was the discovery of a homolog of hsa-miR-219-1 and hsa-miR-3680-3p. hsa-miR-

3680-3p is a miRNA first identified in human periphery blood (Vaz, Ahmad et al. 2010). The discovery of these miRNA further adds to the database of bovine miRNAs.

MicroRNA regulation of transcriptional expression is not a singular event; instead it is part of a larger set of regulatory mechanism which acts in cohort to regulate cellular function. For a more in depth examination of immune regulatory networks, combining additional layers of regulation would add insight to my findings. In chapter 4, a number of inducible intracellular pathways activated in response to infection were described. These pathways cooperate to induce the activation of a large number of transcription factors (TFs). Signal transduction pathways can activate a defined set of TFs that co-assemble into a higher order multi-protein complex. This multi-protein complex termed an enhanceosome (Panne 2008, Smale 2010) can concurrently or synergistically bind to specific DNA binding sites, creating a highly regulated continues recognition surface. During viral induced IFNβ expression, the interferon gene requires the activation and DNA binding of NFκB, IRF3/7, and JUN. Cooperative binding of these activators to a nucleosome-free region of the IFNβ promoter region requires precise positioning of the DNA-binding sites and assembly into an enhanceosome, providing stringent combinatorial regulation and stability (Ellwood, Huang et al. 1999, Panne 2008). Several of the TFs described here, including JUN which was inversely correlated with bta-miR-200b (-0.37095), were predicted to be targets of differentially expressed miRNAs in Chapter 4. However, TF binding is also dependent on the covalent modification of histones. TF entry into the nucleus is highly selective, and allows for the regulation of primary and secondary gene expression immune responses (Smale 2010). By concurrently examining TF

binding, histone action, and non-coding RNA expression, the multiple layers responsible for immune regulation or dysregulation could be more clearly defined.

New technologies that can map complete layers of genetic activity from single samples are allowing us to discover new layers of complexity. However, validating large numbers of computationally predicted hypothesis at the bench is proving more challenging. Simultaneously examining multiple interacting layers of regulation is a useful way of refining predictions prior to functional validation studies, providing multiple lines of evidence for the importance of particular genes or pathways of interest. To complement the miRNA-seq work presented here, integrative platforms for example, sequencing ribosome protected messenger RNA ("Ribo-Seq") would provide a more accurate picture of which transcripts are destined for protein processing, and what effect changes in miRNA expression had on protein levels. However, Ribo-Seq technology has its limitations. Isolating mRNA in ribosome bodies requires the addition of translation elongation inhibitors which prevents the extraction of further RNA products (Ingolia, Brar et al. 2012). In addition mRNA fragments extracted are only 30 nt in length, which will decrease the efficiency of down-stream alignment tools. Another sequencing-based technology that would be particularly useful to this thesis is a method of deciphering protein packaged RNA-RNA interactions directly. Crosslinking immune-precipitation sequencing (CLIP-Seq) or High-throughput sequencing of RNA isolated by crosslinking immune-precipitation (HITS-CLIP) is a method of precipitating proteins directly bound to mRNA~miRNA complexes (Darnell 2010), for example proteins (Argonaute protein) in the RNA induced silencing complex (RISC), and sequencing attached RNA.

This thesis presents significant body of transcriptomic and post-transcriptomic data, examining the host response to *S. uberis*. These findings provide in depth analysis of how monocyte and epithelial cells respond to a particular pathogen. However, numerous bacterial and fungal pathogenic agents have been associated with causing mastitis. (Bannerman, Paape et al. 2004, Petzl, Zerbe et al. 2008, Wellnitz and Bruckmaier 2012). Two such pathogens, *E. coli* and *S. aureus* are associated with significant financial losses to the dairy sector (Oviedo-Boyso, Valdez-Alarcon et al. 2007, Wellnitz and Bruckmaier 2012). As shown in Chapter 3, Gram-positive bacteria present a unique profile in comparison to Gram-negative bacteria. By characterising miRNAs which are induced by a panel of specific pathogens, a miRNA-based signature of the infectious agent could be identified. Using miRNAs as non-invasive biomarkers is not uncommon. They exhibit many properties that have made them of significant interest as non-invasive biomarkers. They are abundantly and stably expressed in a range of accessible tissues including serum, milk, and urine where they can be readily measured (Chen, Gao et al. 2010, Hata, Murakami et al. 2010, Kosaka, Izumi et al. 2010). Importantly for a potential biomarker, miRNAs have a high information content, and the expression profile of panels of miRNAs have been shown to be diagnostic of disease (De Guire, Robitaille et al. 2013). Identifying a diagnostic biomarker for a panel of mastitis causing pathogens would allow tailored treatment strategies, and reduce dependence on broad spectrum antibiotics.

It is undeniable that high-throughput sequencing technology is part of a growing trend of mass data generation and analytics not unique to biology. Indeed, many real world enterprises including, internet security, sport analysis (Arandjelovic 2013), personalised medicine (Balla, Arvai et al. 2014), stock markets

(http://www.bigdatafs.com/), and even policing (http://www.californiareport.org/) now utilise increased server capacity to carry out analytics with the shared goal of identifying biological or human patterns. However, within the agri-food sector there remains both a skill and infrastructure shortage needed to utilise and manage "Big Data". In the context of immune biology, this is an exciting time as more funding is committed to identifying system wide regulatory layers such as the epigenome, transcriptome, and microRNome (Kuchen, Resch et al. 2010). Much as the evolution of qPCR, now known as the gold standard in gene quantification, signalled a push in biological interpretation, so too will high-throughput sequencing become a standardised experimental technique commonly used at the bench (Tam, de Borja et al. 2014).

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Appendix:

Appendix 1: Fluorescence-activated cell sorting (FACS) data for blood and milk CD14+ monocytes in each infected animal and control animal at at A) 0 h, B) 12 h, C) 24 h, D) 36 H, and E) 48 h.

Appendix 2: Manually generated pathway annotations for inflammasome and interferon pathways.

Appendix 3: All miRNA library were assessed using micro-capillary electrophoresis (Agilent Bioanalyzer) on a high sensitivity DNA analysis chip.

Appendix 4: FastQC checks of each library were carried out as a method of quality control checking each miRNA sequencing dataset.

Appendix 5.1: Correlation coefficients of each miRNA normalized read count dataset between samples at each time-point for control and infected technical replicates above 10 tpm.

Appendix 5.2: Gene expression density plots comparing raw and normalised mammary epithelial cell miRNA reads for both control and infected samples.

Appendix 6: Multidimensional scaling (MDS) plots A) 1 hour post infection, B) 2 hour post infection, C) 4 hour post infection, D) 6 hour post infection and E) all samples.

Appendix 7: miRNAs that are expressed at ≥1 tag per million in bovine mammary epithelial cells.

Appendix 8: Differentially expressed miRNAs examined for each mammary epithelial cell sample across full time course as normalised tag counts.

Appendix 9: Summary of lipopolysaccharide (LPS) responsive microRNAs.

Appendix 10: Genes predicted to be targeted by differentially expressed miRNAs.

Appendix 11: Summary of milk volumes, rectal temperatures, ambient temperatures, humidity, bacterial CFU counts, cell counts, CD14+ cells counts, RNA (ng/uL), RIN, 18/28s ratio, and total RNA data for each animal.

Appendix 12: FastQC checks on each library was carried out as a method of quality control checking each sequencing dataset.

Appendix 13: Summary read statistics and number of unique alignments for each blood and milk RNA library.

Appendix 14.1: Average correlation coefficients of blood and milk mRNA normalized read counts between samples at each time-point for control and infected biological replicates.

Appendix 14.2: Gene expression density plots comparing raw and normalised blood & milk CD14+ mRNA reads for both control and infected samples.

Appendix 15.1-15.5: Multidimensional scaling (MDS) plots for milk mRNA (15.1), blood mRNA (15.2), milk miRNA (15.3), blood miRNA sequencing dataset (15.4), and all time-points (15.5). A) 0 hour post infection, B) 12 hour post infection, C) 24 hour post infection, D) 36 hour post infection and E) 48 hour post infection.

Appendix 15.6-15.9: Heatmap of log2 normalised mRNA expression of all A) milk mRNA, B) blood mRNA, and C) milk miRNA differentially expressed genes (tags per million) for infected and control animals at 12, 24, 36, and 48 hours post infection.

Appendix 16: Representative reference genes A) YWHAZ, B) GAPDH, C) PGK1, D) SDHA, and E) HPRT).

Appendix 17: Representative genes of interest A) CCR1, B) IL1β, C) IL8, D) TLR2, E) TLR4 F) TNF-α, G) IL6.

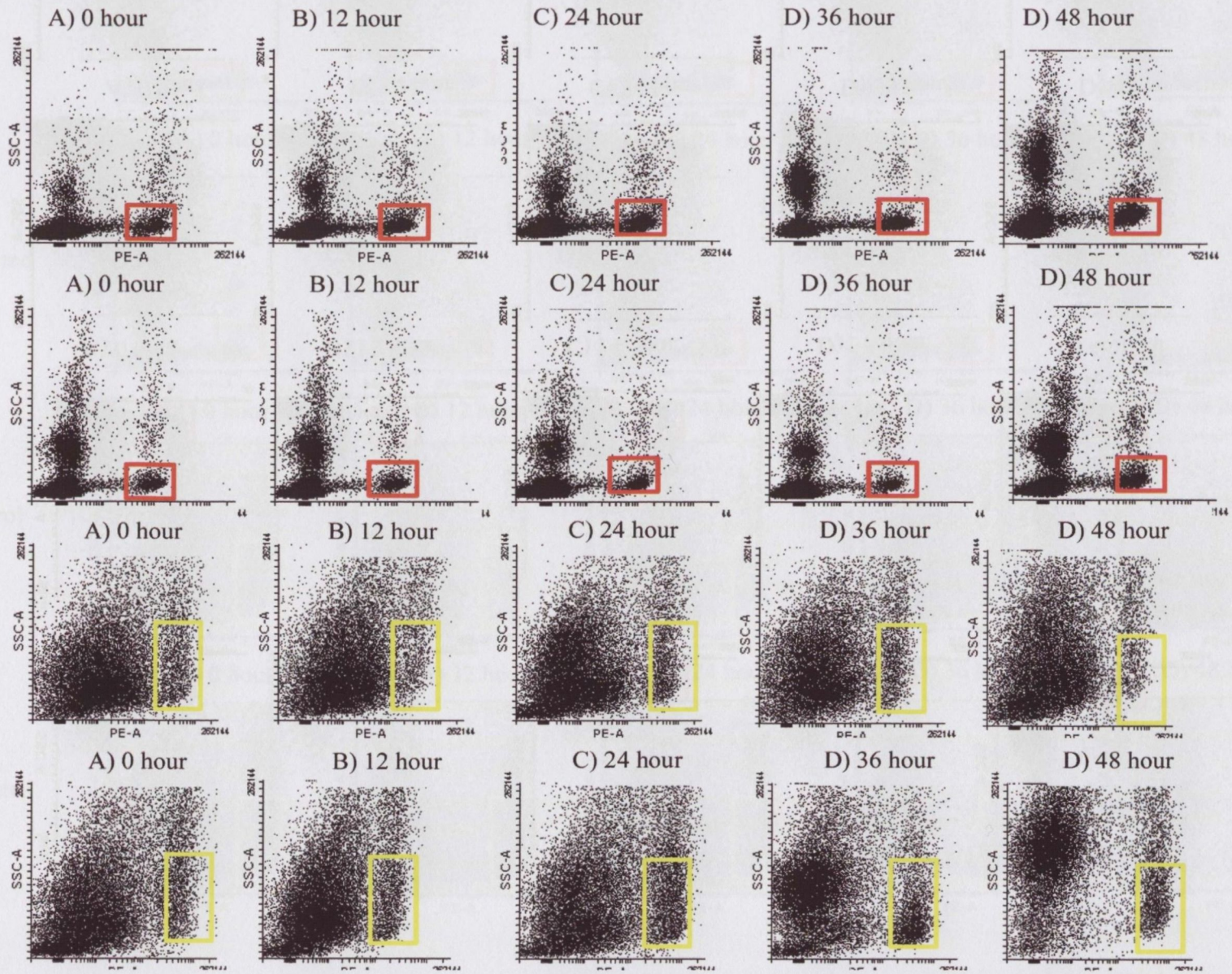
Appendix 18: The top contextual hubs identified using the CHAT software.

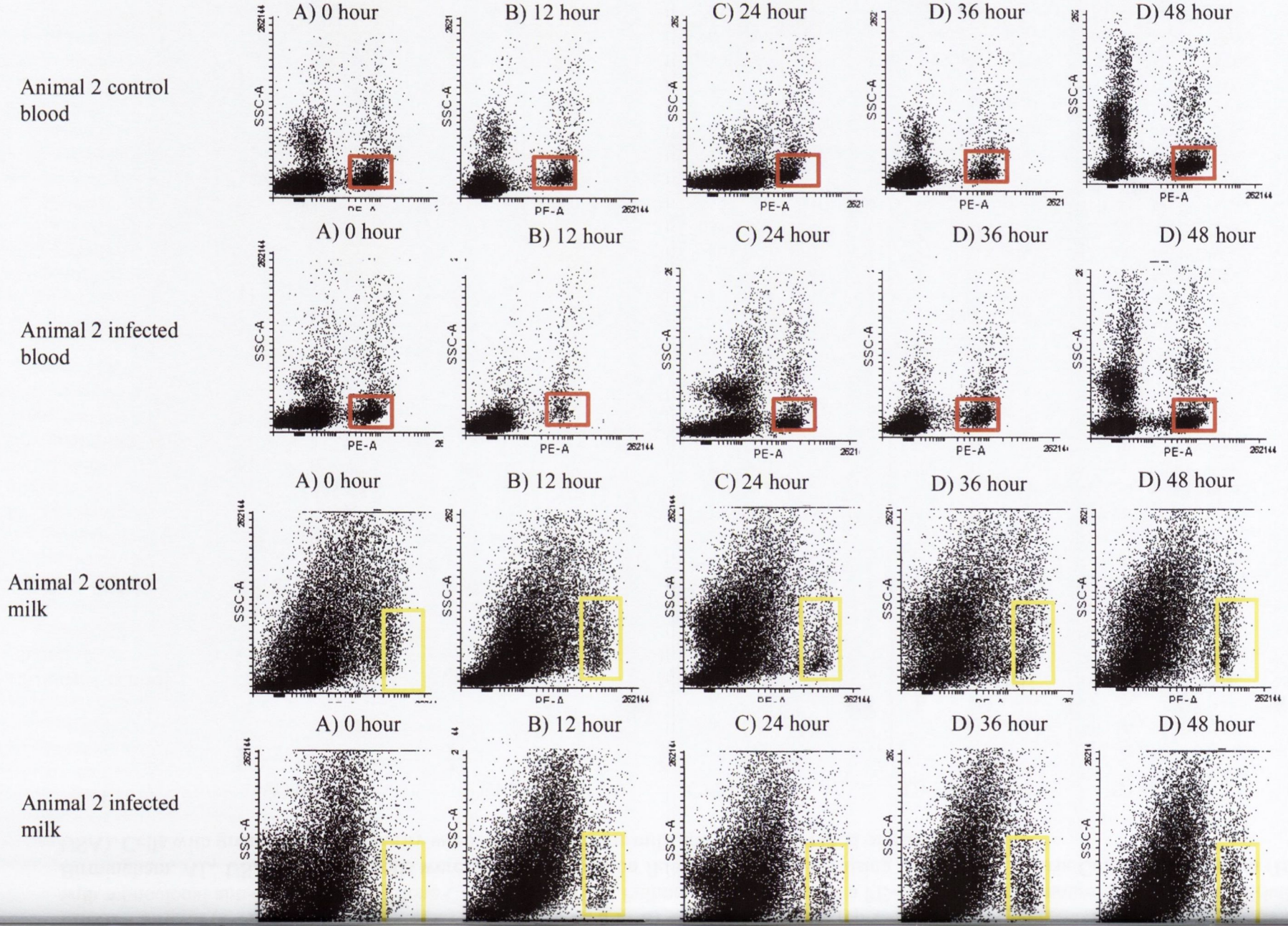
Appendix 19.1: Correlation coefficients of each blood and milk miRNA normalized read count dataset between samples at each time-point for control and infected technical replicates above 100 tpm.

Appendix 19.2: Gene expression density plots comparing raw and normalised blood & milk CD14+ miRNA reads for both control and infected samples.

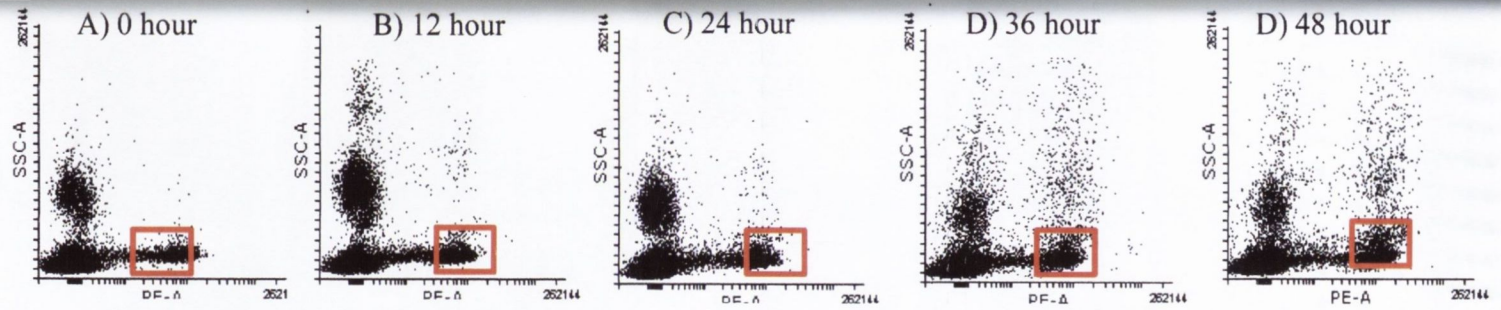
Appendix 21: Differentially expressed miRNAs examined for milk CD14+ samples (A-S), blood CD14+ samples (T) and milk CD14+ reference miRNA genes (U-W) across full time course as normalised tag counts.

Appendix 1.1: Fluorescence-activated cell sorting (FACS) data for blood (red) and milk (yellow) CD14+ monocytes in each control animal (1-5) and infected animal (1-5) at A) 0 h, B) 12 h, C) 24 h, D) 36 H, and E) 48 h. Dot plots are represented on SSC-A (X-axis) and PE-A (Y-axis). Cells were labelled with monoclonal anti-bovine CD14 (Clone CAM36A, VMRD, Pullman, WA, USA) and a PE-conjugated anti-mouse IgG1 antibody (Southern Biotechnology, Birmingham, AL, USA). Labelled cells were separated based on fluorescence intensity using the BD FACS Aria Cell Sorting System (BD Biosciences, CA, USA). Cells with greater than 95% purity were isolated from the milk and peripheral blood of each animal.

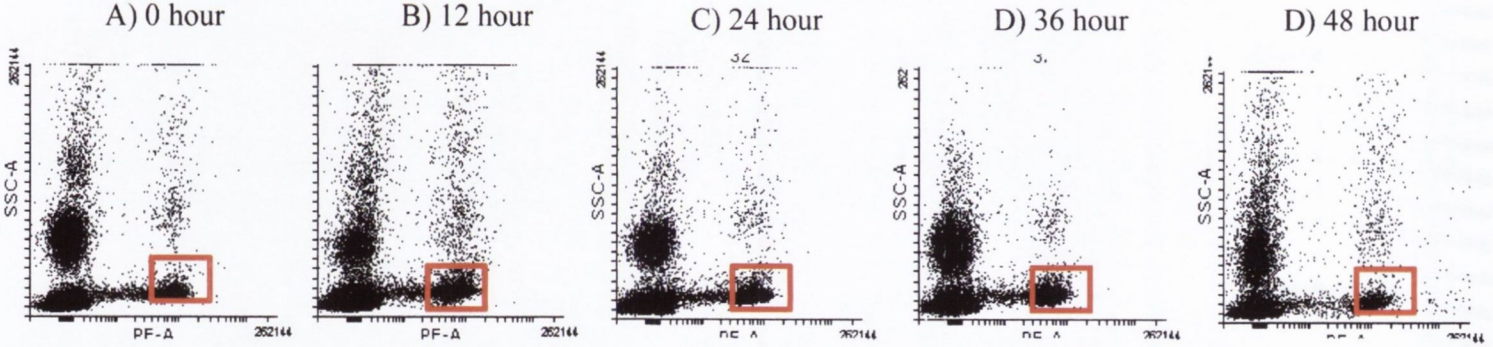



Animal 2 control blood
A) 0 hour
B) 12 hour
C) 24 hour
D) 36 hour
D) 48 hour
Animal 2 infected blood
A) 0 hour
B) 12 hour
C) 24 hour
D) 36 hour
D) 48 hour
Animal 2 control milk
A) 0 hour
B) 12 hour
C) 24 hour
D) 36 hour
D) 48 hour
Animal 2 infected milk
A) 0 hour
B) 12 hour
C) 24 hour
D) 36 hour
D) 48 hour
SSC-A
PE-A

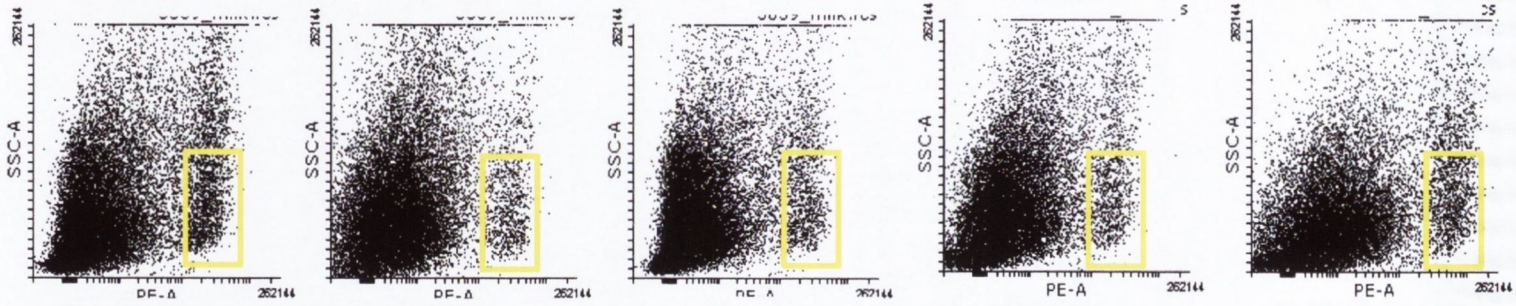
Animal 3 control blood



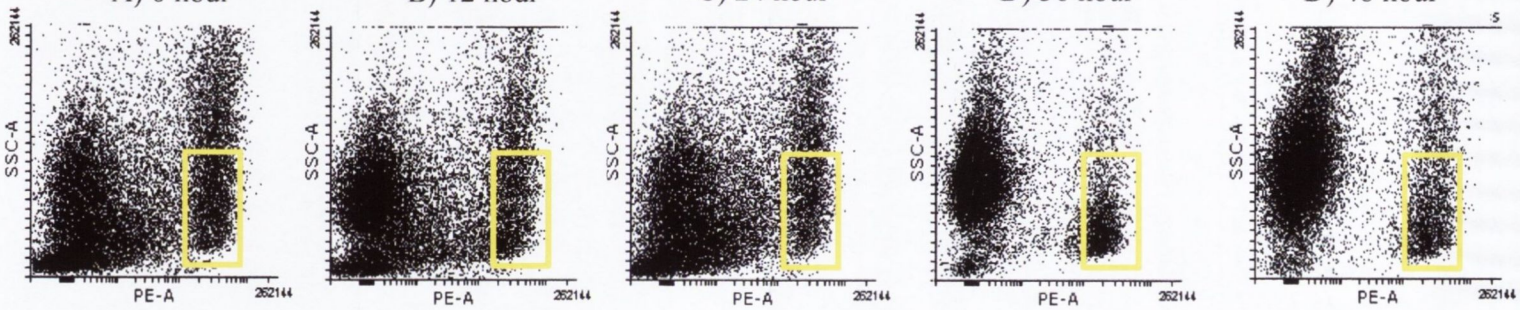
Animal 3 infected blood

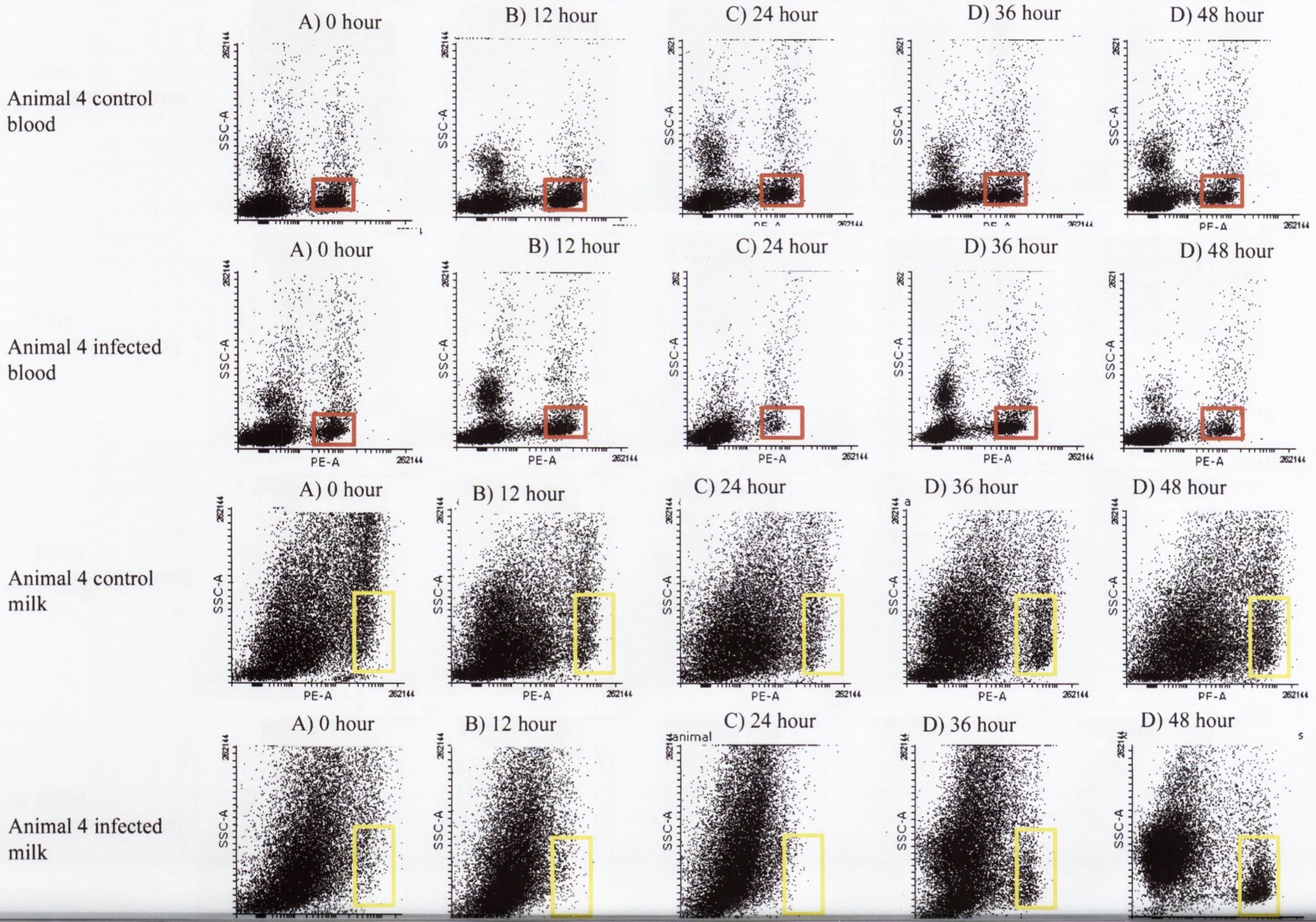


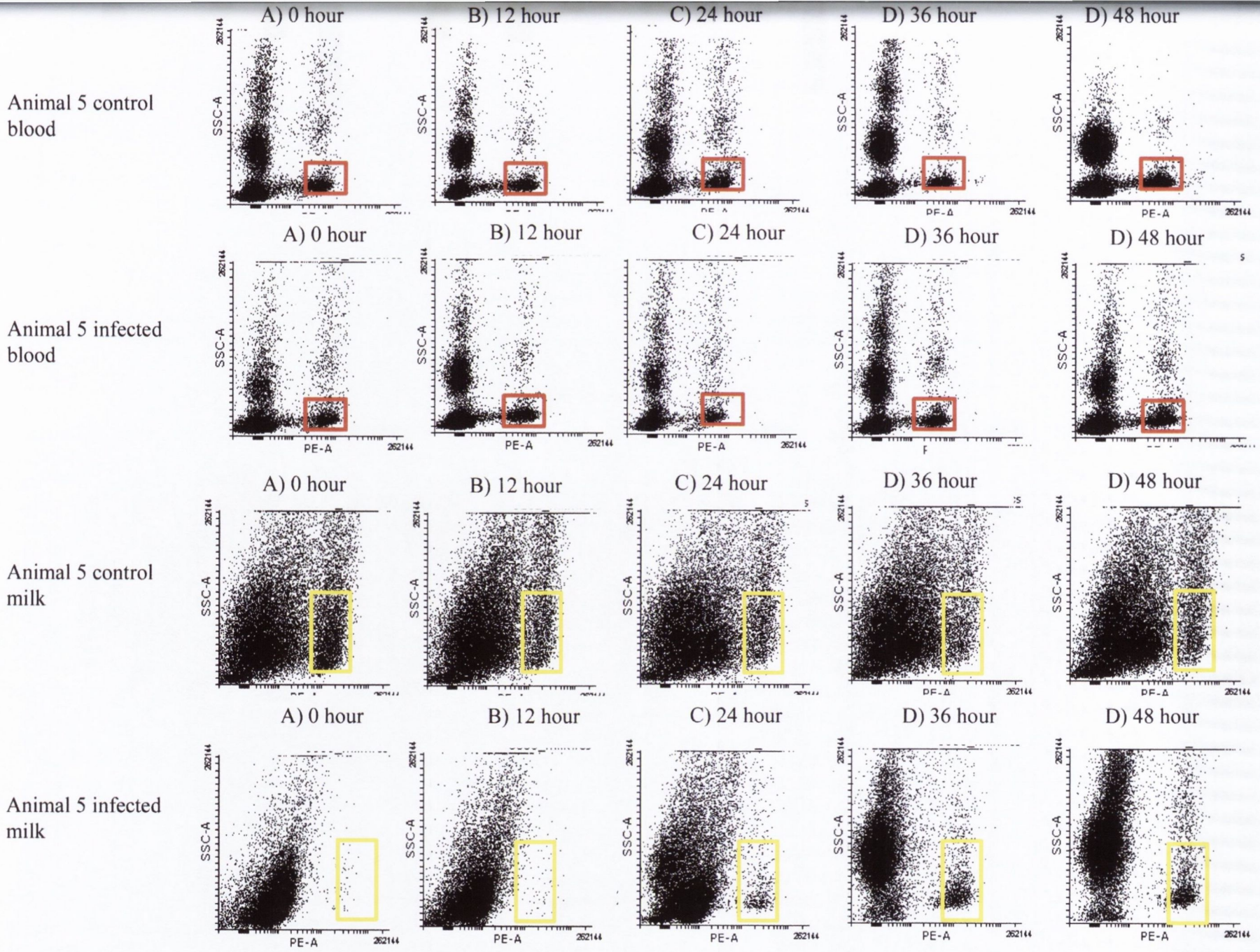
Animal 3 control milk



Animal 3 infected milk

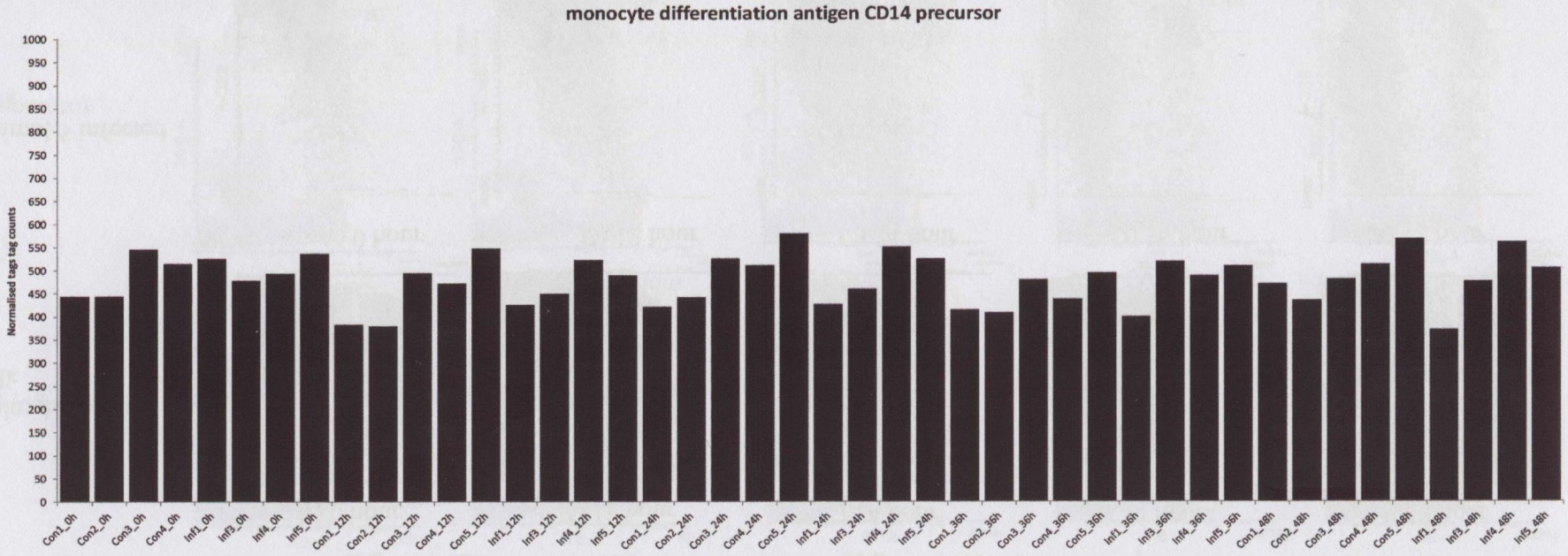



A) 0 hour
B) 12 hour
C) 24 hour
D) 36 hour
D) 48 hour
Animal 4 control blood
Animal 4 infected blood
Animal 4 control milk
Animal 4 infected milk
SSC-A
PE-A


A) 0 hour
B) 12 hour
C) 24 hour
D) 36 hour
D) 48 hour
Animal 5 control blood
Animal 5 infected blood
Animal 5 control milk
Animal 5 infected milk
SSC-A
PE-A
262144

Appendix 1.2: Surface expression markers examined for non-specific cellular contamination of blood A) CD14, B) CD3, C) CD4, D) CCR7, E) CD22, F) CD19, and milk G) CD14, H) CD4, I) CD24, J) CD24, K) CK-8, L) APB, and M) STAT1 derived CD14+ monocyte populations. Genes were plotted for full time course on normalised datasets.

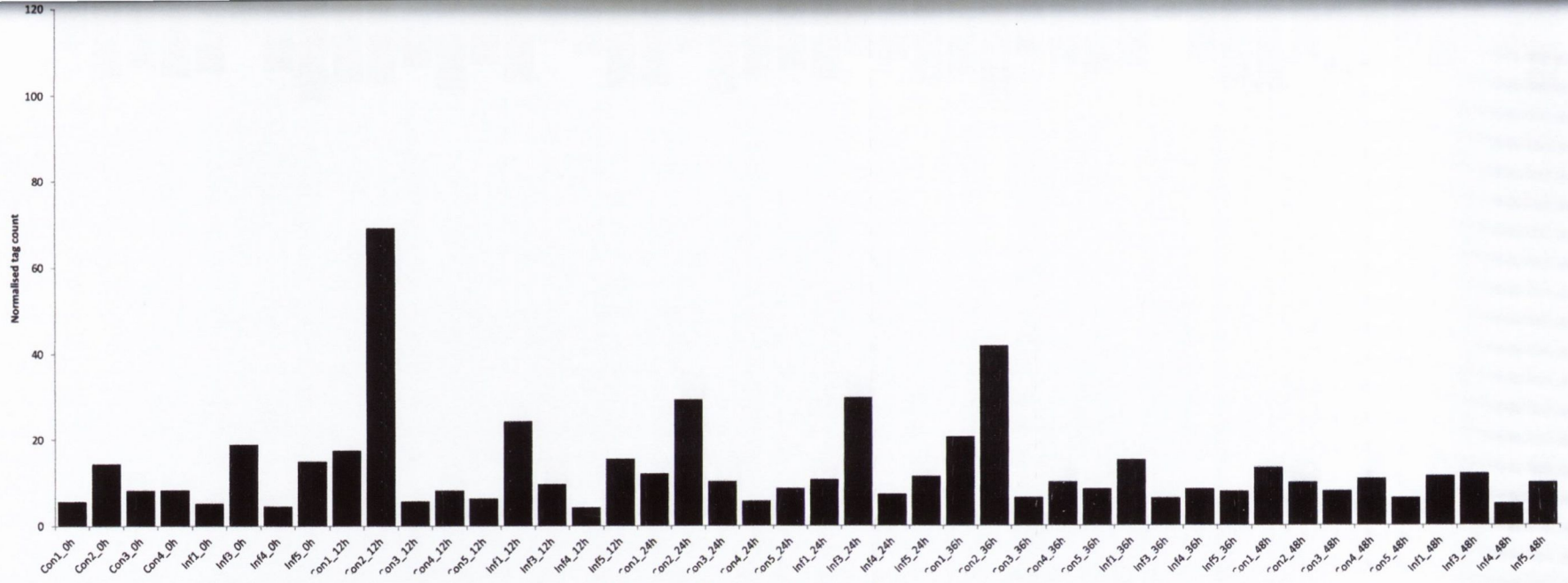
A



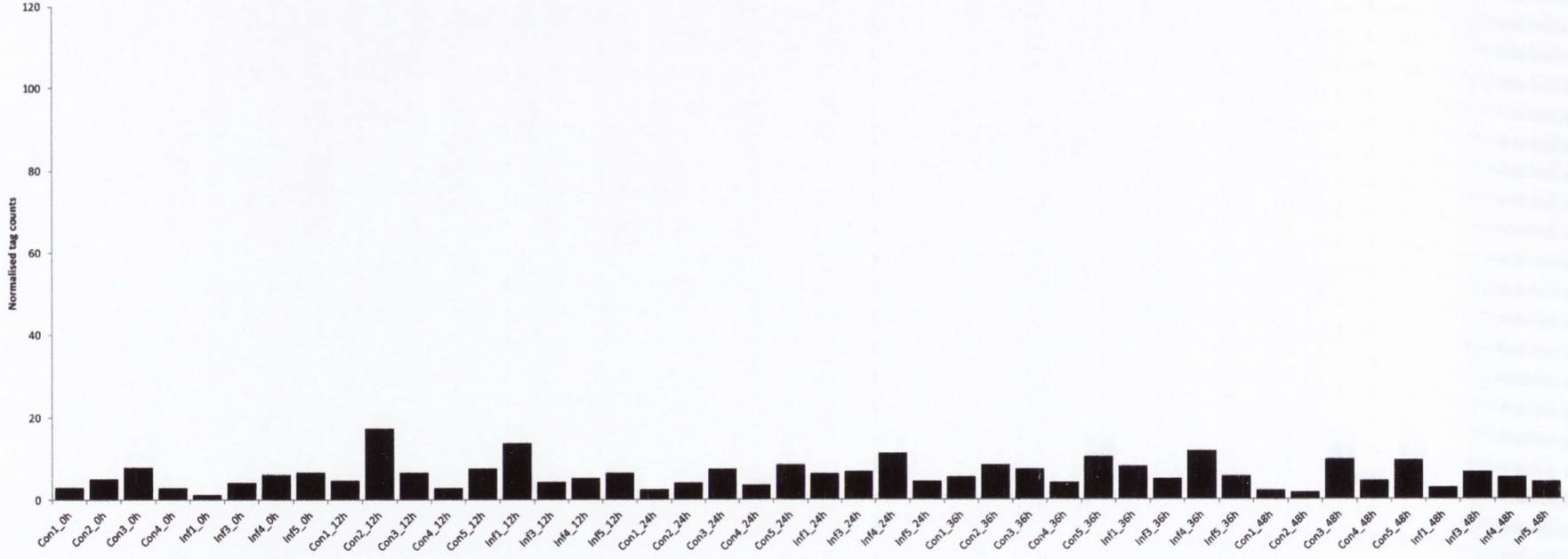
B



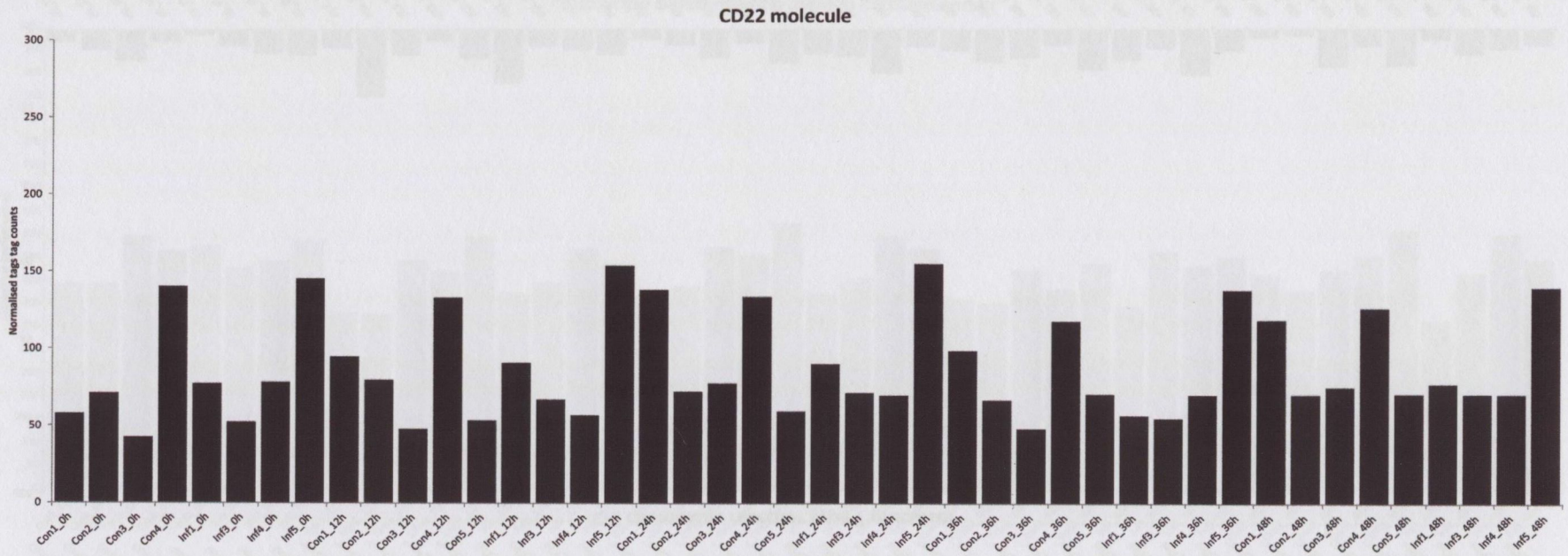
D



C-C chemokine receptor type 7 precursor



E


CD22 molecule
Normalised tags tag counts
300
250
200
150
100
50
0
Con1_0h
Con2_0h
Con3_0h
Con4_0h
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Inf3_0h
Inf4_0h
Inf5_0h
Con1_12h
Con2_12h
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Inf5_36h
Con1_48h
Con2_48h
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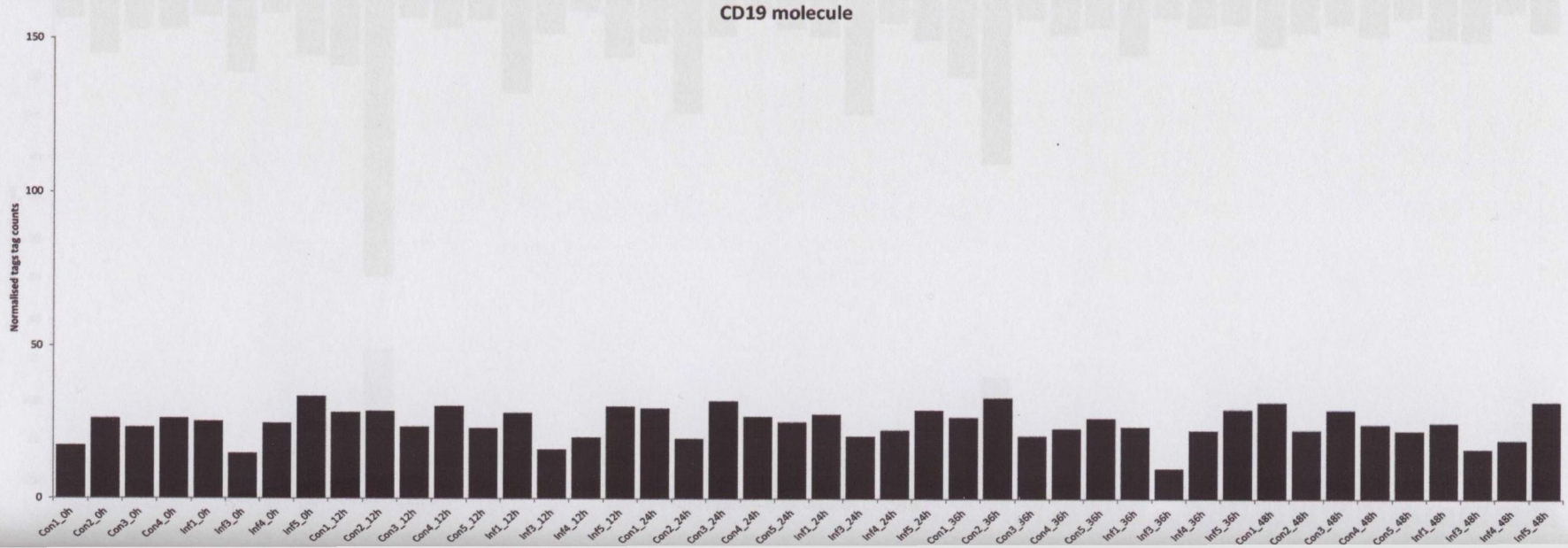
Inf1_48h
Inf3_48h
Inf4_48h
Inf5_48h

F


CD19 molecule
Normalised tags tag counts
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Con2_0h
Con3_0h
Con4_0h
Inf1_0h
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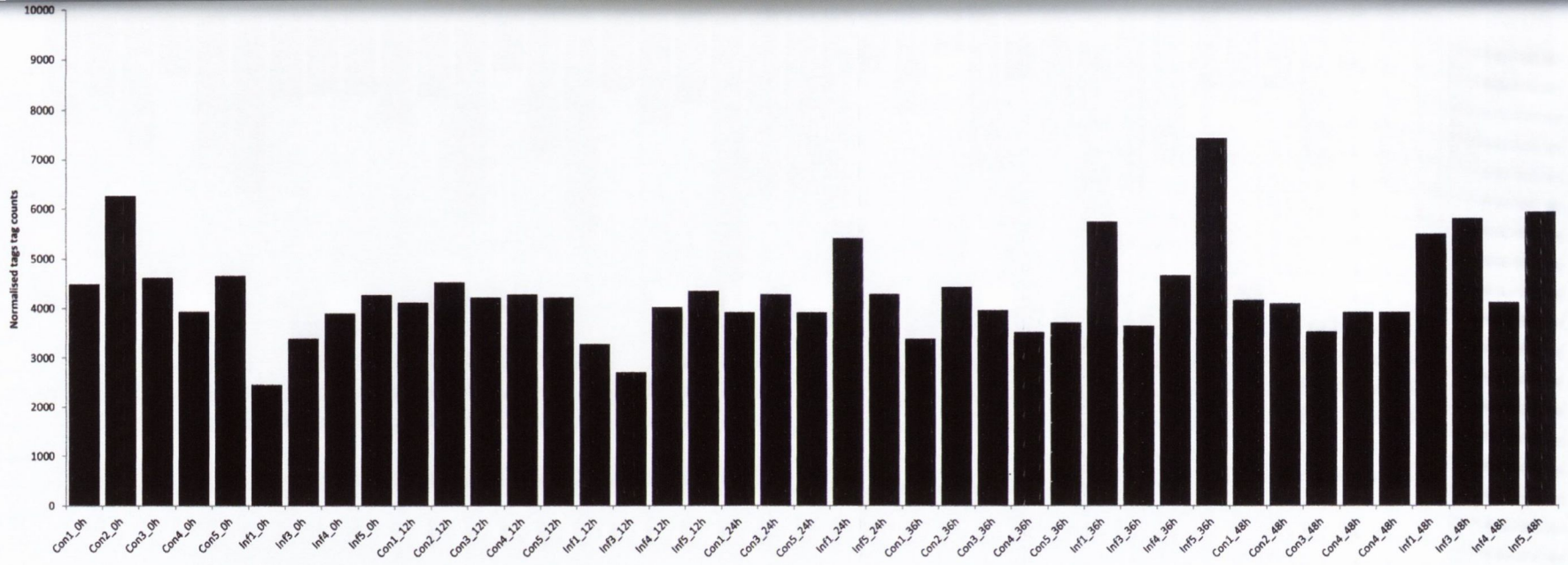
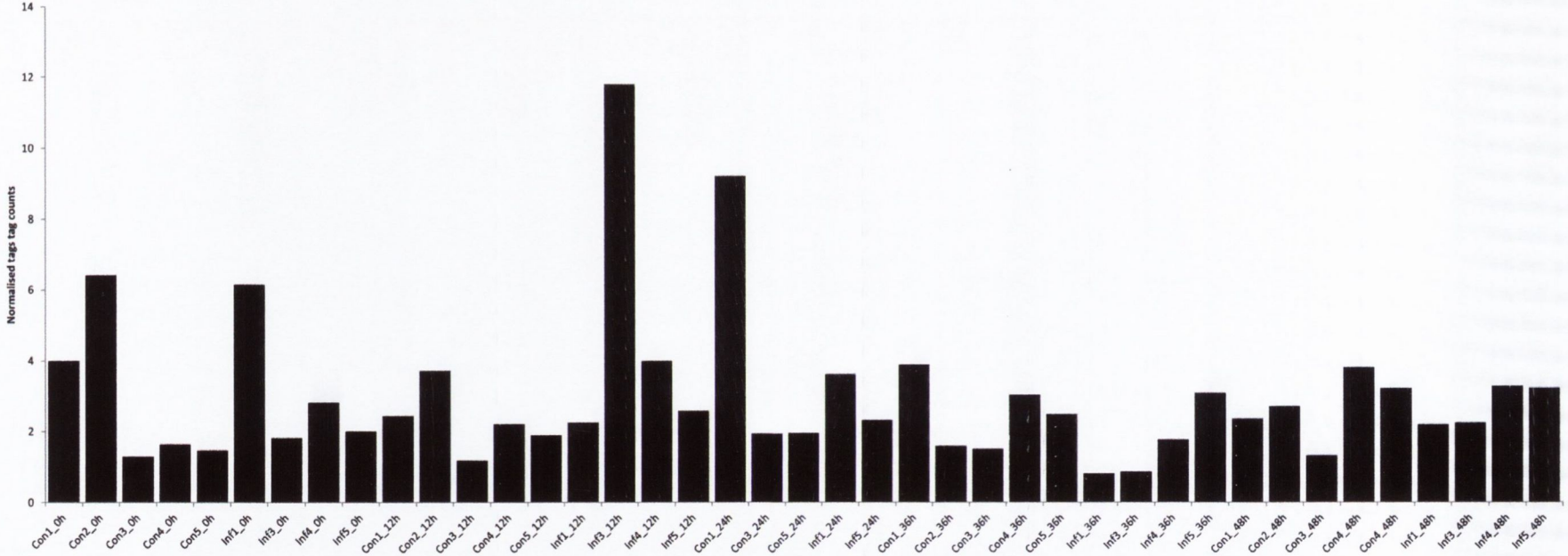
Inf5_48h

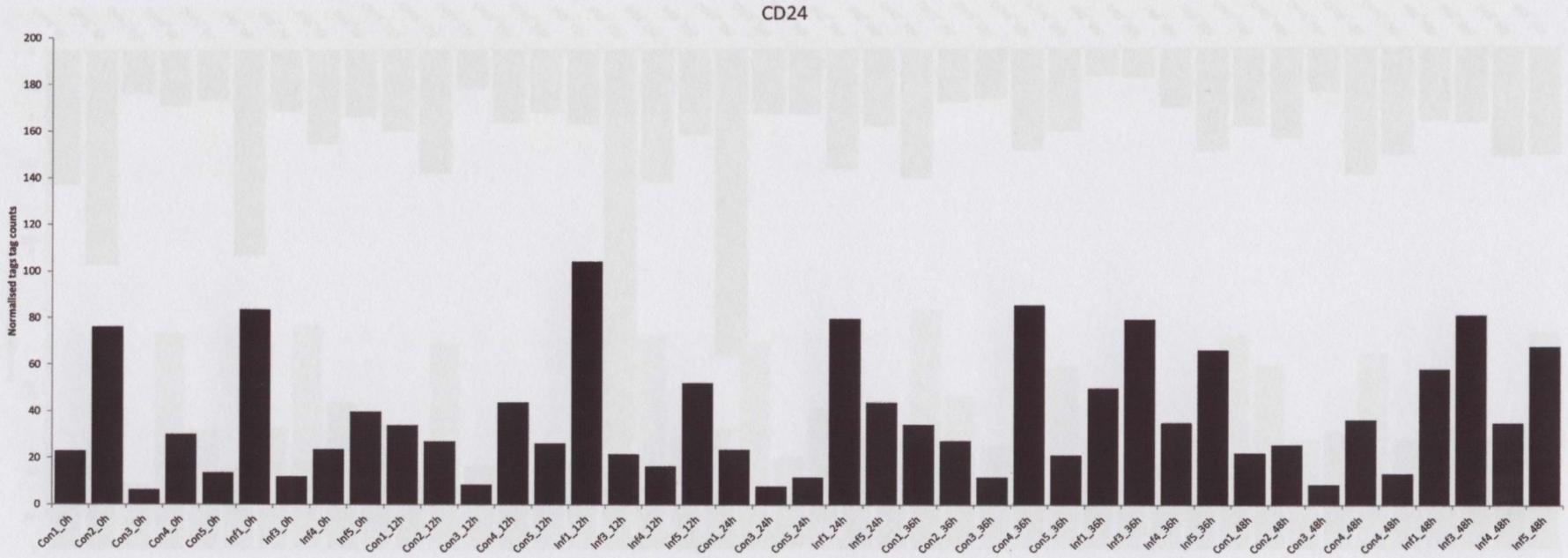


H

T-cell surface glycoprotein CD4 precursor



I


CD24
Normalised tags tag counts
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20
40
60
80
100
120
140
160
180
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Inf5_36h
Con1_48h
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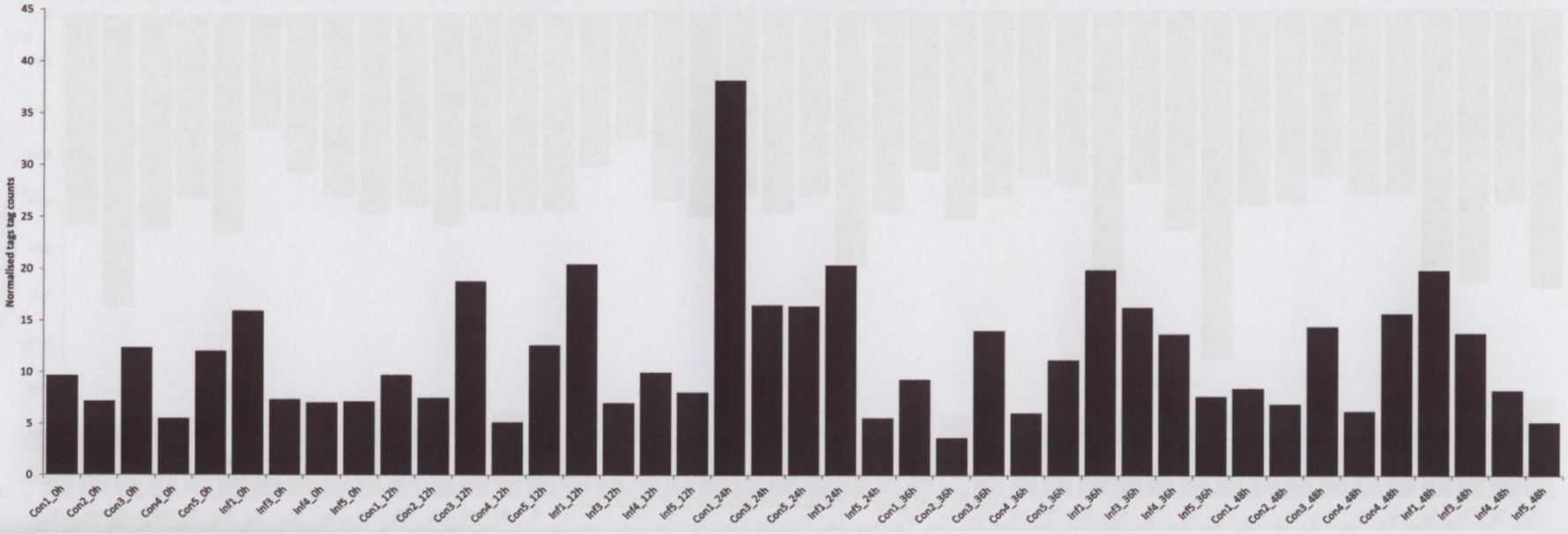
Inf4_48h
Inf5_48h

J


angiotensin I converting enzyme (peptidyl-dipeptidase A) 3 precursor
Normalised tags tag counts
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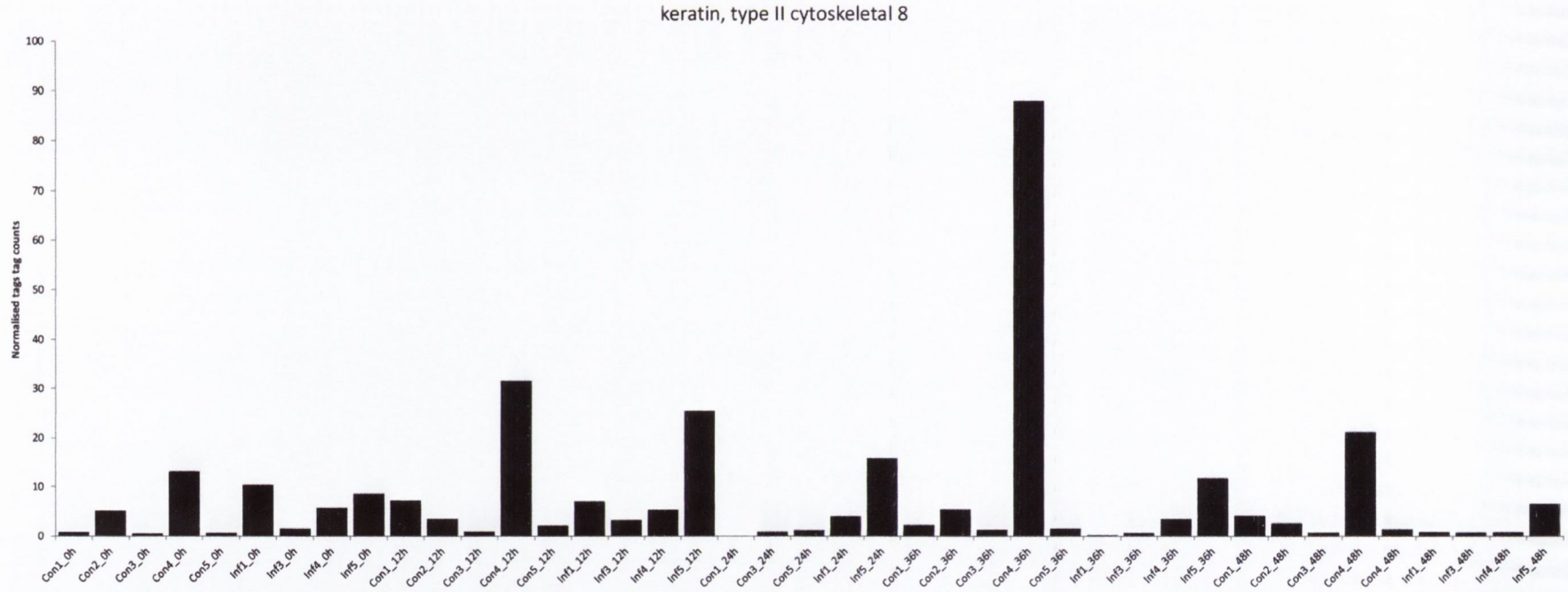
10
15
20
25
30
35
40
45
Con1_0h
Con2_0h
Con3_0h
Con4_0h
Con5_0h
Inf1_0h
Inf3_0h
Inf4_0h
Inf5_0h
Con1_12h
Con2_12h
Con3_12h
Con4_12h
Con5_12h
Inf1_12h
Inf3_12h
Inf4_12h
Inf5_12h
Con1_24h
Con3_24h
Con5_24h
Inf1_24h
Inf5_24h
Con1_36h
Con2_36h
Con3_36h
Con4_36h
Con5_36h
Inf1_36h
Inf3_36h
Inf4_36h
Inf5_36h
Con1_48h
Con2_48h
Con3_48h
Con4_48h
Con4_48h
Inf1_48h
Inf3_48h
Inf4_48h
Inf5_48h

K


keratin, type II cytoskeletal 8
Normalised tags tag counts
100
90
80
70
60
50
40
30
20
10
0
Con1_0h
Con2_0h
Con3_0h
Con4_0h
Con5_0h
Inf1_0h
Inf3_0h
Inf4_0h
Inf5_0h
Con1_12h
Con2_12h
Con3_12h
Con4_12h
Con5_12h
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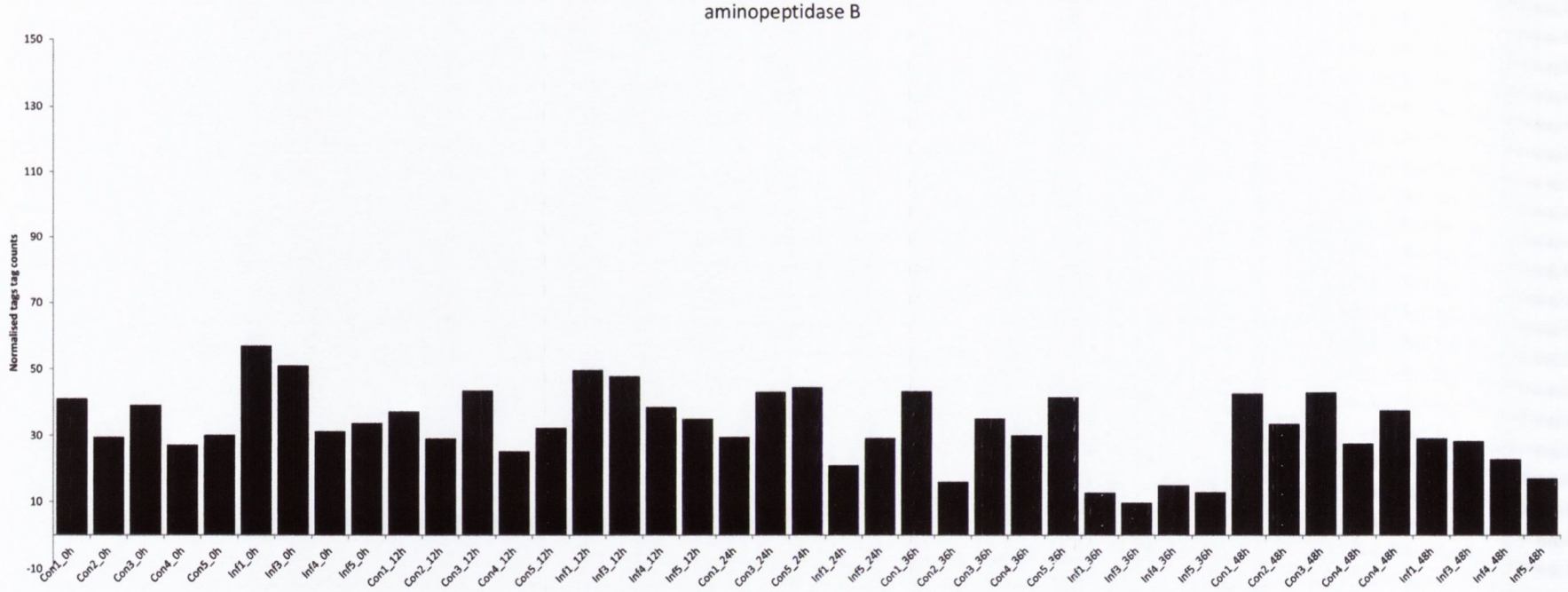
Con4_36h
Con5_36h
Inf1_36h
Inf3_36h
Inf4_36h
Inf5_36h
Con1_48h
Con2_48h
Con3_48h
Con4_48h
Con4_48h
Inf1_48h
Inf3_48h
Inf4_48h
Inf5_48h

L


aminopeptidase B
Normalised tags tag counts
150
130
110
90
70
50
30
10
-10
Con1_0h
Con2_0h
Con3_0h
Con4_0h
Con5_0h
Inf1_0h
Inf3_0h
Inf4_0h
Inf5_0h
Con1_12h
Con2_12h
Con3_12h
Con4_12h
Con5_12h
Inf1_12h
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Inf5_36h
Con1_48h
Con2_48h
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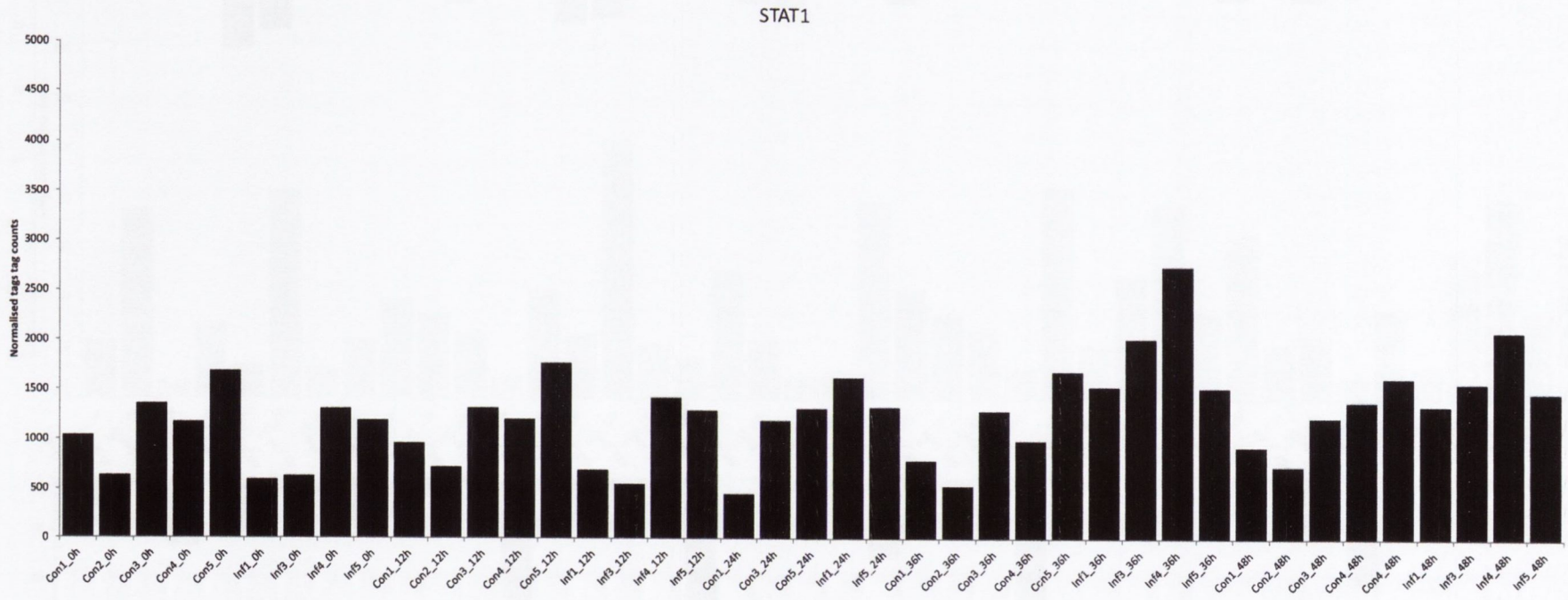
Con4_48h
Con4_48h
Inf1_48h
Inf3_48h
Inf4_48h
Inf5_48h

M


STAT1
Normalised tags tag counts
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4500
4000
3500
3000
2500
2000
1500
1000
500
0
Con1_0h
Con2_0h
Con3_0h
Con4_0h
Con5_0h
Inf1_0h
Inf3_0h
Inf4_0h
Inf5_0h
Con1_12h
Con2_12h
Con3_12h
Con4_12h
Con5_12h
Inf1_12h
Inf3_12h
Inf4_12h
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Con2_36h
Con3_36h
Con4_36h
Con5_36h
Inf1_36h
Inf3_36h
Inf4_36h
Inf5_36h
Con1_48h
Con2_48h
Con3_48h
Con4_48h
Con4_48h
Inf1_48h
Inf3_48h
Inf4_48h
Inf5_48h

Appendix 2: Manually generated pathway annotations for inflammasome and interferon pathways. Sourced from SA biosciences (Qiagen) RT2 Profiler™ PCR Array Human Interferon and Receptors (PAHS-064A), and RT2 Profiler™ PCR Array Human Inflammasome (PAHS-097A) annotations

RT² Profiler™ PCR Array Human Inflammasomes (PAHS-097A) "Inflammasome"	RT² Profiler™ PCR Array Human Interferon and Receptors (PAHS-064A) "Interferon"
ENSG00000163568	ENSG00000006652
ENSG00000171791	ENSG00000016402
ENSG00000171552	ENSG00000027697
ENSG00000110330	ENSG00000068079
ENSG00000023445	ENSG00000077238
ENSG00000255501	ENSG00000089127
ENSG00000132357	ENSG00000091181
ENSG00000137752	ENSG00000092010
ENSG00000196954	ENSG00000100385
ENSG00000137757	ENSG00000103522
ENSG00000064012	ENSG00000105246
ENSG00000108691	ENSG00000110324
ENSG00000161570	ENSG00000111537
ENSG00000108688	ENSG00000113302
ENSG00000102245	ENSG00000115267
ENSG00000003402	ENSG00000116678
ENSG00000213341	ENSG00000117400
ENSG00000179583	ENSG00000117525
ENSG00000164733	ENSG00000117595
ENSG00000163739	ENSG00000119535
ENSG00000081041	ENSG00000119917
ENSG00000168040	ENSG00000119922
ENSG00000080824	ENSG00000120235
ENSG00000096384	ENSG00000120242
ENSG00000166598	ENSG00000122756
ENSG00000171855	ENSG00000124334
ENSG00000111537	ENSG00000125347
ENSG00000104365	ENSG00000126456
ENSG00000073009	ENSG00000126709
ENSG00000269335	ENSG00000128604
ENSG00000168811	ENSG00000131724
ENSG00000113302	ENSG00000135899
ENSG00000150782	ENSG00000136244
ENSG00000125538	ENSG00000137070
ENSG00000137033	ENSG00000137080
ENSG00000136244	ENSG00000137265
ENSG00000184216	ENSG00000137959
ENSG00000268944	ENSG00000137965
ENSG00000125347	ENSG00000140968
ENSG00000168310	ENSG00000142166
ENSG00000135341	ENSG00000147168
ENSG00000100324	ENSG00000147873
ENSG00000055208	ENSG00000147896

ENSG00000100030	ENSG00000155657
ENSG00000185386	ENSG00000157601
ENSG00000188130	ENSG00000159110
ENSG00000156711	ENSG00000159128
ENSG00000102882	ENSG00000160710
ENSG00000107643	ENSG00000160712
ENSG00000050748	ENSG00000163564
ENSG00000103313	ENSG00000163565
ENSG00000172936	ENSG00000164136
ENSG00000249437	ENSG00000164485
ENSG00000263062	ENSG00000164509
ENSG00000109320	ENSG00000165949
ENSG00000100906	ENSG00000168264
ENSG00000104825	ENSG00000168310
ENSG00000091106	ENSG00000168685
ENSG00000140853	ENSG00000169245
ENSG00000091592	ENSG00000170604
ENSG00000142405	ENSG00000171855
ENSG00000162711	ENSG00000174564
ENSG00000160505	ENSG00000177047
ENSG00000171487	ENSG00000184995
ENSG00000174885	ENSG00000185201
ENSG00000185792	ENSG00000185291
ENSG00000160703	ENSG00000185507
ENSG00000167207	ENSG00000185745
ENSG00000089041	ENSG00000185885
ENSG00000110218	ENSG00000187608
ENSG00000162734	ENSG00000188379
ENSG00000140368	ENSG00000197919
ENSG00000073756	ENSG00000198223
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ENSG00000169900	ENSG00000205755
ENSG00000080823	ENSG00000214706
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ENSG00000104312	ENSG00000228083
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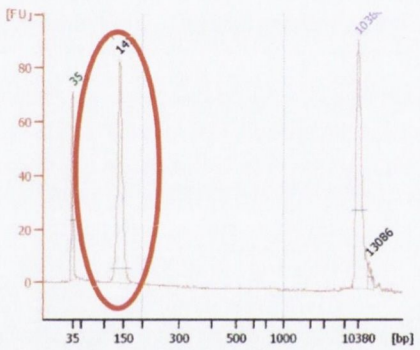
ENSG00000150455	ENSG00000237693
ENSG00000232810;	ENSG00000243646
ENSG00000120659	ENSG00000262795
ENSG00000125735	ENSG00000263093
ENSG00000117586	ENSG00000182393
ENSG00000175104	
ENSG00000117289	
ENSG00000101966	
ENSG00000265972	
ENSG00000230108	

ENSG00000223952	
ENSG00000206439	
ENSG00000228978	
ENSG00000228849	
ENSG00000228321	
ENSG00000204490	
*Note additional 11 human Ensemble ID added	

Appendix 3: All miRNA library were assessed using micro-capillary electrophoresis (Agilent Bioanalyzer) on a high sensitivity DNA analysis chip. Reference ladders are visible at both 35 bp and 10380 bp. Libraries ranged in size from 130-145 bp in length. High sensitivity DNA chips are the most accurate method available to analyse miRNA sequencing libraries for overall quality and ensure the adaptors and barcodes have successfully annealed to RNA based on overall size range. [FU] = fluorescent units [bp] = base pairs. Each miRNA library has been circled in red.

Control 1 h replicate 1



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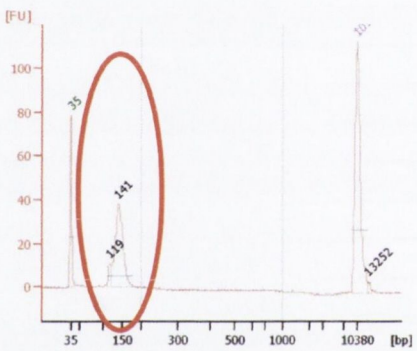

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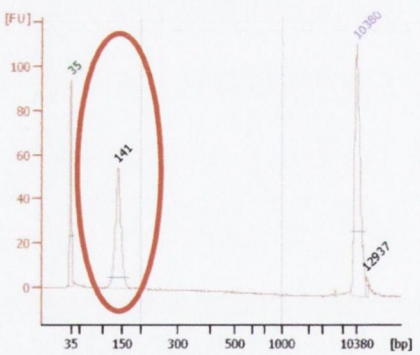

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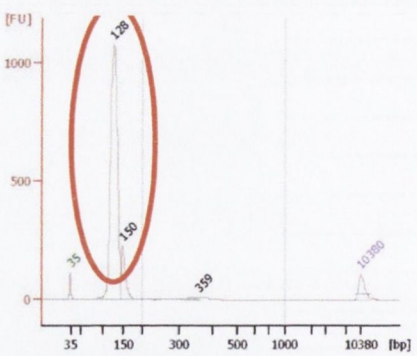
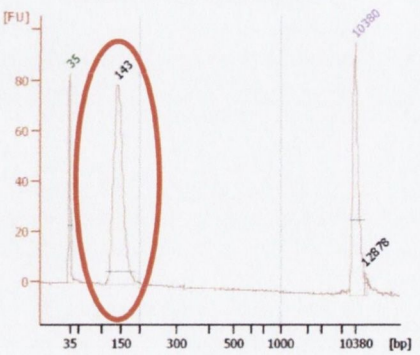


Control 1 h replicate 3



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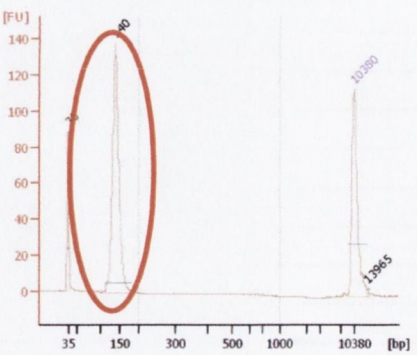

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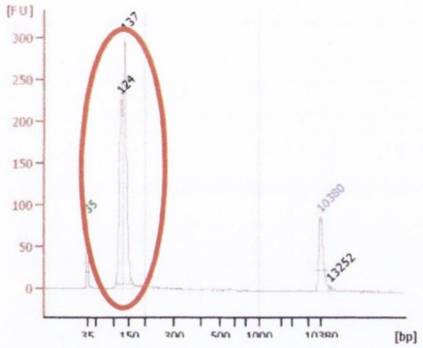

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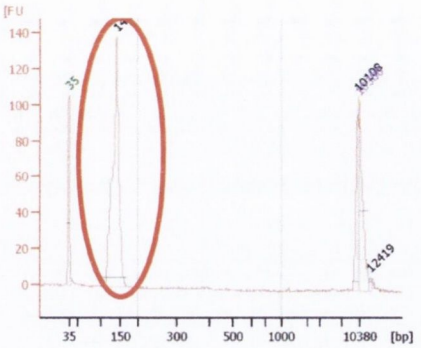

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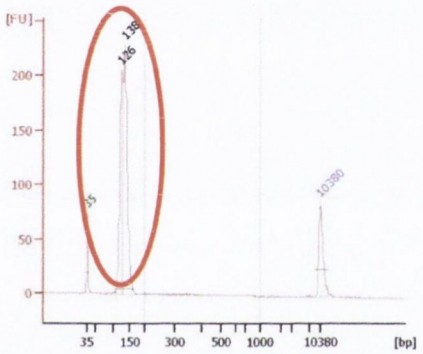

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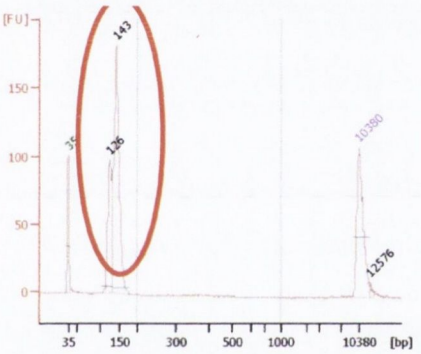

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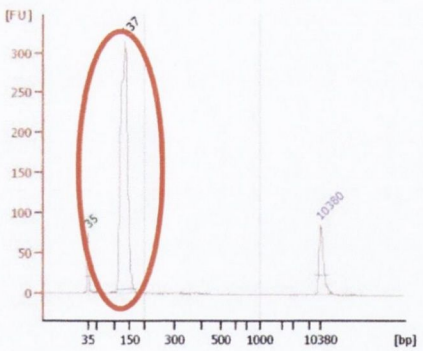

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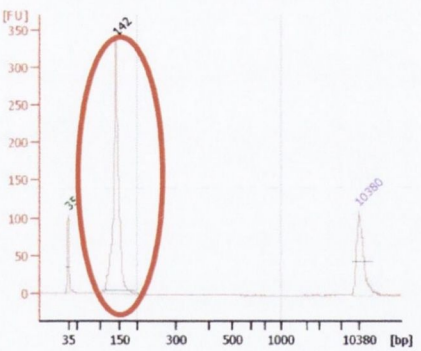

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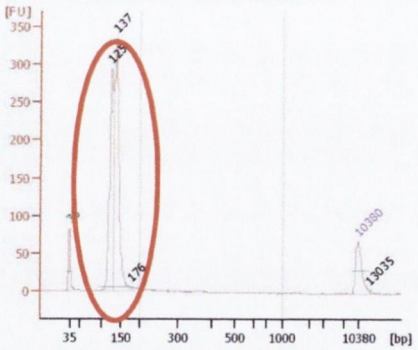

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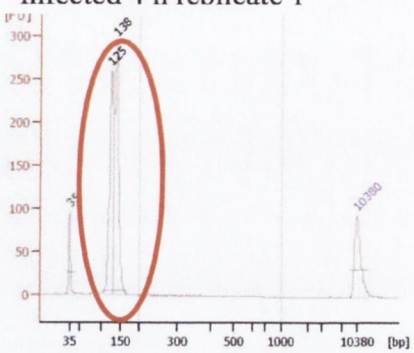

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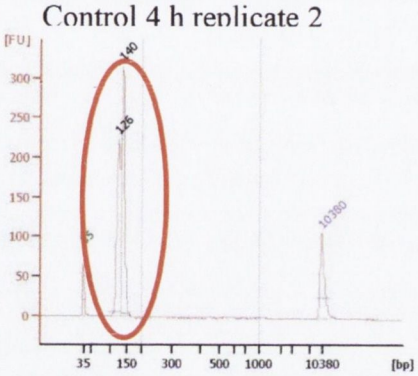

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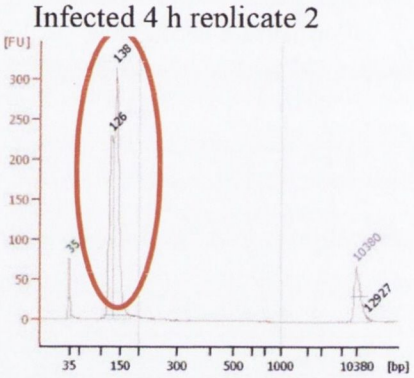

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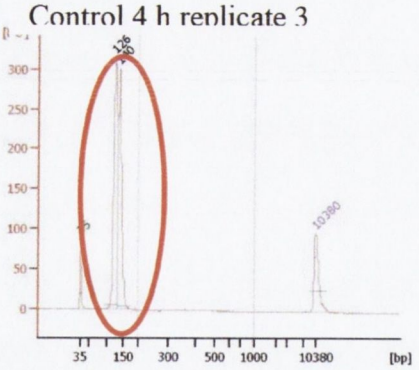

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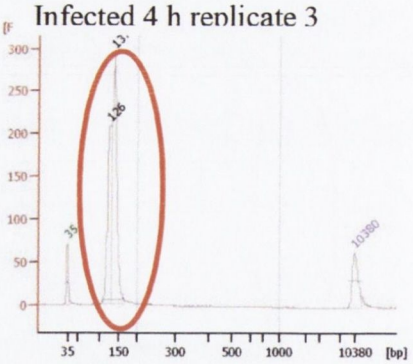

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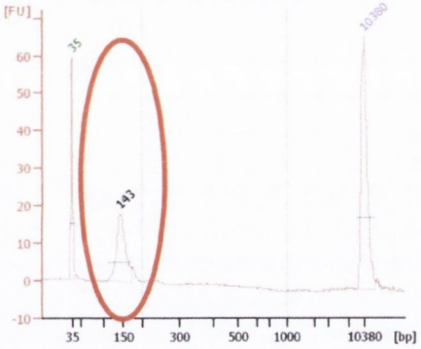

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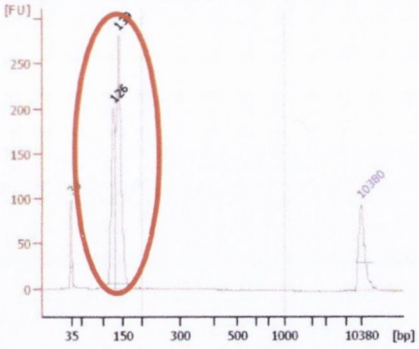

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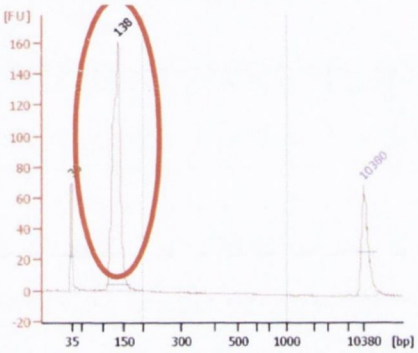

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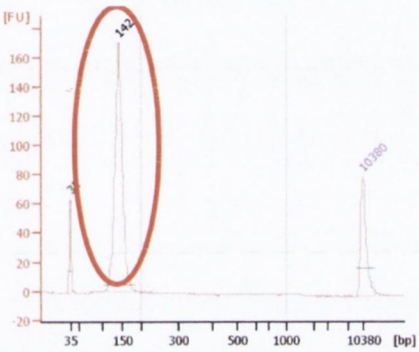

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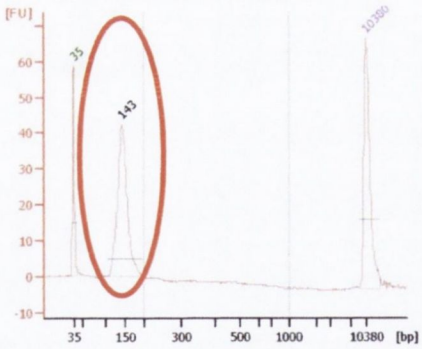

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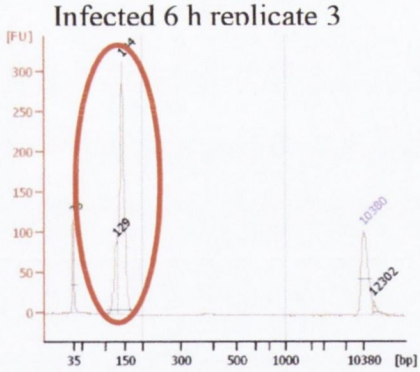
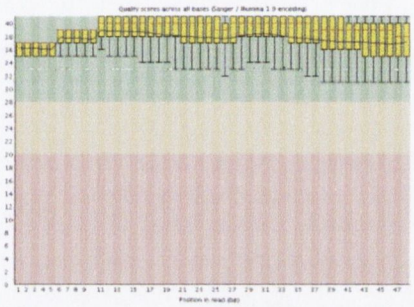


Appendix 4: FastQC checks of each library were carried out as a method of quality control checking each miRNA sequencing dataset. Checks are carried out on raw sequencing data coming from Illumina Hiseq 200 sequencer (Norway sequencing centre). Reads where at least 50% of the bases had a Phred score < 20 were removed (Cock, Fields et al. 2010).). Reads which successfully passed filtering were aligned to the bovine genome (UMD3.1).

Infected 2 h Replicate 1



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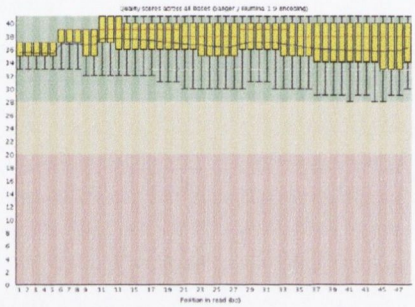
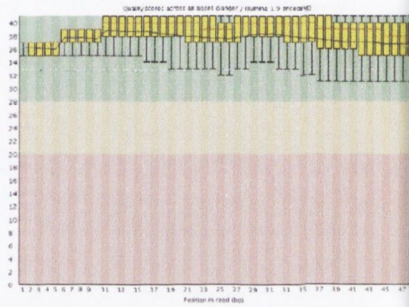
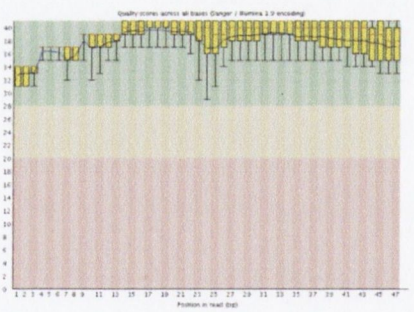


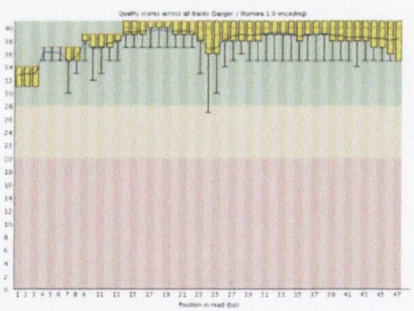
Infected 2 h Replicate 3



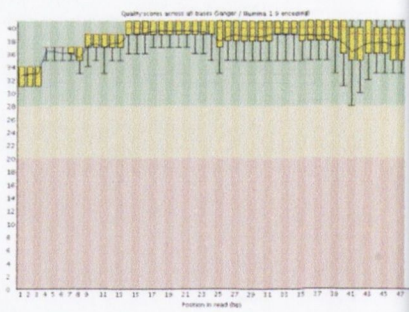
Infected 4 h Replicate 1



Infected 4 h Replicate 2



Infected 4 h Replicate 3



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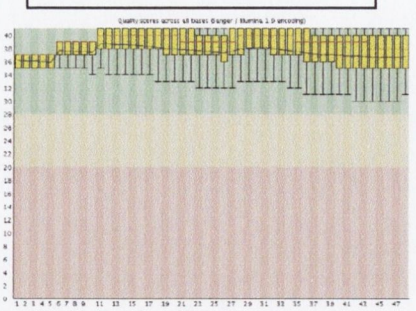

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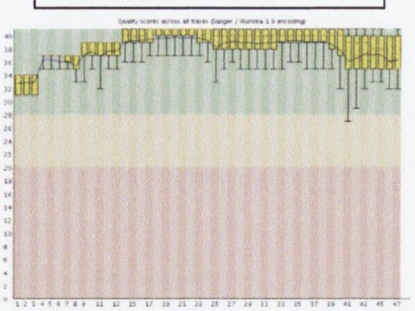
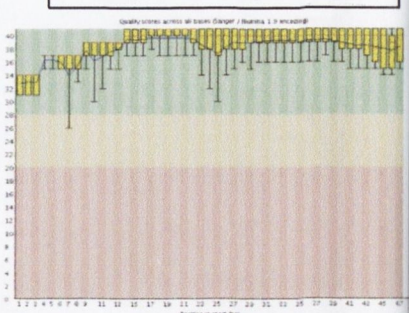
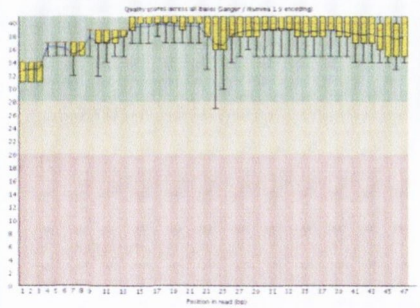


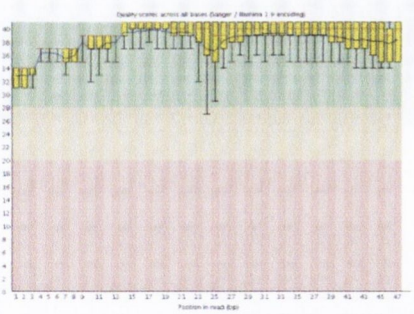
Infected 6 h Replicate 3



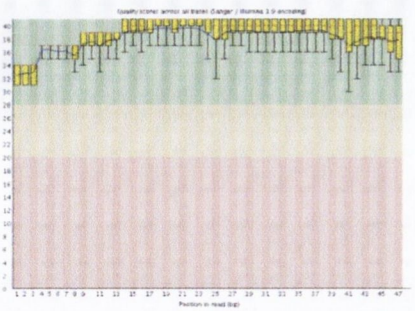
Control 1 h Replicate 1



Control 1 h Replicate 2



Control 1 h Replicate 3



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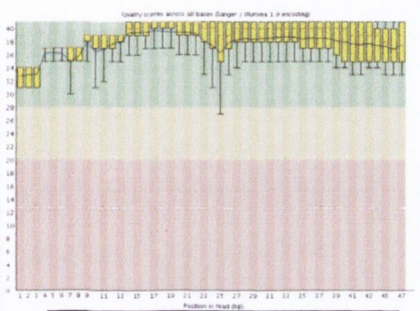
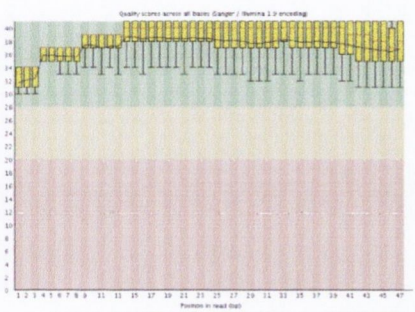
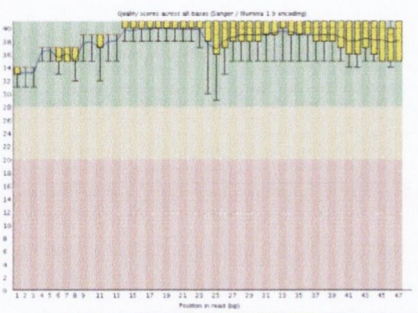


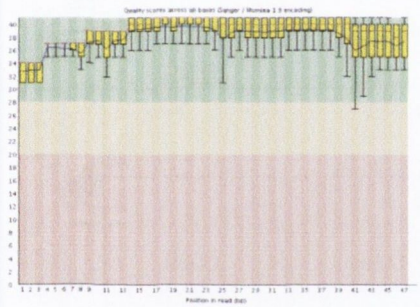
Control 2 h Replicate 2



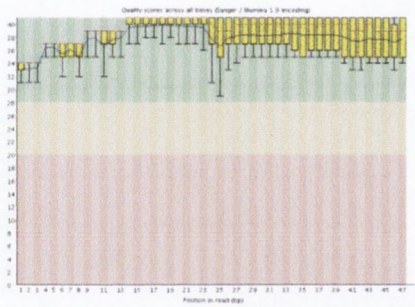
Control 2 h Replicate 3



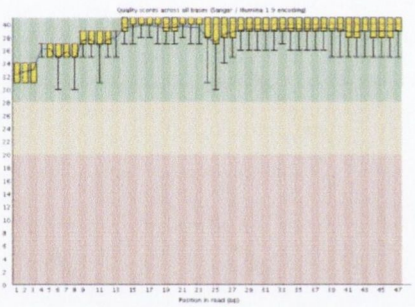
Control 4 h Replicate 1



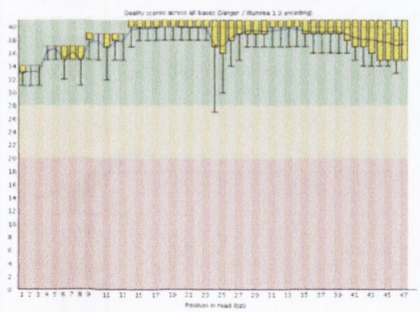
Control 4 h Replicate 2



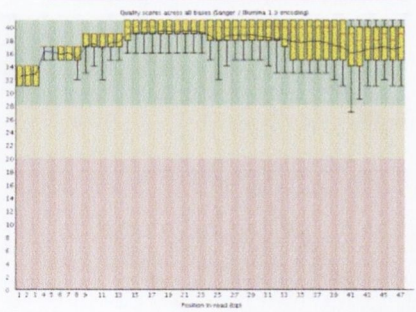
Control 4 h Replicate 3



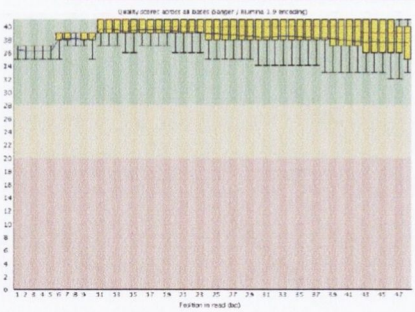
Control 6 h Replicate 1



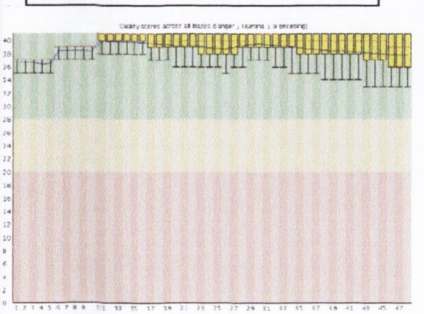
Control 6 h Replicate 2



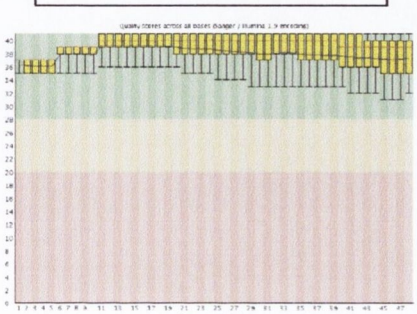
Control 6 h Replicate 3



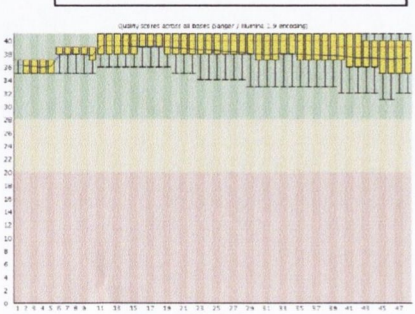
Infected 1 h Replicate 1



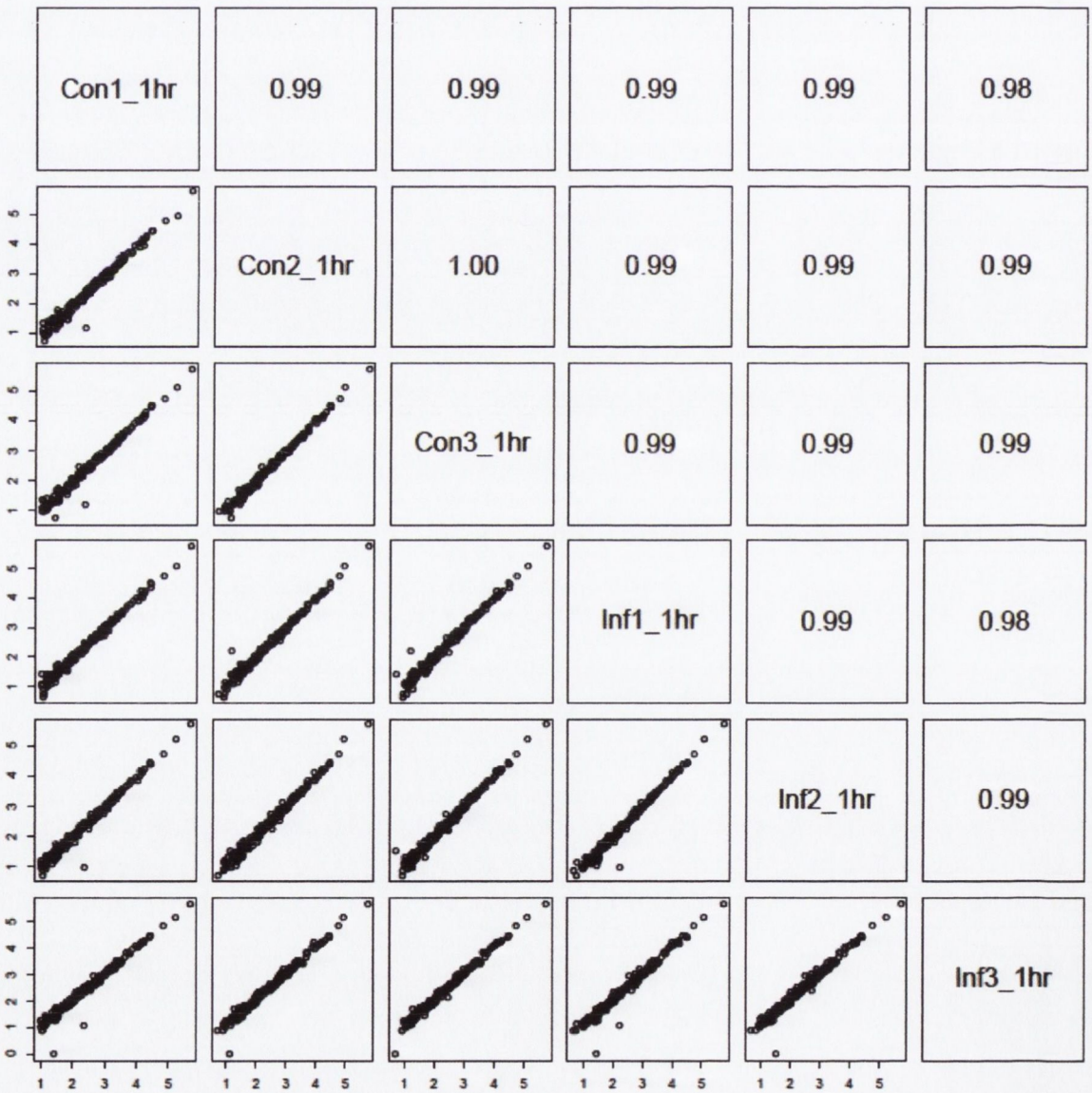
Infected 1 h Replicate 2



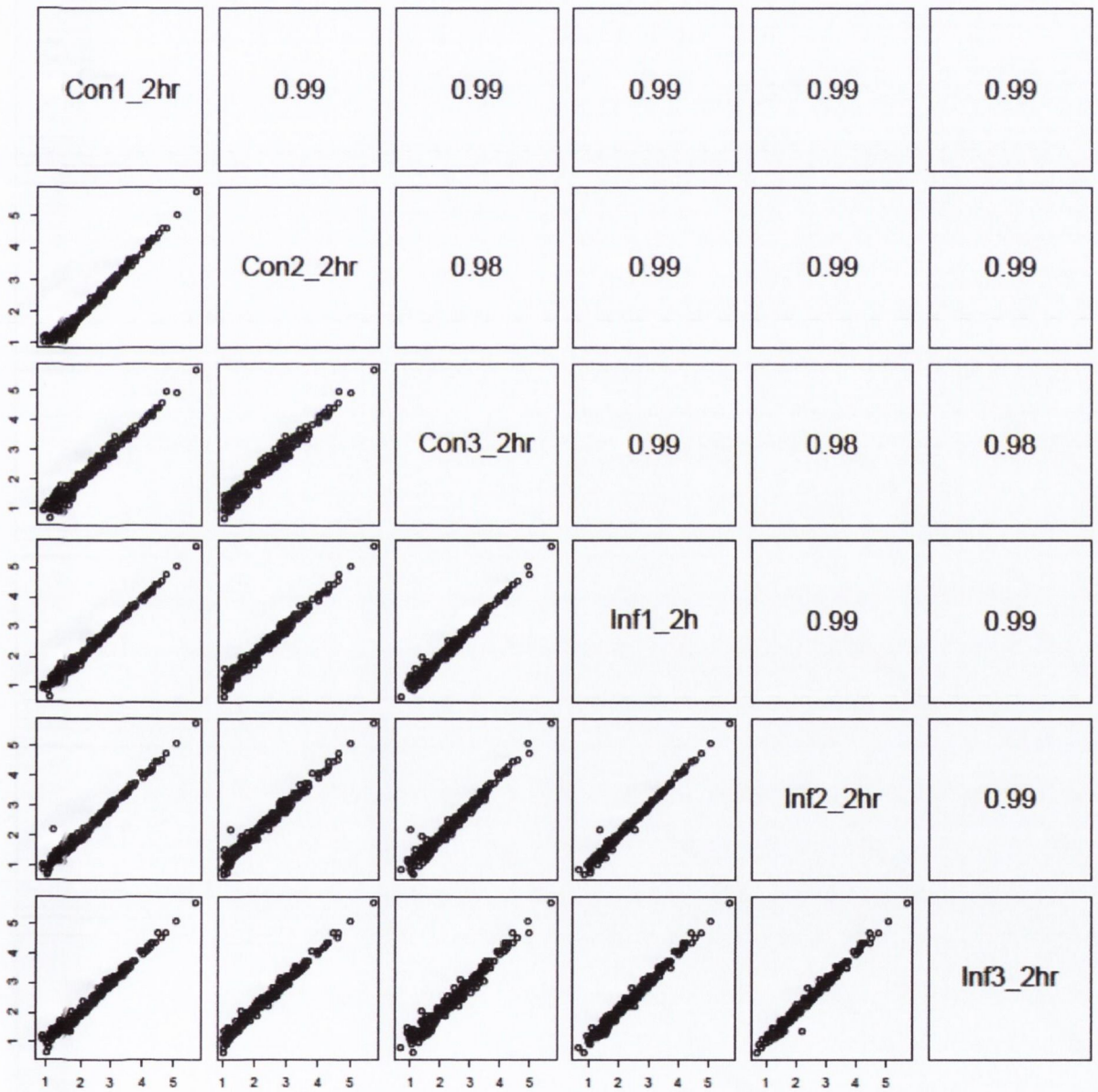
Infected 1 h Replicate 3



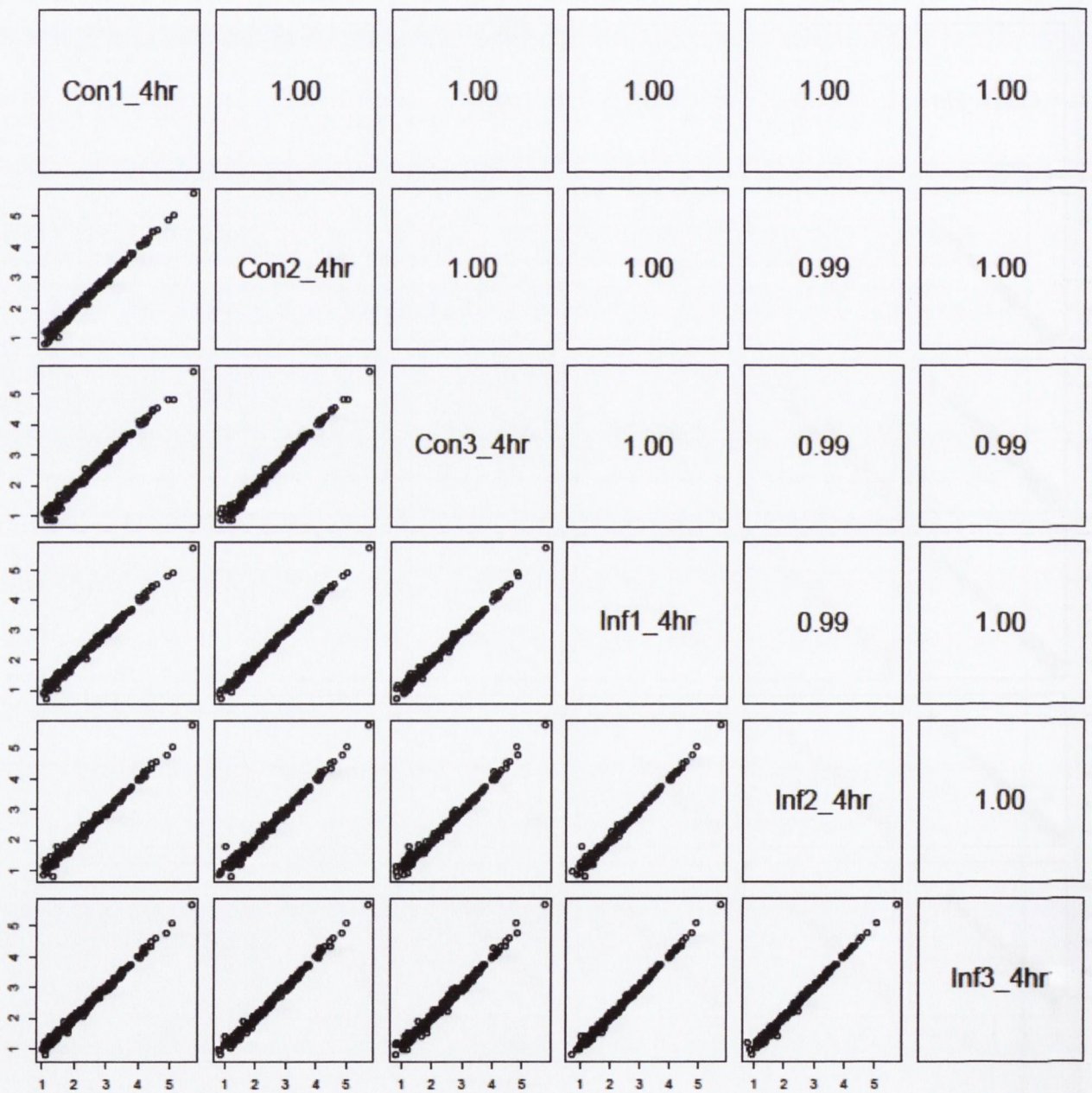
Appendix 5.1: Correlation coefficients of each miRNA normalized read count dataset between samples at each time-point for control and infected technical replicates above 10 tpm. All correlation data was generated in R version 3.0.2 The average correlation between each time-point for both control and infected was > 0.98 indicating reproducibility of the data among replicates. Con1_1hr = 1 h control replicate 1, Con2_1hr = 1 h control replicate 2, Con3_1hr = 1 h control replicate 3 and Inf1_1h = 1 h infected replicate 1, Inf2_1h = 1 h infected replicate 2, Inf3_1h = 1 h infected replicate 3.



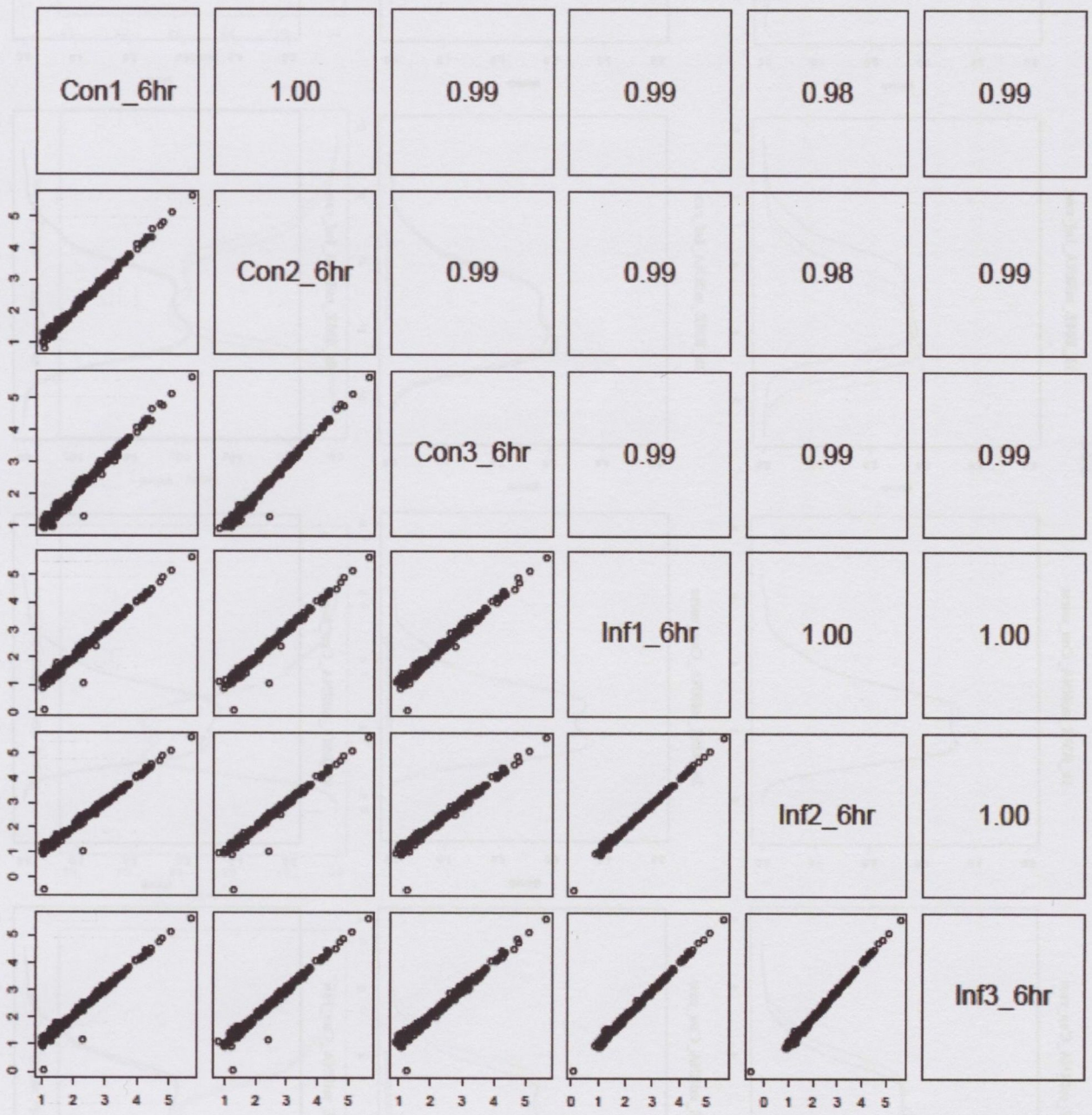
Correlation coefficients data for all 2 h samples. Note Con1_2hr = 2 h control replicate 1, Con2_2hr = 2 h control replicate 2, Con3_2hr = 2 h control replicate 3 and Inf1_2h = 2 h infected replicate 1, Inf2_2h = 2 h infected replicate 2, Inf3_2h = 2 h infected replicate 3.



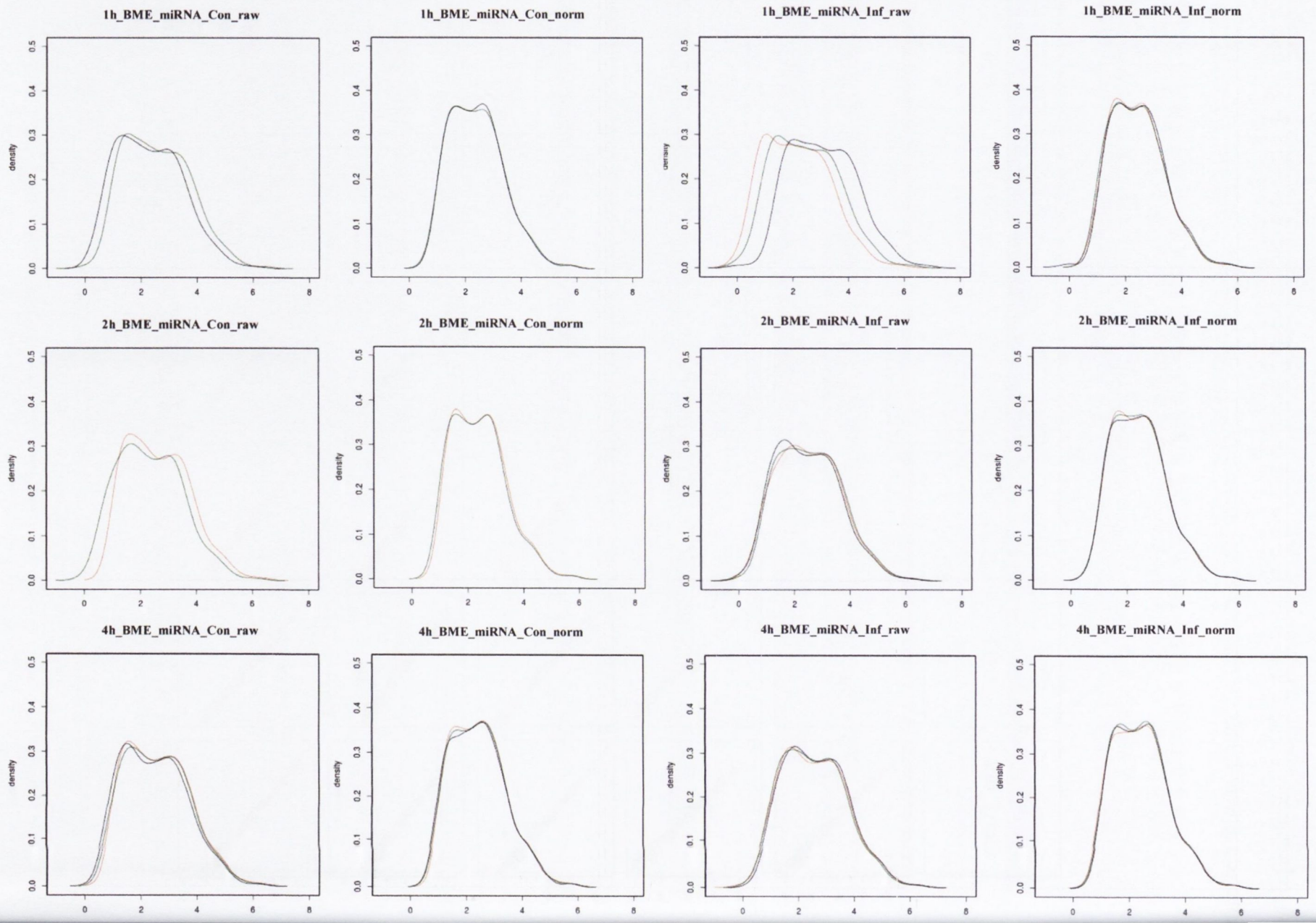
Correlation coefficients data for all 4 h samples. Note Con1_4hr = 4 h control replicate 1, Con2_4hr = 4 h control replicate 2, Con3_4hr = 4 h control replicate 3 and Inf1_4h = 4 h infected replicate 1, Inf2_4h = 4 h infected replicate 2, Inf3_4h = 4 h infected replicate 3.

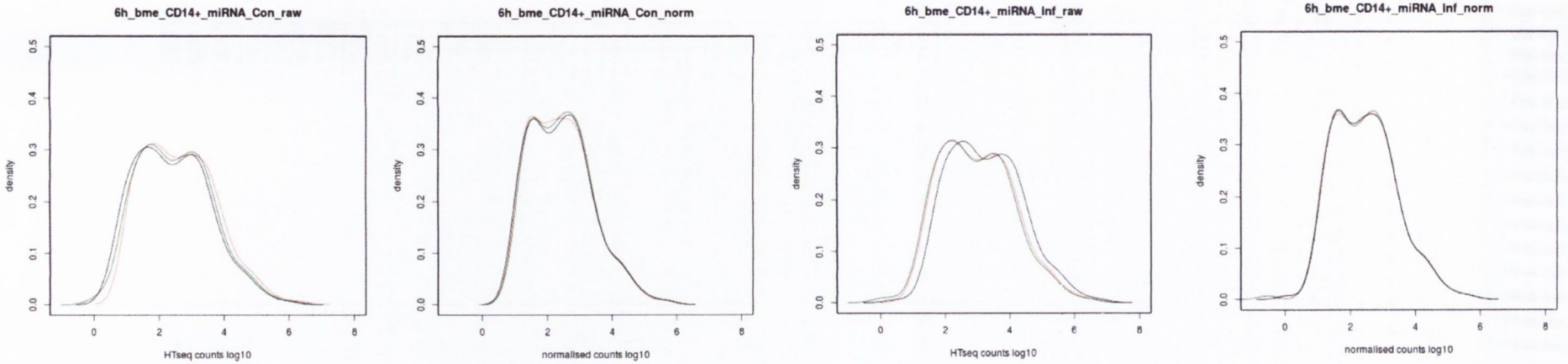


Correlation coefficients data for all 6 h samples. Note Con1_6hr = 6 h control replicate 1, Con2_6hr = 6 h control replicate 2, Con3_6hr = 6 h control replicate 3 and Inf1_6h = 6 h infected replicate 1, Inf2_6h = 6 h infected replicate 2, Inf3_6h = 6 h infected replicate 3.

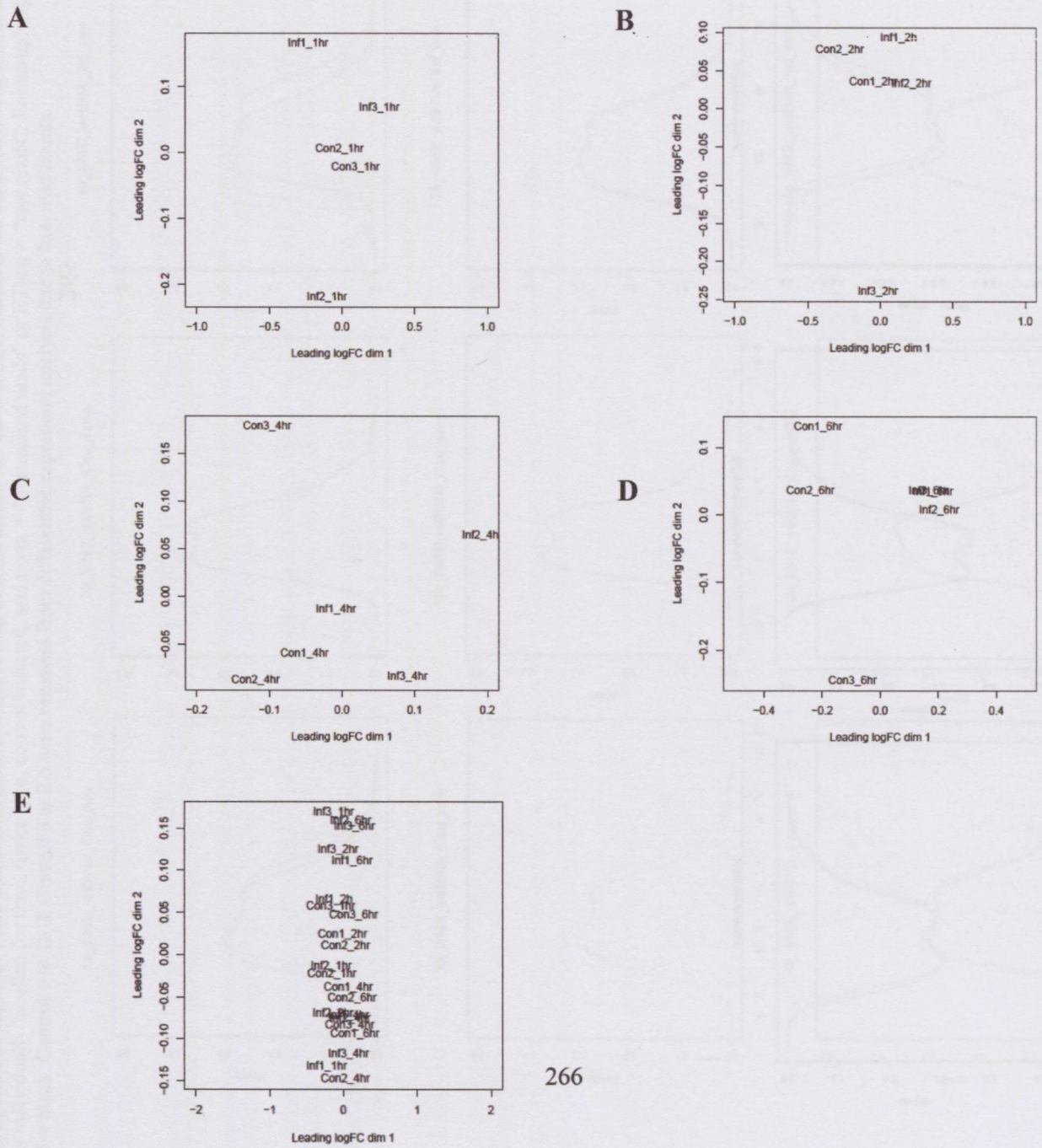


Appendix 5.2. Gene expression density plots comparing raw and normalised (Trimmed Mean of M-values (TMM)) mammary epithelial cell miRNA reads for both control and infected samples. The log10 of count numbers is plotted on the X-axis and the density on the Y-axis to examine the variation of aligned reads and the effect normalisation has had on each sample. Each graph is individually labelled for time, tissue type, control/infected, and norm = "normalised reads" or HTseq = "raw reads". For samples/graphs sample replicate 1 = red, Replicate 2 = green, and Replicate 3 = black. Control 1 at 1h & Control 3 at 2 h were removed from differential expression analysis due to low read counts.




6h_bme_CD14+_miRNA_Con_raw
6h_bme_CD14+_miRNA_Con_norm
6h_bme_CD14+_miRNA_Inf_raw
6h_bme_CD14+_miRNA_Inf_norm
density
HTseq counts log10
normalised counts log10

Appendix 6: Multidimensional scaling (MDS) plots A) 1 hour post infection, B) 2 hour post infection, C) 4 hour post infection, D) 6 hour post infection and E) all samples. Each dataset was visually represented across each time-point and as one complete dataset using MDS. Each dataset was examined across the first and second dimension. From MDS plots A to E it infected samples clearly group together over time as the infection is established and becomes the primary difference between the samples. Con1_1hr = 1 h control replicate 1, Con2_1hr = 1 h control replicate 2, Con3_1hr = 1 h control replicate 3 and Inf1_1h = 1 h infected replicate 1, Inf2_1h = 1 h infected replicate 2, Inf3_1h = 1 h infected replicate 3. Con1_2hr = 2 h control replicate 1, Con2_2hr = 2 h control replicate 2, Con3_2hr = 2 h control replicate 3 and Inf1_2h = 2 h infected replicate 1, Inf2_2h = 2 h infected replicate 2, Inf3_2h = 2 h infected replicate 3. Con1_4hr = 4 h control replicate 1, Con2_4hr = 4 h control replicate 2, Con3_4hr = 4 h control replicate 3 and Inf1_4h = 4 h infected replicate 1, Inf2_4h = 4 h infected replicate 2, Inf3_4h = 4 h infected replicate 3. Con1_6hr = 6 h control replicate 1, Con2_6hr = 6 h control replicate 2, Con3_6hr = 6 h control replicate 3 and Inf1_6h = 6 h infected replicate 1, Inf2_6h = 6 h infected replicate 2, Inf3_6h = 6 h infected replicate 3.

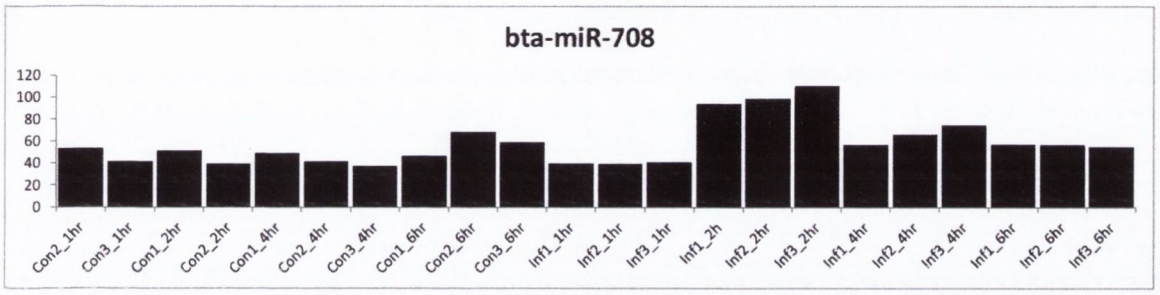


Appendix 7: miRNAs that are expressed at ≥1 tag per million in bovine mammary epithelial cells.

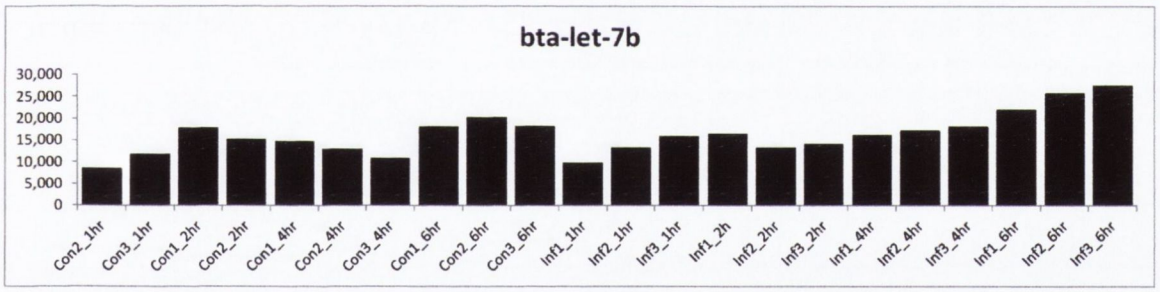
Bovine miRNAs	Average counts	Bovine miRNAs	Average counts	Bovine miRNAs	Average counts
bta-mir-21	494384	bta-let-7e	943	bta-mir-328	189
bta-mir-184	117059	ENSBTAG00000044946	931	bta-mir-183	179
bta-mir-27a	62357	bta-mir-128-1	921	bta-mir-660	175
bta-mir-205	38076	bta-mir-100	899	bta-mir-34b	172
bta-mir-200a	29671	bta-mir-15a	894	bta-mir-147	170
bta-mir-200b	26417	bta-mir-342	856	bta-mir-149	159
bta-mir-27b	19381	bta-mir-374a	825	bta-mir-296	157
bta-let-7b	17735	bta-mir-185	786	bta-mir-1306	156
bta-mir-3596	16224	bta-let-7c	764	bta-mir-33b	152
bta-mir-151	15360	bta-mir-99b	763	bta-mir-194-2	149
bta-mir-23b	13037	bta-mir-224	723	bta-mir-125b-1	144
bta-mir-23a	12793	bta-mir-96	693	bta-mir-20a	140
bta-mir-22	10278	bta-mir-2284x	679	bta-mir-193b	120
bta-mir-200c	9976	bta-mir-186	665	bta-mir-301a	117
bta-mir-99a	9694	bta-let-7d	636	bta-mir-146b	110
bta-mir-16b	5509	bta-mir-98	623	bta-mir-671	104
bta-mir-31	5435	bta-mir-331	589	bta-mir-196b	104
bta-mir-340	4879	bta-mir-532	587	bta-mir-197	101
bta-mir-221	4508	bta-mir-361	544	bta-mir-181d	99
bta-mir-423	3551	bta-mir-15b	517	bta-mir-125b-2	97
bta-mir-29a	3274	bta-mir-191	505	bta-mir-155	94
bta-mir-30d	3268	bta-mir-452	487	bta-mir-30f	92
bta-mir-140	3204	bta-mir-182	483	bta-mir-107	91
bta-mir-26b	2972	bta-mir-192	458	bta-mir-19a	89
bta-mir-222	2866	bta-mir-362	453	bta-let-7f-1	75
bta-mir-210	2619	bta-mir-30b	442	bta-mir-1388	74
bta-mir-193a	2445	bta-mir-421	422	bta-mir-7-2	69
bta-mir-106b	2438	bta-mir-152	416	bta-mir-1249	67
bta-mir-34a	2415	bta-mir-32	384	bta-mir-29e	65
bta-mir-429	2372	bta-mir-3431	378	bta-mir-769	65
bta-mir-17	2256	bta-mir-1271	373	bta-mir-188	64
bta-mir-25	2067	bta-mir-500	372	bta-mir-330	62
bta-mir-16a	1910	bta-mir-29b-2	350	bta-let-7a-1	60
bta-let-7g	1885	bta-let-7f-2	331	bta-mir-545	60
bta-mir-141	1583	bta-mir-130b	317	bta-mir-335	57
bta-mir-425	1576	bta-mir-92b	304	bta-mir-138-1	57
bta-mir-1307	1434	bta-mir-652	289	bta-mir-708	56
bta-mir-28	1429	bta-mir-92a	286	bta-mir-339	54
bta-mir-93	1382	bta-mir-24-1	279	bta-mir-324	53
bta-mir-148b	1338	bta-mir-378	274	bta-mir-374b	48
bta-mir-125a	1317	bta-mir-345	269	bta-mir-126	47
bta-mir-24-2	1200	bta-mir-484	258	bta-mir-143	45
bta-mir-130a	1183	bta-mir-34c	255	bta-mir-181c	45
bta-mir-30a	1116	bta-mir-204	250	bta-mir-139	44
bta-mir-128-2	1074	bta-mir-18a	246	bta-mir-874	43
bta-mir-148a	1029	bta-mir-33a	217	bta-mir-744	40
bta-mir-30e	996	bta-mir-29c	203	bta-mir-2443	40
bta-mir-30c	977	bta-mir-455	196	bta-mir-877	39

Bovine miRNAs	Average counts	Bovine miRNAs	Average counts	Bovine miRNAs	Average counts
bta-mir-195	38	bta-mir-2344	7	bta-mir-2468	2
bta-mir-29b-1	36	bta-mir-129-2	7	bta-mir-2370	2
bta-let-7a-2	34	bta-mir-760	7	ENSBTAG00000048117	2
bta-mir-181a-1	33	ENSBTAG00000030040	7	bta-mir-2455	2
bta-mir-2408	33	bta-mir-92	6	bta-mir-2300a	2
bta-mir-504	32	ENSBTAG00000047677	6	bta-mir-212	2
bta-mir-26a-2	28	bta-mir-2316	6	bta-mir-654	2
bta-mir-365	27	bta-mir-381	6	bta-mir-2285b	2
bta-mir-3613	26	bta-mir-2320	6	bta-mir-103-1	2
bta-mir-375	25	bta-mir-2474	6	bta-mir-2477	2
bta-mir-615	25	ENSBTAG00000037500	6	bta-mir-214	2
bta-mir-103-2	24	bta-mir-10a	6	bta-mir-2368	2
bta-mir-2387	23	bta-mir-2307	5	bta-mir-411c	2
bta-mir-454	23	bta-mir-19b	5	bta-mir-2460	2
bta-mir-499	22	bta-mir-450b	5	bta-mir-2454	2
bta-mir-132	21	bta-mir-2346	5	bta-mir-376b	2
bta-mir-502b	21	btamir-503	5	bta-mir-369	2
bta-mir-196a-2	21	bta-mir-1185	5	bta-mir-154c	2
bta-mir-2299	20	bta-mir-2397	5	bta-mir-2388	2
bta-mir-411	20	bta-mir-2448	5	bta-mir-1224	2
bta-mir-181b-2	20	bta-mir-301b	5	bta-mir-329b	2
bta-mir-2419	20	bta-mir-497	5	bta-mir-3578	2
bta-mir-218-1	19	bta-mir-187	4	bta-mir-106	1
bta-let-7a-3	18	bta-mir-2431	4	bta-mir-2398	1
bta-mir-2336	17	ENSBTAG00000045885	4	bta-mir-95	1
bta-mir-19b-2	17	bta-mir-138-2	4	bta-mir-146a	1
bta-mir-379	17	bta-mir-424	4	bta-mir-2466	1
bta-mir-491	17	bta-mir-376c	4	bta-mir-2342	1
bta-mir-494	16	bta-mir-542	4	bta-mir-338	1
bta-mir-3432-2	16	bta-mir-194-1	4	bta-mir-763	1
bta-mir-122	16	bta-mir-145	4	bta-mir-181b-1	1
bta-mir-1296	14	bta-mir-380	4	bta-mir-10b	1
bta-mir-505	13	bta-mir-495	4	bta-mir-2400	1
bta-mir-101	13	bta-mir-486	3	bta-mir-2483	1
ENSBTAG00000047296	13	bta-mir-592	3	bta-mir-2367	1
bta-mir-1343	13	bta-mir-2308	3	bta-mir-2439	1
bta-mir-2284p	11	bta-mir-2355	3		
ENSBTAG00000046881	11	bta-mir-215	3		
bta-mir-181a-2	10	ENSBTAG00000047440	3		
bta-mir-190a	10	bta-mir-2416	3		
bta-mir-2399	10	bta-mir376e	3		
bta-mir-2313	10	bta-mir-20b	3		
bta-mir-2461	10	bta-mir-2447	3		
bta-mir-628	9	bta-mir-1468	3		
bta-mir-365-2	8	bta-mir-543	2		
bta-mir-26c	8	bta-mir-299	2		
bta-mir-326	8	bta-mir-2284t	2		
bta-mir-409a	7	bta-mir-2339	2		

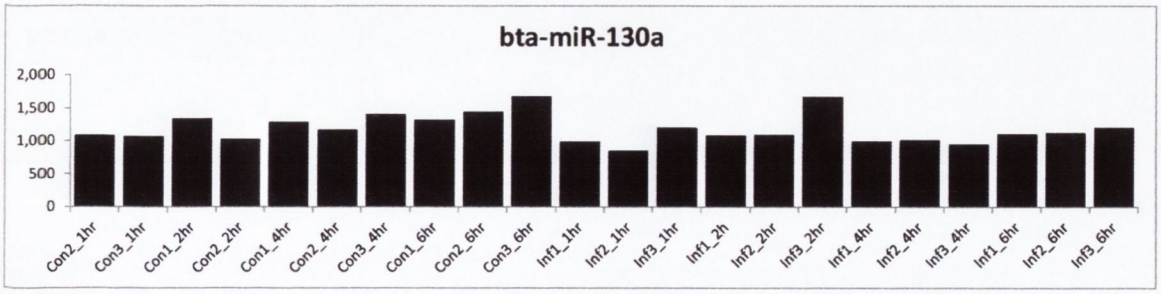
Appendix 8: Differentially expressed miRNAs examined for each mammary epithelial cell sample across full time course as normalised tag counts. Each plot is individually labelled (X-axis) and tag count is plotted on Y-axis (A-J).



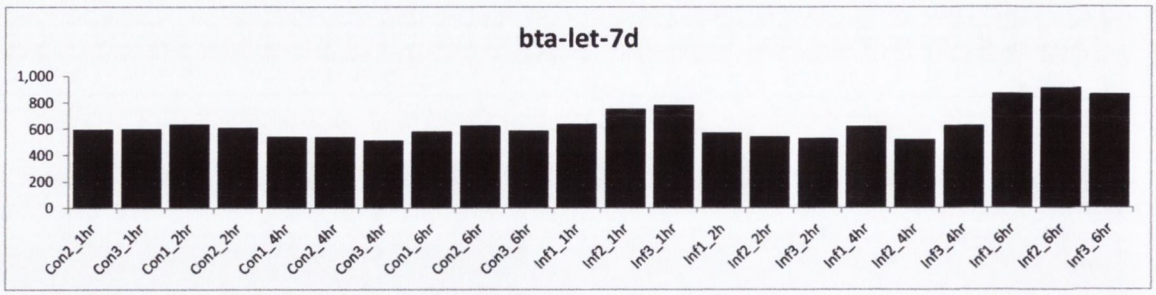
A) **Bta-miR-708:** Identified as up-regulated at 2 hpi by EdgeR, which appears justified when the normalised count data is examined.



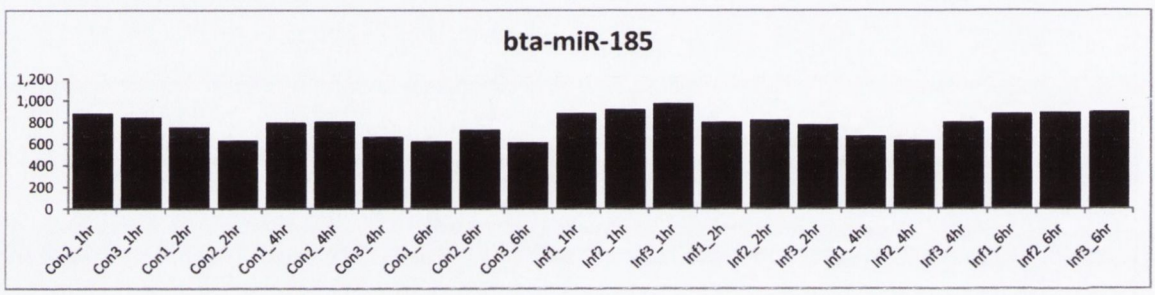
B) bta-let-7b: Identified as upregulated at both 4 hpi and 6 hpi. Counts are more variable across control samples; however there is a gradual induction of bta-let-7b at 4 hpi and 6 hpi.



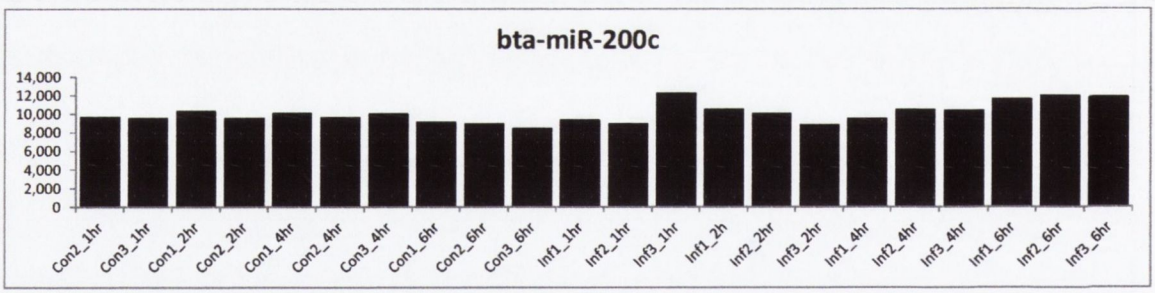
C) bta-miR-130a: Identified as being down-regulated at 4 hpi and 6 hpi.



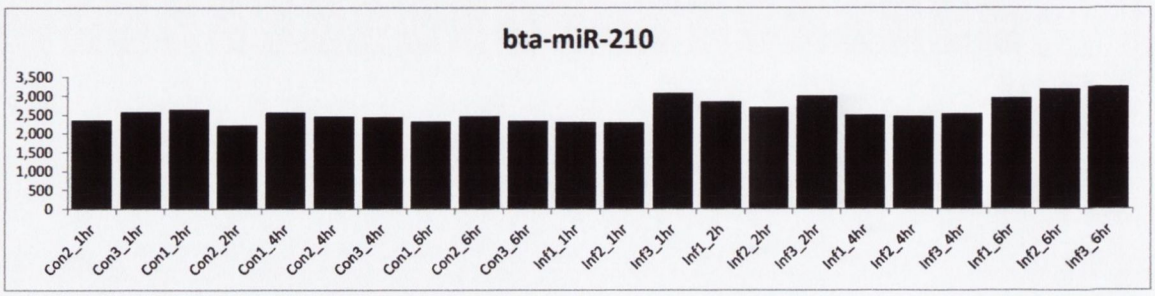
D) bta-let-7d: Identified as up-regulated at 6 hpi by EdgeR.



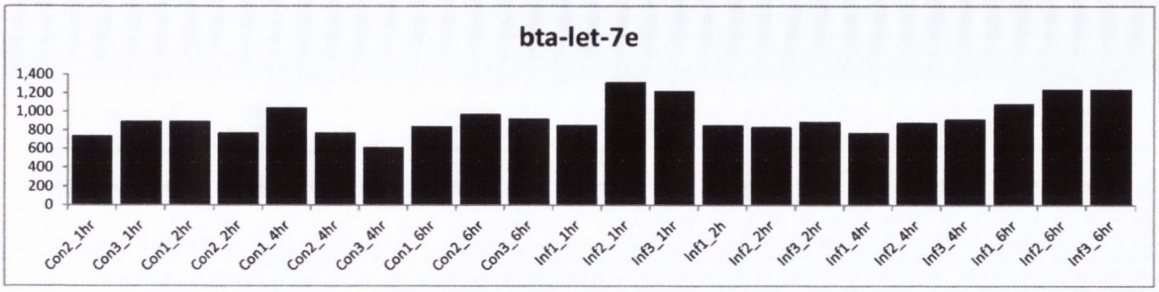
E) bta-miR-185: Identified as up-regulated at 6 hpi. Normalised read counts appear higher in most of the infected samples.



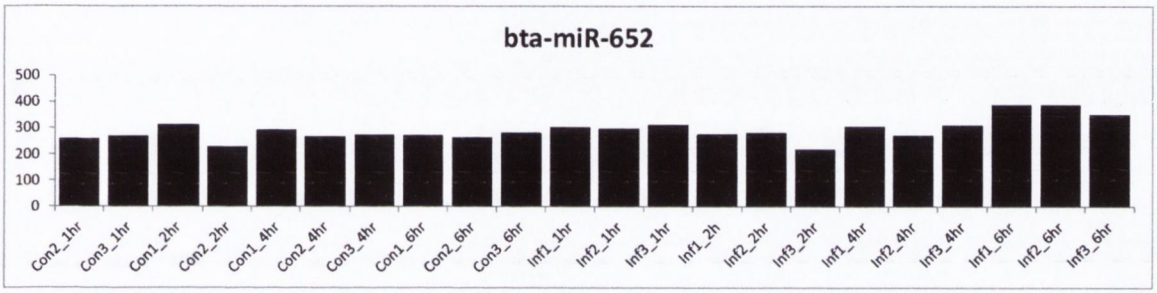
F) bta-miR-200c: Identified as up-regulated at 6 hpi. Normalised read counts appear higher in most of the infected samples.



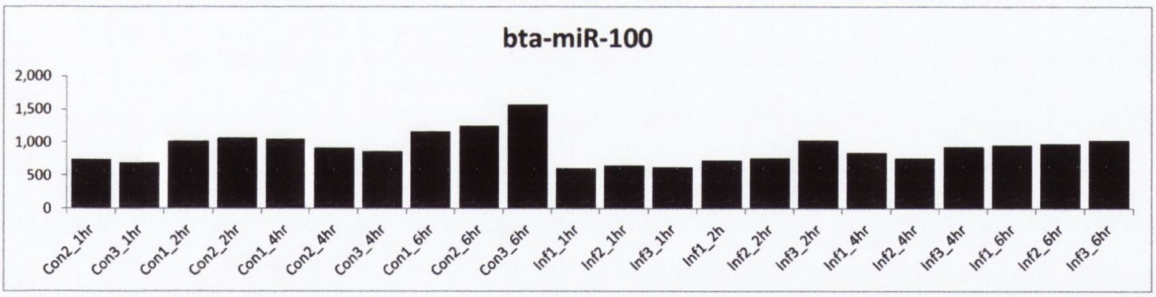
G) bta-miR-210: Identified as up-regulated at 6 Normalised read counts appear higher in most of the infected samples.



H) bta-let-7e: Identified as up-regulated at 6.



I) bta-miR-652: Identified as up-regulated at 6.



J) bta-miR-100: Identified as being down-regulated at 6 hpi, this is reduced in all infected samples in comparison to controls.

Appendix 9: Summary of lipopolysaccharide (LPS) responsive microRNAs. 145 miRNAs that have been identified as being differentially expressed in response to LPS across multiple different species and tissues

MicroRNA	Organism	Response to LPS	Cell-type	MicroRNA response to *S. uberis* hpi	PMID:
mmu-miR-223	Mouse	Down-regulated	Macrophage		22937006
mmu-miR-155	Mouse	Up-regulated	PBMCs		22745684
mmu-miR-34a	Mouse	Down-regulated	Macrophage		22483937
dre-miR-122	Zebrafish	Up-regulated	liver (ZFL) cells		22419229
dre-miR-192	Zebrafish	Up-regulated	liver (ZFL) cells		22419229
dre-miR-194a	Zebrafish	Up-regulated	liver (ZFL) cells		22419229
mmu-miR-146a	Mouse	Up-regulated	Macrophage		22415194
mmu-miR-155	Mouse	Up-regulated	Macrophage		22415194
mmu-miR-27a	Mouse	Down-regulated	Macrophage		22415194
mmu-miR-532-5p	Mouse	Down-regulated	Macrophage		22415194
mmu-miR-155	Mouse	Up-regulated	Macrophage		22326887
bta-miR-155	Cow	Up-regulated	Monocyte		22033210
bta-miR-146a	Cow	Up-regulated	Monocyte		22033210
hsa-miR-155	Human	Up-regulated	Monocyte		21913990
hsa-miR-18a	Human	Up-regulated	Monocyte		21913990
Let-7i	Rat	Up-regulated	Dendritic cells		21742974
hsa-miR-107	Mouse	Down-regulated	pBMDM/PBMC		21628465
has-miR-146a	Mouse	Up-regulated	pBMDM/PBMC		21628465
mmu-miR-98	Mouse	Down-regulated	Macrophage	bta-miR-98/up-regulated/4 hpi	21609717
miR-142-3p	Mouse	Up-regulated	Dendritic cells		21474672
hsa-miR-1282	Human	Up-regulated	human middle ear epithelial cell		21377743
hsa-miR-193a 5p	Human	Up-regulated	human middle ear epithelial cell	bta-miR-193a/down-regulated/4 hpi	21377743
hsa-miR-1248	Human	Up-regulated	human middle ear epithelial cell		21377743
hsa-miR-423 5p	Human	Up-regulated	human middle ear epithelial cell		21377743
hsa-miR-1827	Human	Up-regulated	human middle ear epithelial cell		21377743
hsa-miR-210	Human	Down-regulated	human middle ear epithelial cell	bta-miR-210/up-regulated/6 hpi	21377743
hsa-miR-188 5p	Human	Down-regulated	human middle ear epithelial cell		21377743
hsa-miR-203	Human	Down-regulated	human middle ear epithelial cell		21377743
hsa-miR-494	Human	Down-regulated	human middle ear epithelial cell	bta-miR-494/up-regulated/6 hpi	21377743
hsa-miR-301b	Human	Down-regulated	human middle ear epithelial cell		21377743
hsa-miR-658	Human	Down-regulated	human middle ear epithelial cell		21377743
hsa-miR-675	Human	Down-regulated	human middle ear epithelial cell		21377743
hsa-miR-503	Human	Down-regulated	human middle ear epithelial cell		21377743
hsa-miR-886 5p	Human	Down-regulated	human middle ear		21377743

			epithelial cell		
has-miR-17-3p	Human	Up-regulated	HeLa		21370248
has-miR-106a	Human	Up-regulated	HeLa		21370248
mmu-miR-1244	Mouse	Up-regulated	Spleen		21320120
rno-miR-146a	Rat	Up-regulated	alveolar macrophages		20854734
hsa-miR-525-5p	Human	Up-regulated	Monocytes		20706588
hsa-miR-155	Human	Up-regulated	Monocytes		20622002
has-miR-663	Human	Up-regulated	Monocytes		20622002
hsa-miR-125b	Human	Up-regulated	Macrophage		20351193
hsa-let-7a	Human	Down-regulated	Macrophage		20351193
hsa-miR-27b	Human	Up-regulated	THP1-monocyte		20164187
hsa-miR-21	Mouse	Up-regulated	Macrophage		19946272
hsa-146a	Human	Up-regulated	THP1-monocyte		19918258
hsa-miR-132	Human	Up-regulated	THP1-monocyte		19918258
hsa-miR-155	Human	Up-regulated	THP1-monocyte		19918258
mmu-miR-155	Mouse	Up-regulated	Monocytes		19596814
hsa-miR-98	Human	Down-regulated	Cholangiocyte	bta-miR-98/up-regulated/4 hpi	19592657
hsa-let-7	Human	Down-regulated	Cholangiocyte		19592657
hsa-miR-155	Mouse	Up-regulated	Macrophage		19423639
hsa-miR-146a	Human	Up-regulated	Monocytes		19390647
hsa-miR-155	Mouse	Up-regulated	Macrophage		19359473
hsa-miR-346	Human	Up-regulated	Fibroblast like sinovial cells		19342689
hsa-miR-143	Human	Up-regulated	leukocytes		19284987
hsa-miR-146b	Human	Down-regulated	leukocytes		19284987
hsa-miR-150	Human	Down-regulated	leukocytes		19284987
hsa-miR-342	Human	Down-regulated	leukocytes		19284987
hsa-let-7g	Human	Down-regulated	leukocytes		19284987
hsa-miR-155	Mouse	Up-regulated	Dendritic cells		19193853
mmu-miR-21	Mouse	Up-regulated	Lung tissue		17640343
mmu-miR-25	Mouse	Up-regulated	Lung tissue		17640343
mmu-miR-27b	Mouse	Up-regulated	Lung tissue		17640343
mmu-miR-100	Mouse	Up-regulated	Lung tissue	bta-miR-100/down-regulated/6 hpi	17640343
mmu-miR-140	Mouse	Up-regulated	Lung tissue		17640343
mmu-miR-142-3p	Mouse	Up-regulated	Lung tissue		17640343
mmu-miR-181c	Mouse	Up-regulated	Lung tissue		17640343
mmu-miR-187	Mouse	Up-regulated	Lung tissue		17640343
mmu-miR-194	Mouse	Up-regulated	Lung tissue		17640343
mmu-miR-214	Mouse	Up-regulated	Lung tissue		17640343
mmu-miR-223	Mouse	Up-regulated	Lung tissue		17640343
mmu-miR-224	Mouse	Up-regulated	Lung tissue		17640343
mmu-miR-698		Down-regulated	Lung		22849760
mmu-miR-467b		Down-regulated	Lung		22849760
mmu-miR-466b-3-3p		Down-regulated	Lung		22849760
mmu-miR-376a		Down-regulated	Lung		22849760
mmu-miR-211		Down-regulated	Lung		22849760
mmu-miR-466a-3p		Down-regulated	Lung		22849760

mmu-miR-471	Down-regulated	Lung		22849760
mmu-miR-875-3p	Down-regulated	Lung		22849760
mmu-miR-493	Down-regulated	Lung		22849760
mmu-miR-694	Down-regulated	Lung		22849760
mmu-miR-704	Down-regulated	Lung		22849760
mmu-miR-574-3p	Down-regulated	Lung		22849760
mmu-miR-485	Down-regulated	Lung		22849760
mmu-miR-470	Down-regulated	Lung		22849760
mmu-miR-466d-5p	Down-regulated	Lung		22849760
mmu-let-7a	Down-regulated	Lung		22849760
mmu-miR-34b-3p	Down-regulated	Lung		22849760
mmu-miR-293	Down-regulated	Lung		22849760
mmu-miR-1902	Down-regulated	Lung		22849760
mmu-miR-494	Down-regulated	Lung	bta-miR-494/up-regulated/6 hpi	22849760
mmu-miR-467f	Down-regulated	Lung		22849760
mmu-miR-204	Down-regulated	Lung		22849760
mmu-miR-294	Down-regulated	Lung		22849760
mmu-miR-130a	Down-regulated	Liver	bta-miR-130a/down-regulated/6 hpi	22849760
mmu-miR-126-3p	Down-regulated	Liver		22849760
mmu-miR-193	Down-regulated	Liver		22849760
mmu-miR-690	Down-regulated	Liver		22849760
mmu-miR-805	Down-regulated	Liver		22849760
mmu-miR-221	Down-regulated	Liver		22849760
mmu-miR-29b	Down-regulated	Liver		22849760
mmu-miR-146a	Down-regulated	Liver		22849760
mmu-miR-17	Down-regulated	Liver		22849760
mmu-miR-22	Down-regulated	Liver		22849760
mmu-miR-468	Down-regulated	Spleen		22849760
mmu-miR-672	Down-regulated	Spleen		22849760
mmu-miR-214	Down-regulated	Spleen		22849760
mmu-miR-466d-5p	Down-regulated	Spleen		22849760
mmu-miR-466b-5p	Down-regulated	Spleen		22849760
mmu-miR-18b	Down-regulated	Spleen		22849760
mmu-miR-16	Down-regulated	Spleen		22849760
mmu-miR-669e	Down-regulated	Spleen		22849760
mmu-miR-1195	Down-regulated	Blood		22849760
mmu-miR-667	Down-regulated	Blood		22849760
mmu-miR-690	Down-regulated	Blood		22849760
mmu-miR-880	Down-regulated	Blood		22849760
mmu-miR-34b-3p	Down-regulated	Blood		22849760
mmu-miR-709	Down-regulated	Blood		22849760
mmu-miR-468	Down-regulated	Blood		22849760
mmu-miR-1186	Down-regulated	Blood		22849760
mmu-miR-710	Down-regulated	Blood		22849760
mmu-miR-1897-5p	Down-regulated	Blood		22849760
mmu-miR-466j	Down-regulated	Blood		22849760
mmu-miR-155	Down-regulated	Blood		22849760
mmu-miR-294	Down-regulated	Blood		22849760

mmu-miR-1898	Down-regulated	Blood	22849760
mmu-miR-380-3p	Down-regulated	Blood	22849760
mmu-miR-703	Down-regulated	Blood	22849760
mmu-miR-324-3p	Down-regulated	Blood	22849760
mmu-miR-466d-5p	Down-regulated	Blood	22849760
mmu-miR-206	Down-regulated	Blood	22849760
mmu-miR-678	Down-regulated	Blood	22849760
mmu-miR-691	Down-regulated	Blood	22849760
mmu-miR-532-5p	Down-regulated	Blood	22849760
mmu-miR-685	Down-regulated	Blood	22849760
mmu-miR-1188	Down-regulated	Blood	22849760
mmu-miR-466f-5p	Down-regulated	Blood	22849760
mmu-miR-483	Down-regulated	Blood	22849760
mmu-miR-326	Down-regulated	Blood	22849760
mmu-miR-380-5p	Down-regulated	Blood	22849760
mmu-miR-495	Down-regulated	Blood	22849760
mmu-miR-680	Down-regulated	Blood	22849760
mmu-miR-141	Down-regulated	Blood	22849760
mmu-miR-467e	Down-regulated	Blood	22849760
mmu-miR-1896	Down-regulated	Blood	22849760
mmu-miR-193b	Down-regulated	Blood	22849760
mmu-miR-99b	Down-regulated	Blood	22849760
mmu-miR-673-5p	Down-regulated	Blood	22849760
mmu-miR-882	Down-regulated	Blood	22849760
mmu-miR-1895	Down-regulated	Blood	22849760
mmu-miR-133b	Down-regulated	Blood	22849760
mmu-miR-491	Down-regulated	Blood	22849760
mmu-miR-214	Down-regulated	Blood	22849760
mmu-miR-339-5p	Down-regulated	Blood	22849760
mmu-miR-467h	Down-regulated	Blood	22849760
mmu-miR-134	Down-regulated	Blood	22849760
mmu-miR-466h	Down-regulated	Blood	22849760
mmu-miR-370	Down-regulated	Blood	22849760
mmu-miR-878-5p	Down-regulated	Blood	22849760
mmu-miR-196a	Down-regulated	Blood	22849760
mmu-miR-150	Down-regulated	Blood	22849760
mmu-miR-133a	Down-regulated	Blood	22849760
mmu-miR-186	Down-regulated	Blood	22849760
mmu-miR-223	Up-regulated	Lung	22849760
mmu-miR-155	Up-regulated	Lung	22849760
mmu-miR-21	Up-regulated	Lung	22849760
mmu-miR-667	Up-regulated	Lung	22849760
mmu-let-7a	Up-regulated	Lung	22849760
mmu-miR-146a	Up-regulated	Lung	22849760
mmu-miR-107	Up-regulated	Lung	22849760
mmu-miR-34b-5p	Up-regulated	Lung	22849760
mmu-miR-146b	Up-regulated	Lung	22849760
mmu-miR-133a	Up-regulated	Lung	22849760
mmu-miR-15a	Up-regulated	Lung	22849760

mmu-miR-191	Up-regulated	Lung		22849760
mmu-miR-103	Up-regulated	Lung		22849760
mmu-miR-16	Up-regulated	Lung		22849760
mmu-miR-292-5p	Up-regulated	Liver		22849760
mmu-miR-155	Up-regulated	Spleen		22849760
mmu-miR-21	Up-regulated	Spleen		22849760
mmu-miR-101a	Up-regulated	Spleen		22849760
mmu-miR-15b	Up-regulated	Blood		22849760
mmu-miR-451	Up-regulated	Blood		22849760
mmu-miR-16	Up-regulated	Blood		22849760
mmu-miR-103	Up-regulated	Blood		22849760
mmu-miR-107	Up-regulated	Blood		22849760
mmu-let-7d	Up-regulated	Blood	bta-let-7d/up-regulated/6 hpi	22849760
mmu-miR-92a	Up-regulated	Blood		22849760
mmu-miR-25	Up-regulated	Blood		22849760
mmu-let-7b	Up-regulated	Blood	bta-let-7b/up-regulated/4 hpi	22849760
mmu-let-7a	Up-regulated	Blood		22849760
mmu-let-7i	Up-regulated	Blood		22849760
mmu-miR-652	Up-regulated	Blood	bta-miR-652/up-regulated/6 hpi	22849760
mmu-miR-92b	Up-regulated	Blood		22849760
mmu-miR-590-5p	Up-regulated	Blood		22849760

Appendix 10: Genes predicted to be targeted by differentially expressed miRNAs.

Differentially expressed miRNA	Target Gene Symbol	Ensembl ID of human ortholog
bta-mir-185	CACNA1G	ENSG00000006283
bta-mir-185	ZNF200	ENSG00000010539
bta-mir-185	UBA6	ENSG00000033178
bta-mir-185	GOPC	ENSG00000047932
bta-mir-185	USP36	ENSG00000055483
bta-mir-185	ZC3H11A	ENSG00000058673
bta-mir-185	ALDH18A1	ENSG00000059573
bta-mir-185	MXD1	ENSG00000059728
bta-mir-185	DNAJA2	ENSG00000069345
bta-mir-185	PTPN3	ENSG00000070159
bta-mir-185	TCOF1	ENSG00000070814
bta-mir-185	ATP6AP1	ENSG00000071553
bta-mir-185	HLTF	ENSG00000071794
bta-mir-185	FRY	ENSG00000073910
bta-mir-185	ITCH	ENSG00000078747
bta-mir-185	SCTR	ENSG00000080293
bta-mir-185	SESN1	ENSG00000080546
bta-mir-185	SRCAP	ENSG00000080603
bta-mir-185	NUFIP1	ENSG00000083635
bta-mir-185	OXCT1	ENSG00000083720
bta-mir-185	WDR47	ENSG00000085433
bta-mir-185	SEMA4G	ENSG00000095539
bta-mir-185	HSP90AB1	ENSG00000096384
bta-mir-185	TSPAN15	ENSG00000099282
bta-mir-185	POLDIP3	ENSG00000100227
bta-mir-185	RBM23	ENSG00000100461
bta-mir-185	PIGH	ENSG00000100564
bta-mir-185	SLC10A1	ENSG00000100652
bta-mir-185	BMP7	ENSG00000101144
bta-mir-185	RAE1	ENSG00000101146
bta-mir-185	PORCN	ENSG00000102312
bta-mir-185	PIK3R2	ENSG00000105647
bta-mir-185	WDR91	ENSG00000105875
bta-mir-185	ZC3HAV1	ENSG00000105939
bta-mir-185	CASP2	ENSG00000106144
bta-mir-185	CEP41	ENSG00000106477
bta-mir-185	FKTN	ENSG00000106692
bta-mir-185	GATA3	ENSG00000107485
bta-mir-185	LIPA	ENSG00000107798
bta-mir-185	DRG2	ENSG00000108591
bta-mir-185	SOD3	ENSG00000109610
bta-mir-185	GLRB	ENSG00000109738
bta-mir-185	C11orf58	ENSG00000110696
bta-mir-185	CAMKK2	ENSG00000110931
bta-mir-185	ACSS3	ENSG00000111058
bta-mir-185	ING4	ENSG00000111653
bta-mir-185	CEP85L	ENSG00000111860
bta-mir-185	AIM1	ENSG00000112297
bta-mir-185	HRH2	ENSG00000113749
bta-mir-185	CLCN2	ENSG00000114859
bta-mir-185	LANCL1	ENSG00000115365

bta-mir-185	FN1	ENSG00000115414
bta-mir-185	ELMOD3	ENSG00000115459
bta-mir-185	ARHGEF2	ENSG00000116584
bta-mir-185	FASLG	ENSG00000117560
bta-mir-185	FAM98A	ENSG00000119812
bta-mir-185	NCAPH	ENSG00000121152
bta-mir-185	CXCR4	ENSG00000121966
bta-mir-185	BATF3	ENSG00000123685
bta-mir-185	RBPJL	ENSG00000124232
bta-mir-185	HIF3A	ENSG00000124440
bta-mir-185	BTN1A1	ENSG00000124557
bta-mir-185	ZNF436	ENSG00000125945
bta-mir-185	MACF1	ENSG00000127603
bta-mir-185	ECHS1	ENSG00000127884
bta-mir-185	KIF1C	ENSG00000129250
bta-mir-185	SAT1	ENSG00000130066
bta-mir-185	PGPEP1	ENSG00000130517
bta-mir-185	TRIM28	ENSG00000130726
bta-mir-185	CAPN7	ENSG00000131375
bta-mir-185	C20orf79	ENSG00000132631
bta-mir-185	TMCC2	ENSG00000133069
bta-mir-185	LARGE	ENSG00000133424
bta-mir-185	IMPA1	ENSG00000133731
bta-mir-185	SOX5	ENSG00000134532
bta-mir-185	DTX1	ENSG00000135144
bta-mir-185	ITM2C	ENSG00000135916
bta-mir-185	DNAJB2	ENSG00000135924
bta-mir-185	TMBIM1	ENSG00000135926
bta-mir-185	TM6SF1	ENSG00000136404
bta-mir-185	SMPD4	ENSG00000136699
bta-mir-185	MMP7	ENSG00000137673
bta-mir-185	TRIM29	ENSG00000137699
bta-mir-185	NAB1	ENSG00000138386
bta-mir-185	FAM117B	ENSG00000138439
bta-mir-185	PARP9	ENSG00000138496
bta-mir-185	INHBE	ENSG00000139269
bta-mir-185	LUM	ENSG00000139329
bta-mir-185	MBNL2	ENSG00000139793
bta-mir-185	NIPA2	ENSG00000140157
bta-mir-185	MYOCD	ENSG00000141052
bta-mir-185	CLTC	ENSG00000141367
bta-mir-185	PSMA5	ENSG00000143106
bta-mir-185	RRNAD1	ENSG00000143303
bta-mir-185	CGN	ENSG00000143375
bta-mir-185	CHAC2	ENSG00000143942
bta-mir-185	STK11IP	ENSG00000144589
bta-mir-185	CPLX2	ENSG00000145920
bta-mir-185	PPP1R18	ENSG00000146112
bta-mir-185	ASB15	ENSG00000146809
bta-mir-185	NSDHL	ENSG00000147383
bta-mir-185	CDKN2B	ENSG00000147883
bta-mir-185	FAM57B	ENSG00000149926
bta-mir-185	IFIT5	ENSG00000152778
bta-mir-185	CMTM7	ENSG00000153551

bta-mir-185	DGKE	ENSG00000153933
bta-mir-185	ADAMTS3	ENSG00000156140
bta-mir-185	SLC28A1	ENSG00000156222
bta-mir-185	WDYHV1	ENSG00000156795
bta-mir-185	FUK	ENSG00000157353
bta-mir-185	MX1	ENSG00000157601
bta-mir-185	C2orf29	ENSG00000158435
bta-mir-185	SPATA2	ENSG00000158480
bta-mir-185	ABCG1	ENSG00000160179
bta-mir-185	RRP1B	ENSG00000160208
bta-mir-185	PMF1;PMF1-BGLAP	ENSG00000160783
bta-mir-185	IP6K3	ENSG00000161896
bta-mir-185	TNFSF13	ENSG00000161955
bta-mir-185	PPM1L	ENSG00000163590
bta-mir-185	OTUD4	ENSG00000164164
bta-mir-185	SHROOM1	ENSG00000164403
bta-mir-185	FREM1	ENSG00000164946
bta-mir-185	HGSNAT	ENSG00000165102
bta-mir-185	AMOTL1	ENSG00000166025
bta-mir-185	SGPL1	ENSG00000166224
bta-mir-185	C2	ENSG00000166278
bta-mir-185	MYO1A	ENSG00000166866
bta-mir-185	MS4A8B	ENSG00000166959
bta-mir-185	FOXI1	ENSG00000168269
bta-mir-185	XIRP1	ENSG00000168334
bta-mir-185	KLK7	ENSG00000169035
bta-mir-185	ROR2	ENSG00000169071
bta-mir-185	GKN1	ENSG00000169605
bta-mir-185	FASN	ENSG00000169710
bta-mir-185	JAGN1	ENSG00000171135
bta-mir-185	CANT1	ENSG00000171302
bta-mir-185	RASGRP4	ENSG00000171777
bta-mir-185	NINJ2	ENSG00000171840
bta-mir-185	MLLT3	ENSG00000171843
bta-mir-185	KLF11	ENSG00000172059
bta-mir-185	VEGFB	ENSG00000173511
bta-mir-185	P2RY2	ENSG00000175591
bta-mir-185	TOM1L2	ENSG00000175662
bta-mir-185	PLEKHF2	ENSG00000175895
bta-mir-185	SPRYD4	ENSG00000176422
bta-mir-185	C12orf69	ENSG00000179256
bta-mir-185	R3HDM2	ENSG00000179912
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bta-let-7b	WDR33	ENSG00000136709
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bta-let-7b	HMGA1	ENSG00000137309
bta-let-7b	ARHGAP20	ENSG00000137727
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bta-let-7b	FRAS1	ENSG00000138759
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bta-let-7b	EFHD2	ENSG00000142634
bta-let-7b	POU2F1	ENSG00000143190
bta-let-7b	USP21	ENSG00000143258
bta-let-7b	ABL2	ENSG00000143322
bta-let-7b	RGS16	ENSG00000143333
bta-let-7b	IKBKE	ENSG00000143466
bta-let-7b	VASH2	ENSG00000143494
bta-let-7b	RALB	ENSG00000144118
bta-let-7b	LRIG1	ENSG00000144749
bta-let-7b	OSMR	ENSG00000145623
bta-let-7b	RNF44	ENSG00000146083
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bta-let-7b	BIN3	ENSG00000147439
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bta-let-7b	CRB2	ENSG00000148204
bta-let-7b	MED22	ENSG00000148297
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bta-let-7b	HMGA2	ENSG00000149948
bta-let-7b	ABCB9	ENSG00000150967
bta-let-7b	AKAP6	ENSG00000151320
bta-let-7b	FARP1	ENSG00000152767
bta-let-7b	SLC25A27	ENSG00000153291
bta-let-7b	GDPD1	ENSG00000153982
bta-let-7b	C1orf96	ENSG00000154429
bta-let-7b	ADAMTS5	ENSG00000154736
bta-let-7b	PPARGC1B	ENSG00000155846
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bta-let-7b	SASS6	ENSG00000156876
bta-let-7b	SLC6A1	ENSG00000157103
bta-let-7b	CLDN12	ENSG00000157224
bta-let-7b	UBN2	ENSG00000157741
bta-let-7b	XKR8	ENSG00000158156
bta-let-7b	CNNM4	ENSG00000158158
bta-let-7b	SPATA2	ENSG00000158480
bta-let-7b	PPP1R15B	ENSG00000158615
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bta-let-7b	FAM160B2	ENSG00000158863
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bta-let-7b	C3orf64	ENSG00000163378
bta-let-7b	CLASP2	ENSG00000163539
bta-let-7b	TMEM41A	ENSG00000163900
bta-let-7b	SFMBT1	ENSG00000163935
bta-let-7b	BSN	ENSG00000164061
bta-let-7b	MFSD8	ENSG00000164073
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bta-let-7b	RICTOR	ENSG00000164327
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bta-let-7b	E2F6	ENSG00000169016
bta-let-7b	PARM1	ENSG00000169116
bta-let-7b	CRCT1	ENSG00000169509
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bta-let-7b	RRM2	ENSG00000171848
bta-let-7b	NME6	ENSG00000172113
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bta-let-7b	PHF8	ENSG00000172943
bta-let-7b	TRIB1	ENSG00000173334
bta-let-7b	SMARCC1	ENSG00000173473
bta-let-7b	MGA	ENSG00000174197
bta-let-7b	ATP2A2	ENSG00000174437
bta-let-7b	MFSD4	ENSG00000174514
bta-let-7b	B3GNT1	ENSG00000174684

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bta-let-7b	ZCCHC3	ENSG00000177764
bta-let-7b	CSRNP3	ENSG00000178662
bta-let-7b	C3orf23	ENSG00000179152
bta-let-7b	ARID3B	ENSG00000179361
bta-let-7b	FGD6	ENSG00000180263
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bta-let-7b	RGS6	ENSG00000182732
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bta-let-7b	SF3A3	ENSG00000183431
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bta-let-7b	IRS2	ENSG00000185950
bta-let-7b	SLC36A3	ENSG00000186334
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bta-let-7b	TRIM71	ENSG00000206557
bta-let-7b	NRAS	ENSG00000213281
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bta-let-7b	GRID2IP	ENSG00000215045
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bta-let-7b	PLXNA4	ENSG00000221866
bta-let-7b	MARS2	ENSG00000247626
bta-let-7b	ATXN7L3B	ENSG00000253719
bta-let-7b	MEX3A	ENSG00000254726
bta-let-7b	C5orf62	ENSG00000256235
bta-let-7b	MGAM	ENSG00000257335
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bta-mir-708	RAPGEF4	ENSG00000091428
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bta-mir-708	POFUT1	ENSG00000101346
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bta-mir-708	GTPBP4	ENSG00000107937
bta-mir-708	GIT1	ENSG00000108262
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bta-mir-708	DAP	ENSG00000112977
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bta-mir-708	TMCO6	ENSG00000113119
bta-mir-708	VIPR1	ENSG00000114812
bta-mir-708	FN1	ENSG00000115414
bta-mir-708	DNAJC6	ENSG00000116675
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bta-mir-708	FBXO30	ENSG00000118496
bta-mir-708	PPP6C	ENSG00000119414
bta-mir-708	NR4A3	ENSG00000119508
bta-mir-708	WDR11	ENSG00000120008
bta-mir-708	MSX2	ENSG00000120149
bta-mir-708	WBP4	ENSG00000120688
bta-mir-708	EPHX2	ENSG00000120915
bta-mir-708	POLK	ENSG00000122008
bta-mir-708	MORF4L2	ENSG00000123562
bta-mir-708	PSMB2	ENSG00000126067
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bta-mir-708	PKDREJ	ENSG00000130943
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bta-mir-708	STAB2	ENSG00000136011
bta-mir-708	TMEM8B	ENSG00000137103
bta-mir-708	FAM55D	ENSG00000137634
bta-mir-708	ABCG5	ENSG00000138075
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bta-mir-708	ITGA10	ENSG00000143127
bta-mir-708	PRCC	ENSG00000143294
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bta-mir-708	SLC39A1	ENSG00000143570
bta-mir-708	METTL21A	ENSG00000144401
bta-mir-708	NICN1	ENSG00000145029
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bta-mir-708	CDYL	ENSG00000153046
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bta-mir-708	SFTPC	ENSG00000168484
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bta-mir-708	SYAP1	ENSG00000169895
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bta-mir-708	NINJ2	ENSG00000171840
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bta-mir-708	BPIFC	ENSG00000184459
bta-mir-708	LOC646851	ENSG00000184949
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bta-mir-708	AGBL4	ENSG00000186094
bta-mir-708	FAM212B	ENSG00000197852
bta-mir-708	ZNF192	ENSG00000198315
bta-mir-708	PAPSS2	ENSG00000198682
bta-mir-708	CPLX3	ENSG00000213578
bta-mir-210	DHX33	ENSG00000005100
bta-mir-210	FMO1	ENSG00000010932
bta-mir-210	SRCIN1	ENSG00000017373
bta-mir-210	GCLM	ENSG00000023909
bta-mir-210	IFNGR1	ENSG00000027697
bta-mir-210	SOX30	ENSG00000039600
bta-mir-210	USH2A	ENSG00000042781
bta-mir-210	ADRB1	ENSG00000043591
bta-mir-210	ATP2B4	ENSG00000058668
bta-mir-210	CREB3L3	ENSG00000060566
bta-mir-210	HDAC7	ENSG00000061273
bta-mir-210	WISP2	ENSG00000064205
bta-mir-210	NAV3	ENSG00000067798
bta-mir-210	PDE8A	ENSG00000073417
bta-mir-210	EPB41L3	ENSG00000082397
bta-mir-210	ANGPT2	ENSG00000091879
bta-mir-210	PPM1F	ENSG00000100034
bta-mir-210	TRIM9	ENSG00000100505

Appendix 11: Summary of milk volumes, rectal temperatures, ambient temperatures, humidity, bacterial CFU counts, cell counts, CD14+ cells counts, RNA (ng/uL), RIN, 18/28s ratio, and total RNA data for each animal.

Sample ID	Sample description	Infection week & date	Animal tag number	Milk volume (in litres)	Rectal temperature (in Farenheit)	Ambient temperature (in celsius)	Humidity (%)	Bacterial counts (CFU per 10 mL)*	Cells counted per mL (hemocytometer)	CD14 cell number (FACS sorted)	RNA – ng/mL	RIN	18/28s ratio.	Total RNA in mg.
Milk-mRNA-TC-1-001/Milk-miRNA-TC-1-001	0_hour_control_animal_1	week: 1 date: 18/07/11	3039	3.1	103.6	35	59	0	17,200	1,200,000	21	9.6	2.1	0.7
Milk-mRNA-TC-1-002/Milk-miRNA-TC-1-002	12_hour_control_animal_1	week: 1 date: 18/07/11	3039	2.3	105.5	34	62	210	34,400	2,400,000	38	9.7	2	1.03
Milk-mRNA-TC-1-003/Milk-miRNA-TC-1-003	24_hour_control_animal_1	week: 1 date: 19/07/11	3039	2.2	103.7	25	94	0	64,800	2,430,000	81	10	1.6	1.46
Milk-mRNA-TC-1-004/Milk-miRNA-TC-1-004	36_hour_control_animal_1	week: 1 date: 19/07/11	3039	2.4	105.3	34	90	10	87,000	3,700,000	92	9.4	1.6	2.85
Milk-mRNA-TC-1-005/Milk-miRNA-TC-1-005	48_hour_control_animal_1	week: 1 date: 20/07/11	3039	2.5	103.2	30	53	17	29,000	2,280,000	20	10	2.1	0.82
Milk-mRNA-TI-1-001/Milk-miRNA-TI-1-001	0_hour_infected_animal_1	week: 1 date: 18/07/11	3029	2.8	104.4	35	59	290	43,200	4,100,000	30	7.7	1.8	0.78
Milk-mRNA-TI-1-002/Milk-miRNA-TI-1-002	12_hour_infected_animal_1	week: 1 date: 18/07/11	3029	2.4	105.5	34	62	200	67,200	4,030,000	25	8.7	2.2	0.65
Milk-mRNA-TI-1-003/Milk-miRNA-TI-1-003	24_hour_infected_animal_1	week: 1 date: 19/07/11	3029	2.07	104.7	25	94	11,400	210,000	4,030,000	52	9.4	2	1.4
Milk-mRNA-TI-1-004/Milk-miRNA-TI-1-004	36_hour_infected_animal_1	week: 1 date: 19/07/11	3029	1.8	105.5	34	90	22,000	2,400,000	4,030,000	54	10	1.7	1.73
Milk-mRNA-TI-1-005/Milk-miRNA-TI-1-005	48_hour_infected_animal_1	week: 1 date: 20/07/11	3029	1.25	103.4	30	53	1,350	3,350,000	4,030,000	45	10	1.9	1.76
Milk-mRNA-TC-2-001/Milk-miRNA-TC-2-001	0_hour_control_animal_2	week: 2 date: 02/08/11	3523	3	102.6	23	87	14	23,000	500,000	3	9.1	2.3	0.14
Milk-mRNA-TC-2-002/Milk-miRNA-TC-2-002	12_hour_control_animal_2	week: 2 date: 02/08/11	3523	3.2	103.2	31	75	10	46,000	900,000	9	7.3	2	0.26
Milk-mRNA-TC-2-003/Milk-miRNA-TC-2-003	24_hour_control_animal_2	week: 2 date: 03/08/11	3523	3.4	101.9	23	65	11	18,400	280,000	5	9.6	2.2	0.19
Milk-mRNA-TC-2-004/Milk-miRNA-TC-2-004	36_hour_control_animal_2	week: 2 date: 03/08/11	3523	2.05	102.4	26	71	63	18,000	1,300,000	25	9.7	2.2	0.98
Milk-mRNA-TC-2-005/Milk-miRNA-TC-2-005	48_hour_control_animal_2	week: 2 date: 04/08/11	3523	3.7	101.6	22	81	31	2,600	1,300,000	20	9.9	2.3	0.7
Milk-mRNA-TI-2-001/Milk-miRNA-TI-2-001	0_hour_infected_animal_2	week: 2 date: 02/08/11	3206	1.8	101.8	23	87	0	30,000	2,500,000	50	10	1.6	2.1
Milk-mRNA-TI-2-002/Milk-miRNA-TI-2-002	12_hour_infected_animal_2	week: 2 date: 02/08/11	3206	2	103.1	31	75	80	11,400	1,300,000	26	10	2.1	0.91
Milk-mRNA-TI-2-003/Milk-miRNA-TI-2-003	24_hour_infected_animal_2	week: 2 date: 03/08/11	3206	1.6	101.6	23	65	196	27,600	710,000	5	8.9	2.1	0.19
Milk-mRNA-TI-2-004/Milk-miRNA-TI-2-004	36_hour_infected_animal_2	week: 2 date: 03/08/11	3206	2.7	102.3	26	71	56	460,000	2,800,000	38	9.6	1.6	1.25
Milk-mRNA-TI-2-005/Milk-miRNA-TI-2-005	48_hour_infected_animal_2	week: 2 date: 04/08/11	3206	2.3	101.4	22	81	107	89,000	2,200,000	35	10	1.9	1.23
Milk-mRNA-TC-3-001/Milk-miRNA-TC-3-001	0_hour_control_animal_3	Week: 5 date:28/08/12	3132	3.5	101.8	18	85	8	79,000	4,010,000	54	8.5	2.6	2.05
Milk-mRNA-TC-3-002/Milk-miRNA-TC-3-002	12_hour_control_animal_3	Week: 5 date:28/08/12	3132	2.8	102.1	30	67	10	73,000	4,040,000	69	8.3	1.9	2.69
Milk-mRNA-TC-3-003/Milk-miRNA-TC-3-003	24_hour_control_animal_3	Week: 5 date:01/09/12	3132	2.05	101.6	21	100	16	49,000	4,000,000	68	8.7	2.1	2.45
Milk-mRNA-TC-3-004/Milk-miRNA-TC-3-004	36_hour_control_animal_3	Week: 5 date:01/09/12	3132	1.7	102.4	34	46	108	24,000	4,000,000	36	8.8	2.1	1.26
Milk-mRNA-TC-3-005/Milk-miRNA-TC-3-005	48_hour_control_animal_3	Week: 5 date:02/09/12	3132	3.8	102.7	22	82	274	46,000	4,000,000	86	8.9	2.9	3.09
Milk-mRNA-TI-3-001/Milk-miRNA-TI-3-001	0_hour_infected_animal_3	Week: 5 date:28/08/12	3658	2.15	102.1	18	85	1	14,000	260,000	1	8.4	2	0.32
Milk-mRNA-TI-3-002/Milk-miRNA-TI-3-002	12_hour_infected_animal_3	Week: 5 date:28/08/12	3658	1.6	101.9	30	67	912	15,000	290,000	1	7.5	1.9	0.26
Milk-mRNA-TI-3-003/Milk-miRNA-TI-3-003	24_hour_infected_animal_3	Week: 5 date:01/09/12	3658	3.3	102.4	21	100	31,300	31,000	560,000	1	9	2.2	0.43
Milk-mRNA-TI-3-004/Milk-miRNA-TI-3-004	36_hour_infected_animal_3	Week: 5 date:01/09/12	3658	2.7	101.5	34	46	5,760	1,000,000	4,000,000	62	9.1	2.1	2.17
Milk-mRNA-TI-3-005/Milk-miRNA-TI-3-005	48_hour_infected_animal_3	Week: 5 date:02/09/12	3658	2	101.6	22	82	5,810	850,000	4,000,000	61	8.9	2	1.58
Milk-mRNA-TC-4-001/Milk-miRNA-TC-4-001	0_hour_control_animal_4	Week: 6 date:07/09/12	3039	2.8	100.9	10	62	21	54,000	4,900,000	68	9.7	2.5	1.7
Milk-mRNA-TC-4-002/Milk-miRNA-TC-4-002	12_hour_control_animal_4	Week: 6 date:07/09/12	3039	1.95	101.3	24	46	2	59,000	2,510,000	16	9.7	2	0.4
Milk-mRNA-TC-4-003/Milk-miRNA-TC-4-003	24_hour_control_animal_4	Week: 6 date:08/09/12	3039	2.7	101.6	9	96	6	56,000	1,700,000	23	9.9	2	0.575
Milk-mRNA-TC-4-004/Milk-miRNA-TC-4-004	36_hour_control_animal_4	Week: 6 date:08/09/12	3039	2	101.6	26	45	1	46,000	4,000,000	36	9.9	2.1	0.9
Milk-mRNA-TC-4-005/Milk-miRNA-TC-4-005	48_hour_control_animal_4	Week: 6 date:09/09/12	3039	2.6	101.4	13	93	11	54,000	4,000,000	69	9.6	2.1	1.7
Milk-mRNA-TI-4-001/Milk-miRNA-TI-4-001	0_hour_infected_animal_4	Week: 6 date:07/09/12	3207	4.4	100.6	10	62	0	35,000	4,030,000	19	9.6	1.5	0.475
Milk-mRNA-TI-4-002/Milk-miRNA-TI-4-002	12_hour_infected_animal_4	Week: 6 date:07/09/12	3207	2.8	101.2	24	46	97	30,000	4,000,000	41	9.7	2.1	1.025
Milk-mRNA-TI-4-003/Milk-miRNA-TI-4-003	24_hour_infected_animal_4	Week: 6 date:08/09/12	3207	4.55	100.9	9	96	2,060	56,000	3,700,000	46	10	2.1	1.15
Milk-mRNA-TI-4-004/Milk-miRNA-TI-4-004	36_hour_infected_animal_4	Week: 6 date:08/09/12	3207	3.05	102.1	26	45	6,900	650,000	4,000,000	56	10	2.1	1.4
Milk-mRNA-TI-4-005/Milk-miRNA-TI-4-005	48_hour_infected_animal_4	Week: 6 date:09/09/12	3207	3.8	103.1	13	93	3,460	1,200,000	4,000,000	69	9.5	9.6	1.73
Milk-mRNA-TC-5-001/Milk-miRNA-TC-5-001	0_hour_control_animal_5	week: 7 date: 13/09/11	3132	3.6	101.1	10	90	0	53,000	4,000,000	52	9.5	2.3	1.3
Milk-mRNA-TC-5-002/Milk-miRNA-TC-5-002	12_hour_control_animal_5	week: 7 date: 13/09/11	3132	2.9	101.1	25	44	10	33,000	4,000,000	26	10	2	0.65
Milk-mRNA-TC-5-003/Milk-miRNA-TC-5-003	24_hour_control_animal_5	week: 7 date: 14/09/11	3132	3.5	100.8	9	49	11	70,000	4,000,000	57	9.8	2.4	1.43
Milk-mRNA-TC-5-004/Milk-miRNA-TC-5-004	36_hour_control_animal_5	week: 7 date: 14/09/11	3132	2.6	101.4	16	31	63	84,000	4,000,000	77	9.8	2.4	1.92
Milk-mRNA-TC-5-005/Milk-miRNA-TC-5-005	48_hour_control_animal_5	week: 7 date: 15/09/11	3132	3.3	102.4	3	89	31	45,000	4,002,000	61	9.9	2.4	1.53
Milk-mRNA-TI-5-001/Milk-miRNA-TI-5-001	0_hour_infected_animal_5	week: 7 date: 13/09/11	3039	3.6	101.6	10	90	0	49,000	4,000,000	85	9.3	2.2	2.13
Milk-mRNA-TI-5-002/Milk-miRNA-TI-5-002	12_hour_infected_animal_5	week: 7 date: 13/09/11	3039	2.9	101.7	25	44	63	53,000	4,000,000	46	9.9	1.9	1.15
Milk-mRNA-TI-5-003/Milk-miRNA-TI-5-003	24_hour_infected_animal_5	week: 7 date: 14/09/11	3039	3.5	101.2	9	49	1,860	58,000	4,001,000	48	10	1.8	1.2
Milk-mRNA-TI-5-004/Milk-miRNA-TI-5-004	36_hour_infected_animal_5	week: 7 date: 14/09/11	3039	2.6	101.5	16	31	6,260	180,000	4,000,000	44	10	1.7	1.1
Milk-mRNA-TI-5-005/Milk-miRNA-TI-5-005	48_hour_infected_animal_5	week: 7 date: 15/09/11	3039	3.3	101.7	3	89	12,200	680,000	4,000,000	59	9.1	2.6	1.48

Sample ID	Sample description	Infection week & date	Animal tag number	Time	Blood volume (in mL)	Rectal temperature (in celsius)	Ambient temperature (in celsius)	Humidity (in percentage)	Total cells counted (hemocytometer).	CD14 cell number (FACS sorted).	RNA – ng/mL	RIN	18/28s ratio.	Total RNA in ng.
Blood-mRNA-TC-1-001/Blood-miRNA-TC-1-001	0_hour_control_animal_1	week: 1 date: 18/07/11	3039	0	50	103.6	35	59	14,000,000	1,500,000	119	9.7	1.7	2.38
Blood-mRNA-TC-1-002/Blood-miRNA-TC-1-002	12_hour_control_animal_1	week: 1 date: 18/07/11	3039	12	40	105.5	34	62	87,000,000	2,300,000	53	10	2.2	1.48
Blood-mRNA-TC-1-003/Blood-miRNA-TC-1-003	24_hour_control_animal_1	week: 1 date: 19/07/11	3039	24	60	103.7	25	94	180,000,000	4,100,000	114	10	2	2.51
Blood-mRNA-TC-1-004/Blood-miRNA-TC-1-004	36_hour_control_animal_1	week: 1 date: 19/07/11	3039	36	60	105.3	34	90	176,000,000	4,001,000	136	10	2.2	3.54
Blood-mRNA-TC-1-005/Blood-miRNA-TC-1-005	48_hour_control_animal_1	week: 1 date: 20/07/11	3039	48	60	103.2	30	53	62,000,000	4,050,000	90	9.8	2.3	2.79
Blood-mRNA-TI-1-001/Blood-miRNA-TI-1-001	0_hour_infected_animal_1	week: 1 date: 18/07/11	3029	0	50	104.4	35	59	190,000,000	4,060,000	108	10	1.9	2.27
Blood-mRNA-TI-1-002/Blood-miRNA-TI-1-002	12_hour_infected_animal_1	week: 1 date: 18/07/11	3029	12	40	105.5	34	62	170,000,000	1,120,000	52.1	9.8	2	1.3
Blood-mRNA-TI-1-003/Blood-miRNA-TI-1-003	24_hour_infected_animal_1	week: 1 date: 19/07/11	3029	24	60	104.7	25	94	160,000,000	4,070,000	114	9.9	1.9	2.62
Blood-mRNA-TI-1-004/Blood-miRNA-TI-1-004	36_hour_infected_animal_1	week: 1 date: 19/07/11	3029	36	60	105.5	34	90	127,000,000	3,100,000	81	10	2.2	2.34
Blood-mRNA-TI-1-005/Blood-miRNA-TI-1-005	48_hour_infected_animal_1	week: 1 date: 20/07/11	3029	48	60	103.4	30	53	53,000,000	3,300,000	86	10	2.4	3.35
Blood-mRNA-TC-2-001/Blood-miRNA-TC-2-001	0_hour_control_animal_2	week: 2 date: 02/08/11	3523	0	60	102.6	23	87	140,000,000	4,120,000	88	9.4	2.6	3.78
Blood-mRNA-TC-2-002/Blood-miRNA-TC-2-002	12_hour_control_animal_2	week: 2 date: 02/08/11	3523	12	60	103.2	31	75	190,000,000	1,260,000	53	9.7	1.4	1.48
Blood-mRNA-TC-2-003/Blood-miRNA-TC-2-003	24_hour_control_animal_2	week: 2 date: 03/08/11	3523	24	60	101.9	23	65	120,000,000	4,090,000	85	9.7	1.5	2.98
Blood-mRNA-TC-2-004/Blood-miRNA-TC-2-004	36_hour_control_animal_2	week: 2 date: 03/08/11	3523	36	60	102.4	26	71	190,000,000	1,920,000	60	9.9	1.7	2.04
Blood-mRNA-TC-2-005/Blood-miRNA-TC-2-005	48_hour_control_animal_2	week: 2 date: 04/08/11	3523	48	60	101.6	22	81	330,000,000	4,020,000	51	10	1.6	1.84
Blood-mRNA-TI-2-001/Blood-miRNA-TI-2-001	0_hour_infected_animal_2	week: 2 date: 02/08/11	3206	0	60	101.8	23	87	120,000,000	4,100,000	52	9.6	1.6	2.29
Blood-mRNA-TI-2-002/Blood-miRNA-TI-2-002	12_hour_infected_animal_2	week: 2 date: 02/08/11	3206	12	60	103.1	31	75	170,000,000	2,000,000	46	10	2.1	1.24
Blood-mRNA-TI-2-003/Blood-miRNA-TI-2-003	24_hour_infected_animal_2	week: 2 date: 03/08/11	3206	24	60	101.6	23	65	130,000,000	4,100,000	90	10	2	3.51
Blood-mRNA-TI-2-004/Blood-miRNA-TI-2-004	36_hour_infected_animal_2	week: 2 date: 03/08/11	3206	36	60	102.3	26	71	515,000,000	2,500,000	83	10	1.5	2.82
Blood-mRNA-TI-2-005/Blood-miRNA-TI-2-005	48_hour_infected_animal_2	week: 2 date: 04/08/11	3206	48	60	101.4	22	81	270,000,000	4,040,000	88	9.8	1.3	2.9
Blood-mRNA-TC-3-001/Blood-miRNA-TC-3-001	0_hour_control_animal_3	Week: 5 date:28/08/12	3132	0	40	101.8	18	85	332,000,000	4,030,000	95	8.7	2.4	3.51
Blood-mRNA-TC-3-002/Blood-miRNA-TC-3-002	12_hour_control_animal_3	Week: 5 date:28/08/12	3132	12	30	102.1	30	67	136,000,000	3,200,000	41	9.1	1.6	1.27
Blood-mRNA-TC-3-003/Blood-miRNA-TC-3-003	24_hour_control_animal_3	Week: 5 date:01/09/12	3132	24	60	101.6	21	100	348,000,000	4,000,000	65	9.2	2.2	2.6
Blood-mRNA-TC-3-004/Blood-miRNA-TC-3-004	36_hour_control_animal_3	Week: 5 date:01/09/12	3132	36	60	102.4	34	46	373,000,000	4,010,000	90	9.1	2	3.15
Blood-mRNA-TC-3-005/Blood-miRNA-TC-3-005	48_hour_control_animal_3	Week: 5 date:02/09/12	3132	48	60	102.7	22	82	300,000,000	4,000,000	57	8.6	1.8	1.65
Blood-mRNA-TI-3-001/Blood-miRNA-TI-3-001	0_hour_infected_animal_3	Week: 5 date:28/08/12	3658	0	60	102.1	18	85	201,000,000	4,000,000	77	9.2	1.9	2.77
Blood-mRNA-TI-3-002/Blood-miRNA-TI-3-002	12_hour_infected_animal_3	Week: 5 date:28/08/12	3658	12	30	101.9	30	67	183,000,000	4,000,000	64	9.8	2.2	2.36
Blood-mRNA-TI-3-003/Blood-miRNA-TI-3-003	24_hour_infected_animal_3	Week: 5 date:01/09/12	3658	24	60	102.4	21	100	334,000,000	4,000,000	107	9.4	2.2	4.17
Blood-mRNA-TI-3-004/Blood-miRNA-TI-3-004	36_hour_infected_animal_3	Week: 5 date:01/09/12	3658	36	60	101.5	34	46	331,000,000	4,000,000	80	9.7	2.9	2.1
Blood-mRNA-TI-3-005/Blood-miRNA-TI-3-005	48_hour_infected_animal_3	Week: 5 date:02/09/12	3658	48	60	101.6	22	82	245,000,000	4,000,000	66	9.5	1.9	2.5
Blood-mRNA-TC-4-001/Blood-miRNA-TC-4-001	0_hour_control_animal_4	Week: 6 date:07/09/12	3039	0	40	100.9	10	62	176,000,000	4,030,000	36	10	2.4	0.9
Blood-mRNA-TC-4-002/Blood-miRNA-TC-4-002	12_hour_control_animal_4	Week: 6 date:07/09/12	3039	12	40	101.3	24	46	144,000,000	4,000,000	52	10	2	1.3
Blood-mRNA-TC-4-003/Blood-miRNA-TC-4-003	24_hour_control_animal_4	Week: 6 date:08/09/12	3039	24	40	101.6	9	96	219,000,000	4,000,000	66	10	2.3	1.6
Blood-mRNA-TC-4-004/Blood-miRNA-TC-4-004	36_hour_control_animal_4	Week: 6 date:08/09/12	3039	36	40	101.6	26	45	223,000,000	4,110,000	56	10	2.4	1.4
Blood-mRNA-TC-4-005/Blood-miRNA-TC-4-005	48_hour_control_animal_4	Week: 6 date:09/09/12	3039	48	40	101.4	13	93	248,000,000	4,000,000	57	9.9	1.7	1.4
Blood-mRNA-TI-4-001/Blood-miRNA-TI-4-001	0_hour_infected_animal_4	Week: 6 date:07/09/12	3207	0	40	100.6	10	62	163,000,000	4,020,000	38	10	2.7	0.95
Blood-mRNA-TI-4-002/Blood-miRNA-TI-4-002	12_hour_infected_animal_4	Week: 6 date:07/09/12	3207	12	40	101.2	24	46	225,000,000	4,000,000	52	9.9	1.6	1.3
Blood-mRNA-TI-4-003/Blood-miRNA-TI-4-003	24_hour_infected_animal_4	Week: 6 date:08/09/12	3207	24	40	100.9	9	96	204,000,000	4,000,000	51	10	2.2	1.28
Blood-mRNA-TI-4-004/Blood-miRNA-TI-4-004	36_hour_infected_animal_4	Week: 6 date:08/09/12	3207	36	40	102.1	26	45	214,000,000	4,000,000	48	10	2.2	1.2
Blood-mRNA-TI-4-005/Blood-miRNA-TI-4-005	48_hour_infected_animal_4	Week: 6 date:09/09/12	3207	48	40	103.1	13	93	145,000,000	3,220,000	28	10	1.6	0.7
Blood-mRNA-TC-5-001/Blood-miRNA-TC-5-001	0_hour_control_animal_5	week: 7 date: 13/09/11	3132	0	60	101.1	10	90	256,000,000	3,900,000	135	9.7	2.2	3.38
Blood-mRNA-TC-5-002/Blood-miRNA-TC-5-002	12_hour_control_animal_5	week: 7 date: 13/09/11	3132	12	40	101.1	25	44	269,000,000	3,110,000	68	10	2.2	1.7
Blood-mRNA-TC-5-003/Blood-miRNA-TC-5-003	24_hour_control_animal_5	week: 7 date: 14/09/11	3132	24	40	100.8	9	49	210,000,000	4,000,000	125	9.8	2.2	3.13
Blood-mRNA-TC-5-004/Blood-miRNA-TC-5-004	36_hour_control_animal_5	week: 7 date: 14/09/11	3132	36	40	101.4	16	31	307,000,000	4,000,000	101	9.7	2.5	2.53
Blood-mRNA-TC-5-005/Blood-miRNA-TC-5-005	48_hour_control_animal_5	week: 7 date: 15/09/11	3132	48	40	102.4	3	89	280,000,000	4,000,000	94	10	2.1	2.35
Blood-mRNA-TI-5-001/Blood-miRNA-TI-5-001	0_hour_infected_animal_5	week: 7 date: 13/09/11	3039	0	60	101.1	10	90	262,000,000	4,000,000	121	9.7	2	3.03
Blood-mRNA-TI-5-002/Blood-miRNA-TI-5-002	12_hour_infected_animal_5	week: 7 date: 13/09/11	3039	12	40	101.1	25	44	236,000,000	4,000,000	103	10	1.9	2.58
Blood-mRNA-TI-5-003/Blood-miRNA-TI-5-003	24_hour_infected_animal_5	week: 7 date: 14/09/11	3039	24	40	100.8	9	49	210,000,000	4,010,000	85	10	2.5	2.13
Blood-mRNA-TI-5-004/Blood-miRNA-TI-5-004	36_hour_infected_animal_5	week: 7 date: 14/09/11	3039	36	40	101.4	16	31	264,000,000	4,030,000	77	9.9	2.2	1.93
Blood-mRNA-TI-5-005/Blood-miRNA-TI-5-005	48_hour_infected_animal_5	week: 7 date: 15/09/11	3039	48	40	102.4	3	89	271,000,000	4,000,000	113	10	2.4	2.83

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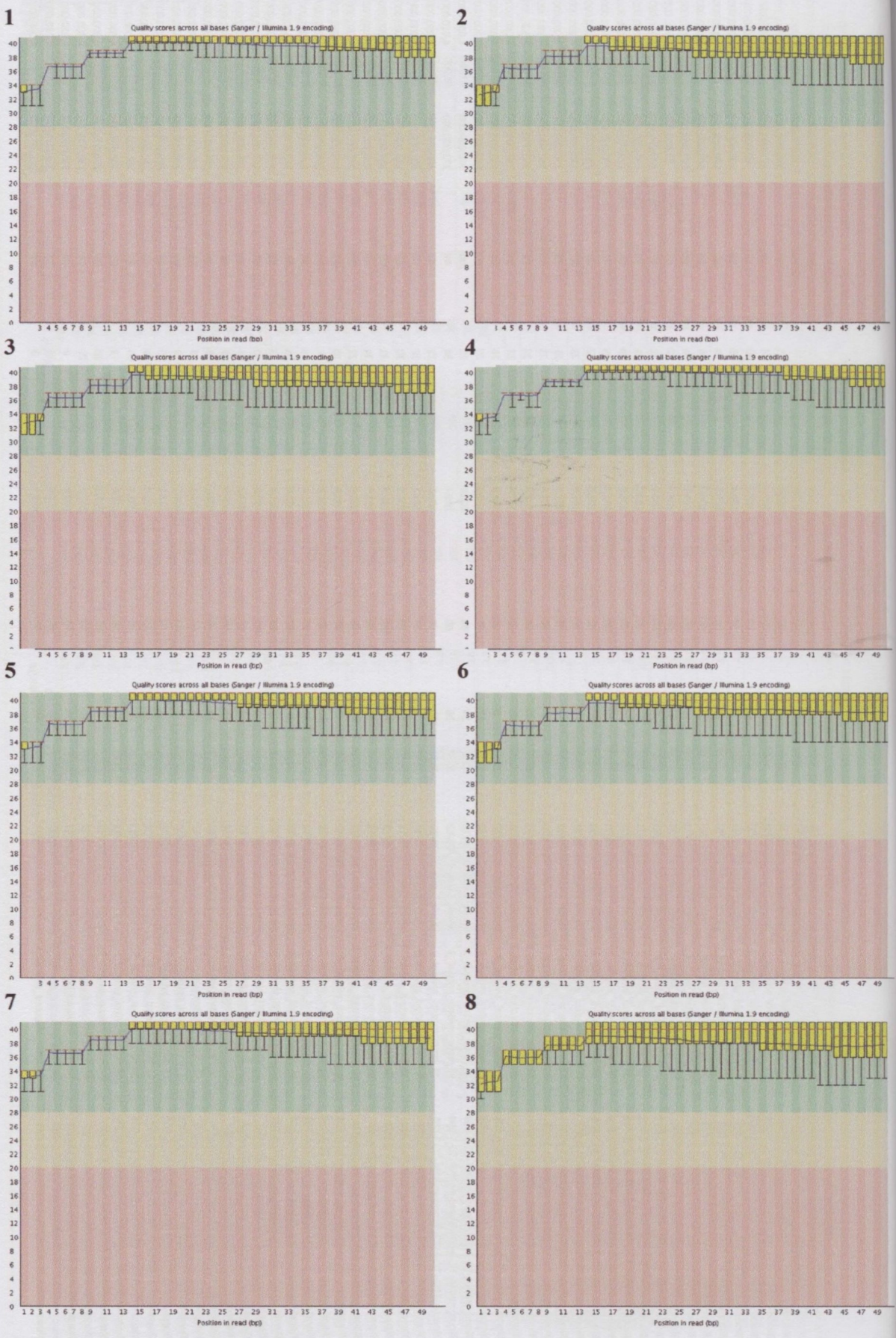
Appendix 12: FastQC checks on each library was carried out as a method of quality control checki sequencing dataset. Checks are carried out on sequencing data from Illumina Hiseq 2000 sequencer (Io Sequencing centre). Reads where at least 50% of the bases had a Phred score < 20 were removed (Cock, Fie 2010). Reads which successfully passed filtering were aligned to the bovine genome (UMD3.1). Fig. 1-5 = 1 control 0, 12, 24, 36, & 48. 6-10 = Animal 2 control 0, 12, 24, 36, & 48. 11-15 = Animal 3 control 0, 12, 2 48. 16-20 = Animal 4 control 0, 12, 24, 36, & 48. 21-25 = Animal 5 control 0, 12, 24, 36, & 48. 26-30 = A infected 0, 12, 24, 36, & 48. 31-35 = Animal 2 infected 0, 12, 24, 36, & 48. 31-35 = Animal 3 infected 0, 12 & 48. 31-35 = Animal 4 infected 0, 12, 24, 36, & 48. 36-40 = Animal 5 infected 0, 12, 24, 36, & 48.



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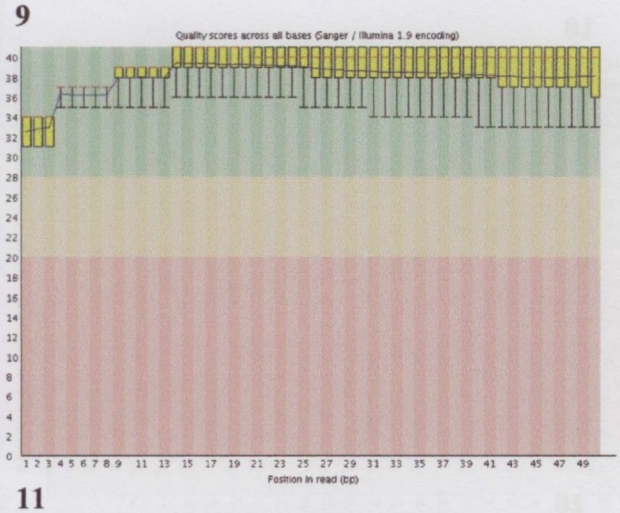

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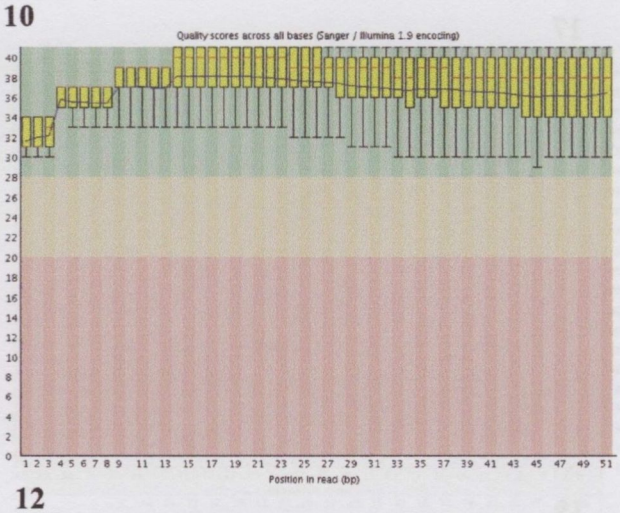

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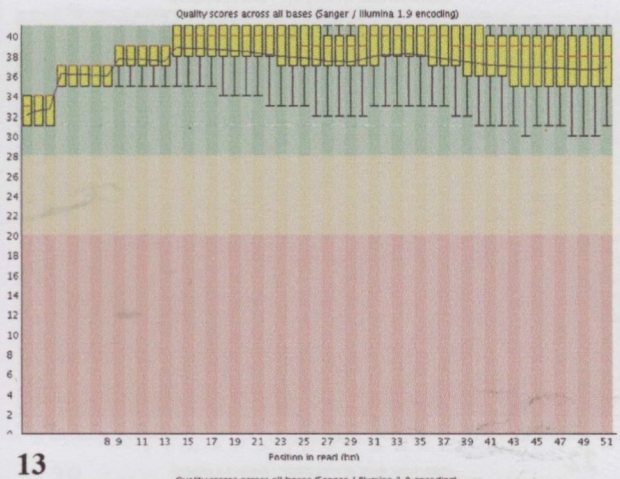

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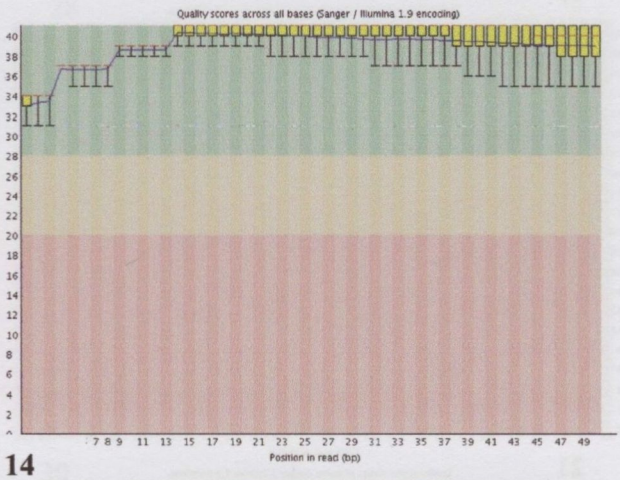

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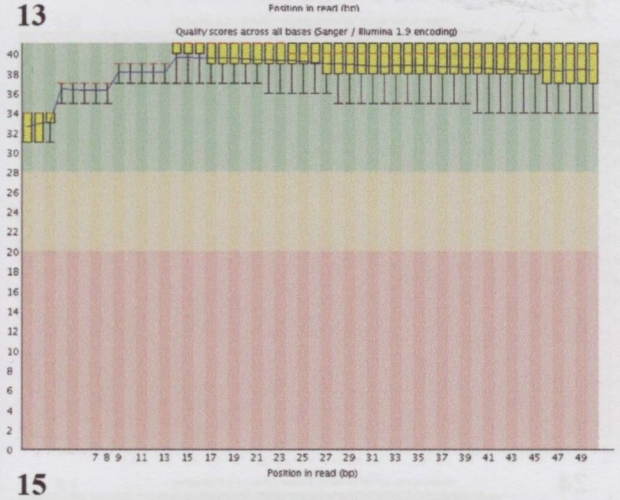

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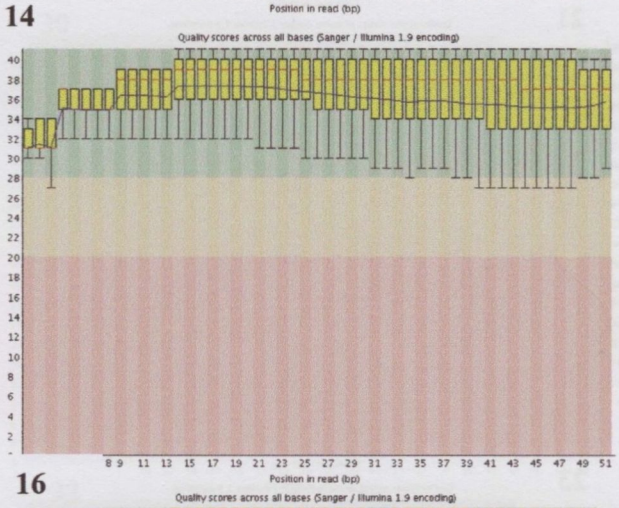

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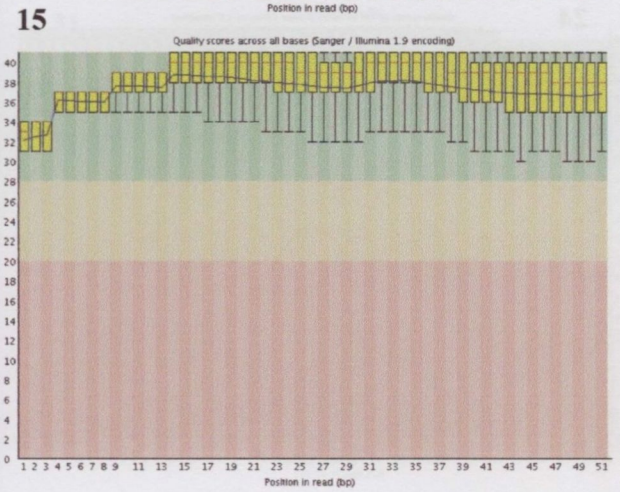

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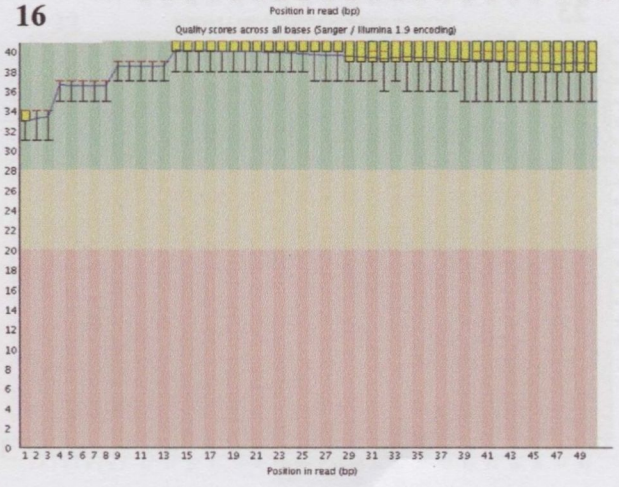

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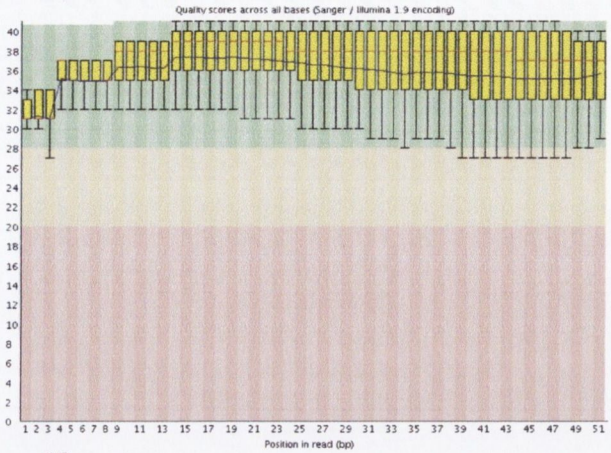

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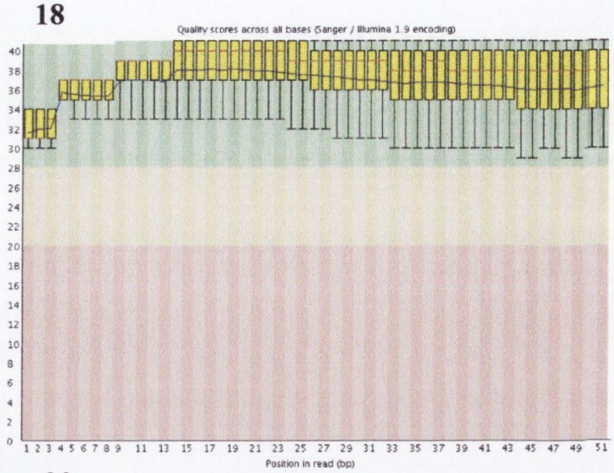

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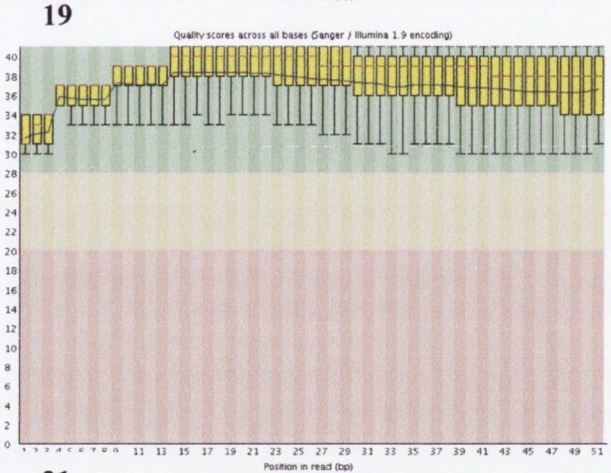

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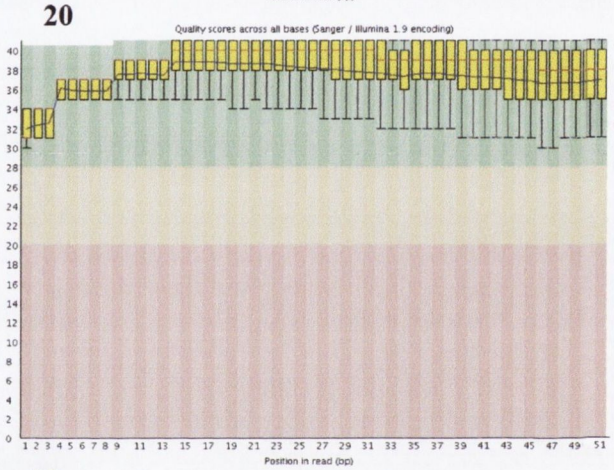

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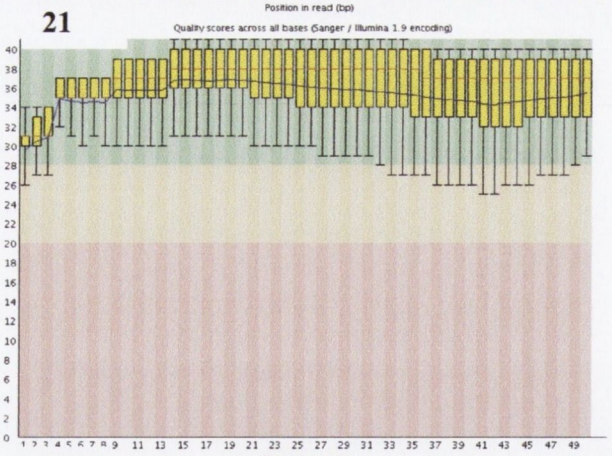

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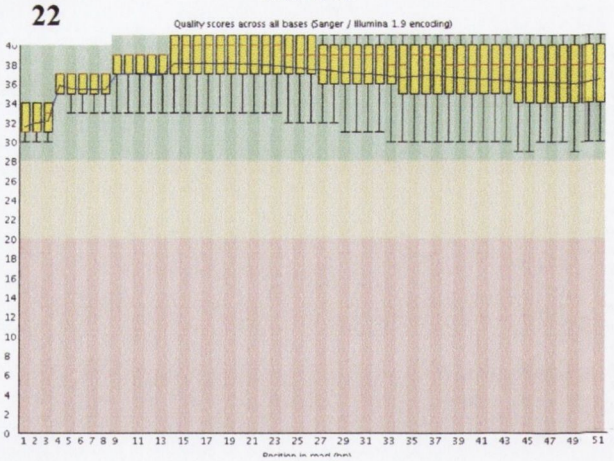

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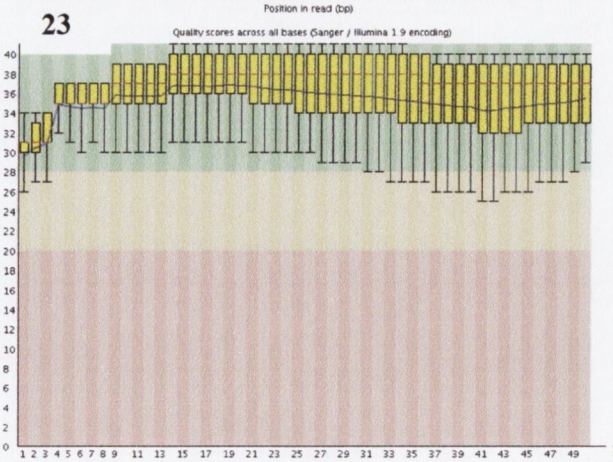

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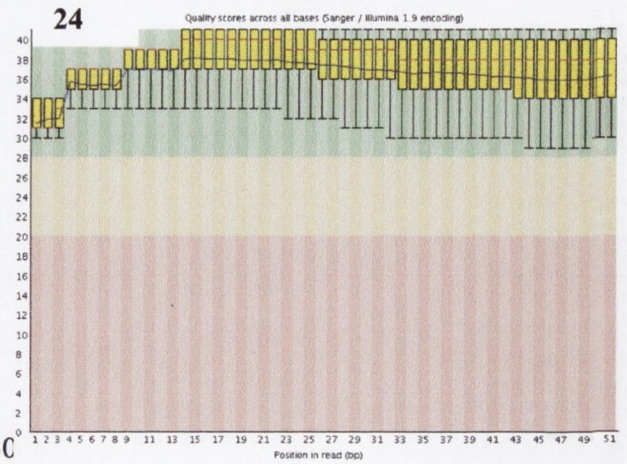

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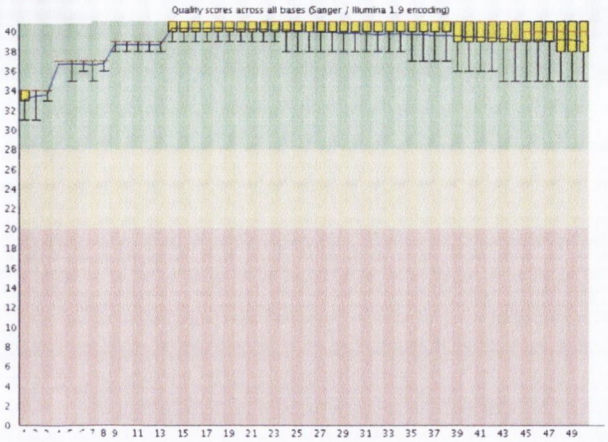

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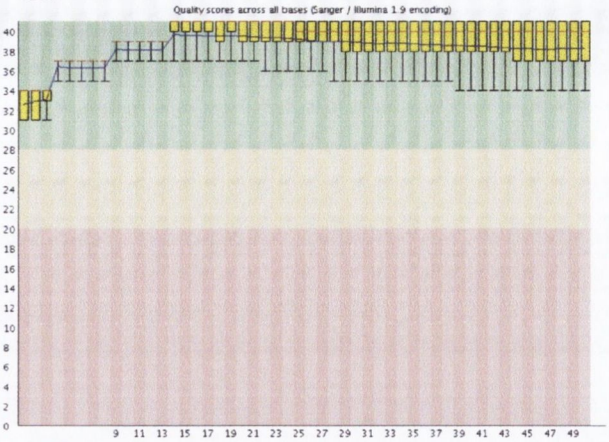

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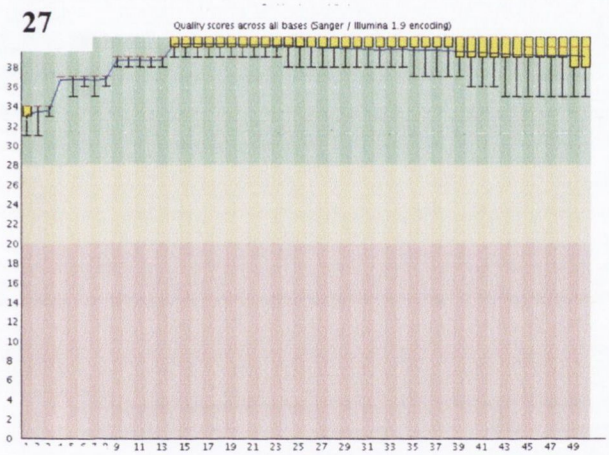

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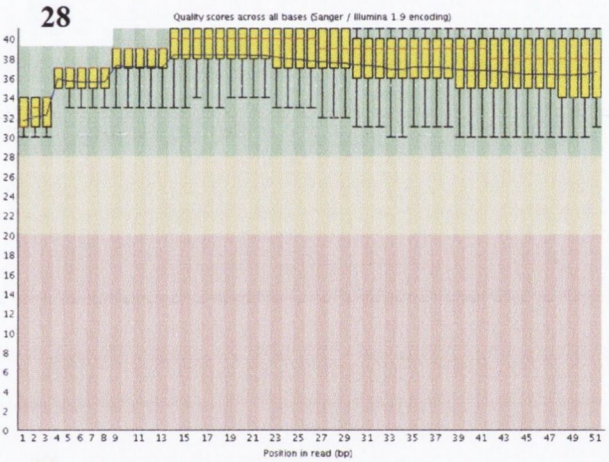

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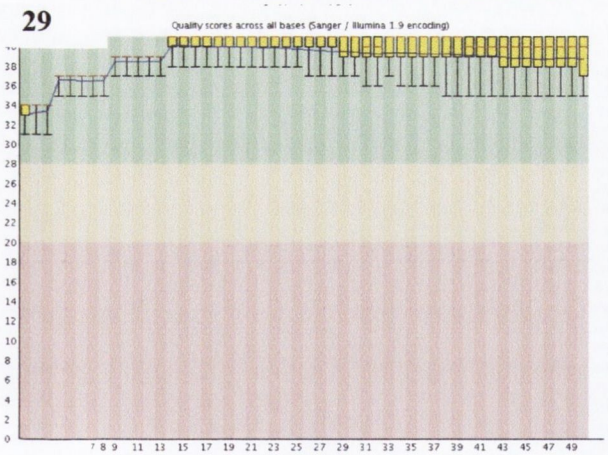

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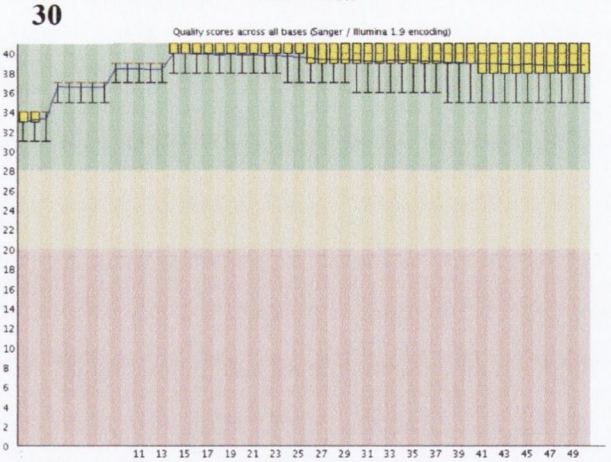

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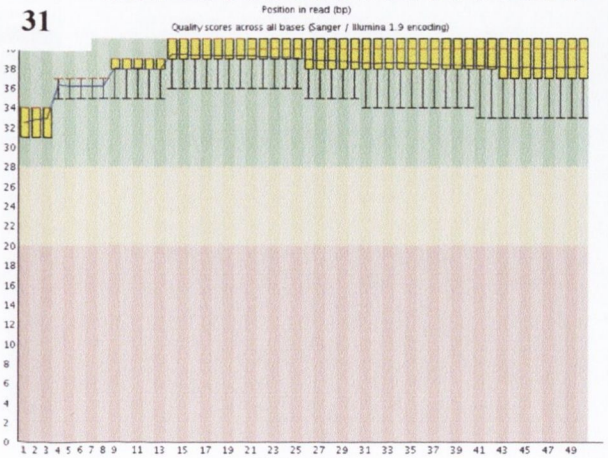

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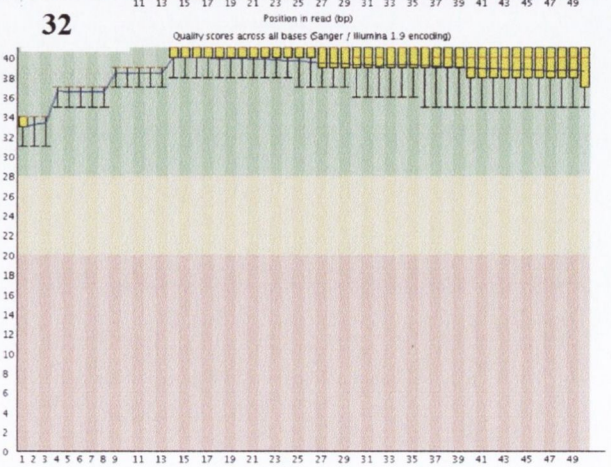

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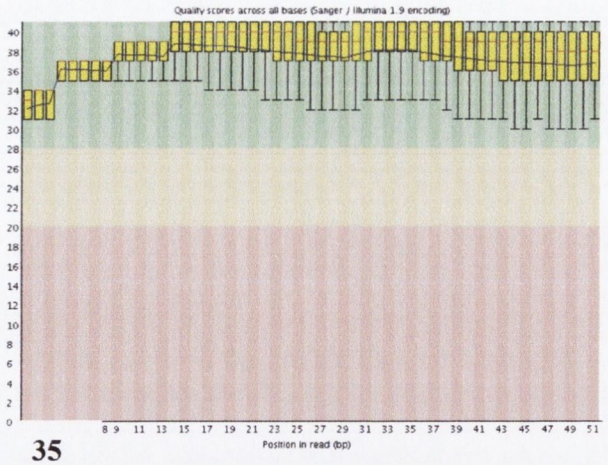

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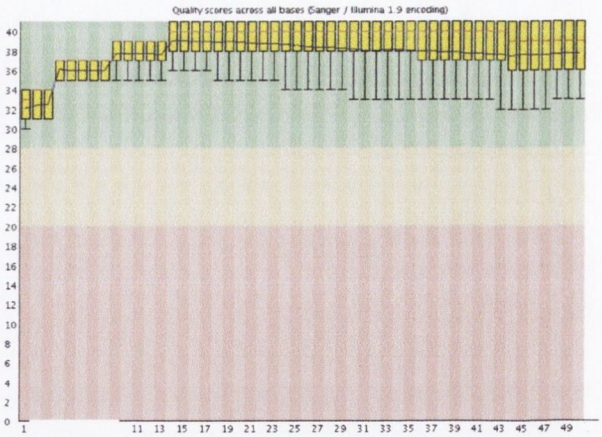

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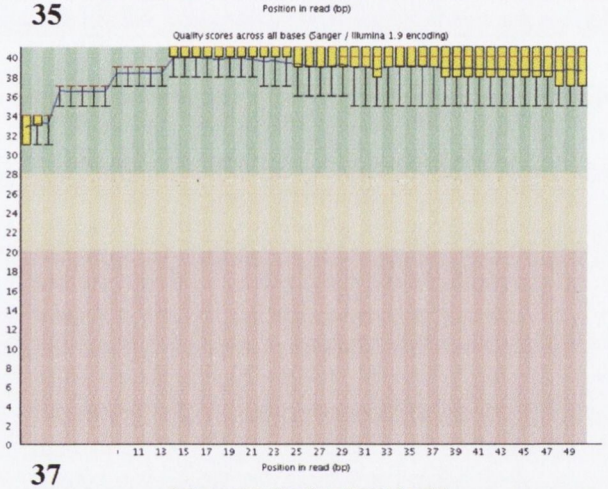

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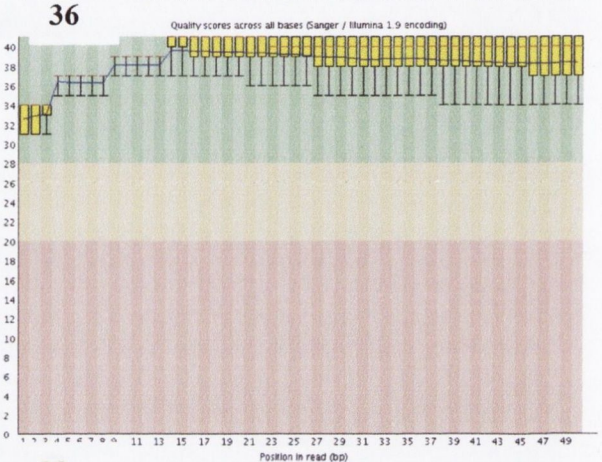

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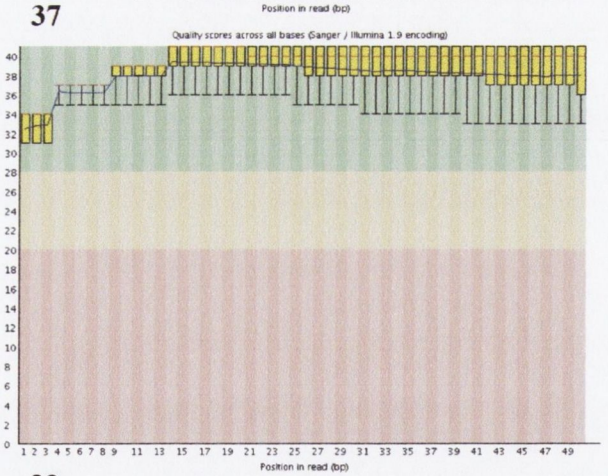

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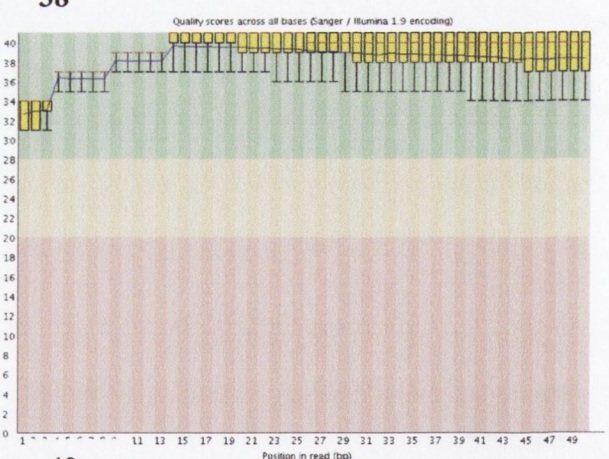

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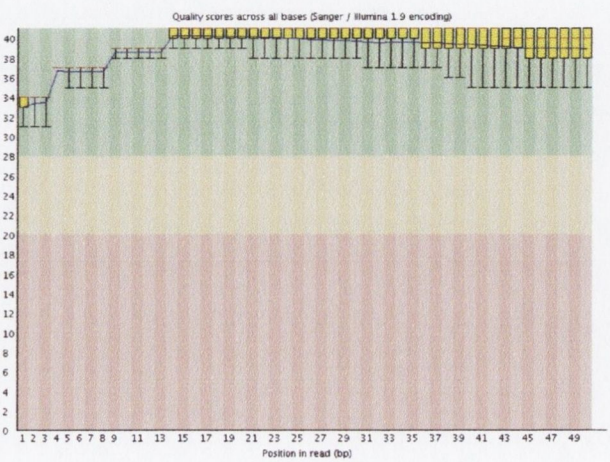

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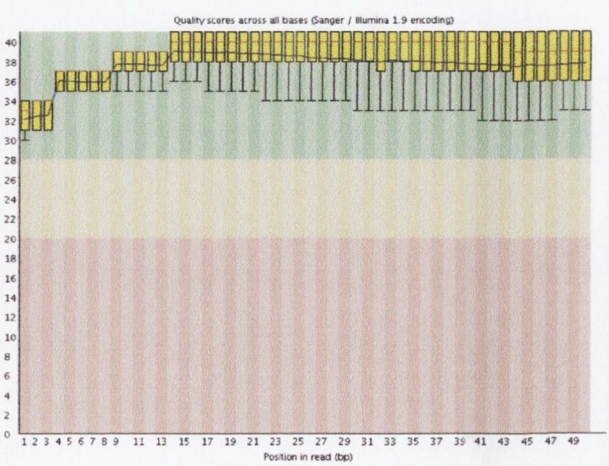

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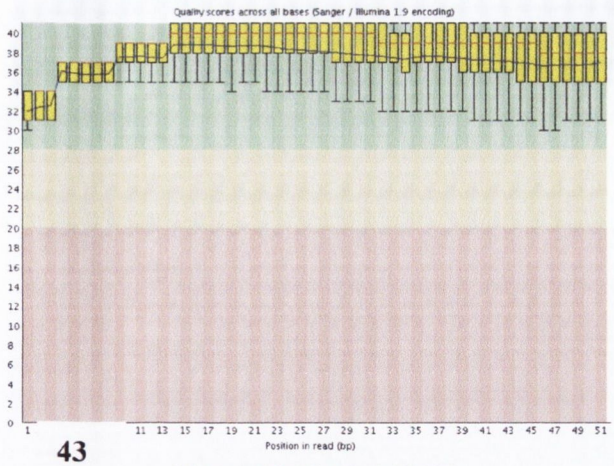

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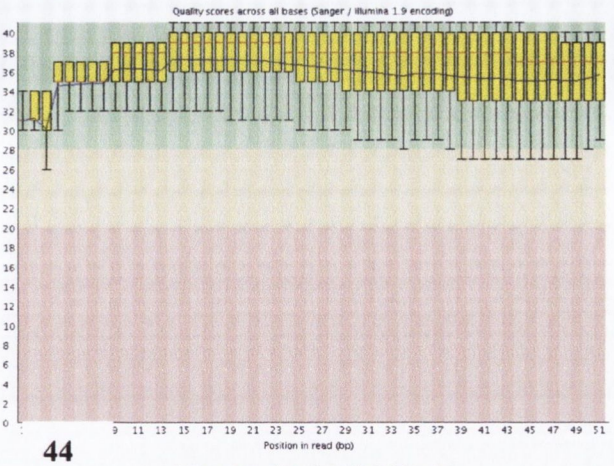

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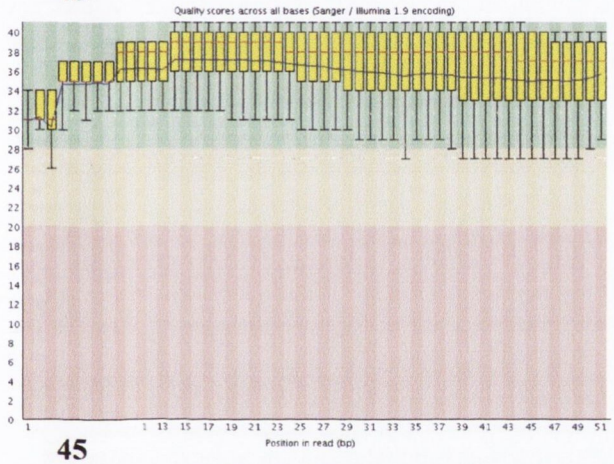

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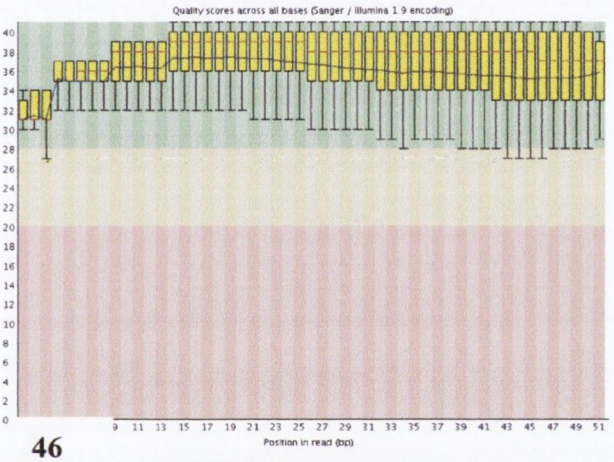

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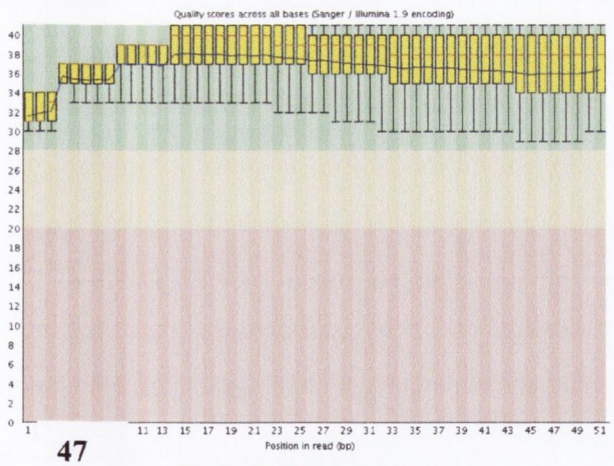

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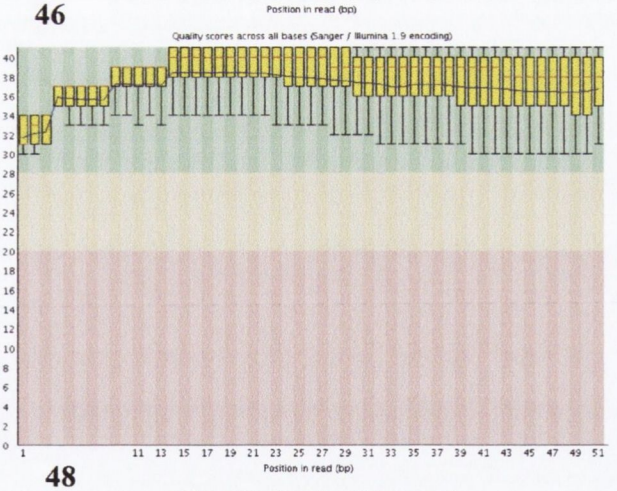

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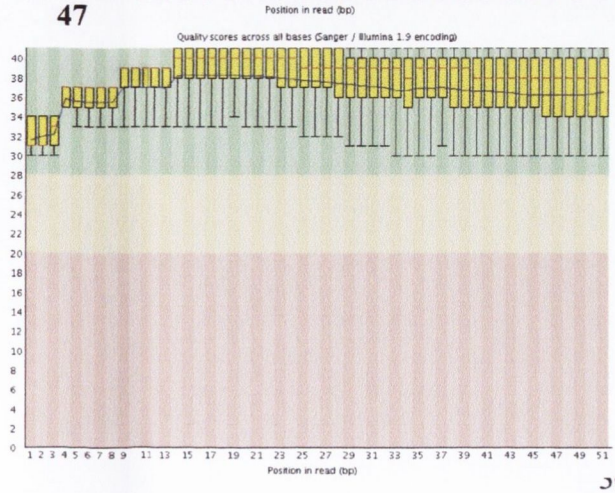

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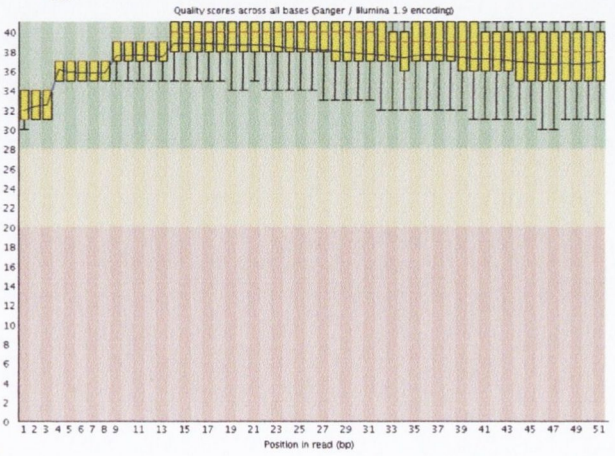

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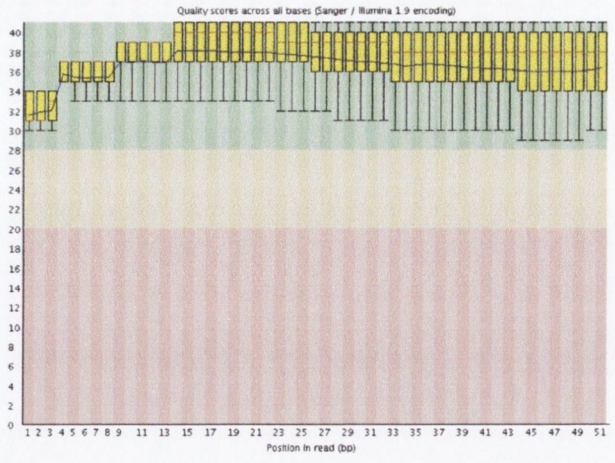
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Position in read (bp)

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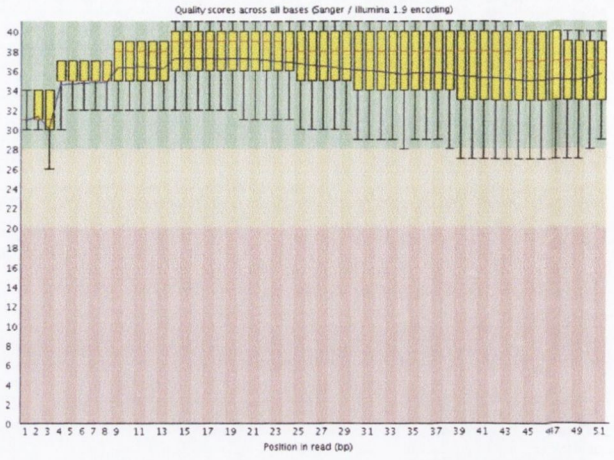
Quality scores across all bases (Sanger / Illumina 1.9 encoding)
Position in read (bp)

Blood mRNA FastQC library checks Fig. 1-5 = Animal 1 control 0, 12, 24, 36, & 48. 6-10 = Animal 2 control 0, 12, 24, 36, & 48. 11-15 = Animal 3 control 0, 12, 24, 36, & 48. 16-20 = Animal 4 control 0, 12, 24, 36, & 48. 21-25 = Animal 5 control 0, 12, 24, 36, & 48. 26-30 = Animal 1 infected 0, 12, 24, 36, & 48. 31-35 = Animal 2 infected 0, 12, 24, 36, & 48. 31-35 = Animal 3 infected 0, 12, 24, 36, & 48. 31-35 = Animal 4 infected 0, 12, 24, 36, & 48. 36-40 = Animal 5 infected 0, 12, 24, 36, & 48.

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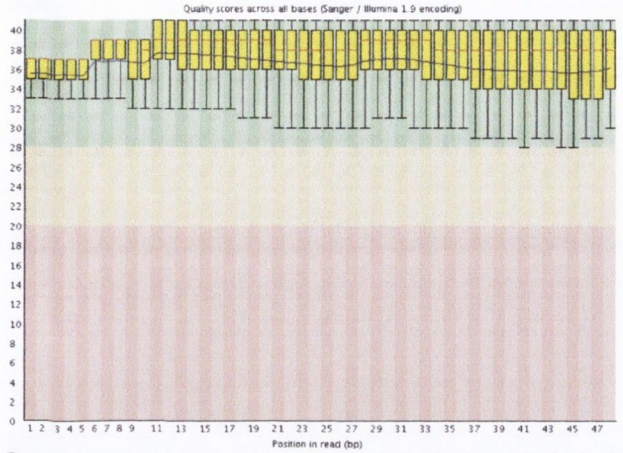

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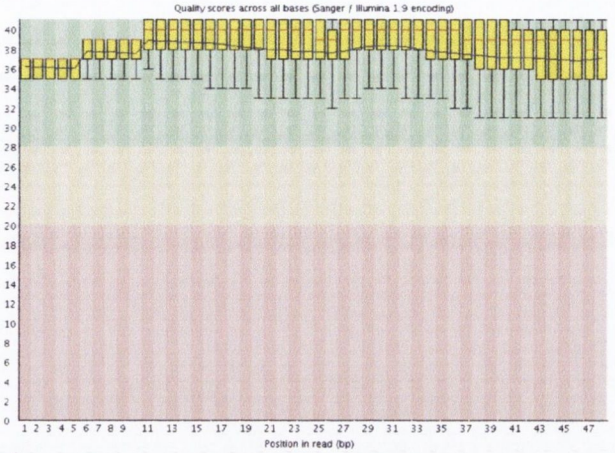

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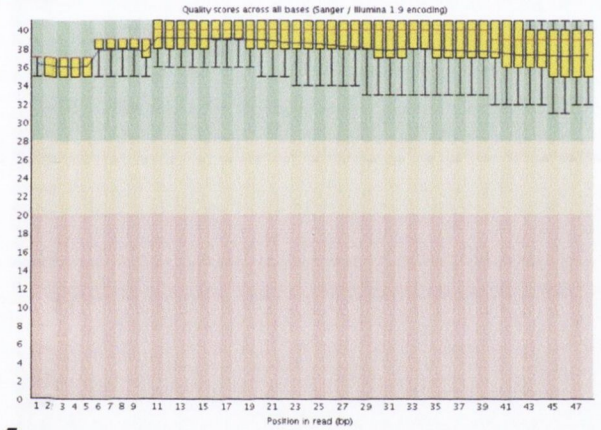

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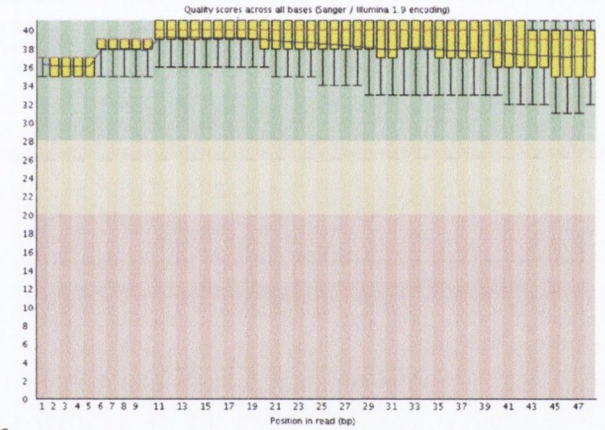

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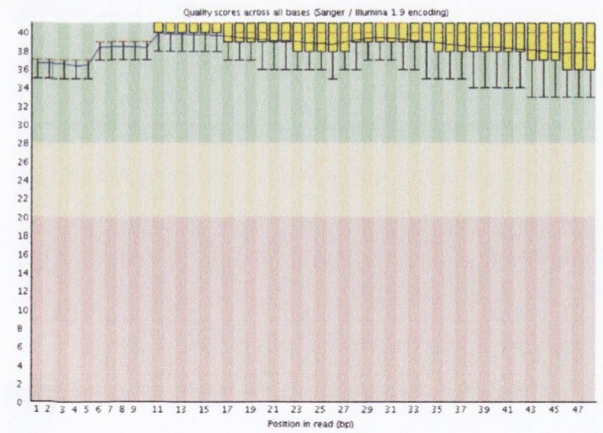

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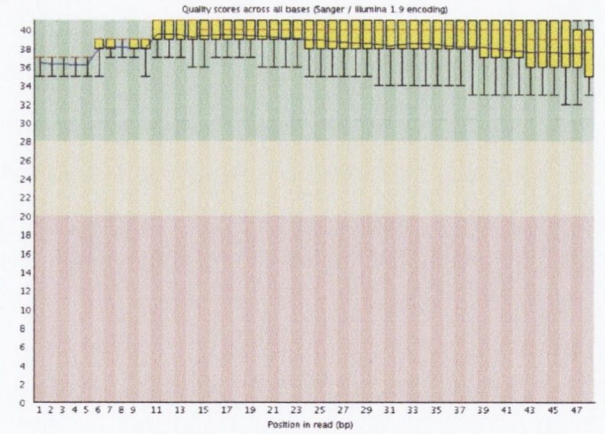

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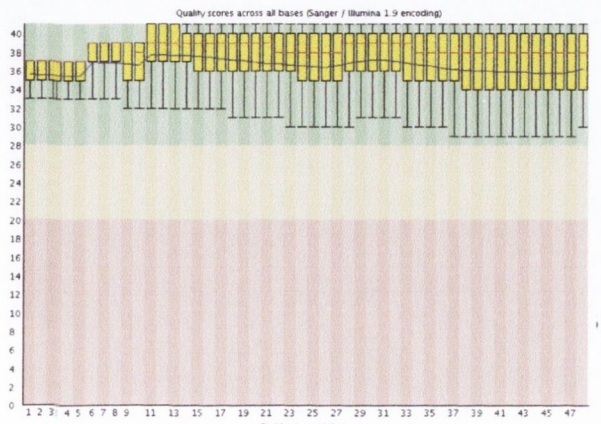

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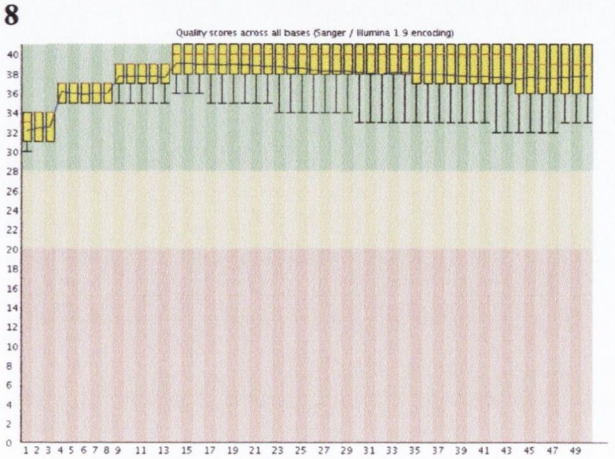

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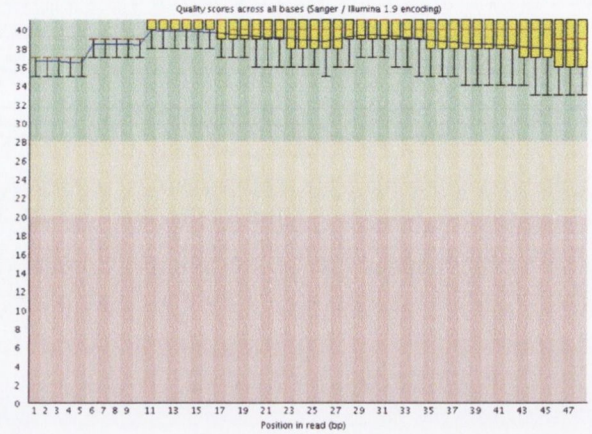

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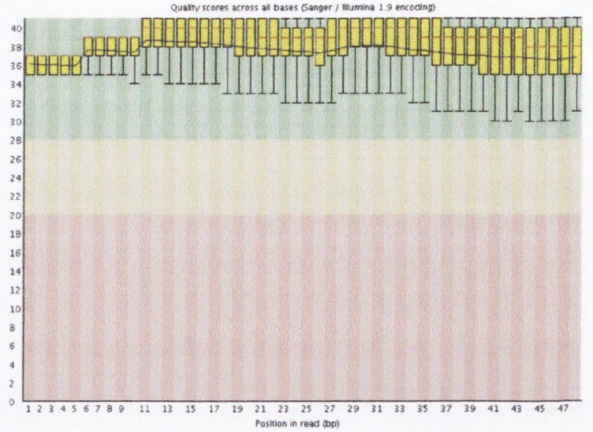

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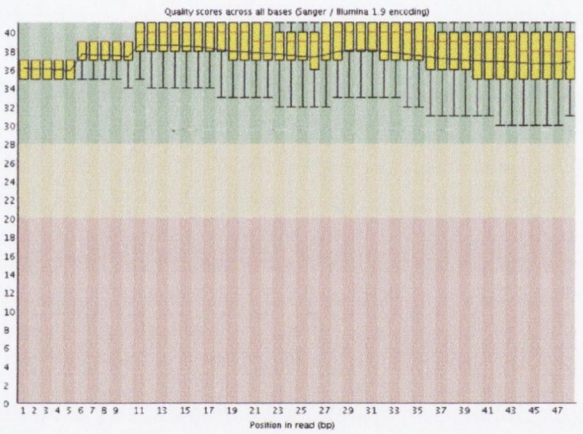

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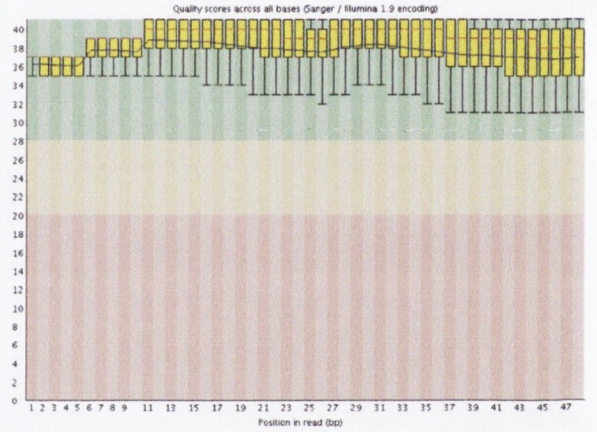

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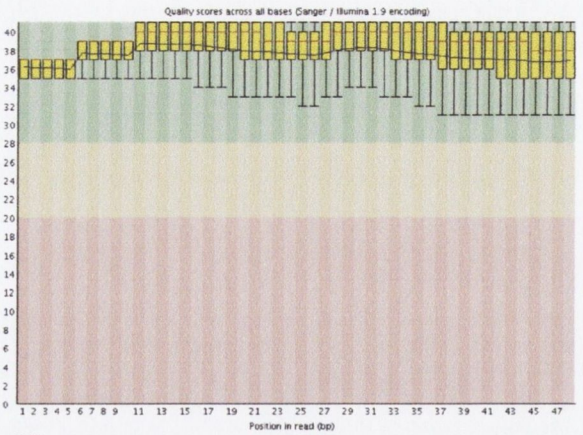

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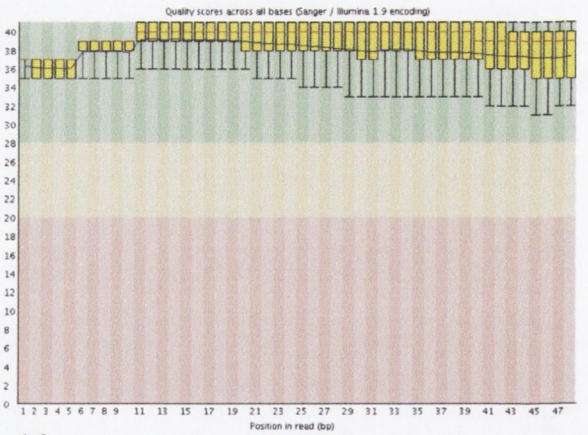

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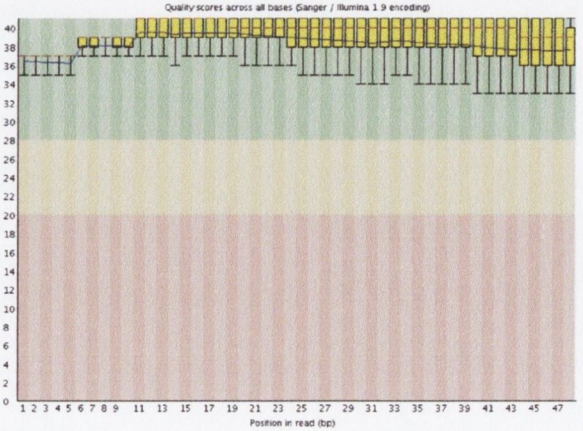

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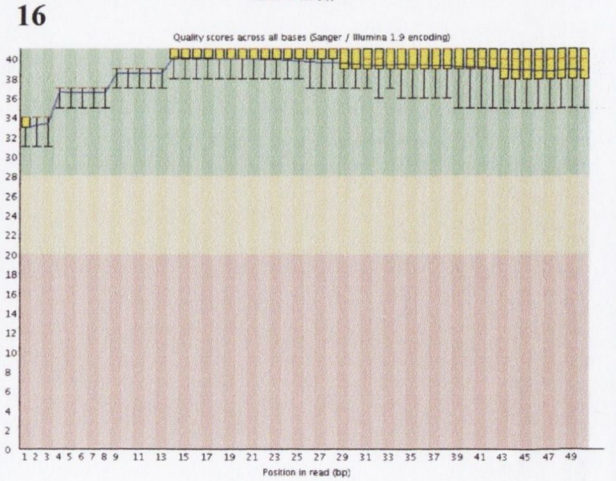

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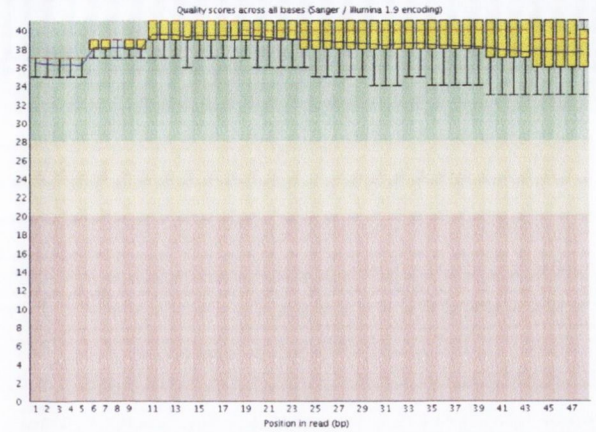

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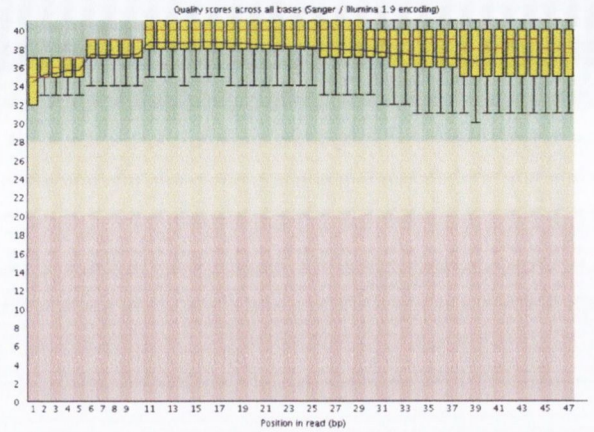

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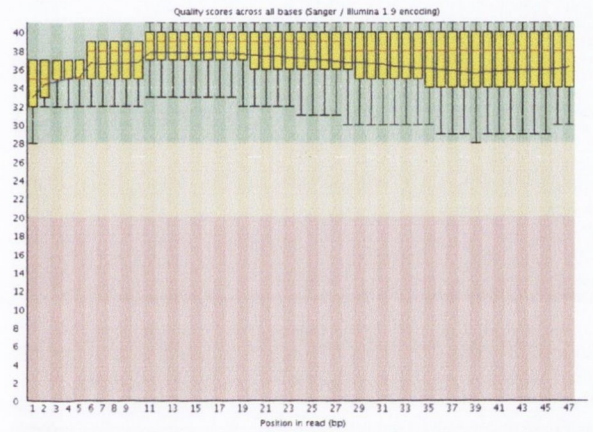

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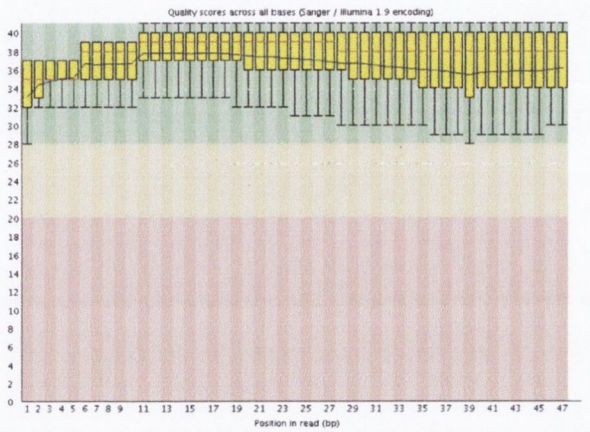

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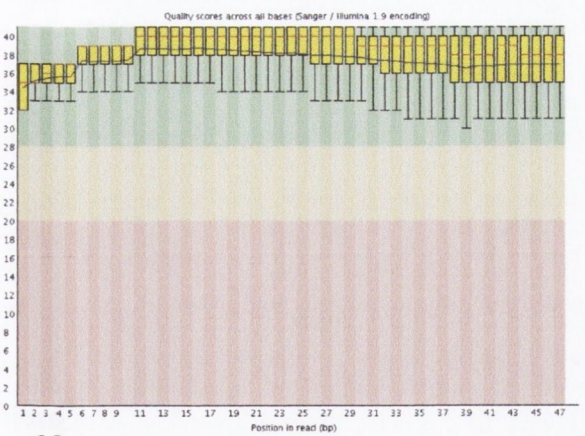

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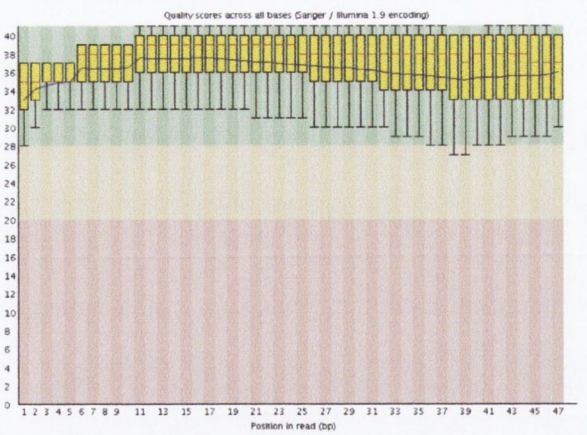

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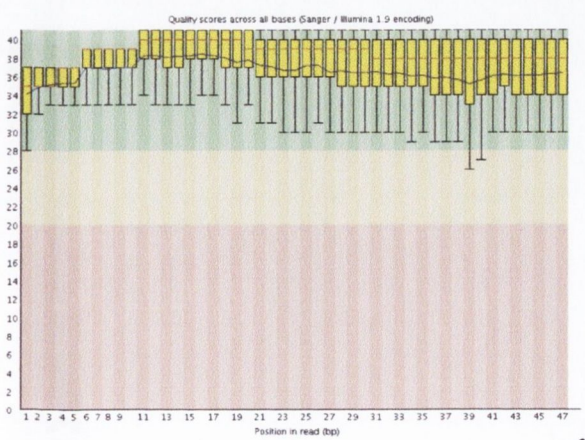

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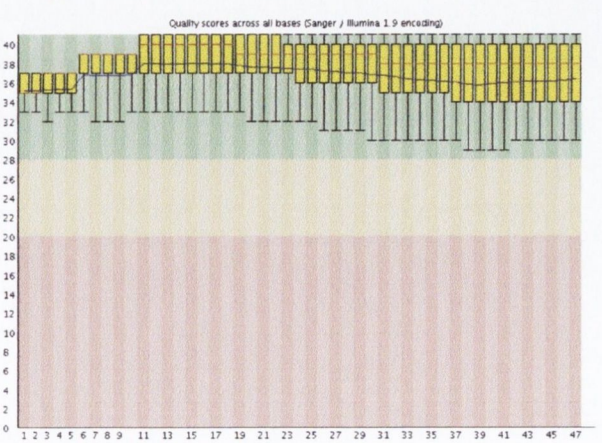

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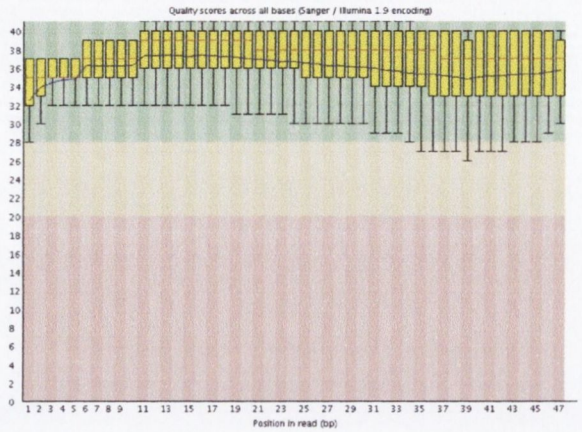

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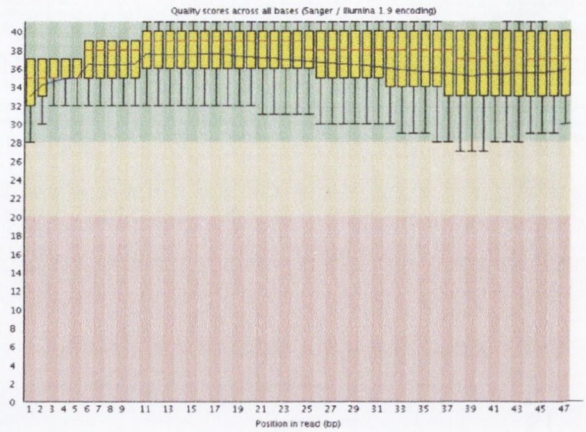

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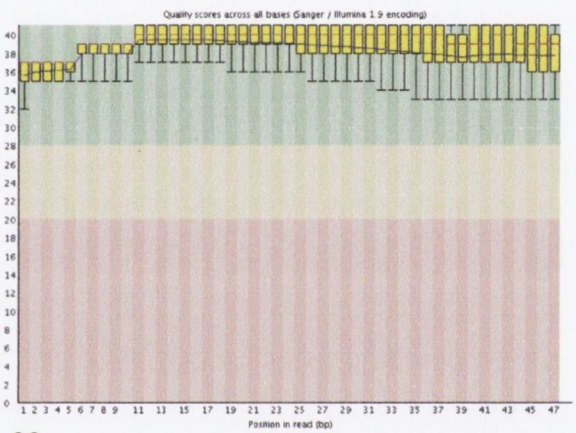

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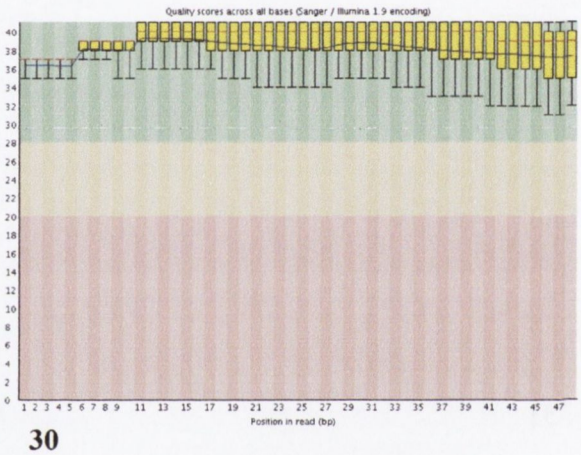

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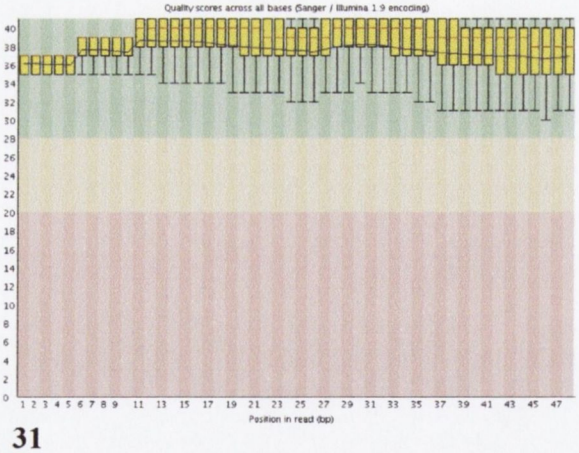

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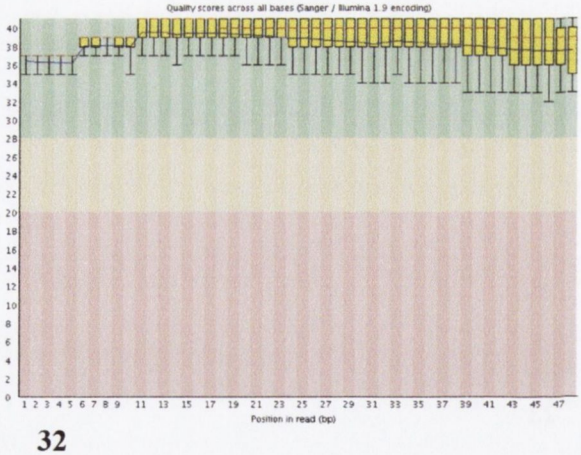

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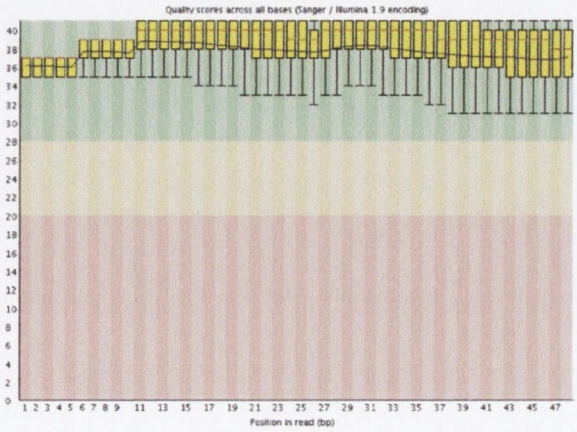

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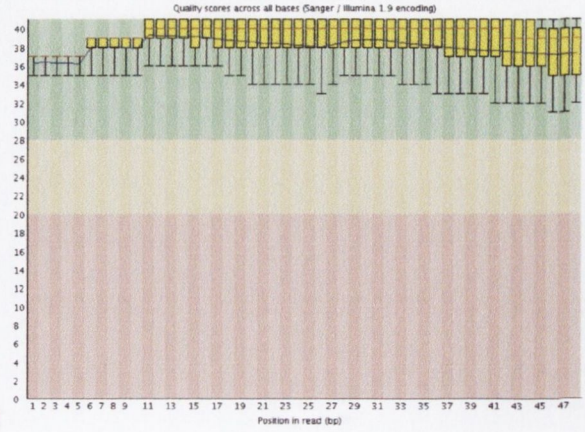

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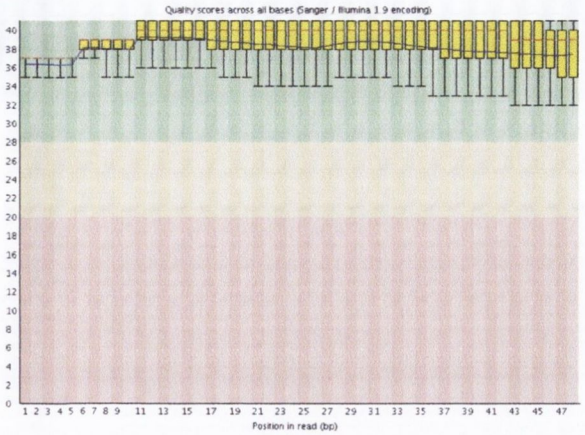

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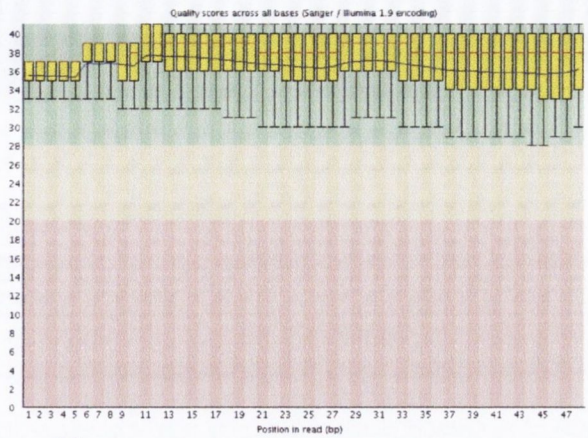

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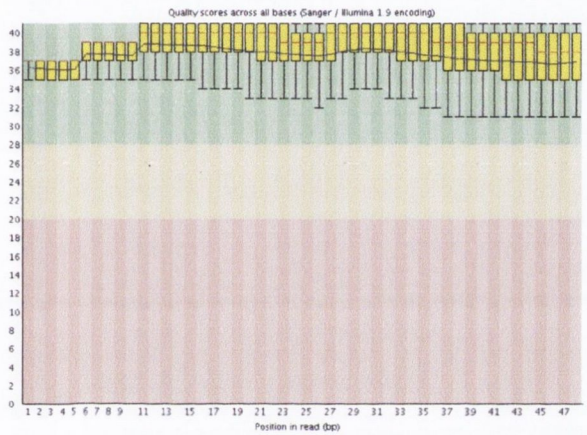

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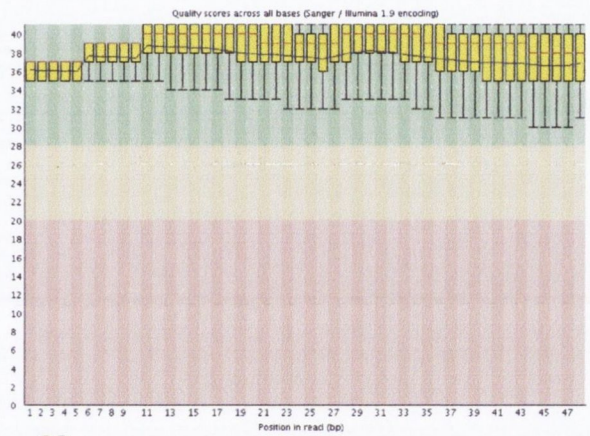

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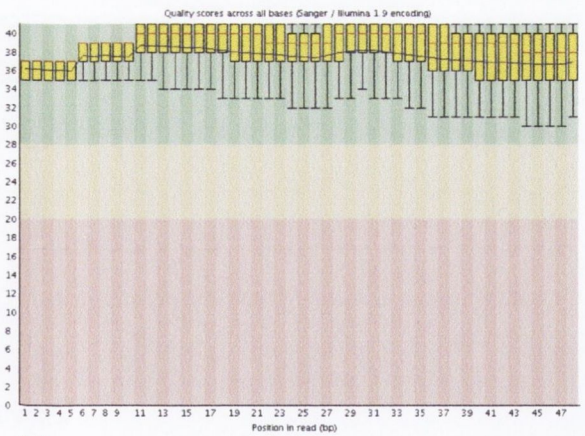

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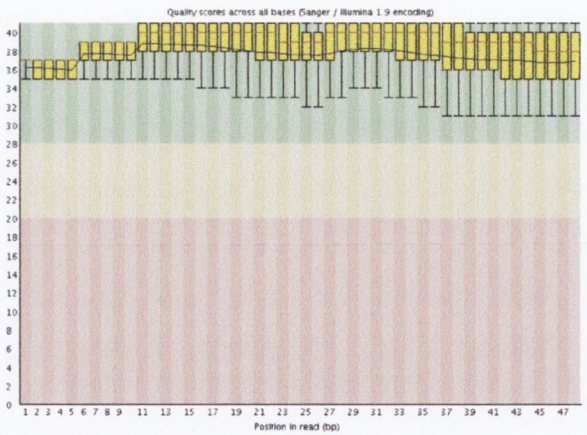

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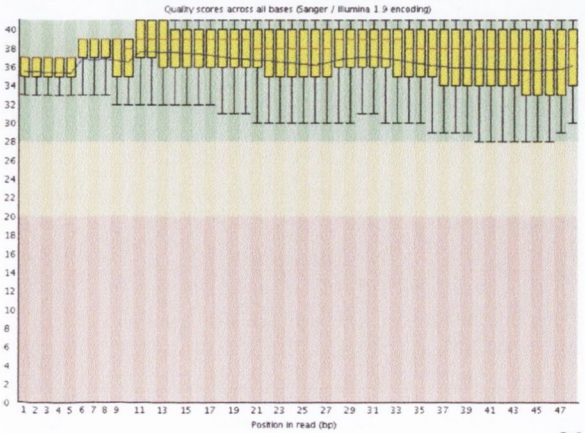

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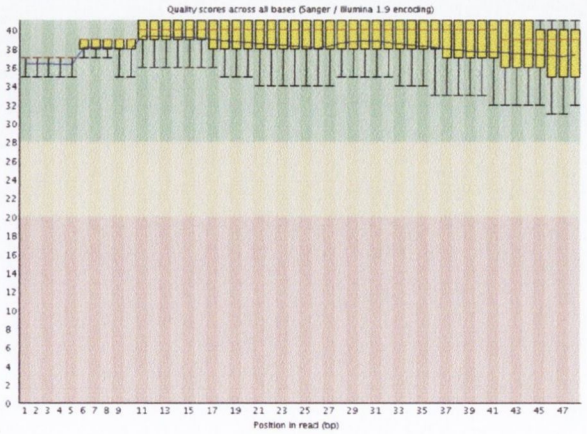

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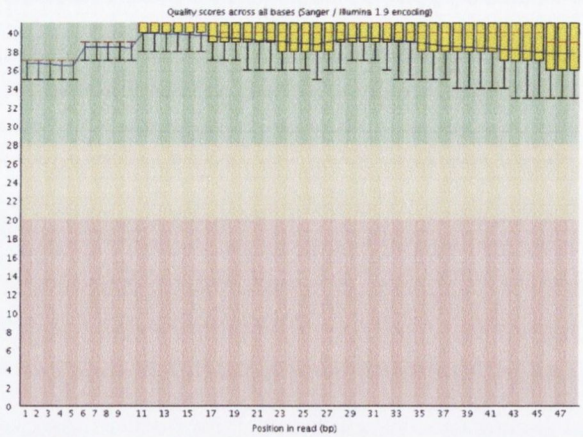

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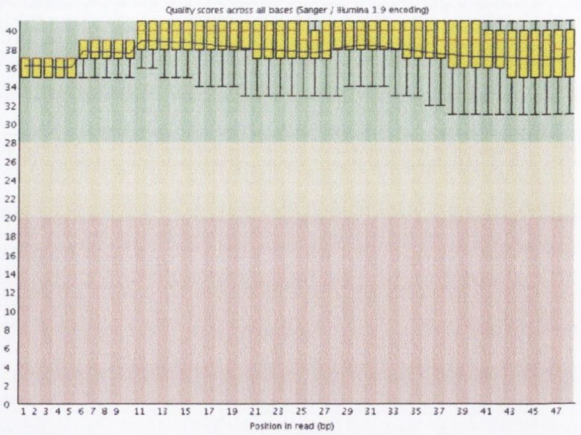

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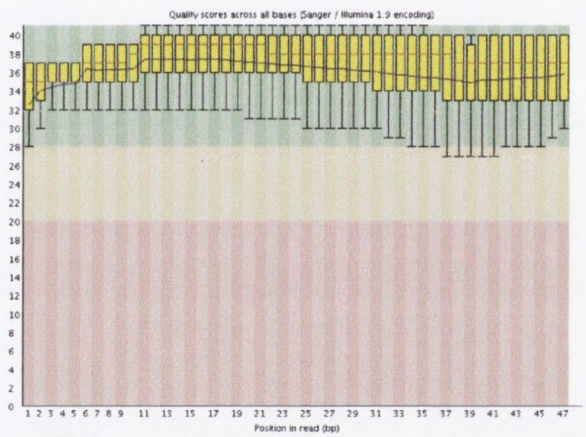

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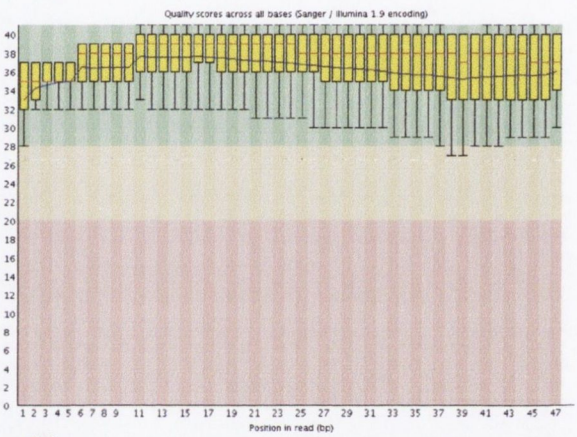

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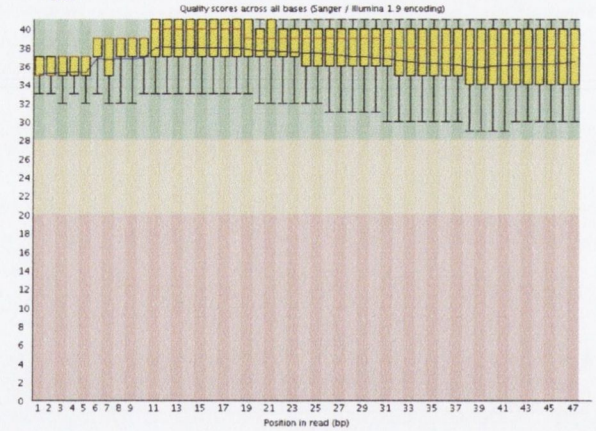

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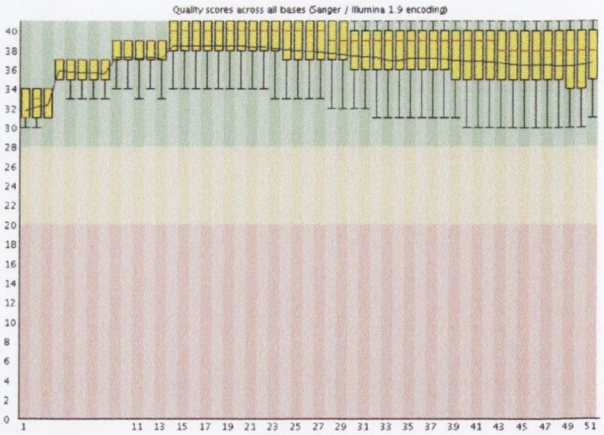

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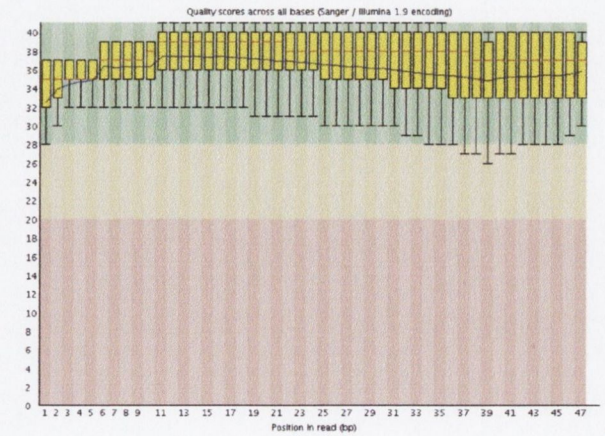

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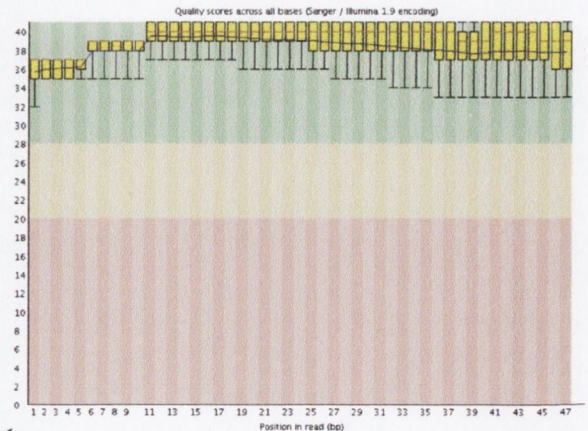

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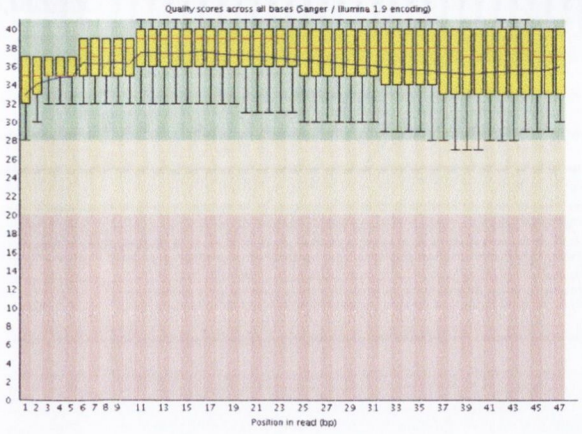
Quality scores across all bases (Sanger / Illumina 1.9 encoding)
Position in read (bp)

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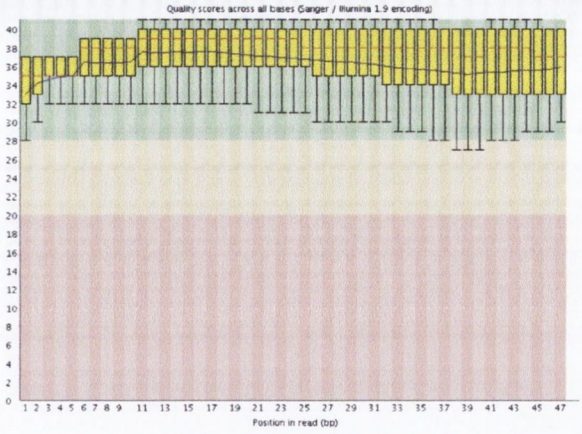
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Milk miRNA FastQC library checks Fig. 1-5 = Animal 1 control 0, 12, 24, 36, & 48. 6-10 = Animal 2 control 0, 12, 24, 36, & 48. 11-15 = Animal 3 control 0, 12, 24, 36, & 48. 16-20 = Animal 4 control 0, 12, 24, 36, & 48. 21-25 = Animal 5 control 0, 12, 24, 36, & 48. 26-30 = Animal 1 infected 0, 12, 24, 36, & 48. 31-35 = Animal 2 infected 0, 12, 24, 36, & 48. 31-35 = Animal 3 infected 0, 12, 24, 36, & 48. 31-35 = Animal 4 infected 0, 12, 24, 36, & 48. 36-40 = Animal 5 infected 0, 12, 24, 36, & 48.

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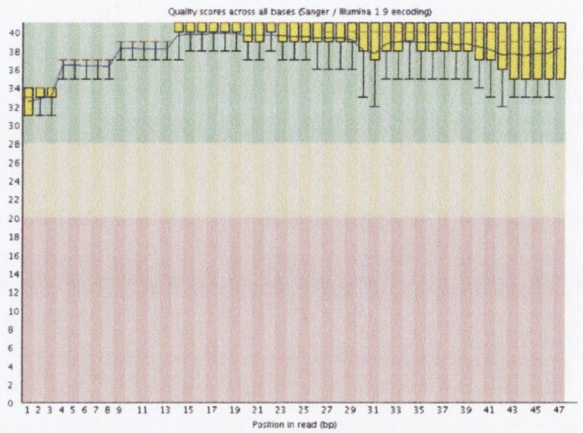

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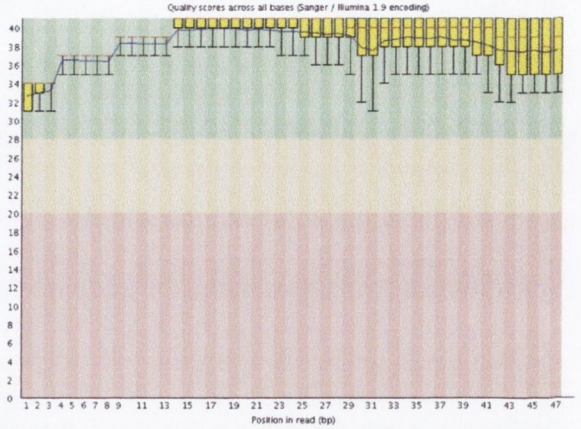

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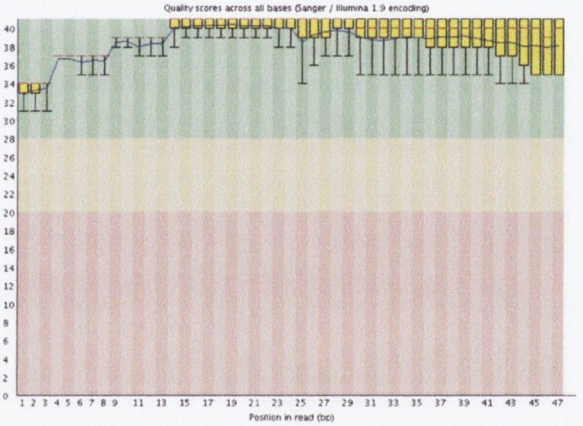

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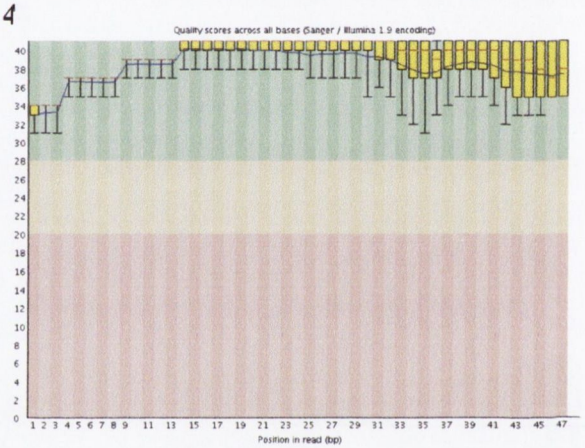

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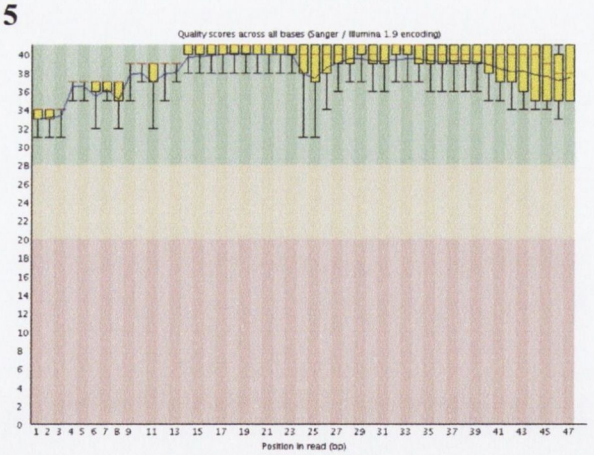

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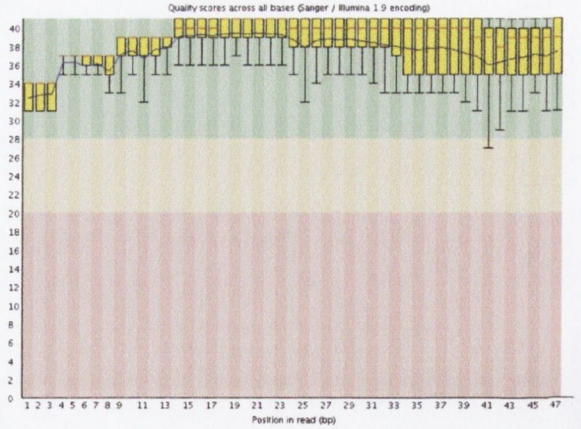

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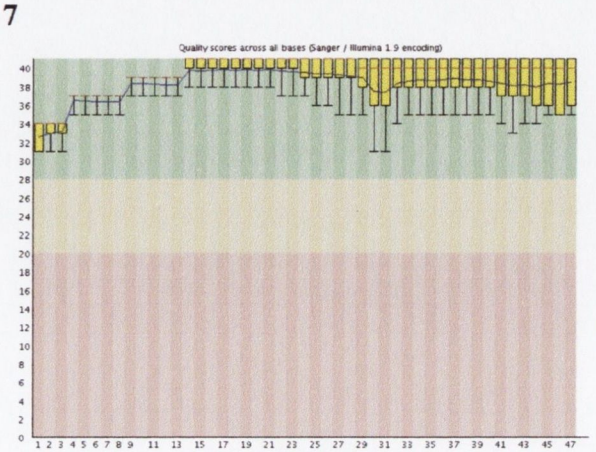

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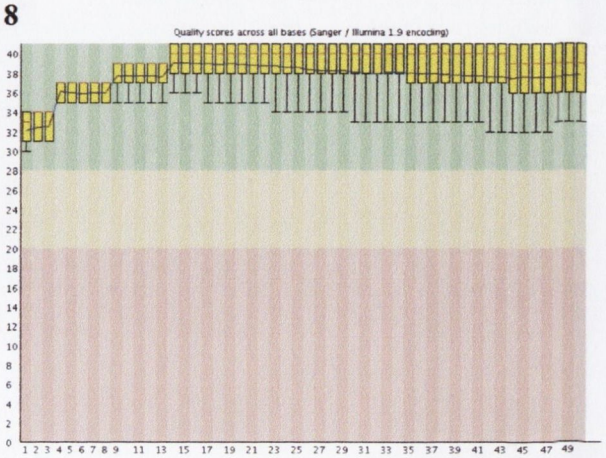

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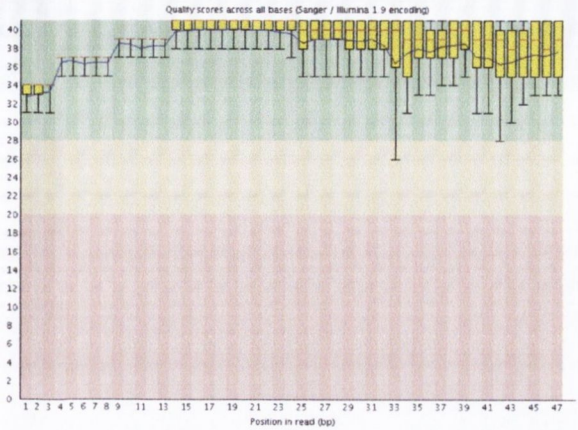

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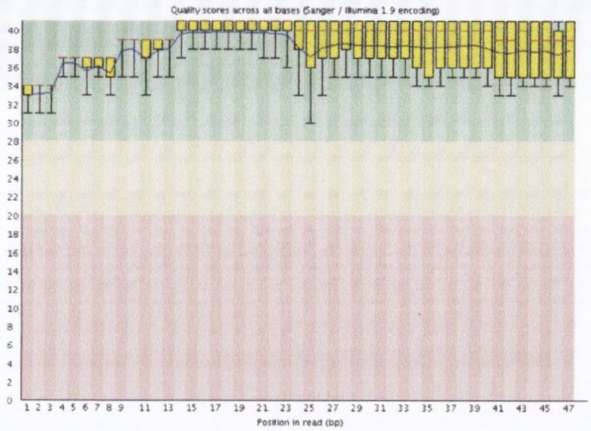

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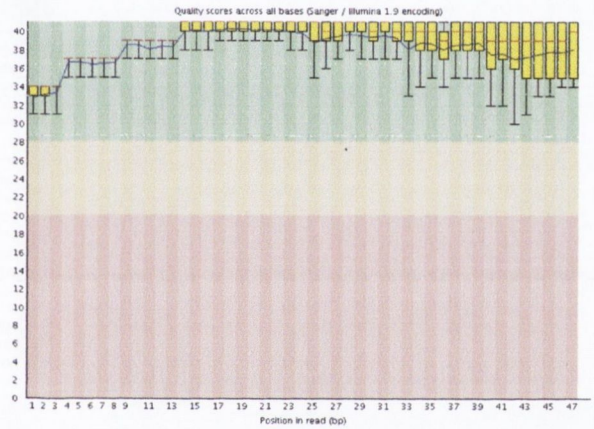

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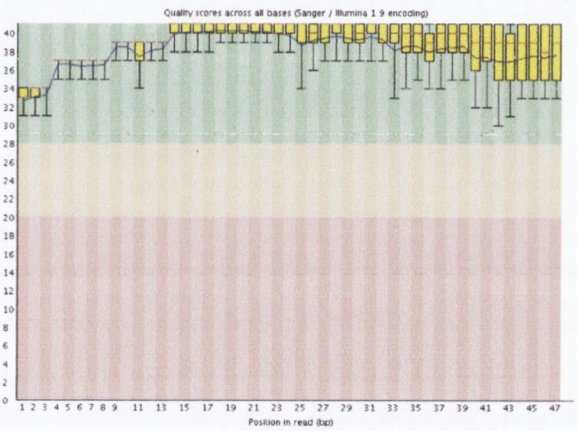

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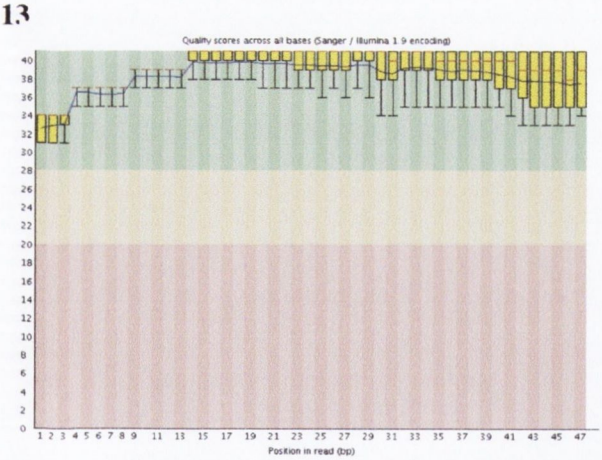

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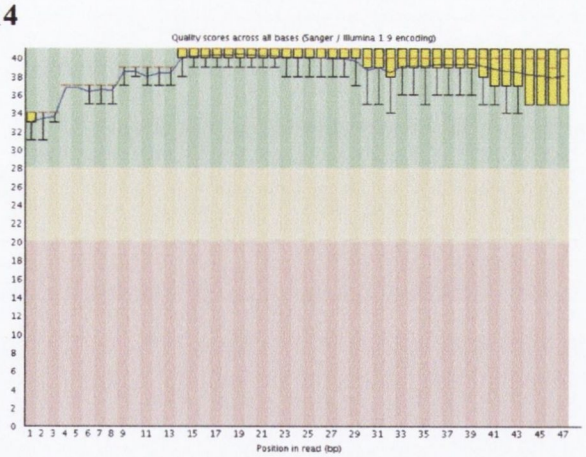

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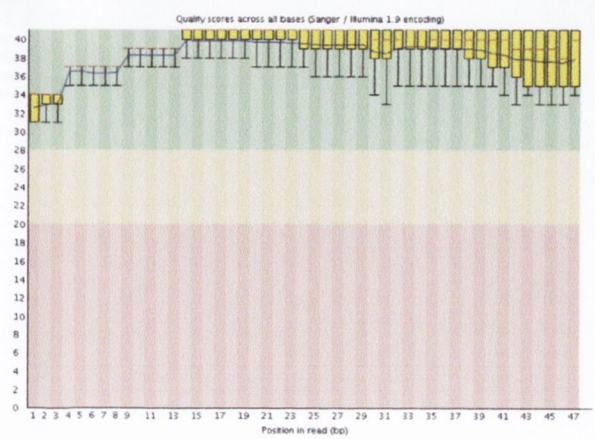

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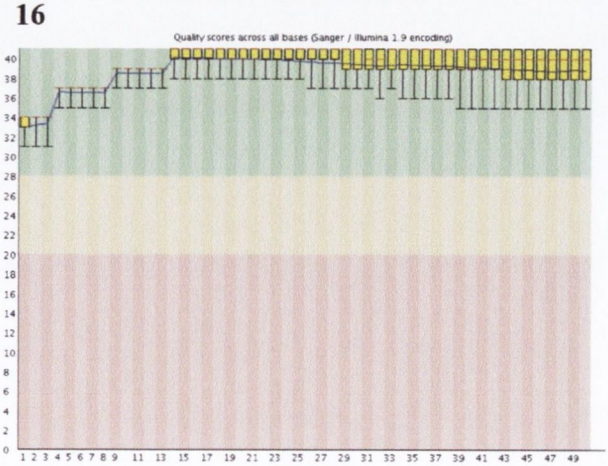

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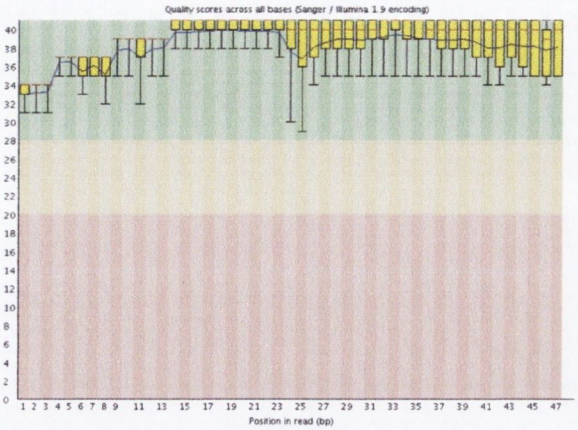

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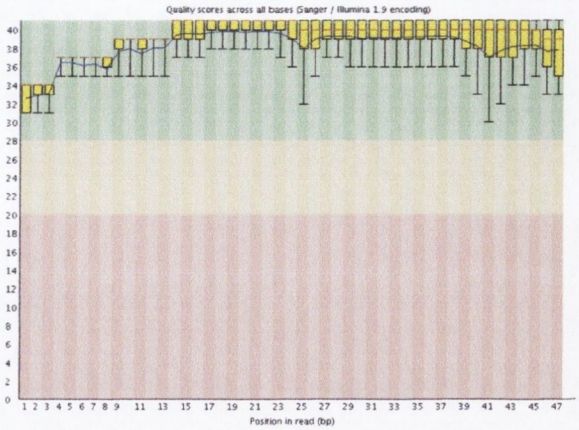

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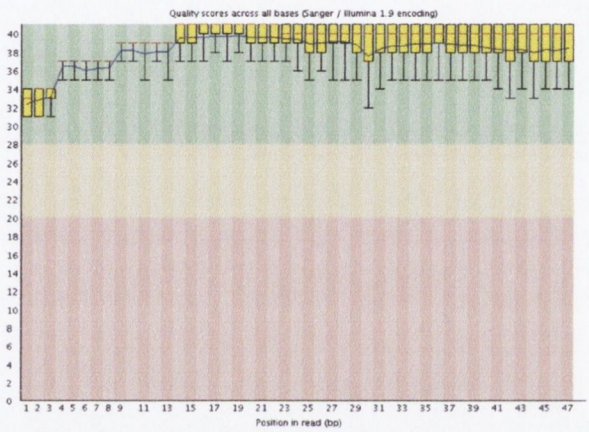

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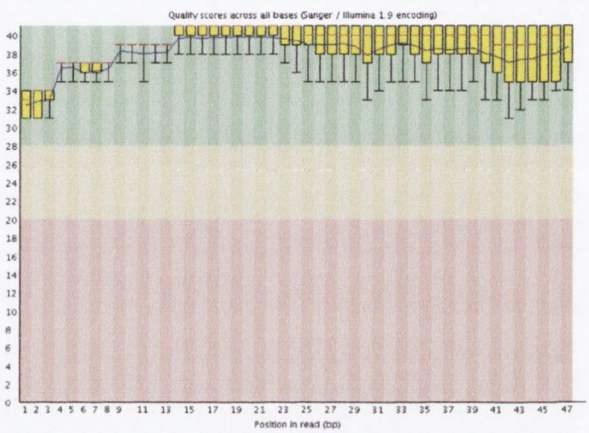

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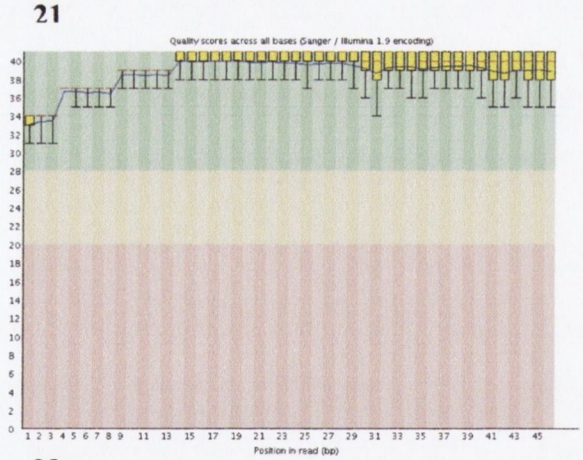

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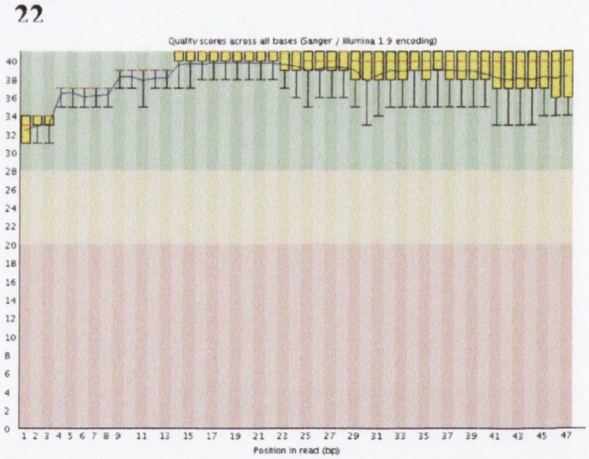

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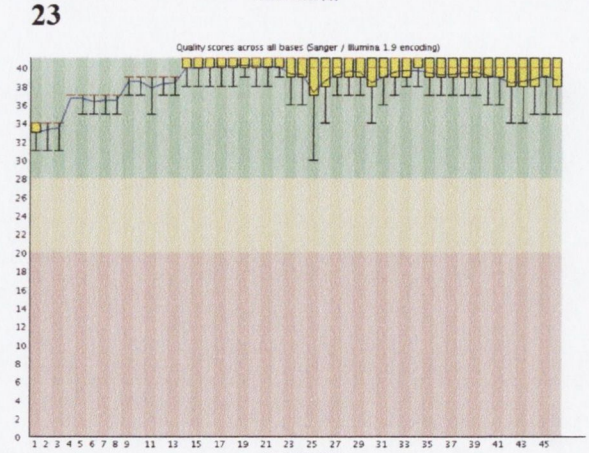

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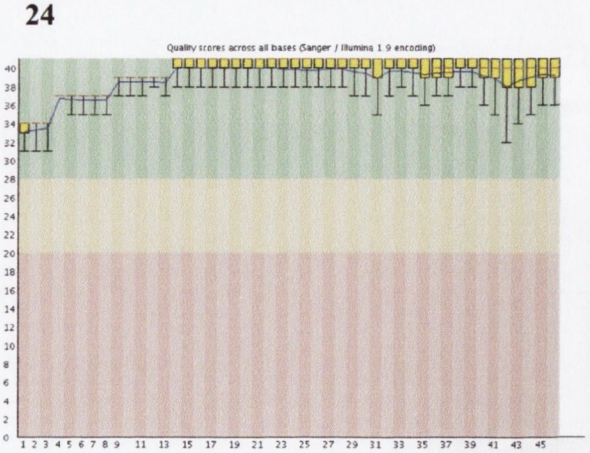

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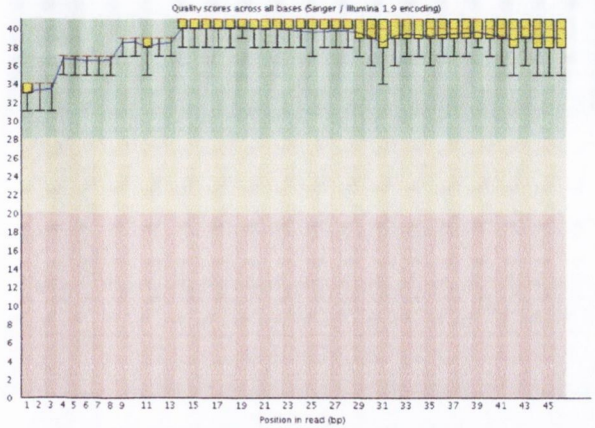

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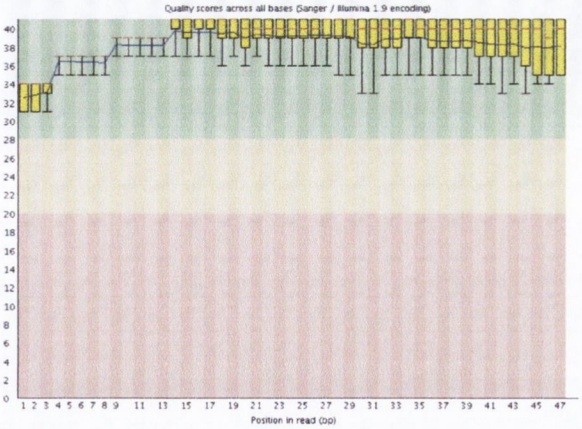

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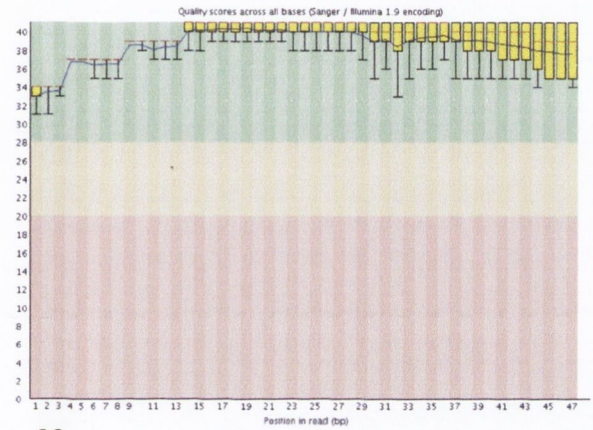

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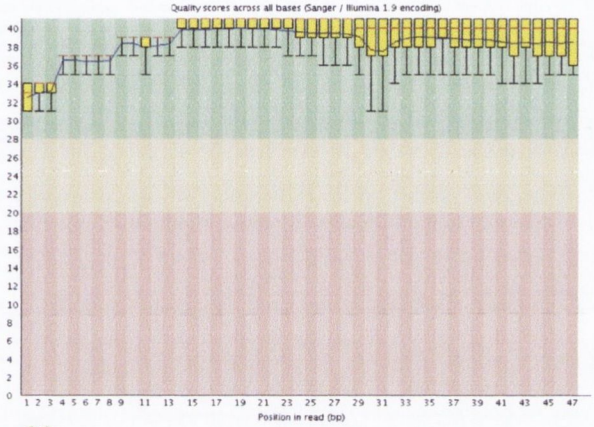

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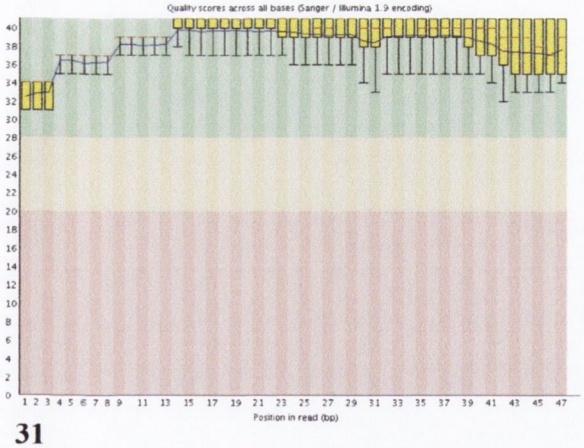

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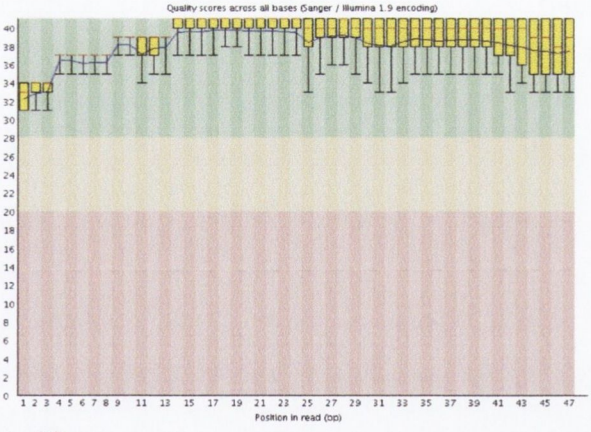

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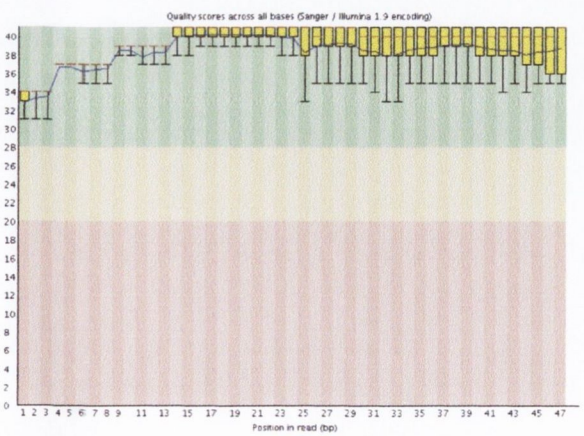

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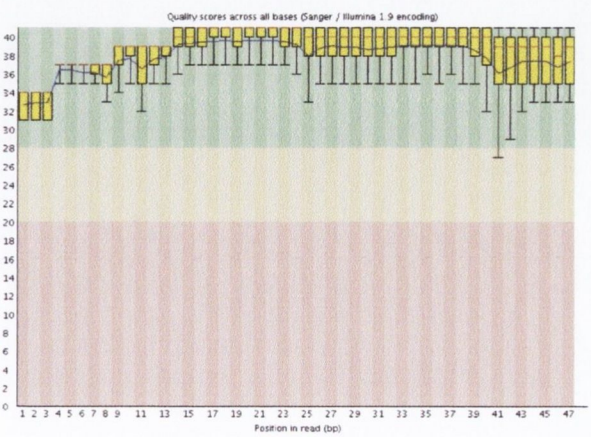

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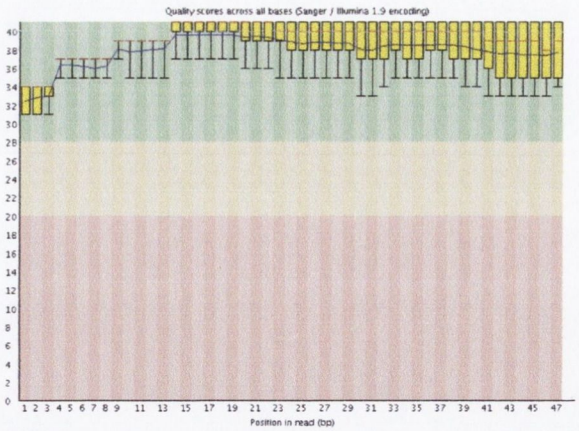

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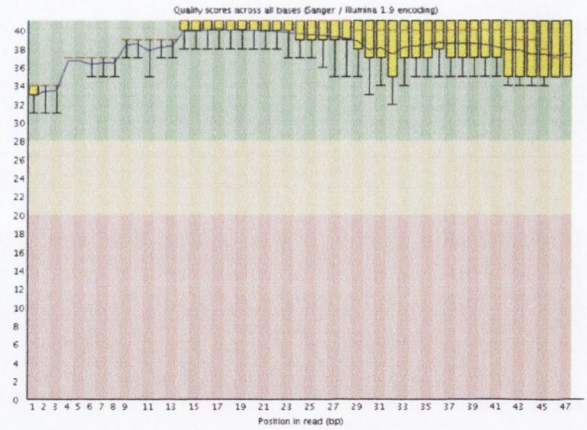

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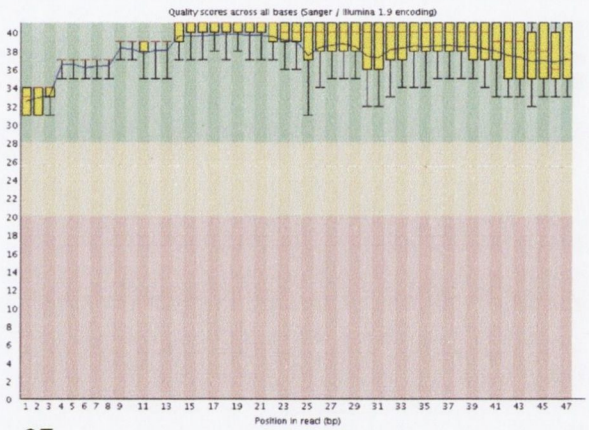

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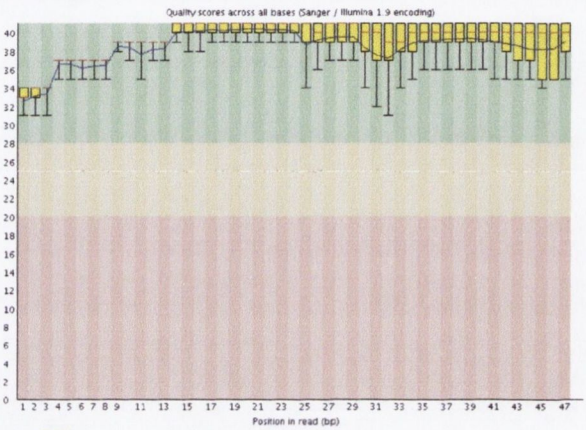

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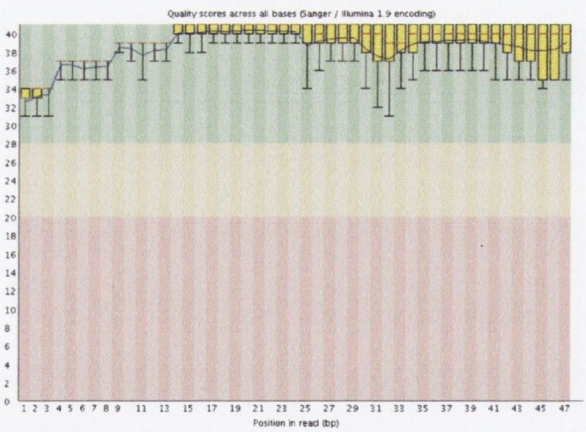

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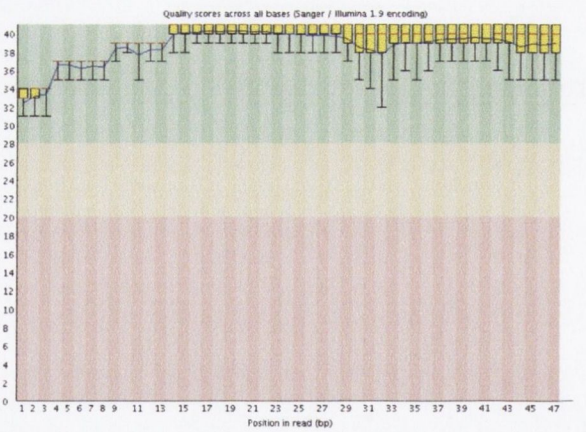

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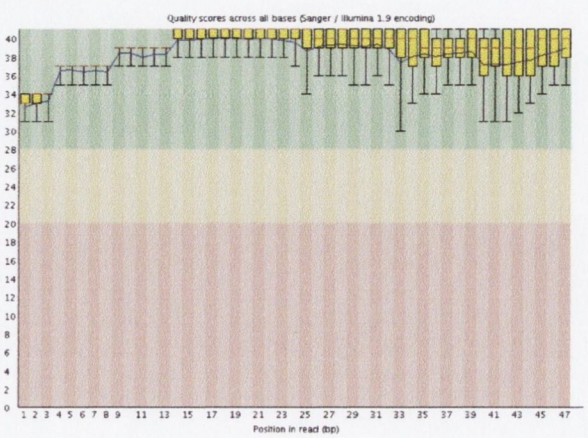

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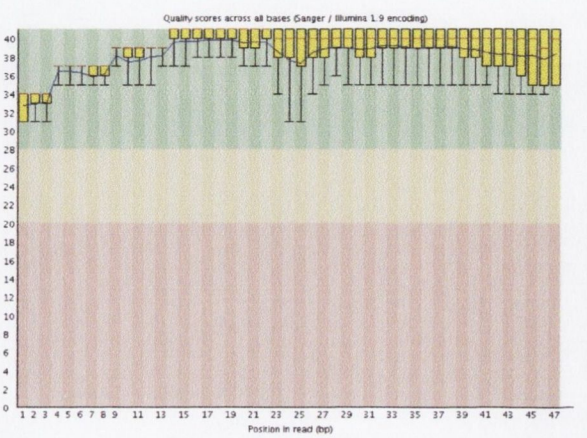

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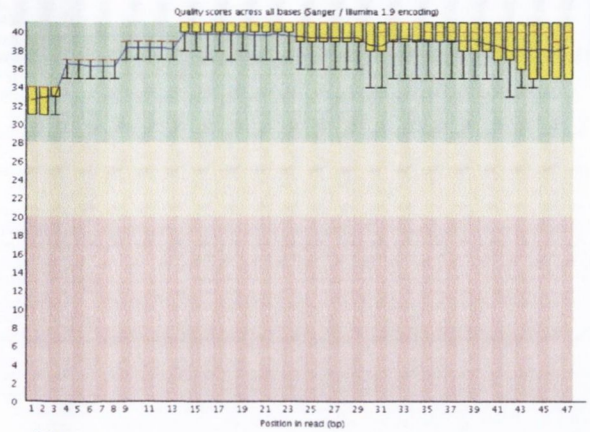

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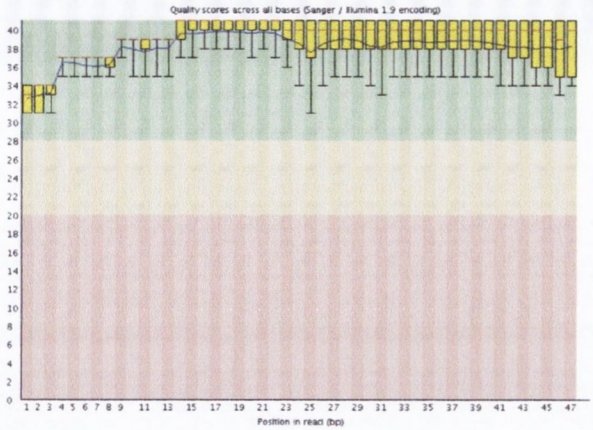

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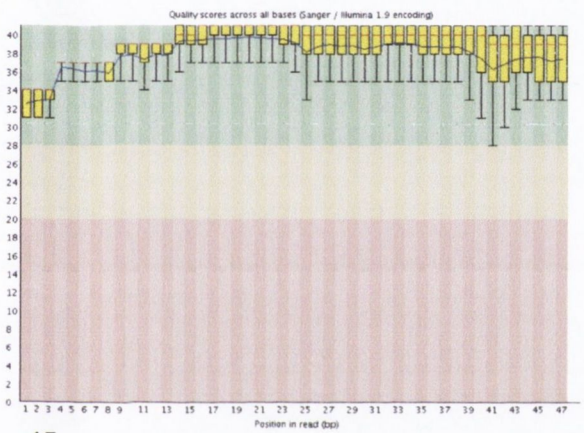

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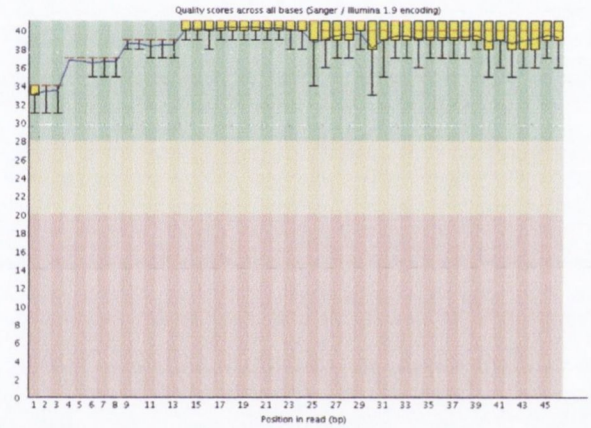

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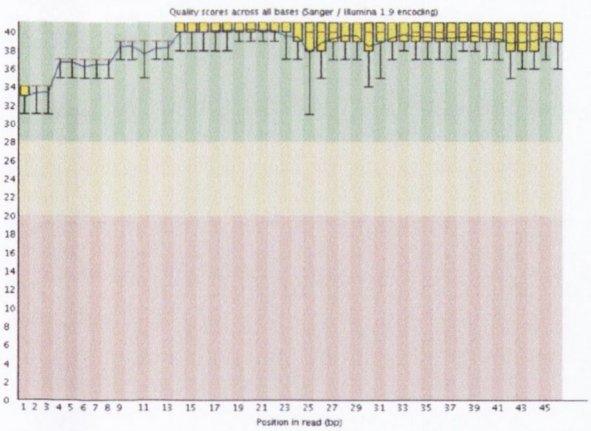

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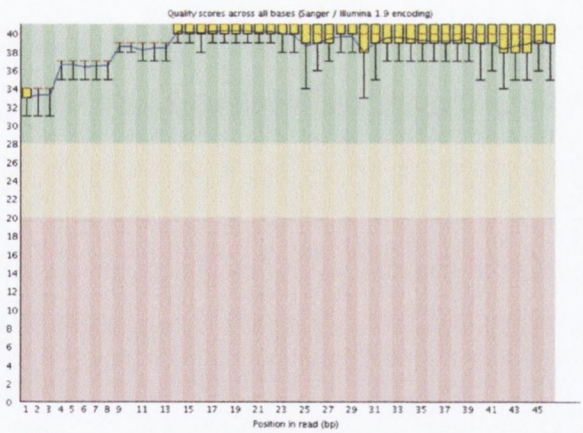

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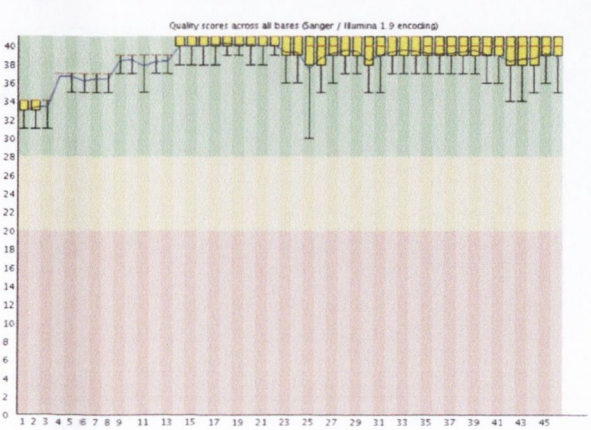

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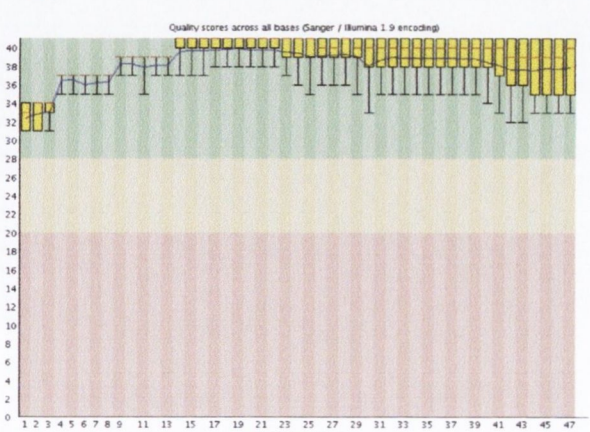

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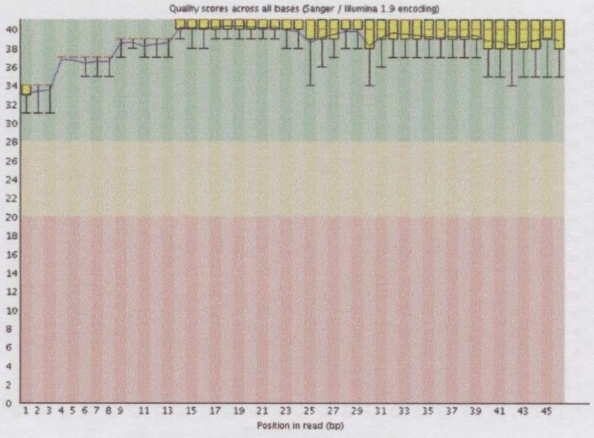
Quality scores across all bases (Sanger / Illumina 1.9 encoding)
Position in read (bp)

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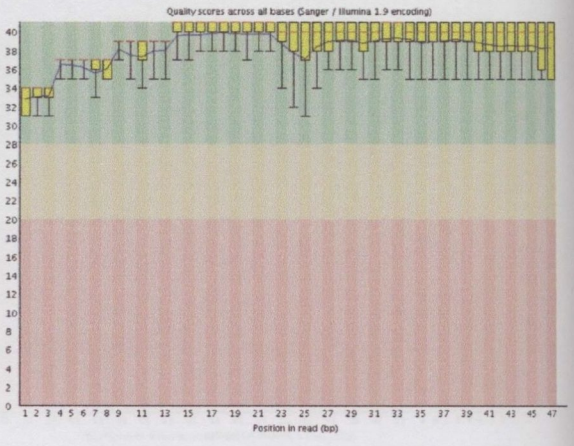
Quality scores across all bases (Sanger / Illumina 1.9 encoding)
Position in read (bp)

Blood miRNA FastQC library checks Fig. 1-5 = Animal 1 control 0, 12, 24, 36, & 48. 6-10 = Animal 2 control 0, 12, 24, 36, & 48. 11-15 = Animal 3 control 0, 12, 24, 36, & 48. 16-20 = Animal 4 control 0, 12, 24, 36, & 48. 21-25 = Animal 5 control 0, 12, 24, 36, & 48. 26-30 = Animal 1 infected 0, 12, 24, 36, & 48. 31-35 = Animal 2 infected 0, 12, 24, 36, & 48. 31-35 = Animal 3 infected 0, 12, 24, 36, & 48. 31-35 = Animal 4 infected 0, 12, 24, 36, & 48. 36-40 = Animal 5 infected 0, 12, 24, 36, & 48.

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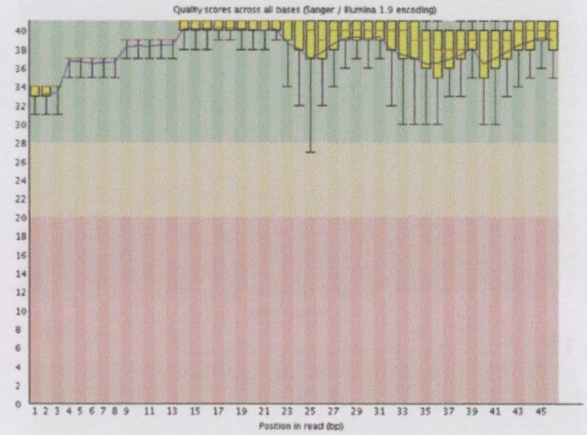

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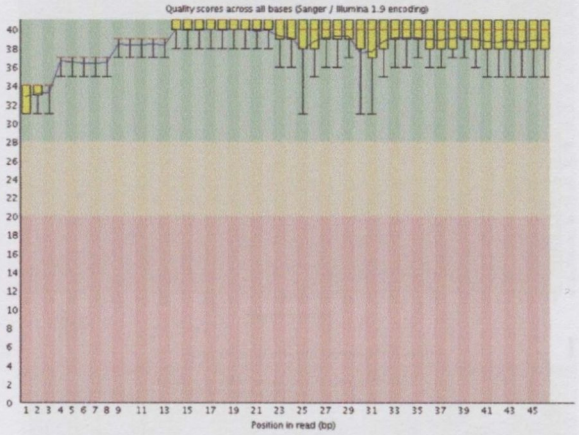

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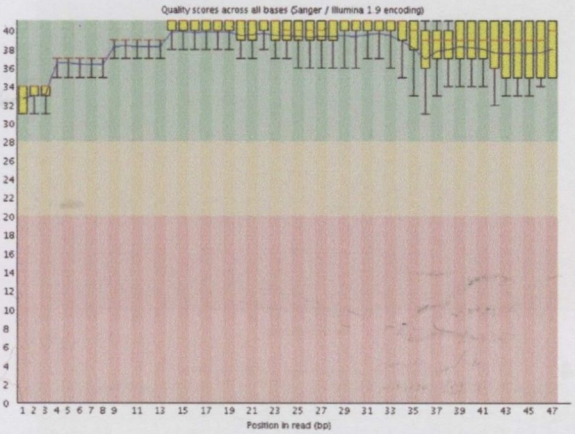

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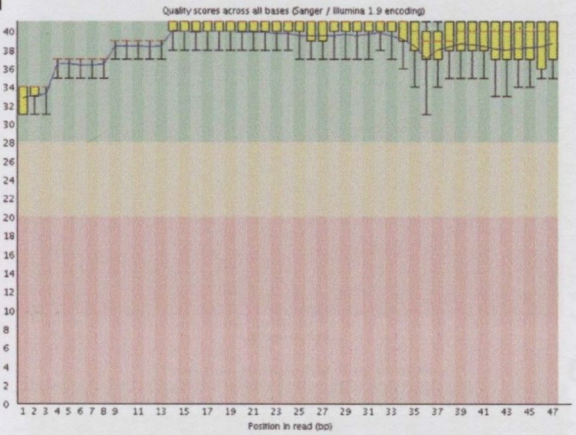

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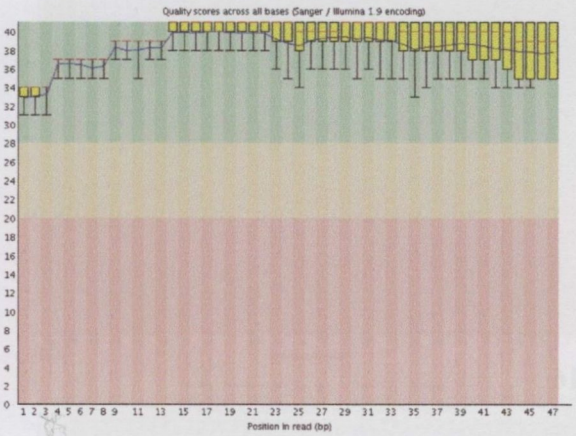

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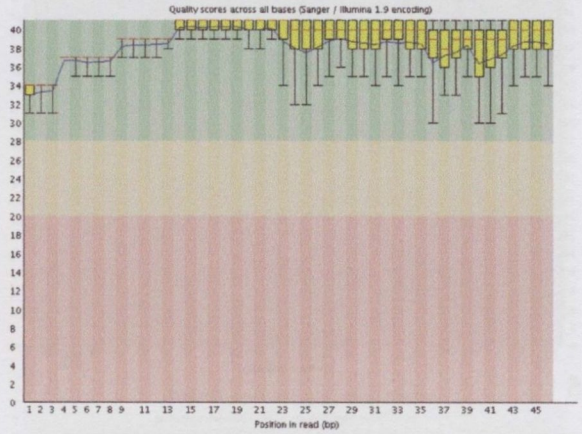

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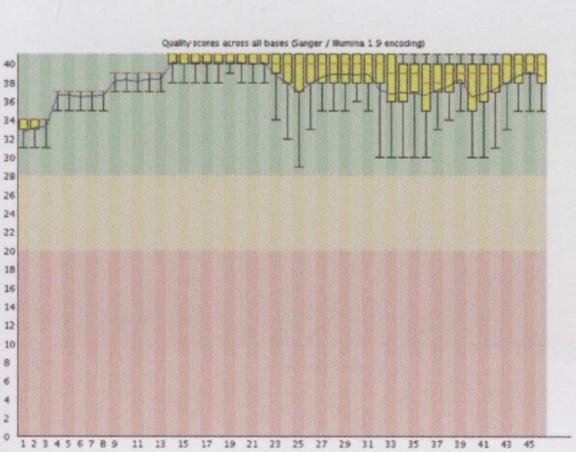

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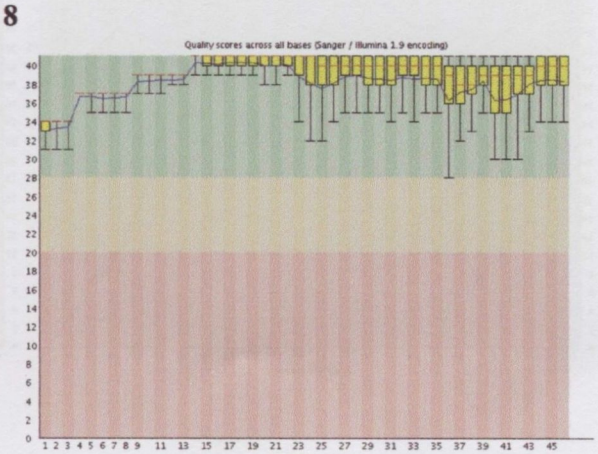

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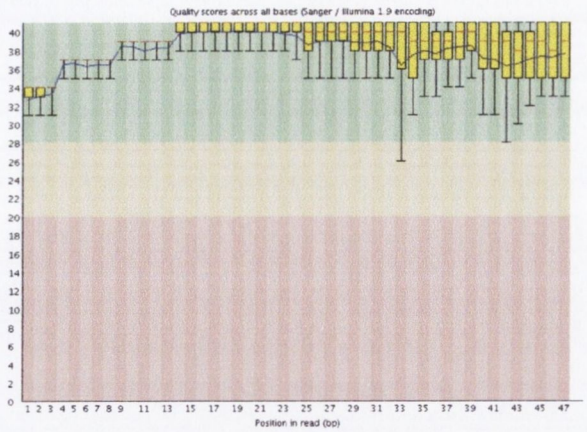

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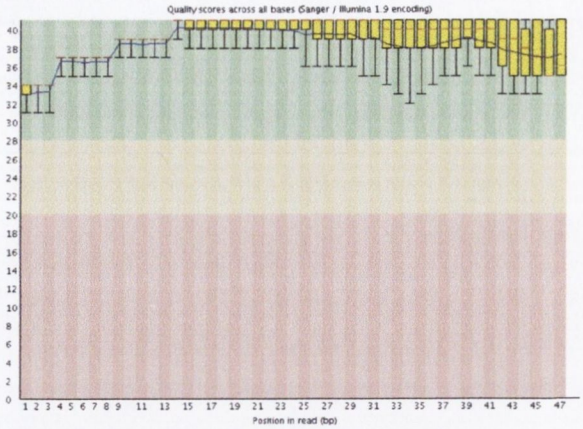

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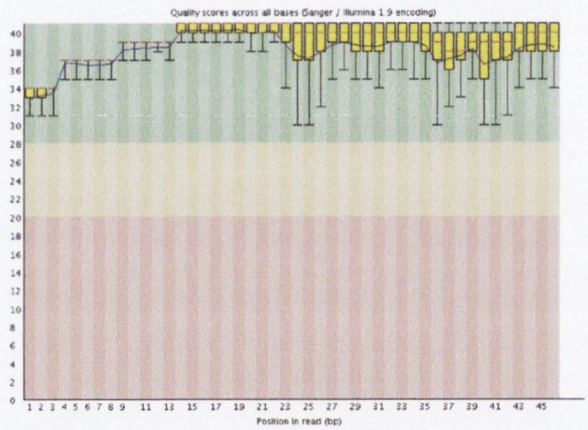

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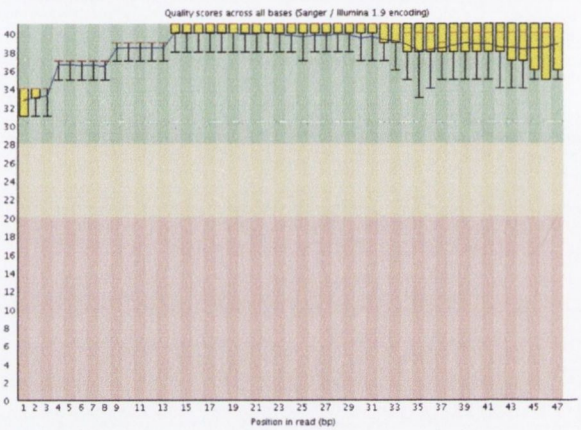

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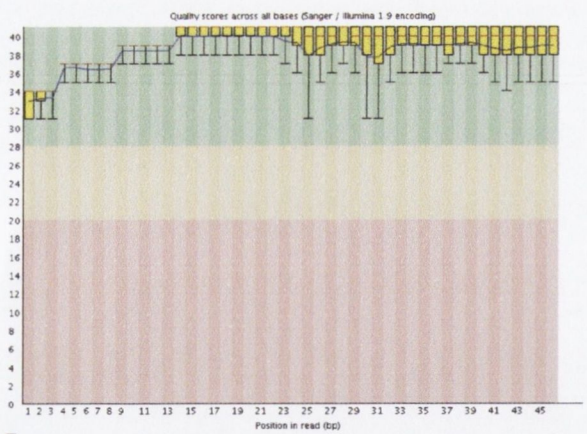

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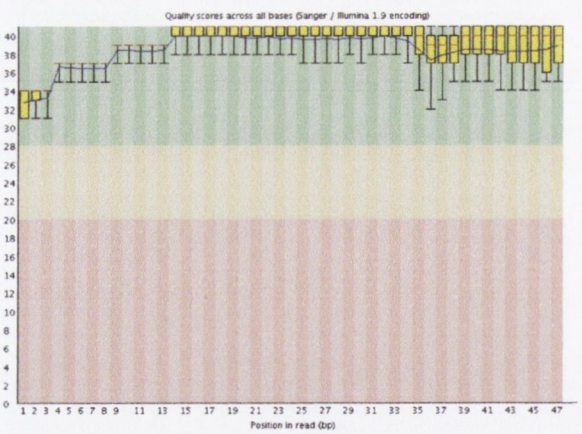

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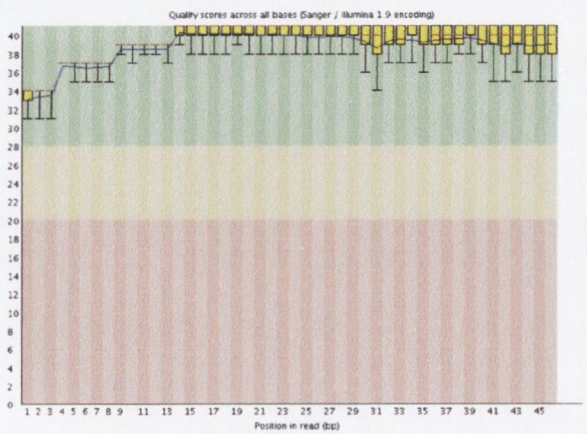

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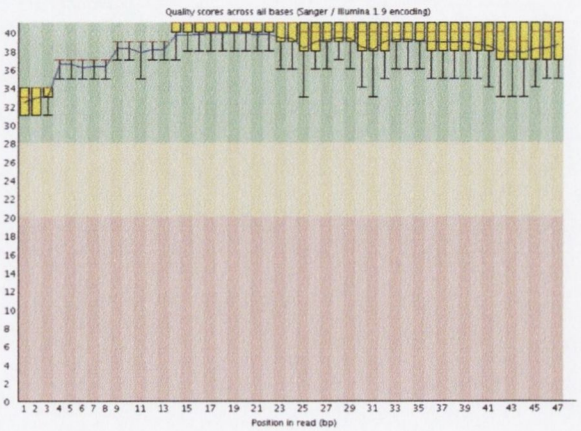

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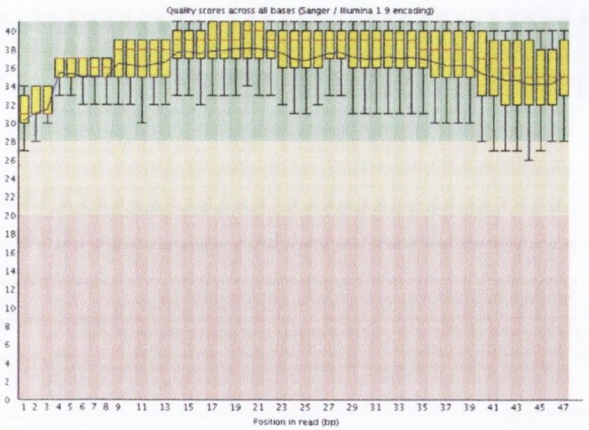

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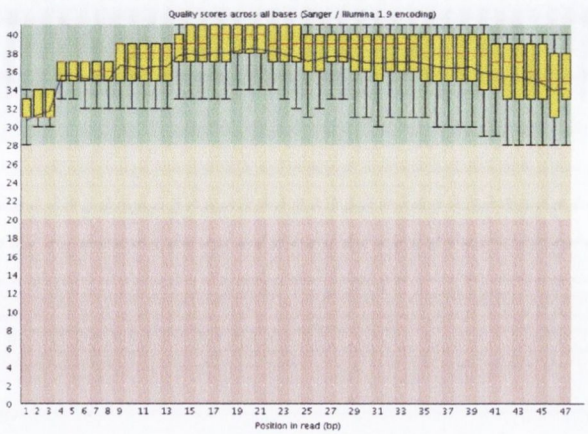

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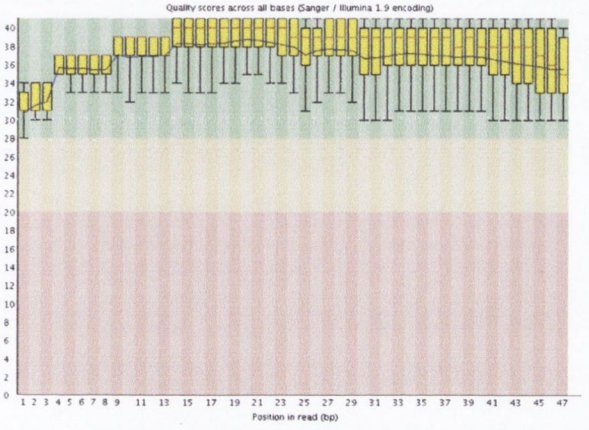

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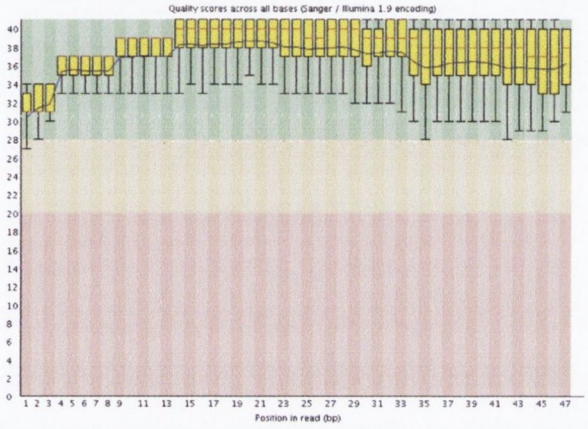

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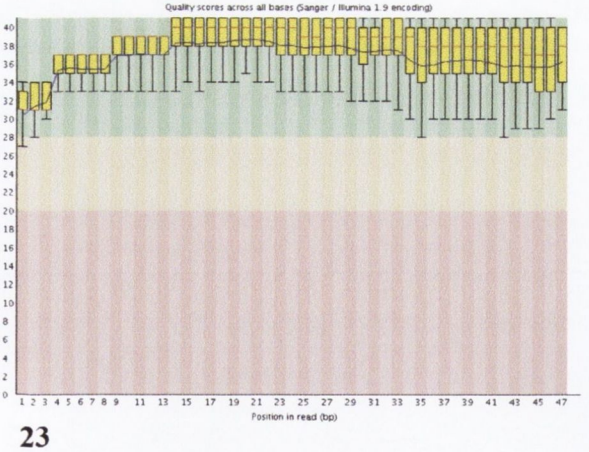

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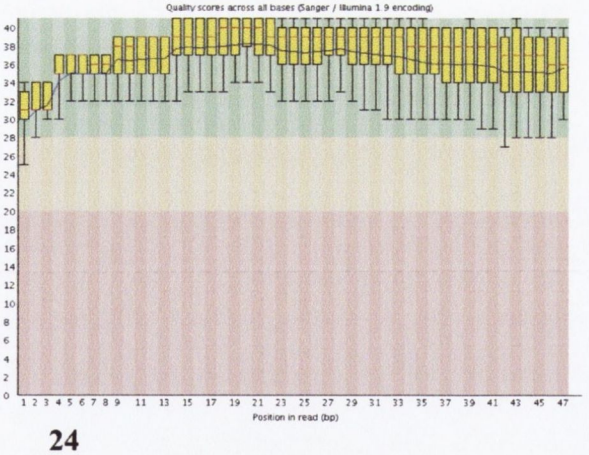

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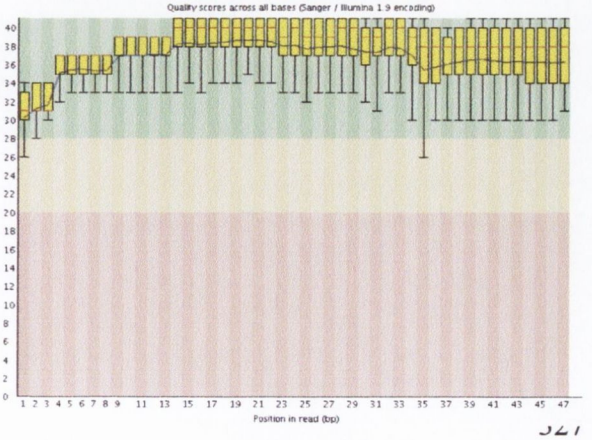

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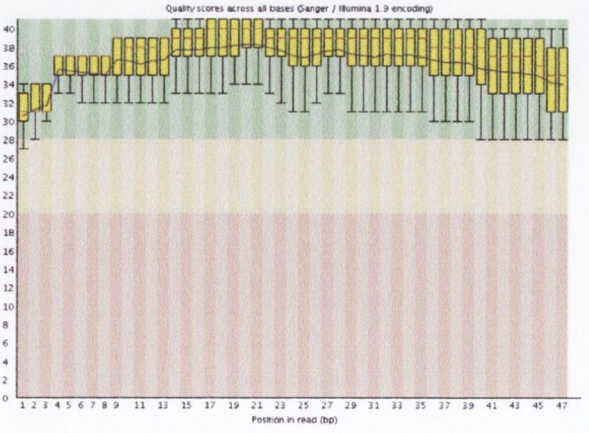

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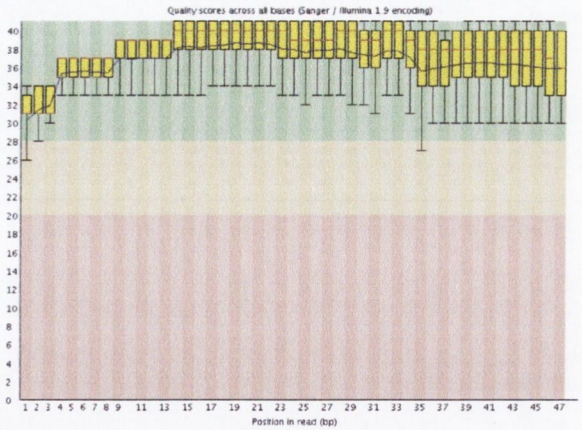

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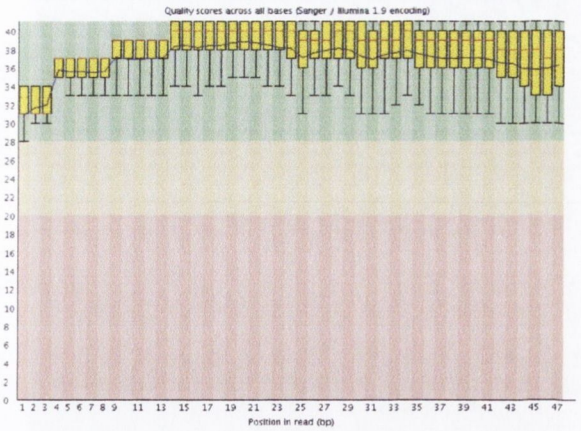

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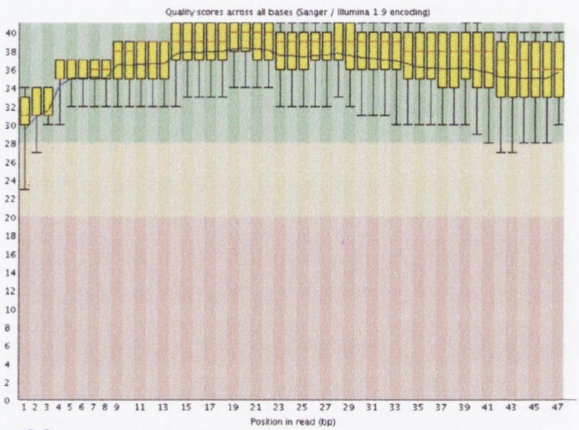

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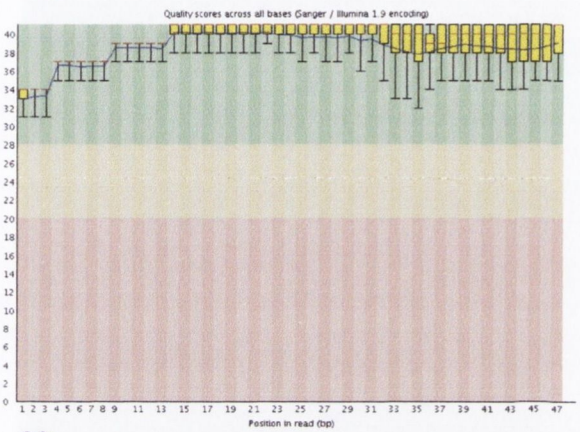

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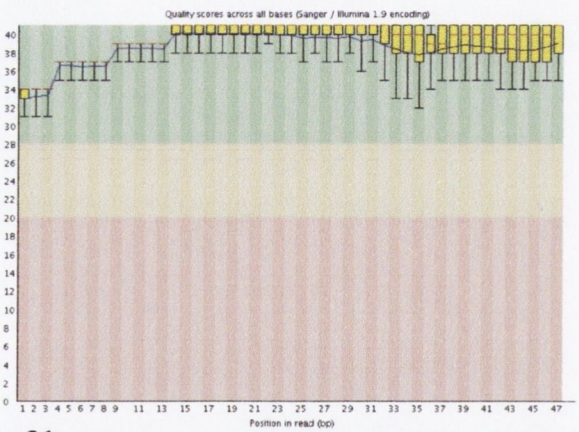

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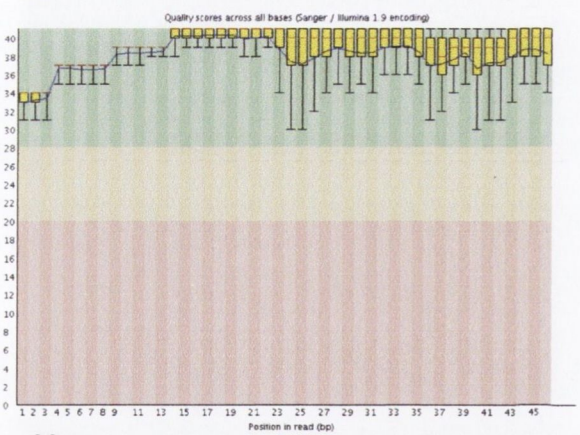

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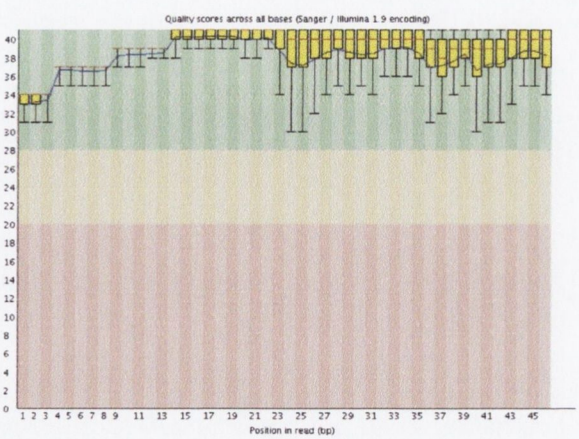

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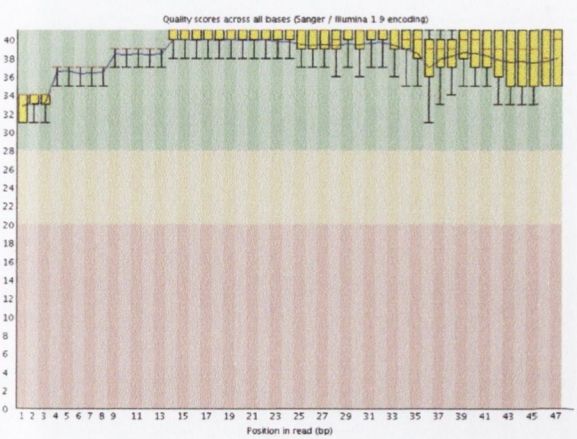

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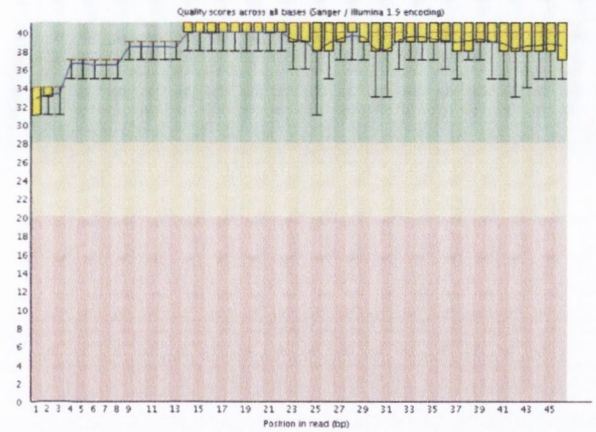

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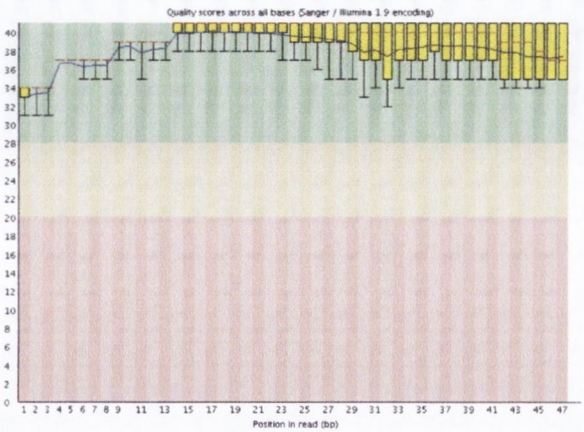

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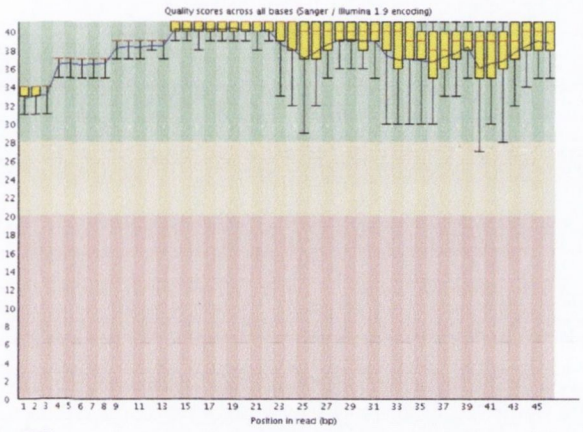

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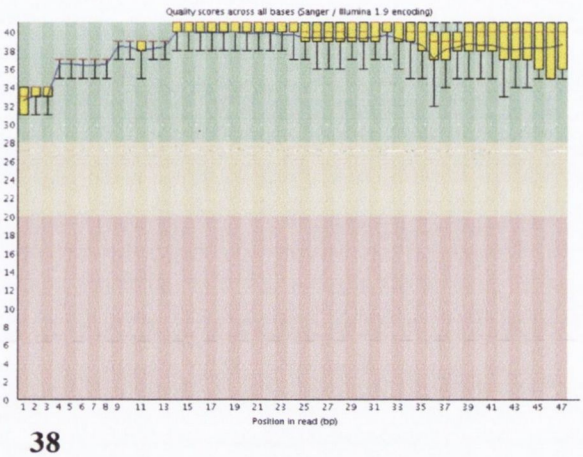

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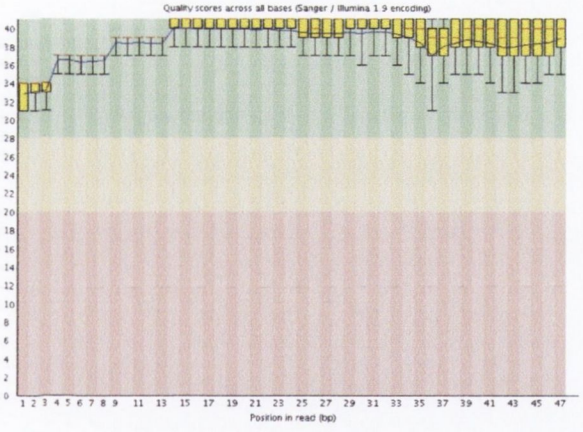

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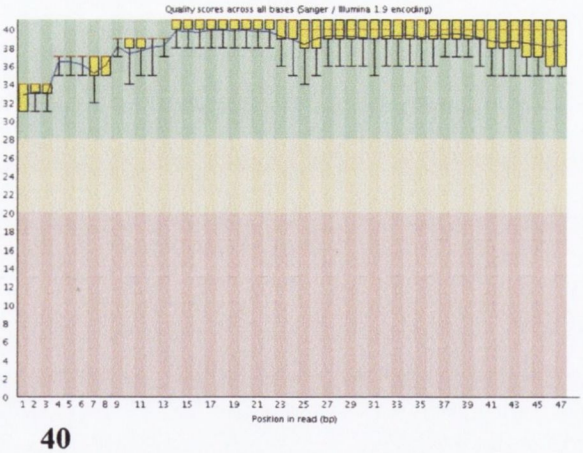

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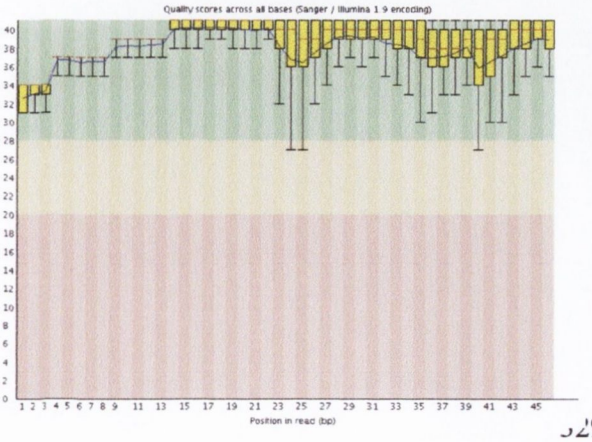

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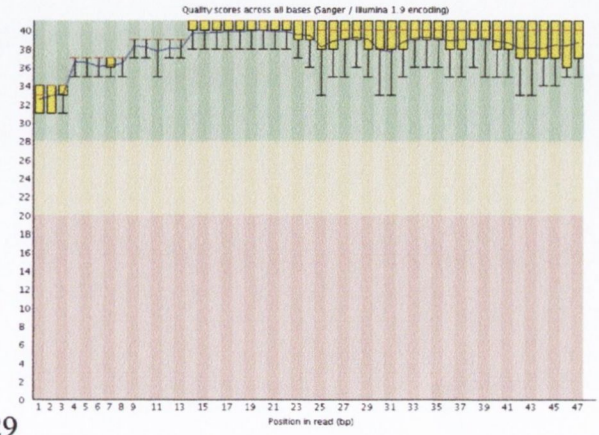

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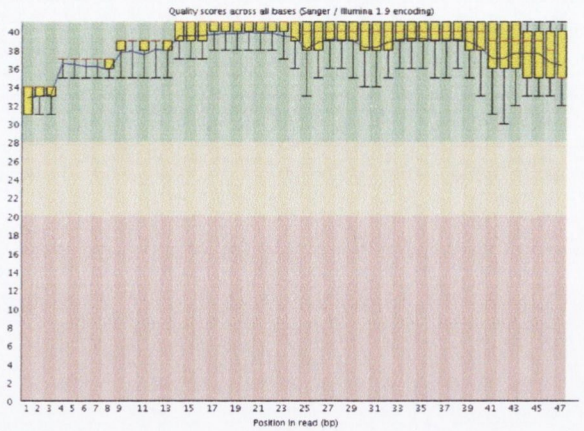

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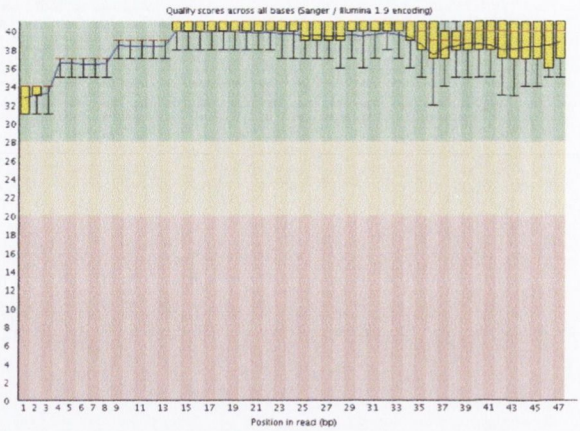

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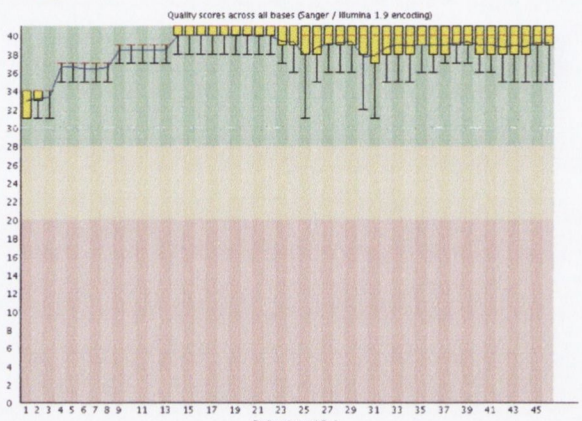

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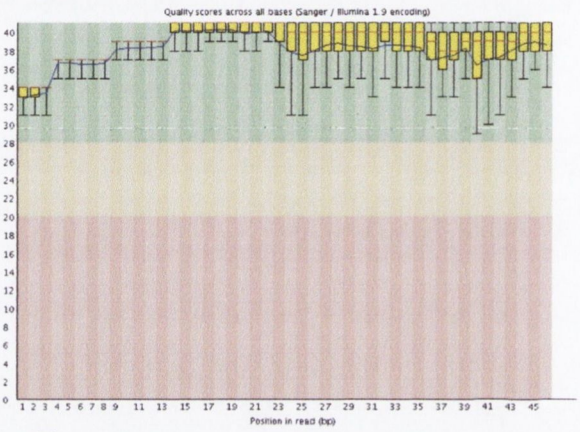

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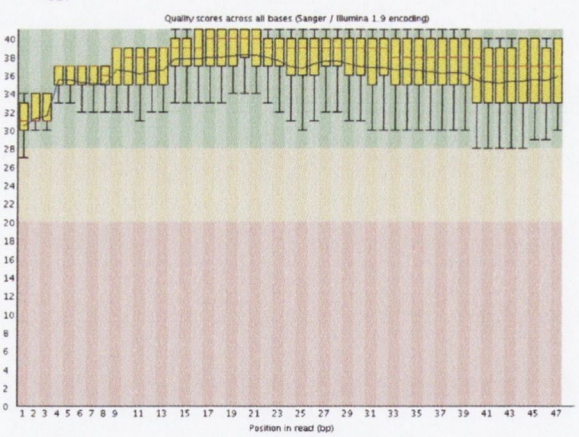

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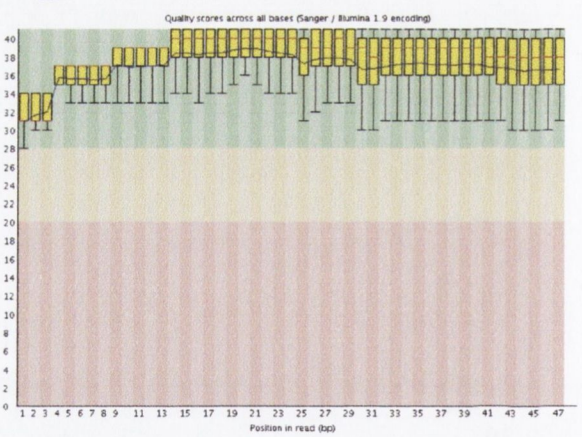

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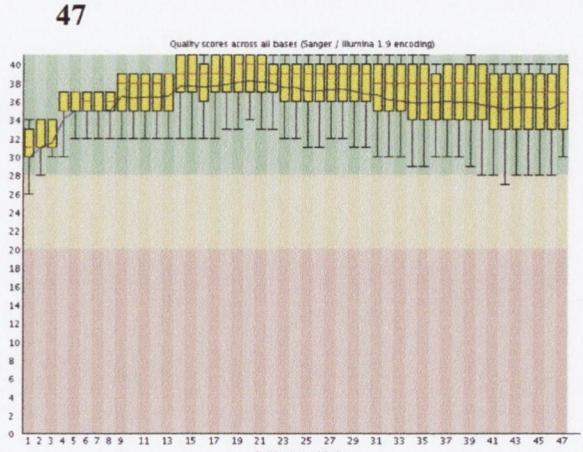

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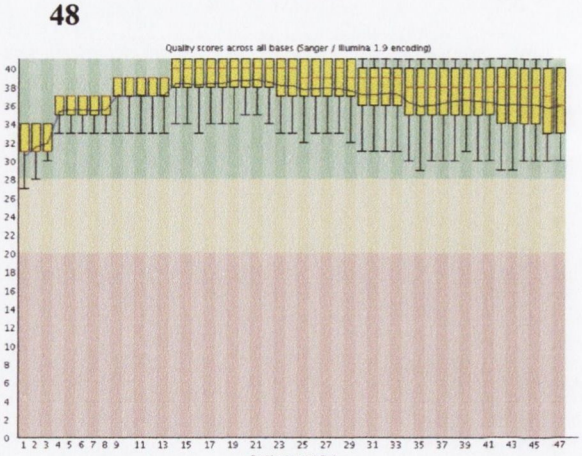

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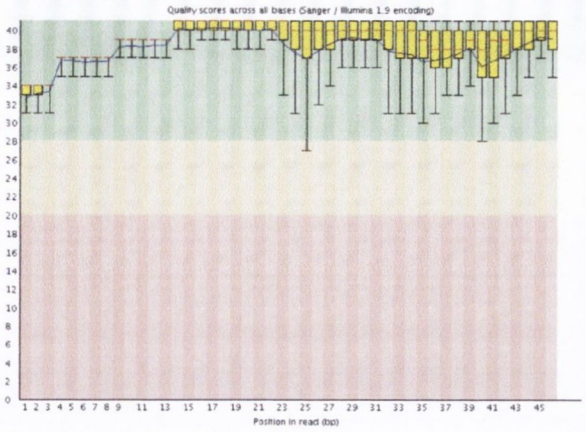
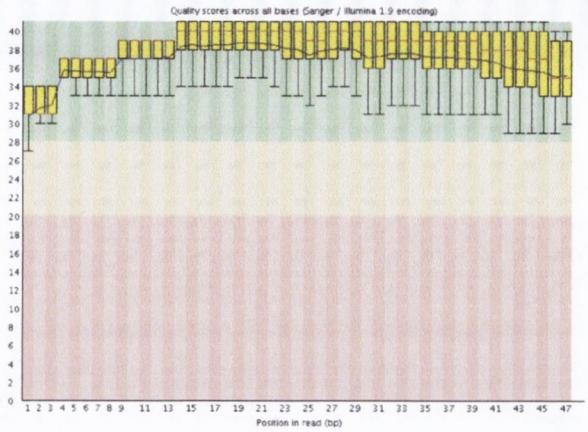

Quality scores across all bases (Sanger / Illumina 1.9 encoding)
Position in read (bp)

50


Quality scores across all bases (Sanger / Illumina 1.9 encoding)
Position in read (bp)

Appendix 13: Summary read statistics and number of unique alignments for each blood and m

mRNA library

Sample ID	Sample name	Raw Reads	Post cutadapt Trimming	Post Fastx Trimmer	Post Fastq quality Filter	Uniquely align UMD 3.1
Blood-mRNA-TC-1-001	0_hour_control_animal_1	47,346,827	47,137,909	47,137,909	46,917,379	46,914,379
Blood-mRNA-TC-1-002	12_hour_control_animal_1	41,691,610	41,072,129	41,072,129	40,954,260	40,951,623
Blood-mRNA-TC-1-003	24_hour_control_animal_1	50,432,332	50,324,562	50,324,562	50,247,733	50,243,361
Blood-mRNA-TC-1-004	36_hour_control_animal_1	23,763,122	23,739,286	23,739,286	23,703,385	23,701,476
Blood-mRNA-TC-1-005	48_hour_control_animal_1	19,016,424	18,980,500	18,980,500	18,953,574	18,952,199
Blood-mRNA-TC-2-001	0_hour_control_animal_2	29,536,306	29,364,403	29,364,403	29,321,249	29,319,686
Blood-mRNA-TC-2-002	12_hour_control_animal_2	28,409,993	28,333,817	28,333,817	28,209,328	28,207,479
Blood-mRNA-TC-2-003	24_hour_control_animal_2	33,015,304	32,898,121	32,898,121	32,812,593	32,810,631
Blood-mRNA-TC-2-004	36_hour_control_animal_2	7,002,584	6,939,258	6,939,258	6,927,503	6,927,015
Blood-mRNA-TC-2-005	48_hour_control_animal_2	38,668,107	38,481,146	38,481,146	38,378,703	38,376,176
Blood-mRNA-TC-3-001	0_hour_control_animal_3	71,550,870	71,215,791	71,215,791	71,006,739	71,002,673
Blood-mRNA-TC-3-002	12_hour_control_animal_3	38,466,549	38,103,568	38,103,568	38,006,737	38,004,321
Blood-mRNA-TC-3-003	24_hour_control_animal_3	14,199,178	14,120,276	14,120,276	14,087,086	14,086,147
Blood-mRNA-TC-3-004	36_hour_control_animal_3	26,970,719	26,935,128	26,935,128	26,893,008	26,890,788
Blood-mRNA-TC-3-005	48_hour_control_animal_3	24,263,808	23,489,143	23,489,143	23,455,587	23,454,372
Blood-mRNA-TC-4-001	0_hour_control_animal_4	53,849,823	53,397,279	53,397,279	53,318,062	53,314,967
Blood-mRNA-TC-4-002	12_hour_control_animal_4	26,184,144	26,098,794	26,098,794	26,021,055	26,018,362
Blood-mRNA-TC-4-003	24_hour_control_animal_4	57,094,436	57,034,394	57,034,394	56,872,091	56,868,746
Blood-mRNA-TC-4-004	36_hour_control_animal_4	52,678,779	51,761,986	51,761,986	51,660,346	51,657,660
Blood-mRNA-TC-4-005	48_hour_control_animal_4	44,742,880	44,713,537	44,713,537	44,566,246	44,563,566
Blood-mRNA-TC-5-001	0_hour_control_animal_5	10,063,154	2,095,049	2,095,049	2,066,010	2,065,400
Blood-mRNA-TC-5-002	12_hour_control_animal_5	66,228,330	65,224,947	65,224,947	64,965,352	64,961,785
Blood-mRNA-TC-5-003	24_hour_control_animal_5	46,098,977	46,081,072	46,081,072	45,890,760	45,887,231
Blood-mRNA-TC-5-004	36_hour_control_animal_5	42,427,878	42,415,902	42,415,902	42,276,131	42,273,931
Blood-mRNA-TC-5-005	48_hour_control_animal_5	24,350,504	24,068,932	24,068,932	24,045,691	24,043,707
Blood-mRNA-TI-1-001	0_hour_infected_animal_1	26,088,257	23,948,116	23,948,116	23,894,664	23,892,917
Blood-mRNA-TI-1-002	12_hour_infected_animal_1	41,726,671	35,449,825	35,449,825	35,351,974	35,349,489
Blood-mRNA-TI-1-003	24_hour_infected_animal_1	44,539,549	44,218,488	44,218,488	44,106,697	44,104,094
Blood-mRNA-TI-1-004	36_hour_infected_animal_1	20,392,340	20,075,662	20,075,662	20,039,679	20,038,584
Blood-mRNA-TI-1-005	48_hour_infected_animal_1	19,901,376	19,725,257	19,725,257	19,682,068	19,680,928
Blood-mRNA-TI-2-001	0_hour_infected_animal_2	18,959,051	18,897,203	18,897,203	18,860,105	18,858,927
Blood-mRNA-TI-2-002	12_hour_infected_animal_2	38,676,539	38,553,805	38,553,805	38,386,116	38,383,599
Blood-mRNA-TI-2-003	24_hour_infected_animal_2	35,578,817	35,504,879	35,504,879	35,418,715	35,416,626
Blood-mRNA-TI-2-004	36_hour_infected_animal_2	20,088,857	20,033,032	20,033,032	19,976,941	19,975,706
Blood-mRNA-TI-2-005	48_hour_infected_animal_2	28,677,442	28,407,309	28,407,309	28,329,694	28,327,903
Blood-mRNA-TI-3-001	0_hour_infected_animal_3	18,482,556	17,578,374	17,578,374	17,520,607	17,519,590
Blood-mRNA-TI-3-002	12_hour_infected_animal_3	41,643,785	40,995,486	40,995,486	40,786,859	40,784,076
Blood-mRNA-TI-3-003	24_hour_infected_animal_3	19,202,741	19,112,217	19,112,217	19,068,202	19,067,152
Blood-mRNA-TI-3-004	36_hour_infected_animal_3	13,292,992	13,249,649	13,249,649	13,229,572	13,228,438
Blood-mRNA-TI-3-005	48_hour_infected_animal_3	27,603,963	27,574,656	27,574,656	27,509,678	27,508,144
Blood-mRNA-TI-4-001	0_hour_infected_animal_4	53,670,166	53,574,133	53,574,133	53,422,292	53,418,856
Blood-mRNA-TI-4-002	12_hour_infected_animal_4	34,977,843	34,378,601	34,378,601	34,245,971	34,241,221
Blood-mRNA-TI-4-003	24_hour_infected_animal_4	42,897,211	42,827,437	42,827,437	42,664,573	42,661,864
Blood-mRNA-TI-4-004	36_hour_infected_animal_4	47,008,426	46,968,142	46,968,142	46,773,249	46,770,344
Blood-mRNA-TI-4-005	48_hour_infected_animal_4	15,648,618	15,204,191	15,204,191	15,187,439	15,185,461
Blood-mRNA-TI-5-001	0_hour_infected_animal_5	25,906,130	25,608,626	25,608,626	25,580,338	25,578,079
Blood-mRNA-TI-5-002	12_hour_infected_animal_5	58,267,346	58,200,437	58,200,437	57,963,587	57,959,966
Blood-mRNA-TI-5-003	24_hour_infected_animal_5	48,101,479	47,421,667	47,421,667	47,260,415	47,256,695
Blood-mRNA-TI-5-004	36_hour_infected_animal_5	47,646,187	47,573,376	47,573,376	47,380,832	47,378,197

lood-mRNA-TI-5-005	48_hour_infected_animal_5	54,896,392	54,816,516	54,816,516	46,917,379	54,657,174
ilk-mRNA-TC-1-001	0_hour_control_animal_1	20,718,878	19,336,153	19,328,079	19,307,150	18,064,353
ilk-mRNA-TC-1-002	12_hour_control_animal_1	19,811,944	18,281,223	18,273,406	18,258,550	17,235,919
ilk-mRNA-TC-1-003	24_hour_control_animal_1	25,858,517	24,471,548	24,461,463	24,438,687	22,886,053
ilk-mRNA-TC-1-004	36_hour_control_animal_1	18,640,732	17,252,508	17,245,314	17,233,010	16,290,330
ilk-mRNA-TC-1-005	48_hour_control_animal_1	17,609,857	16,612,787	16,604,414	16,589,194	15,676,892
ilk-mRNA-TC-2-001	0_hour_control_animal_2	33,798,892	31,489,259	31,474,610	31,443,234	29,832,937
ilk-mRNA-TC-2-002	12_hour_control_animal_2	17,280,881	16,488,827	16,483,101	16,470,089	15,372,259
ilk-mRNA-TC-2-003	24_hour_control_animal_2	52,006,852	49,608,093	49,585,966	49,543,606	46,922,688
ilk-mRNA-TC-2-004	36_hour_control_animal_2	27,355,722	25,841,082	25,830,494	25,809,413	24,418,831
ilk-mRNA-TC-2-005	48_hour_control_animal_2	9,829,401	9,291,256	9,286,537	9,278,328	8,725,386
ilk-mRNA-TC-3-001	0_hour_control_animal_3	17,143,701	16,216,940	16,209,067	16,195,417	15,197,532
ilk-mRNA-TC-3-002	12_hour_control_animal_3	18,841,300	16,656,377	16,645,740	16,622,914	15,282,952
ilk-mRNA-TC-3-003	24_hour_control_animal_3	12,573,120	11,856,321	11,851,235	11,840,541	11,066,341
ilk-mRNA-TC-3-004	36_hour_control_animal_3	15,470,012	14,173,745	14,167,898	14,154,127	13,269,377
ilk-mRNA-TC-3-005	48_hour_control_animal_3	36,051,781	34,312,448	34,299,185	34,271,203	32,507,286
ilk-mRNA-TC-4-001	0_hour_control_animal_4	17,968,186	16,775,662	16,766,049	16,748,828	15,810,728
ilk-mRNA-TC-4-002	12_hour_control_animal_4	12,002,000	11,412,043	11,407,724	11,398,851	10,775,671
ilk-mRNA-TC-4-003	24_hour_control_animal_4	19,366,295	17,919,083	17,912,093	17,893,477	16,571,786
ilk-mRNA-TC-4-004	36_hour_control_animal_4	45,770,860	41,823,865	41,801,582	41,762,701	39,524,002
ilk-mRNA-TC-4-005	48_hour_control_animal_4	43,264,871	39,688,122	39,666,313	39,615,100	37,458,314
ilk-mRNA-TC-5-001	0_hour_control_animal_5	24,902,268	21,746,965	21,730,601	21,709,141	20,420,966
ilk-mRNA-TC-5-002	12_hour_control_animal_5	38,440,720	36,392,440	36,373,229	36,339,589	34,460,755
ilk-mRNA-TC-5-003	24_hour_control_animal_5	70,750,168	66,486,723	66,455,121	66,392,147	62,928,962
ilk-mRNA-TC-5-004	36_hour_control_animal_5	66,078,829	62,609,567	62,579,944	62,530,343	59,286,612
ilk-mRNA-TC-5-005	48_hour_control_animal_5	77,385,014	70,875,606	70,830,344	70,764,520	66,918,192
ilk-mRNA-TI-1-001	0_hour_infected_animal_1	18,370,591	15,883,319	15,871,040	15,856,522	14,682,372
ilk-mRNA-TI-1-002	12_hour_infected_animal_1	24,563,421	21,980,593	21,964,725	21,940,776	19,906,558
ilk-mRNA-TI-1-003	24_hour_infected_animal_1	70,796,017	53,932,070	53,891,623	53,688,372	50,589,454
ilk-mRNA-TI-1-004	36_hour_infected_animal_1	38,934,888	36,463,122	36,444,676	36,408,730	34,202,483
ilk-mRNA-TI-1-005	48_hour_infected_animal_1	116,133,346	105,585,632	105,514,392	105,384,190	97,987,933
ilk-mRNA-TI-2-001	0_hour_infected_animal_2	69,439,723	54,006,139	53,964,799	53,758,291	50,793,099
ilk-mRNA-TI-2-002	12_hour_infected_animal_2	47,933,807	41,261,334	41,241,357	41,157,538	34,943,039
ilk-mRNA-TI-2-003	24_hour_infected_animal_2	68,995,891	54,120,302	54,077,217	53,875,165	50,037,902
ilk-mRNA-TI-2-004	36_hour_infected_animal_2	49,224,600	42,097,158	42,074,048	41,981,041	37,848,968
ilk-mRNA-TI-2-005	48_hour_infected_animal_2	74,685,983	54,971,895	54,934,789	54,699,358	50,196,712
ilk-mRNA-TI-3-001	0_hour_infected_animal_3	55,867,931	45,525,441	45,498,797	45,337,559	42,591,858
ilk-mRNA-TI-3-002	12_hour_infected_animal_3	44,902,024	40,757,651	40,741,900	40,683,159	37,719,466
ilk-mRNA-TI-3-003	24_hour_infected_animal_3	4,303,612	3,733,435	3,730,528	3,723,848	3,334,191
ilk-mRNA-TI-3-004	36_hour_infected_animal_3	53,998,739	50,091,282	50,073,243	50,020,617	47,131,363
ilk-mRNA-TI-3-005	48_hour_infected_animal_3	39,319,518	36,290,815	36,276,560	36,234,661	34,049,103
ilk-mRNA-TI-4-001	0_hour_infected_animal_4	49,159,957	42,843,279	42,821,343	42,736,524	40,577,407
ilk-mRNA-TI-4-002	12_hour_infected_animal_4	66,381,407	57,237,773	57,208,853	57,084,742	54,171,364
ilk-mRNA-TI-4-003	24_hour_infected_animal_4	38,731,025	35,752,101	35,738,358	35,699,147	33,621,378
ilk-mRNA-TI-4-004	36_hour_infected_animal_4	86,666,760	64,184,128	64,143,794	63,877,074	60,179,393
ilk-mRNA-TI-4-005	48_hour_infected_animal_4	54,446,900	48,044,020	48,018,862	47,941,082	45,205,761
ilk-mRNA-TI-5-001	0_hour_infected_animal_5	83,696,307	70,931,831	70,892,881	70,727,231	66,472,040
ilk-mRNA-TI-5-002	12_hour_infected_animal_5	72,428,423	63,671,056	63,637,843	63,525,984	59,594,120
ilk-mRNA-TI-5-003	24_hour_infected_animal_5	61,824,286	50,622,243	50,591,345	50,417,646	47,529,066
ilk-mRNA-TI-5-004	36_hour_infected_animal_5	41,858,195	34,504,939	34,484,442	34,368,557	32,310,388
ilk-mRNA-TI-5-005	48_hour_infected_animal_5	50,193,787	44,128,651	44,108,324	44,024,330	41,371,143

Appendix 13: Summary read statistics and number of unique alignments for each blood and milk miRNA library

Sample ID	Sample name	Raw Reads	Post cutadapt Trimming	Post Fastx Trimmer	Post Fastq quality Filter	Uniquely aligned to UMD 3.1
Blood-miRNA-TC-1-001	0_hour_control_animal_1	20,718,878	19,336,153	19,328,079	19,307,150	18,064,353
Blood-miRNA-TC-1-002	12_hour_control_animal_1	19,811,944	18,281,223	18,273,406	18,258,550	17,235,919
Blood-miRNA-TC-1-003	24_hour_control_animal_1	25,858,517	24,471,548	24,461,463	24,438,687	22,886,053
Blood-miRNA-TC-1-004	36_hour_control_animal_1	18,640,732	17,252,508	17,245,314	17,233,010	16,290,330
Blood-miRNA-TC-1-005	48_hour_control_animal_1	17,609,857	16,612,787	16,604,414	16,589,194	15,676,892
Blood-miRNA-TC-1-005(R)	48_hour_control_animal_1	33,798,892	31,489,259	31,474,610	31,443,234	29,832,937
Blood-miRNA-TC-2-001	0_hour_control_animal_2	17,280,881	16,488,827	16,483,101	16,470,089	15,372,259
Blood-miRNA-TC-2-002	12_hour_control_animal_2	52,006,852	49,608,093	49,585,966	49,543,606	46,922,688
Blood-miRNA-TC-2-003	24_hour_control_animal_2	27,355,722	25,841,082	25,830,494	25,809,413	24,418,831
Blood-miRNA-TC-2-004	36_hour_control_animal_2	9,829,401	9,291,256	9,286,537	9,278,328	8,725,386
Blood-miRNA-TC-2-005	48_hour_control_animal_2	17,143,701	16,216,940	16,209,067	16,195,417	15,197,532
Blood-miRNA-TC-3-001	0_hour_control_animal_3	18,841,300	16,656,377	16,645,740	16,622,914	15,282,952
Blood-miRNA-TC-3-002	12_hour_control_animal_3	12,573,120	11,856,321	11,851,235	11,840,541	11,066,341
Blood-miRNA-TC-3-003	24_hour_control_animal_3	15,470,012	14,173,745	14,167,898	14,154,127	13,269,377
Blood-miRNA-TC-3-004	36_hour_control_animal_3	36,051,781	34,312,448	34,299,185	34,271,203	32,507,286
Blood-miRNA-TC-3-005	48_hour_control_animal_3	17,968,186	16,775,662	16,766,049	16,748,828	15,810,728
Blood-miRNA-TC-4-001	0_hour_control_animal_4	12,002,000	11,412,043	11,407,724	11,398,851	10,775,671
Blood-miRNA-TC-4-002	12_hour_control_animal_4	19,366,295	17,919,083	17,912,093	17,893,477	16,571,786
Blood-miRNA-TC-4-003	24_hour_control_animal_4	45,770,860	41,823,865	41,801,582	41,762,701	39,524,002
Blood-miRNA-TC-4-004	36_hour_control_animal_4	43,264,871	39,688,122	39,666,313	39,615,100	37,458,314
Blood-miRNA-TC-4-005	48_hour_control_animal_4	24,902,268	21,746,965	21,730,601	21,709,141	20,420,966
Blood-miRNA-TC-5-001	0_hour_control_animal_5	38,440,720	36,392,440	36,373,229	36,339,589	34,460,755
Blood-miRNA-TC-5-002	12_hour_control_animal_5	70,750,168	66,486,723	66,455,121	66,392,147	62,928,962
Blood-miRNA-TC-5-003	24_hour_control_animal_5	66,078,829	62,609,567	62,579,944	62,530,343	59,286,612
Blood-miRNA-TC-5-004	36_hour_control_animal_5	77,385,014	70,875,606	70,830,344	70,764,520	66,918,192
Blood-miRNA-TC-5-005	48_hour_control_animal_5	18,370,591	15,883,319	15,871,040	15,856,522	14,682,372
Blood-miRNA-TI-1-001	0_hour_infected_animal_1	24,563,421	21,980,593	21,964,725	21,940,776	19,906,558
Blood-miRNA-TI-1-002	12_hour_infected_animal_1	70,796,017	53,932,070	53,891,623	53,688,372	50,589,454
Blood-miRNA-TI-1-003	24_hour_infected_animal_1	38,934,888	36,463,122	36,444,676	36,408,730	34,202,483
Blood-miRNA-TI-1-004	36_hour_infected_animal_1	116,133,346	105,585,632	105,514,392	105,384,190	97,987,933
Blood-miRNA-TI-1-005	48_hour_infected_animal_1	69,439,723	54,006,139	53,964,799	53,758,291	50,793,099
Blood-miRNA-TI-2-001	0_hour_infected_animal_2	47,933,807	41,261,334	41,241,357	41,157,538	34,943,039
Blood-miRNA-TI-2-002	12_hour_infected_animal_2	68,995,891	54,120,302	54,077,217	53,875,165	50,037,902
Blood-miRNA-TI-2-003	24_hour_infected_animal_2	49,224,600	42,097,158	42,074,048	41,981,041	37,848,968
Blood-miRNA-TI-2-004	36_hour_infected_animal_2	74,685,983	54,971,895	54,934,789	54,699,358	50,196,712
Blood-miRNA-TI-2-004(R)	36_hour_infected_animal_2	55,867,931	45,525,441	45,498,797	45,337,559	42,591,858
Blood-miRNA-TI-2-005	48_hour_infected_animal_2	44,902,024	40,757,651	40,741,900	40,683,159	37,719,466
Blood-miRNA-TI-3-001	0_hour_infected_animal_3	4,303,612	3,733,435	3,730,528	3,723,848	3,334,191
Blood-miRNA-TI-3-002	12_hour_infected_animal_3	53,998,739	50,091,282	50,073,243	50,020,617	47,131,363
Blood-miRNA-TI-3-002(R)	12_hour_infected_animal_3	39,319,518	36,290,815	36,276,560	36,234,661	34,049,103
Blood-miRNA-TI-3-003	24_hour_infected_animal_3	49,159,957	42,843,279	42,821,343	42,736,524	40,577,407
Blood-miRNA-TI-3-004	36_hour_infected_animal_3	66,381,407	57,237,773	57,208,853	57,084,742	54,171,364
Blood-miRNA-TI-3-005	48_hour_infected_animal_3	38,731,025	35,752,101	35,738,358	35,699,147	33,621,378
Blood-miRNA-TI-4-001	0_hour_infected_animal_4	86,666,760	64,184,128	64,143,794	63,877,074	60,179,393
Blood-miRNA-TI-4-002	12_hour_infected_animal_4	54,446,900	48,044,020	48,018,862	47,941,082	45,205,761
Blood-miRNA-TI-4-003	24_hour_infected_animal_4	83,696,307	70,931,831	70,892,881	70,727,231	66,472,040
Blood-miRNA-TI-4-004	36_hour_infected_animal_4	72,428,423	63,671,056	63,637,843	63,525,984	59,594,120
Blood-miRNA-TI-4-005	48_hour_infected_animal_4	61,824,286	50,622,243	50,591,345	50,417,646	47,529,066
Blood-miRNA-TI-5-001	0_hour_infected_animal_5	41,858,195	34,504,939	34,484,442	34,368,557	32,310,388
Blood-miRNA-TI-5-002	12_hour_infected_animal_5	50,193,787	44,128,651	44,108,324	44,024,330	41,371,143
Blood-miRNA-TI-5-003	24_hour_infected_animal_5	46,726,602	41,150,537	41,038,387	41,036,537	18,665,758
Blood-miRNA-TI-5-004	36_hour_infected_animal_5	34,796,220	21,611,147	21,520,049	21,503,669	12,823,491
Blood-miRNA-TI-5-005	48_hour_infected_animal_5	36,947,773	32,842,467	32,740,887	32,734,061	17,096,502
Milk-miRNA-TC-1-001	0_hour_control_animal_1	13,822,969	13,074,656	12,999,791	12,999,003	8,461,837
Milk-miRNA-TC-1-002	12_hour_control_animal_1	24,286,872	17,382,210	17,353,959	17,352,514	10,258,453
Milk-miRNA-TC-1-003	24_hour_control_animal_1	15,990,154	13,661,106	13,648,002	13,646,664	6,413,875

ilk-miRNA-TC-1-004	36_hour_control_animal_1	21,922,591	19,858,178	19,840,063	19,838,811	12,006,361
ilk-miRNA-TC-1-005	48_hour_control_animal_1	5,884,282	3,717,758	3,714,152	3,713,640	2,439,656
ilk-miRNA-TC-1-005(R)	48_hour_control_animal_1	3,168,444	1,803,045	1,802,824	1,802,510	
ilk-miRNA-TC-2-001	0_hour_control_animal_2	15,690,575	13,685,212	13,583,269	13,582,404	8,789,698
ilk-miRNA-TC-2-001(R)	0_hour_control_animal_2	9,236,304	5,018,012	4,977,744	4,977,395	3,117,455
ilk-miRNA-TC-2-002	12_hour_control_animal_2	16,099,455	14,701,703	14,600,825	14,600,061	9,809,966
ilk-miRNA-TC-2-003	24_hour_control_animal_2	18,124,354	15,621,075	15,608,295	15,607,101	9,833,478
ilk-miRNA-TC-2-003(R)	24_hour_control_animal_2	16,685,141	15,117,092	15,011,248	15,010,506	9,334,217
ilk-miRNA-TC-2-004	36_hour_control_animal_2	12,617,137	11,331,219	11,322,301	11,321,386	5,520,412
ilk-miRNA-TC-2-005	48_hour_control_animal_2	18,828,581	10,923,711	10,906,450	10,905,417	6,583,618
ilk-miRNA-TC-2-005(R)	48_hour_control_animal_2	10,315,741	9,108,892	9,100,860	9,100,131	4,185,972
ilk-miRNA-TC-3-001	0_hour_control_animal_3	21,577,760	13,619,622	13,586,686	13,585,781	8,330,314
ilk-miRNA-TC-3-002	12_hour_control_animal_3	21,287,070	19,234,294	19,211,505	19,209,651	9,466,484
ilk-miRNA-TC-3-003	24_hour_control_animal_3	51,852,053	6,712,327	6,687,272	6,677,437	3,011,865
ilk-miRNA-TC-3-004	36_hour_control_animal_3	29,831,309	17,668,629	17,610,818	17,597,603	11,067,853
ilk-miRNA-TC-3-005	48_hour_control_animal_3	17,902,290	4,643,436	4,630,828	4,625,978	2,381,119
ilk-miRNA-TC-4-001	0_hour_control_animal_4	43,230,322	38,678,004	38,563,409	38,553,940	22,659,515
ilk-miRNA-TC-4-002	12_hour_control_animal_4	55,105,904	48,130,080	47,934,631	47,917,326	26,701,342
ilk-miRNA-TC-4-002(R)	12_hour_control_animal_4	38,917,580	33,388,721	33,288,548	33,276,897	19,695,961
ilk-miRNA-TC-4-003	24_hour_control_animal_4	7,185,340	6,656,614	6,635,662	6,634,701	4,055,942
ilk-miRNA-TC-4-003(R)	24_hour_control_animal_4	19,090,064	15,280,483	15,236,780	15,229,639	8,097,581
ilk-miRNA-TC-4-004	36_hour_control_animal_4	11,747,207	9,975,277	9,950,546	9,946,871	6,067,705
ilk-miRNA-TC-4-005	48_hour_control_animal_4	44,522,320	39,350,252	39,188,789	39,177,052	20,896,022
ilk-miRNA-TC-5-001	0_hour_control_animal_5	22,612,310	18,573,941	18,558,439	18,556,404	10,226,911
ilk-miRNA-TC-5-002	12_hour_control_animal_5	9,082,348	6,499,999	6,493,995	6,493,449	3,807,528
ilk-miRNA-TC-5-003	24_hour_control_animal_5	16,240,629	13,932,193	13,824,130	13,823,195	8,631,862
ilk-miRNA-TC-5-004	36_hour_control_animal_5	15,248,782	13,768,116	13,755,060	13,754,212	8,582,755
ilk-miRNA-TC-5-005	48_hour_control_animal_5	22,403,304	19,596,469	19,566,484	19,564,652	10,874,685
ilk-miRNA-TI-1-001	0_hour_infected_animal_1	14,667,549	13,617,164	13,527,764	13,526,739	8,857,451
ilk-miRNA-TI-1-002	12_hour_infected_animal_1	13,933,578	12,534,535	12,522,382	12,521,403	7,479,714
ilk-miRNA-TI-1-003	24_hour_infected_animal_1	23,602,107	21,831,608	21,811,723	21,810,053	13,839,401
ilk-miRNA-TI-1-004	36_hour_infected_animal_1	20,853,996	7,682,457	7,662,354	7,662,250	9,514,542
ilk-miRNA-TI-1-005	48_hour_infected_animal_1	22,967,209	7,805,628	7,804,947	7,804,652	
ilk-miRNA-TI-1-005(R)	48_hour_infected_animal_1	15,032,519	13,916,986	13,820,935	13,819,744	7,792,249
ilk-miRNA-TI-2-001	0_hour_infected_animal_2	20,884,166	13,873,024	13,854,486	13,852,421	7,863,626
ilk-miRNA-TI-2-002	12_hour_infected_animal_2	19,927,395	9,423,083	9,407,211	9,405,973	13,934,104
ilk-miRNA-TI-2-002(R)	12_hour_infected_animal_2	26,116,056	12,815,003	12,814,425	12,811,900	
ilk-miRNA-TI-2-003	24_hour_infected_animal_2	14,557,130	11,706,773	11,695,044	11,693,986	6,758,643
ilk-miRNA-TI-2-003(R)	24_hour_infected_animal_2	18,578,111	14,688,958	14,666,686	14,665,092	7,797,393
ilk-miRNA-TI-2-004	36_hour_infected_animal_2	9,726,173	7,023,950	6,966,032	6,965,501	4,409,800
ilk-miRNA-TI-2-005	48_hour_infected_animal_2	20,800,706	9,417,185	9,382,529	9,373,020	4,615,787
ilk-miRNA-TI-3-001	0_hour_infected_animal_3	14,382,228	7,004,968	6,986,517	6,980,549	4,106,922
ilk-miRNA-TI-3-002	12_hour_infected_animal_3	36,267,991	30,993,770	30,849,760	30,840,536	18,073,806
ilk-miRNA-TI-3-003	24_hour_infected_animal_3	11,132,248	10,510,012	10,480,009	10,479,236	6,733,089
ilk-miRNA-TI-3-004	36_hour_infected_animal_3	17,622,054	12,208,157	12,119,299	12,118,008	7,535,424
ilk-miRNA-TI-3-004(R)	36_hour_infected_animal_3	50,172,091	29,792,756	29,699,730	29,675,438	16,702,521
ilk-miRNA-TI-3-005	48_hour_infected_animal_3	51,042,523	48,840,824	48,721,800	29,675,438	16,702,521
ilk-miRNA-TI-4-001	0_hour_infected_animal_4	46,726,602	41,150,537	41,038,387	41,036,537	18,665,758
ilk-miRNA-TI-4-001(R)	0_hour_infected_animal_4	34,796,220	21,611,147	21,520,049	21,503,669	12,823,491
ilk-miRNA-TI-4-002	12_hour_infected_animal_4	36,947,773	32,842,467	32,740,887	32,734,061	17,096,502
ilk-miRNA-TI-4-002(R)	12_hour_infected_animal_4	13,822,969	13,074,656	12,999,791	12,999,003	8,461,837
ilk-miRNA-TI-4-003	24_hour_infected_animal_4	24,286,872	17,382,210	17,353,959	17,352,514	10,258,453
ilk-miRNA-TI-4-004	36_hour_infected_animal_4	15,990,154	13,661,106	13,648,002	13,646,664	6,413,875
ilk-miRNA-TI-4-005	48_hour_infected_animal_4	21,922,591	19,858,178	19,840,063	19,838,811	12,006,361
ilk-miRNA-TI-5-001	0_hour_infected_animal_5	5,884,282	3,717,758	3,714,152	3,713,640	2,439,656
ilk-miRNA-TI-5-002	12_hour_infected_animal_5	3,168,444	1,803,045	1,802,824	1,802,510	
ilk-miRNA-TI-5-003	24_hour_infected_animal_5	15,690,575	13,685,212	13,583,269	13,582,404	8,789,698
ilk-miRNA-TI-5-004	36_hour_infected_animal_5	9,236,304	5,018,012	4,977,744	4,977,395	3,117,455
ilk-miRNA-TI-5-004(R)	36_hour_infected_animal_5	16,099,455	14,701,703	14,600,825	14,600,061	9,809,966
ilk-miRNA-TI-5-005	48_hour_infected_animal_5	18,124,354	15,621,075	15,608,295	15,607,101	9,833,478
ilk-miRNA-TI-3-003	0_hour_control_animal_1	11,132,248	10,510,012	10,480,009	10,479,236	6,733,089

Appendix 14.1: Average correlation coefficients of mRNA normalised read counts between samples at each time-point for control and infected biological replicates. Table 1: Milk mRNA correlation coefficients. Table 2: Blood mRNA correlation coefficients.

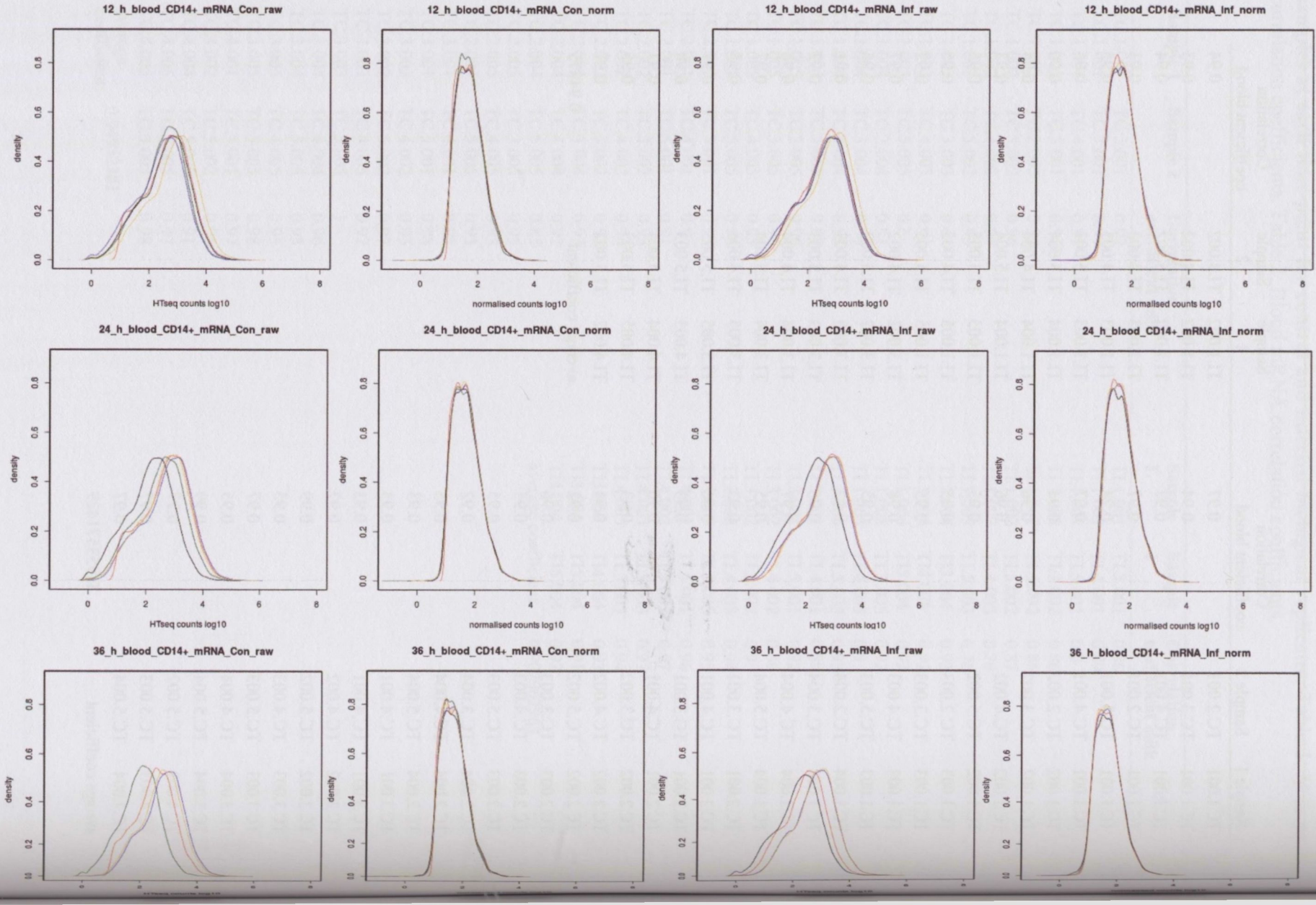
Sample 1	Sample 2	Correlation coefficient milk
TC.1.001	TC.2.001	0.92
TC.1.001	TC.3.001	0.97
TC.1.001	TC.4.001	0.95
TC.1.001	TC.5.001	0.95
TC.1.002	TC.2.002	0.94
TC.1.002	TC.3.002	0.96
TC.1.002	TC.4.002	0.95
TC.1.002	TC.5.002	0.93
TC.1.003	TC.3.003	0.6
TC.1.003	TC.4.005	0.61
TC.1.003	TC.5.005	0.6
TC.1.004	TC.2.004	0.91
TC.1.004	TC.3.004	0.94
TC.1.004	TC.4.004	0.95
TC.1.004	TC.5.005	0.88
TC.1.005	TC.2.005	0.96
TC.1.005	TC.3.005	0.96
TC.1.005	TC.4.005	0.95
TC.1.005	TC.5.005	0.96
TC.2.001	TC.3.001	0.93
TC.2.001	TC.4.001	0.9
TC.2.001	TC.5.001	0.91
TC.2.002	TC.3.002	0.95
TC.2.002	TC.4.002	0.94
TC.2.002	TC.5.002	0.92
TC.2.004	TC.3.004	0.93
TC.2.004	TC.4.004	0.92
TC.2.004	TC.5.005	0.85
TC.2.005	TC.3.005	0.95
TC.2.005	TC.4.005	0.93
TC.2.005	TC.5.005	0.93
TC.3.001	TC.4.001	0.95
TC.3.001	TC.5.001	0.98
TC.3.002	TC.4.002	0.95
TC.3.002	TC.5.002	0.97
TC.3.003	TC.4.005	0.97
TC.3.003	TC.5.005	1
TC.3.004	TC.4.004	0.96
TC.3.004	TC.5.005	0.93
TC.3.005	TC.4.005	0.95
TC.3.005	TC.5.005	0.98
TC.4.001	TC.5.001	0.97
TC.4.002	TC.5.002	0.96
TC.4.004	TC.5.005	0.91
TC.4.005	TC.5.005	0.97
TC.4.005	TC.5.005	0.98
average coefficient	0.94952381	

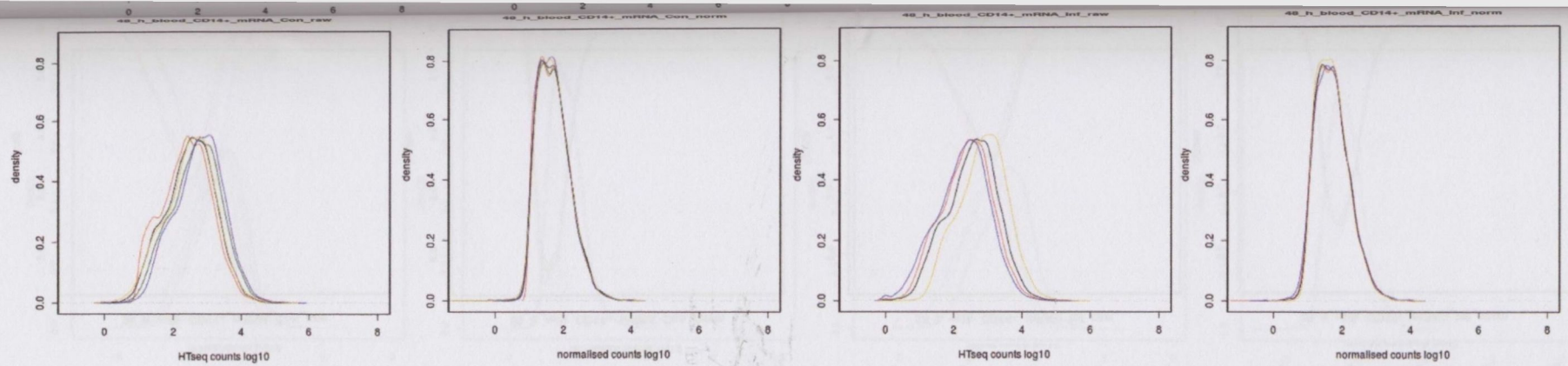
Sample 1	Sample 2	Correlation coefficient milk
TI.1.001	TI.3.001	0.86
TI.1.001	TI.4.001	0.89
TI.1.001	TI.5.001	0.9
TI.1.002	TI.3.002	0.86
TI.1.002	TI.4.002	0.86
TI.1.002	TI.5.002	0.87
TI.1.003	TI.4.003	0.79
TI.1.003	TI.5.003	0.76
TI.1.004	TI3.004	0.93
TI.1.004	TI4.004	0.91
TI.1.004	TI5.004	0.92
TI.1.005	TI.3.005	0.92
TI.1.005	TI.4.005	0.9
TI.1.005	TI.5.005	0.93
TI.3.001	TI.4.001	0.89
TI.3.001	TI.5.001	0.92
TI.3.002	TI.4.002	0.88
TI.3.002	TI.5.002	0.9
TI.3.005	TI.4.005	0.94
TI.3.005	TI.5.005	0.95
TI.4.001	TI.5.001	0.96
TI.4.002	TI.5.002	0.97
TI.4.003	TI.5.003	0.97
TI.4.005	TI.5.005	0.94
TI3.004	TI4.004	0.93
TI3.004	TI5.004	0.91
TI4.004	TI5.004	0.92
average coefficient		0.902962963

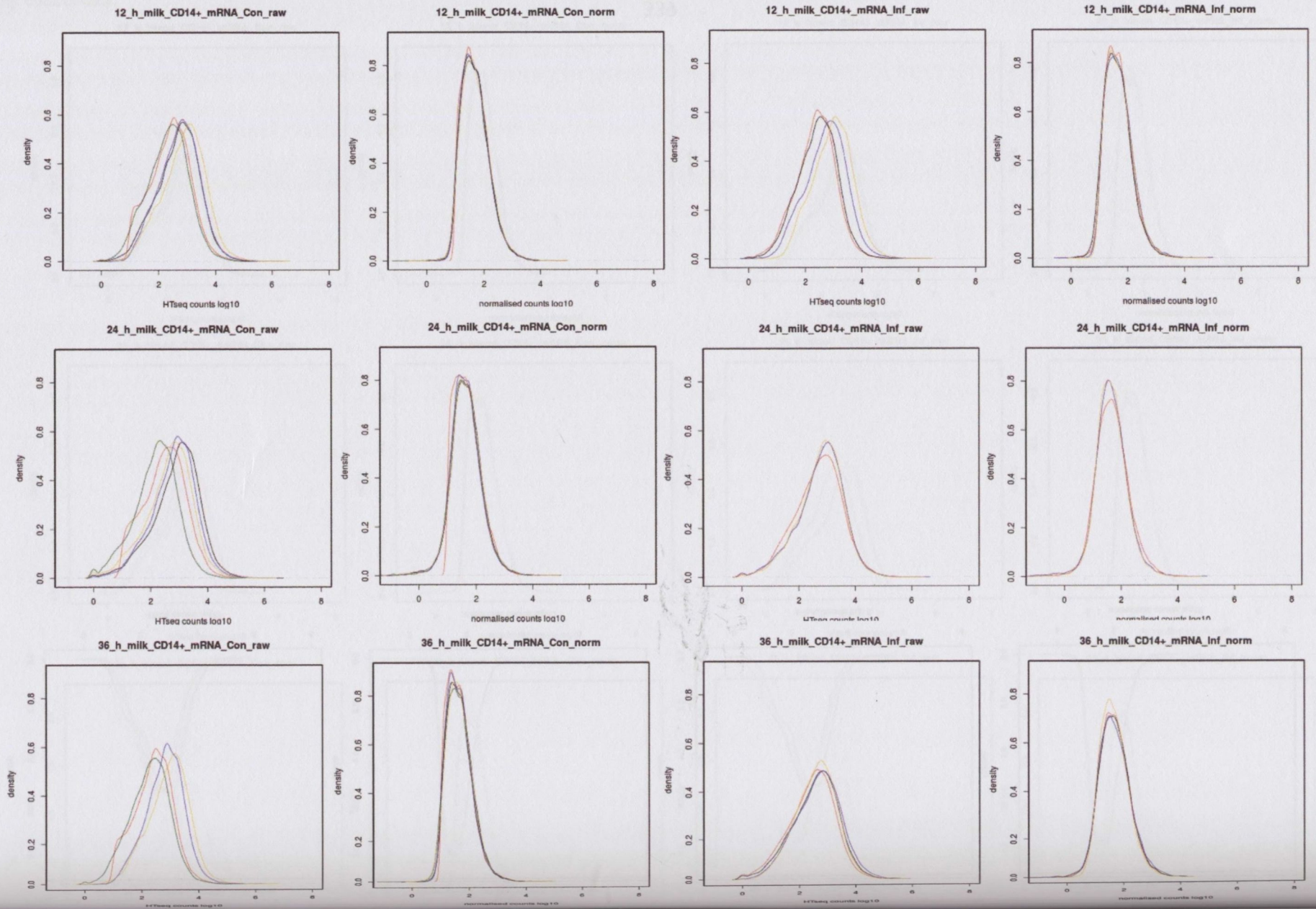
Sample 1	Sample 2	Correlation coefficient blood
TC.1.001	TC.2.001	0.97
TC.1.001	TC.3.001	0.94
TC.1.001	TC.4.001	0.97
TC.1.001	TC.2.001	0.97
TC.1.001	TC.3.001	0.94
TC.1.001	TC.4.001	0.97
TC.1.002	TC.2.002	0.94
TC.1.002	TC.3.002	0.96
TC.1.002	TC.4.002	0.96
TC.1.002	TC.5.002	0.95
TC.1.003	TC.2.003	0.95
TC.1.003	TC.3.003	0.95
TC.1.003	TC.4.003	0.98
TC.1.003	TC.5.003	0.95
TC.1.004	TC.2.004	0.95
TC.1.004	TC.3.004	0.96
TC.1.004	TC.4.004	0.97
TC.1.004	TC.5.004	0.95
TC.2.001	TC.3.001	0.93
TC.2.001	TC.4.001	0.96
TC.2.001	TC.3.001	0.93
TC.2.001	TC.4.001	0.96
TC.2.002	TC.3.002	0.93
TC.2.002	TC.4.002	0.94
TC.2.002	TC.5.002	0.92
TC.2.003	TC.3.003	0.96
TC.2.003	TC.4.003	0.95
TC.2.003	TC.5.003	0.95
TC.2.004	TC.3.004	0.92
TC.2.004	TC.4.004	0.95
TC.2.004	TC.5.004	0.93
TC.3.001	TC.4.001	0.93
TC.3.001	TC.4.001	0.93
TC.3.002	TC.4.002	0.97
TC.3.002	TC.5.002	0.99
TC.3.003	TC.4.003	0.95
TC.3.003	TC.5.003	0.97
TC.3.004	TC.4.004	0.95
TC.3.004	TC.5.004	0.99
TC.4.002	TC.5.002	0.97
TC.4.003	TC.5.003	0.97
TC.4.004	TC.5.004	0.97
average coefficient		0.953571429

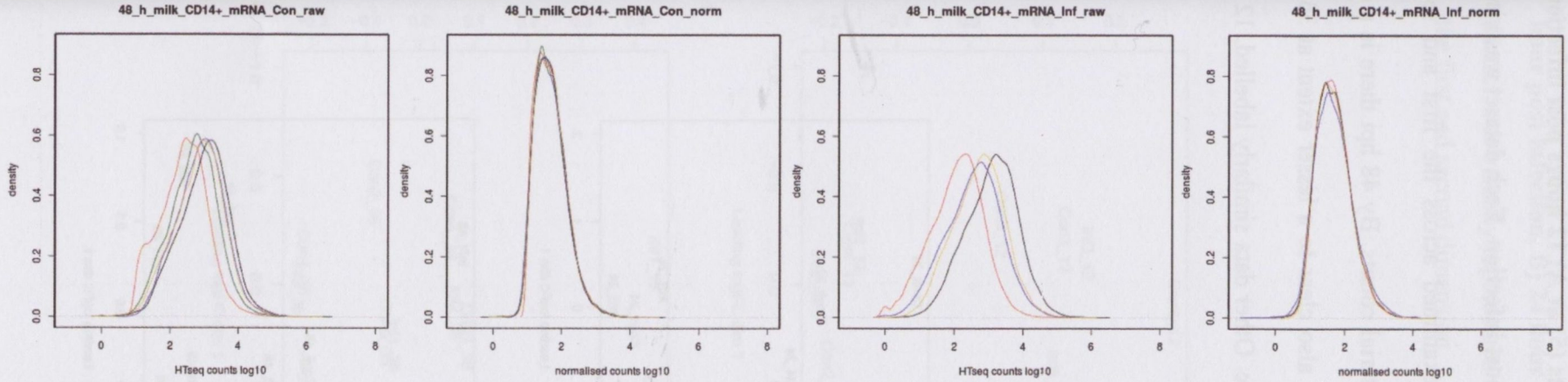
Sample 1	Sample 2	Correlation coefficient blood
TI.1.002	TI.3.002	0.94
TI.1.002	TI.4.002	0.93
TI.1.002	TI.5.002	0.94
TI.1.003	TI.3.003	0.93
TI.1.003	TI.4.003	0.95
TI.1.003	TI.5.003	0.95
TI.1.004	TI.3.004	0.96
TI.1.004	TI.4.004	0.94
TI.1.004	TI.5.004	0.93
TI.1.005	TI.3.005	0.95
TI.1.005	TI.4.005	0.95
TI.1.005	TI.5.005	0.92
TI.3.002	TI.4.002	0.97
TI.3.002	TI.5.002	0.96
TI.3.003	TI.4.003	0.91
TI.3.003	TI.5.003	0.92
TI.3.004	TI.4.004	0.96
TI.3.004	TI.5.004	0.97
TI.3.005	TI.4.005	0.95
TI.3.005	TI.5.005	0.97
TI.4.003	TI.5.003	0.98
TI.4.004	TI.5.004	0.97
TI.4.005	TI.5.005	0.95
TI.4.002	TI.5.002	0.97
average coefficient		0.94875

Appendix 14.2. Gene expression density plots comparing raw and normalised (Trimmed Mean of M-values (TMM)) blood & milk CD14+ mRNA reads for both control and infected samples. The log10 of count numbers is plotted on the X-axis and the density on the Y-axis to examine the variation of aligned reads and the effect normalisation has had on each sample. Each graph is individually labelled for time, tissue type, control/infected, and norm = "normalised reads" or HTseq = "raw reads". For samples/graphs sample animal 1 = red, animal 2 = green, and animal 3 = black, animal 4 = blue, animal 5 = yellow. Infected animal 2 samples, control animal 2, -4 at 24 hpi (milk) infected animal 3, -4 at 24 hpi (milk) were removed from differential expression analysis based on CFU and quality control data.

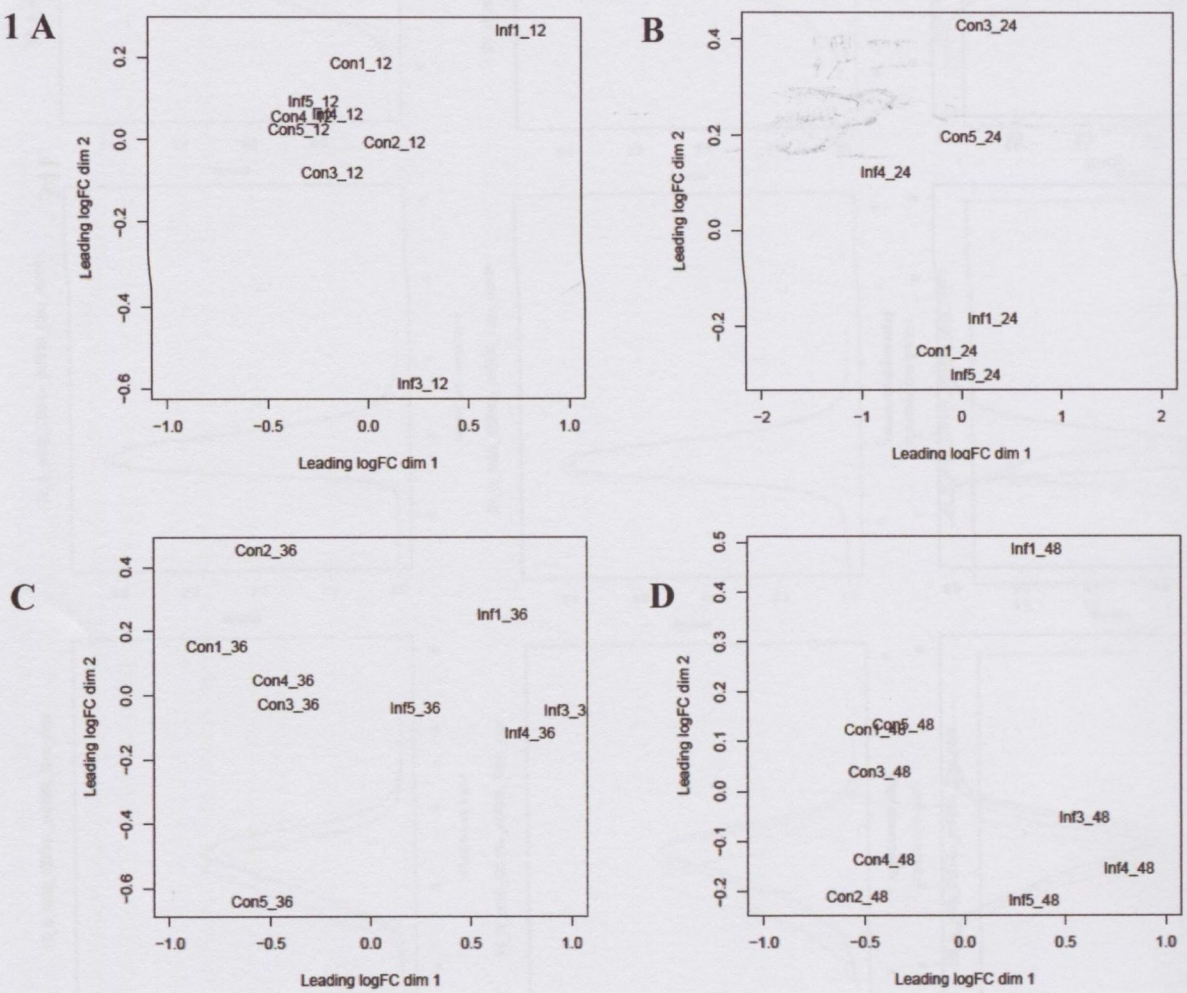



48_h_blood_CD14+_mRNA_Con_raw
density
HTseq counts log10
48_h_blood_CD14+_mRNA_Con_norm
density
normalised counts log10
48_h_blood_CD14+_mRNA_Inf_raw
density
HTseq counts log10
48_h_blood_CD14+_mRNA_Inf_norm
density
normalised counts log10

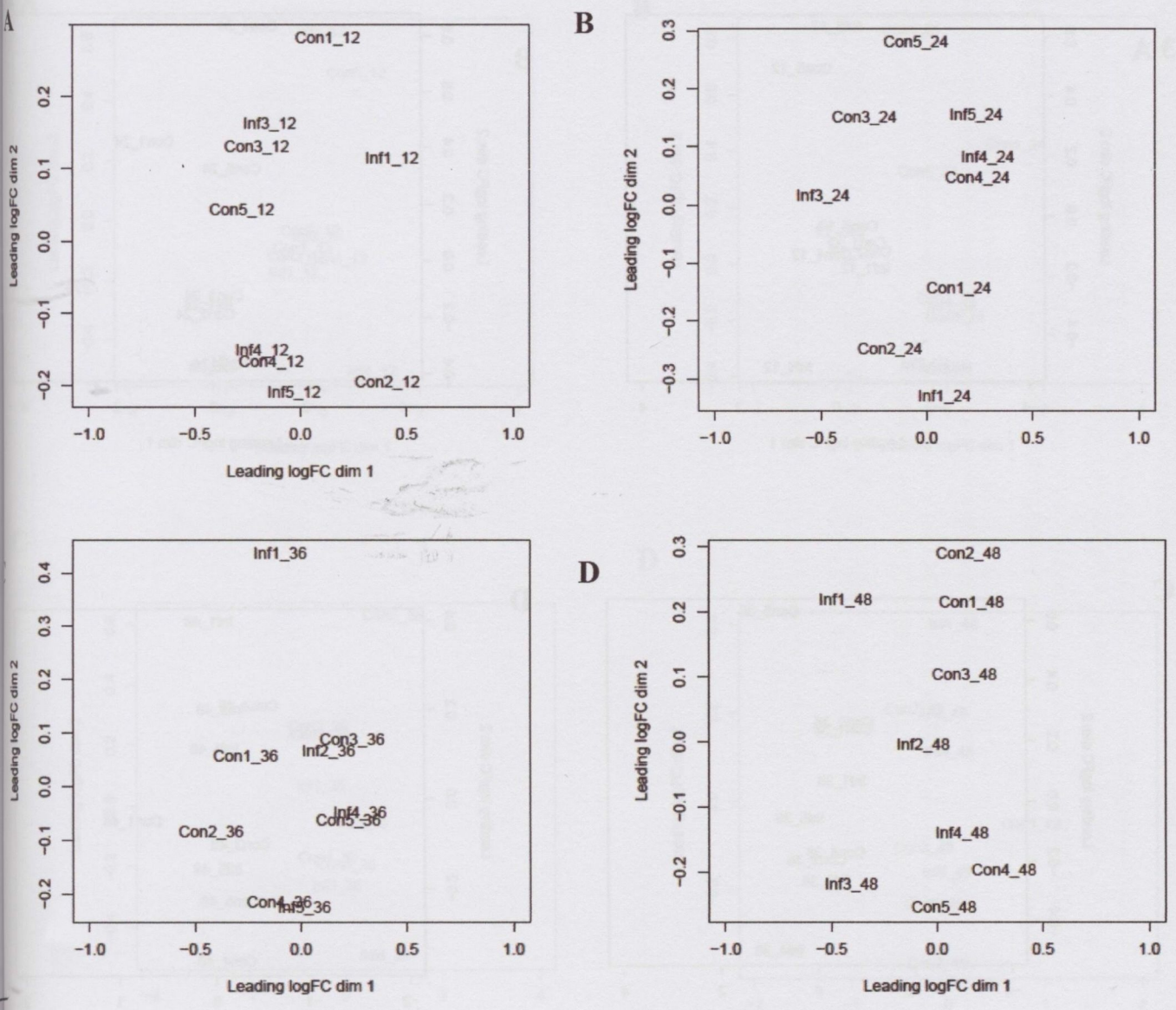

12_h_milk_CD14+_mRNA_Con_raw
12_h_milk_CD14+_mRNA_Con_norm
12_h_milk_CD14+_mRNA_Inf_raw
12_h_milk_CD14+_mRNA_Inf_norm
24_h_milk_CD14+_mRNA_Con_raw
24_h_milk_CD14+_mRNA_Con_norm
24_h_milk_CD14+_mRNA_Inf_raw
24_h_milk_CD14+_mRNA_Inf_norm
36_h_milk_CD14+_mRNA_Con_raw
36_h_milk_CD14+_mRNA_Con_norm
36_h_milk_CD14+_mRNA_Inf_raw
36_h_milk_CD14+_mRNA_Inf_norm
density
HTseq counts log10
normalised counts log10


48_h_milk_CD14+_mRNA_Con_raw
48_h_milk_CD14+_mRNA_Con_norm
48_h_milk_CD14+_mRNA_Inf_raw
48_h_milk_CD14+_mRNA_Inf_norm
density
HTseq counts log10
normalised counts log10

Appendix 15: Multidimensional scaling (MDS) analysis plots for milk mRNA (1), blood mRNA (2), mi miRNA (3), blood miRNA sequencing datasets (4) and all time-points (5) at A) 12 hours post infection, B 24 hours post infection, C) 36 hours post infection and D) 48 hours post infection. Each dataset was visual represented across each time-point using MDS. Each dataset was examined across the first and secon dimension, samples showing largest separation showed the highest bacterial counts. By 48 hpi there is a cle separation between of infected and control animals. This separation is also clear to a lesser extent at 36 hp Con1_12hr = 12 h control replicate 1; Inf1_12h = 12 h infected replicate. Other data similarly labelled. 12 hr= 12 hour post-infection/mock infection.

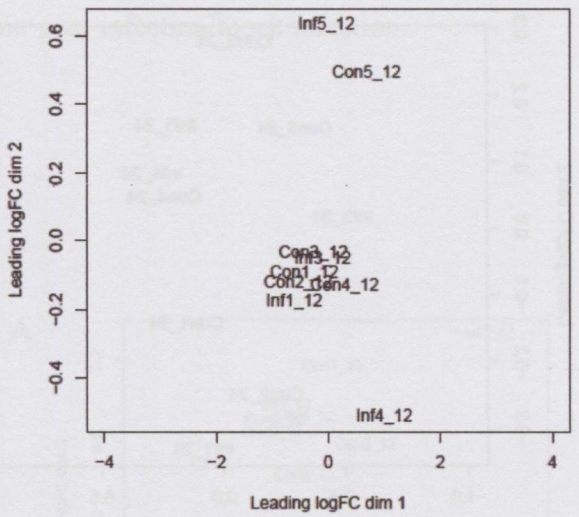


Appendix 15.2: Multidimensional scaling (MDS) plots for blood mRNA sequencing dataset A) 12 hour post infection, B) 24 hour post infection, C) 36 hour post infection and D) 48 hour post. Each dataset was visually represented across each time point using MDS. Each dataset was examined across the first and second dimension.

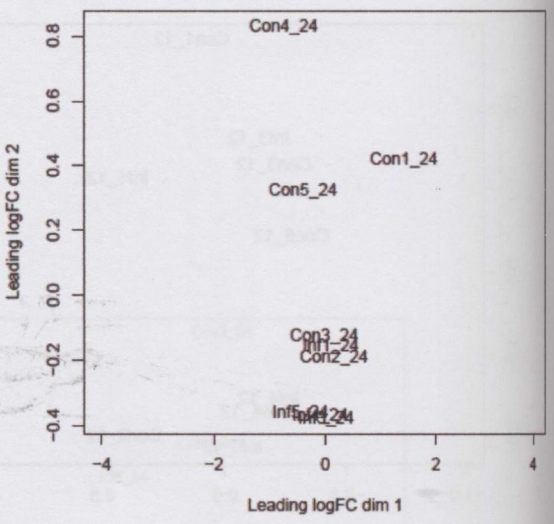


Appendix 15.3: Multidimensional scaling (MDS) plots for milk miRNA sequencing dataset A) 12 hour post infection, B) 24 hour post infection, C) 36 hour post infection, D) 48 hour post infection and. Each dataset was visually represented across each time-point and as one complete dataset using MDS. Each dataset was examined across the first and second dimension. In plot C (36 hpi) infected samples separate from control samples across the first dimension.

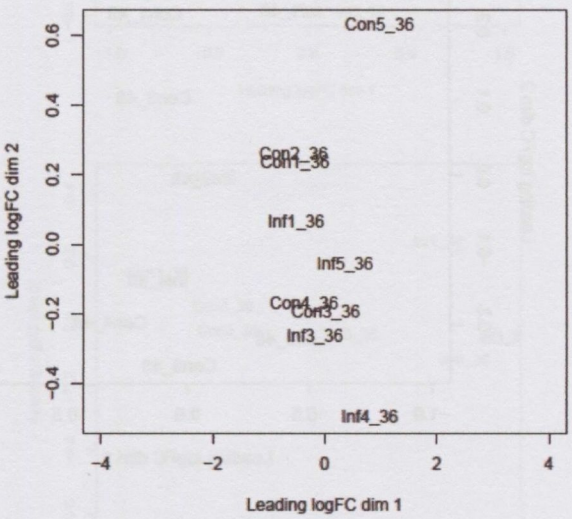
3 A



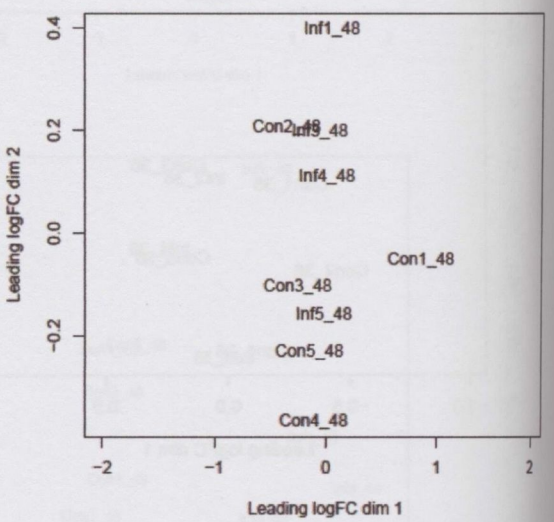
B



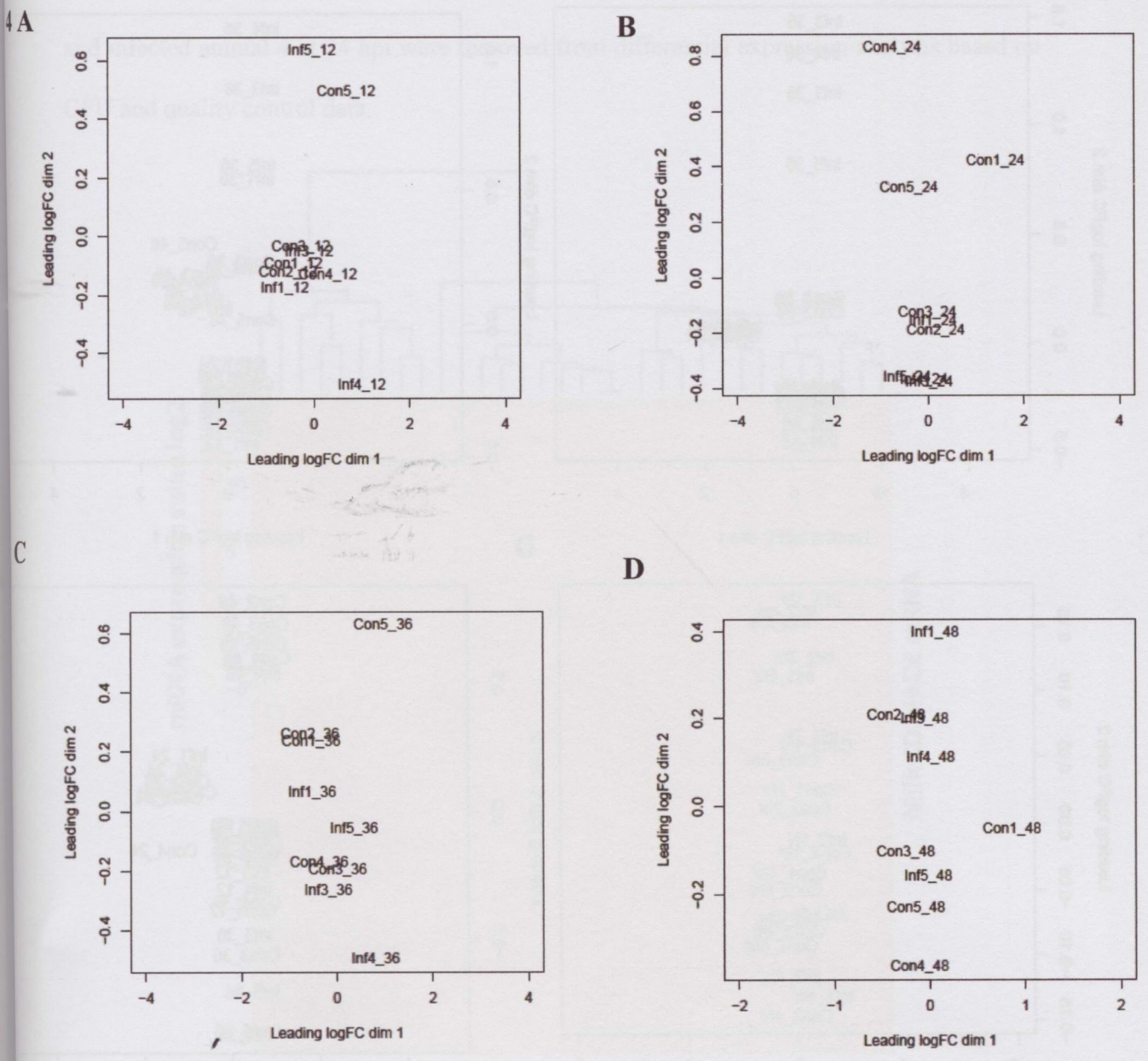
C



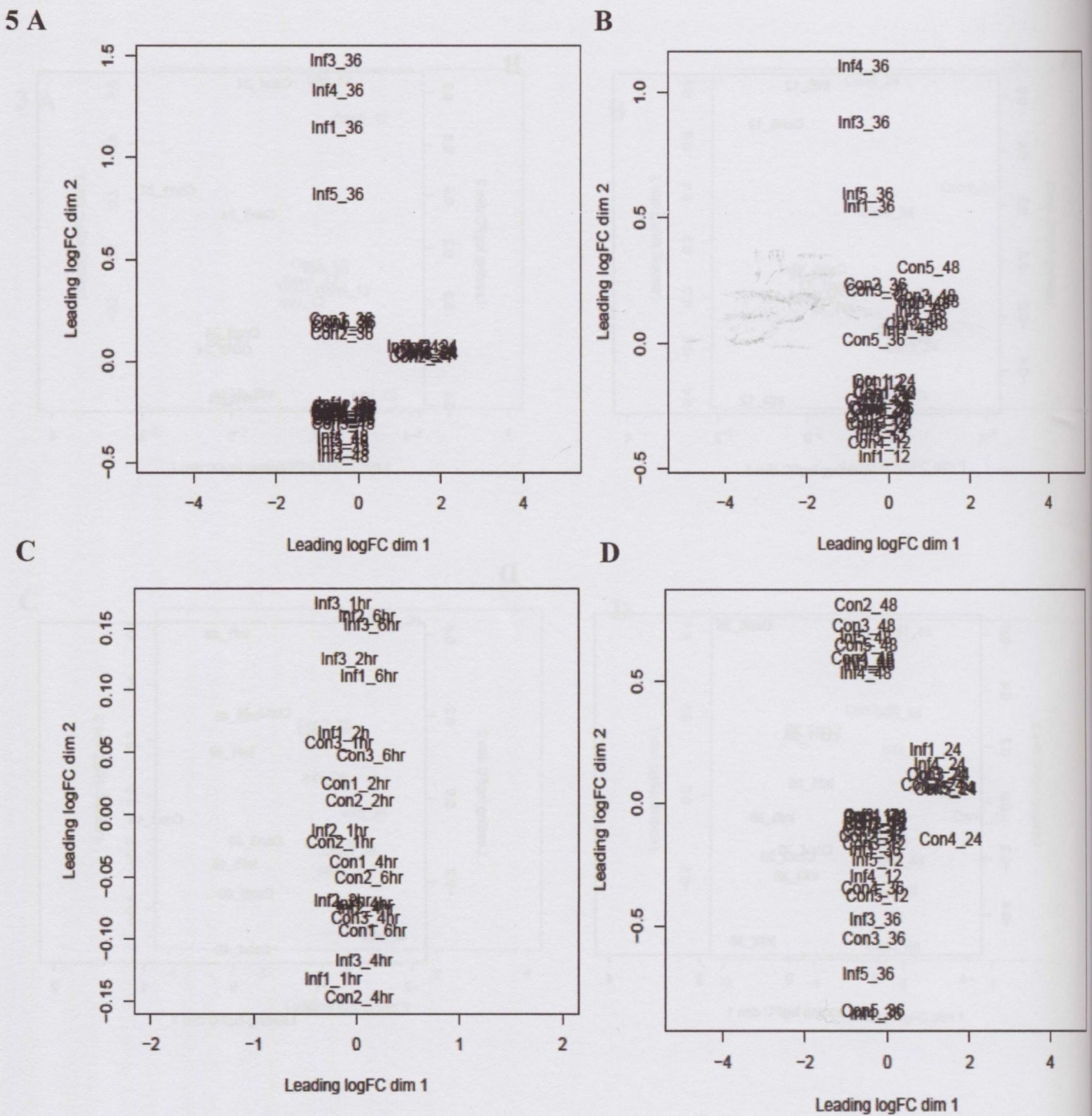
D



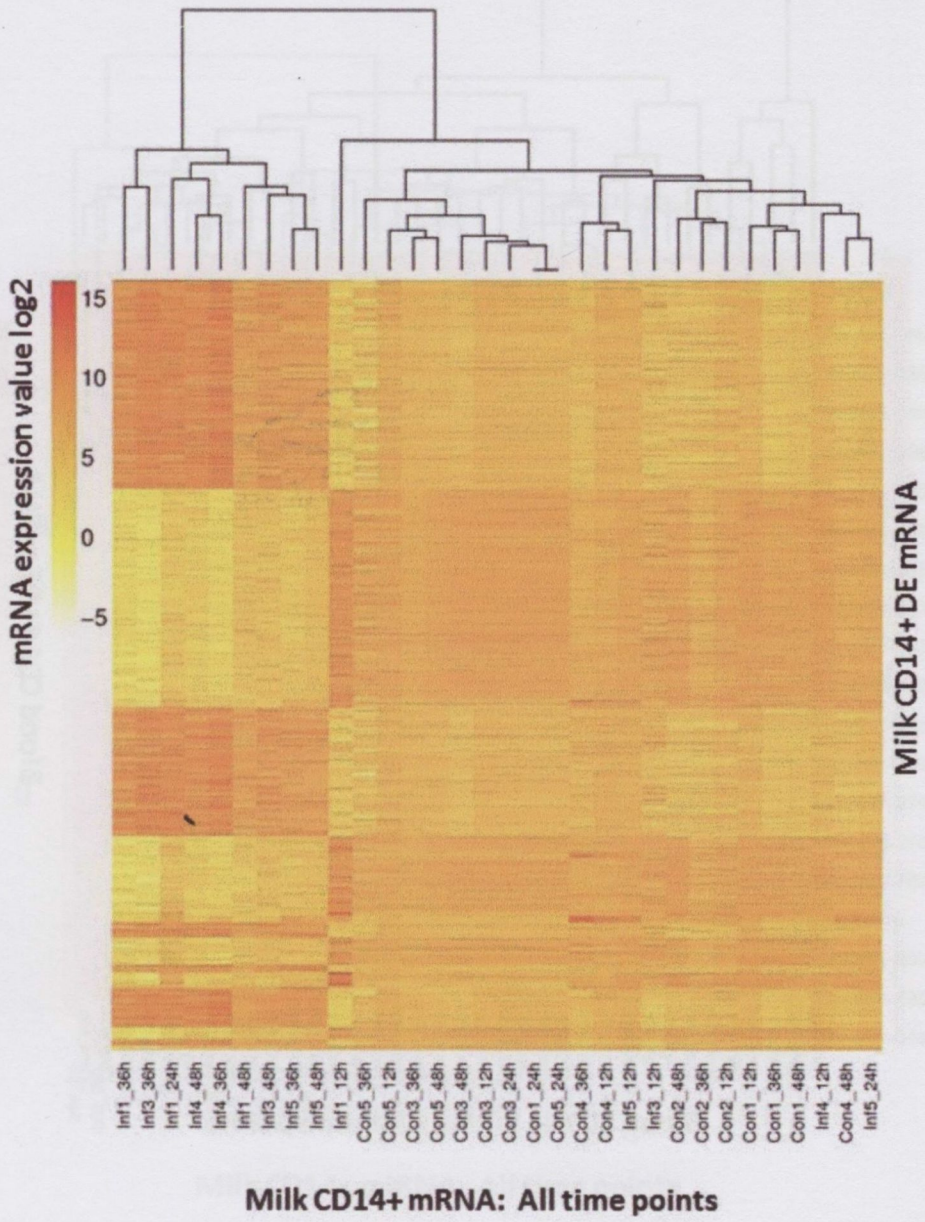
Appendix 15.4: Multidimensional scaling (MDS) plots for blood miRNA sequencing dataset A) 12 hour post infection, B) 24 hour post infection, C) 36 hour post infection and D) 48 hour post infection. Each dataset was visually represented across each time using MDS. Each dataset was examined across the first and second dimension. Only one miRNA was identified as being differentially expressed in this dataset corroborating MDS plots.



Appendix 15.5: Multidimensional scaling (MDS) plots for each CD14+ sequencing dataset A) milk CD14 + mRNA, B) milk CD14 + miRNA, C) blood CD14 + miRNA and D) blood CD14 + mRNA. Each dataset was visually represented across each time using MDS. Each dataset was examined across the first and second dimension.

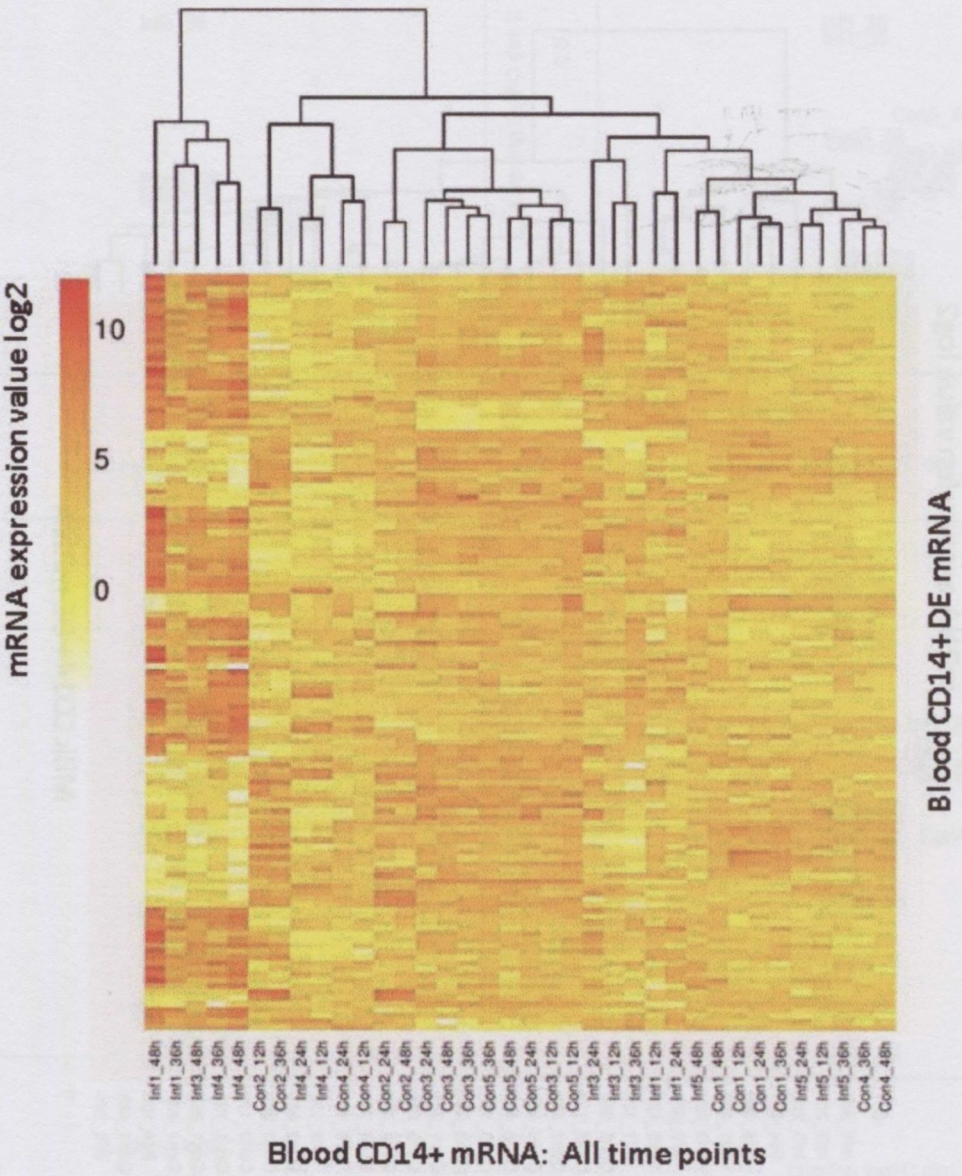


Appendix 15.6: Heatmap of log2 normalised mRNA expression levels CD14+ monocytes isolated from the milk of S. uberis infected and control animals at 12, 24, 36, and 48 hours post infection (hpi). Transcripts that were differentially expressed for at least one time-point are shown. At 36 hpi and 48 hpi, infected samples clearly cluster separately from control samples. Heatmaps were generated using the hclust package in R (v2.15.2). Infected animal 2 samples, control animal 2, control animal 4 at 24 hpi infected, infected animal 3, and infected animal 4 at 24 hpi were removed from differential expression analysis based on CFU and quality control data.

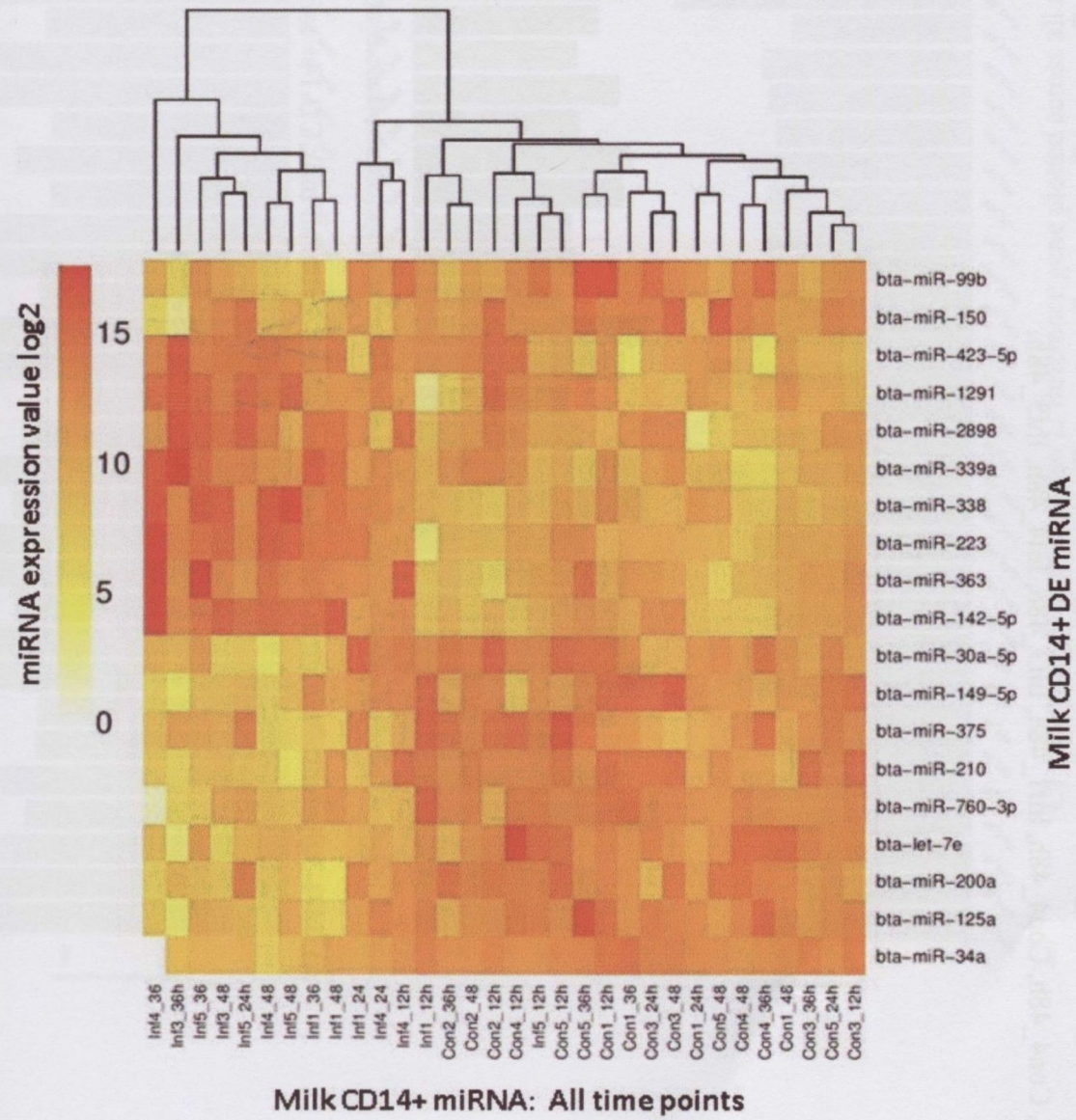


Milk CD14+ mRNA: All time points

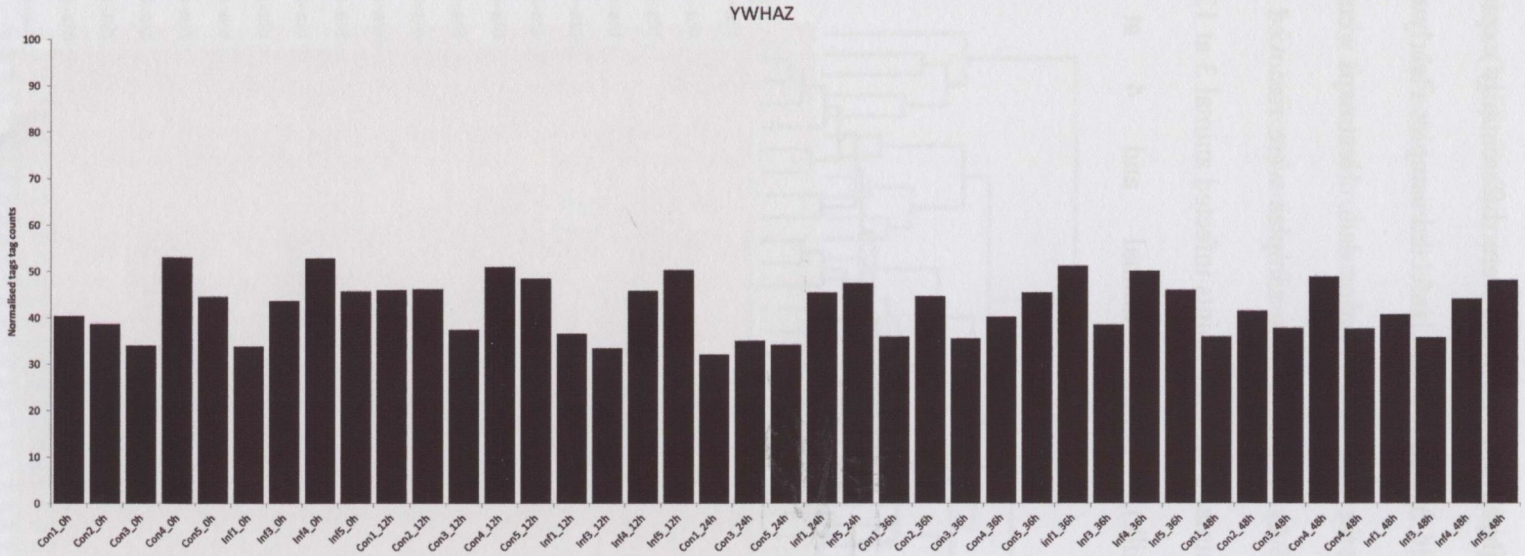
Appendix 15.7: Heatmap of log2 normalised mRNA expression levels CD14+ monocytes isolated from the blood of S. uberis infected and control animals at 12, 24, 36, and 48 hours post infection (hpi). Transcripts that were differentially expressed for at least one time-point are shown. Infected animal 1 and 4 at 36 and 48 hpi and infected animal 3 at 48 hpi cluster separately from control samples. Heatmaps were generated using the hclust package in R (v2.15.2). Infected animal 2 samples were removed from differential expression analysis.



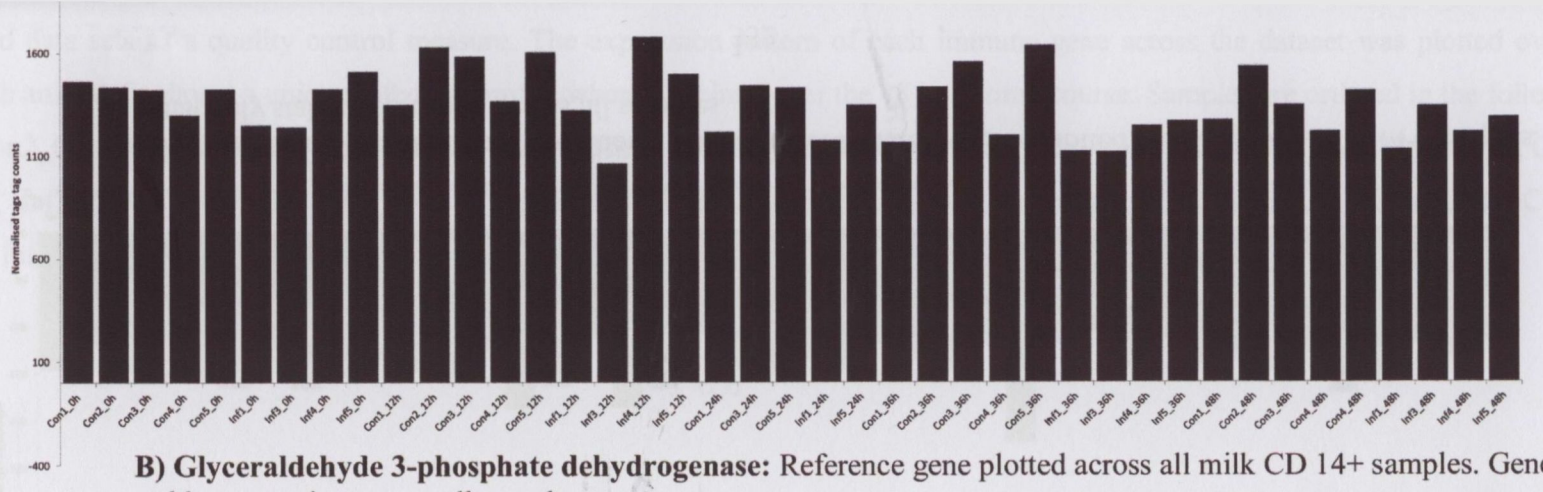
Appendix 15.8: Heatmap of log2 normalised miRNA expression levels CD14+ monocytes isolated from the milk of S. uberis infected and control animals at 12, 24, 36, and 48 hours post infection (hpi). miRNAs that were differentially expressed for at least one time-point are shown. At 36 hpi and 48 hpi, infected samples clearly cluster separately from control samples mirroring the mRNA expression data. Heatmaps were generated using the hclust package in R (v2.15.2). The following samples were removed from differential expression analysis, infected animal 2 all-time points infected animal 3 at 12h (milk), control animal 2 at 24h (milk), control animal and 4 at 24h (milk).



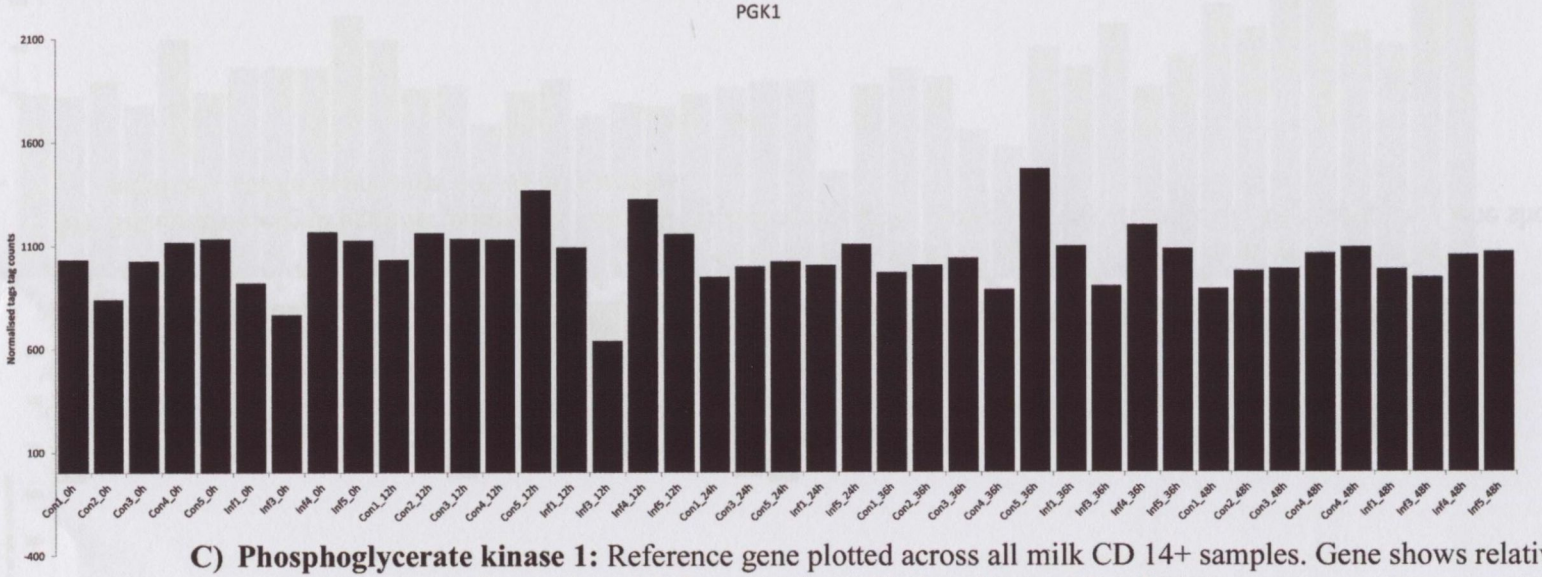
Appendix 16: Representative reference genes A) YWHAZ, B) GAPDH, C) PGK1, D) SDHA, and E) HPRT). Genes were plotted over the full time course on normalised data sets as a quality control measure. Samples are ordered in the following order from left to right Con1_0h, Con2_0h, Con3_0h, Con4_0h, Con5_0h, Inf1_0h, Inf3_0h, Inf4_0h, Inf5_0h, Con1_12h, Con2_12h, Con3_12h, Con4_12h, Con5_12h, Inf1_12h, Inf3_12h, Inf4_12h, Inf5_12h, Con1_24h, Con3_24h, Con5_24h, Inf1_24h, Inf5_24h, Con1_36h, Con2_36h, Con3_36h, Con4_36h, Con5_36h, Inf1_36h, Inf3_36h, Inf4_36h, Inf5_36h, Con1_48h, Con2_48h, Con3_48h, Con4_48h, Con4_48h, Inf1_48h, Inf3_48h, Inf4_48h, Inf5_48h.



A) 14-3-3 protein zeta/delta: Reference gene plotted across all milk CD 14+ samples. Gene shows relatively stable expression across all samples.



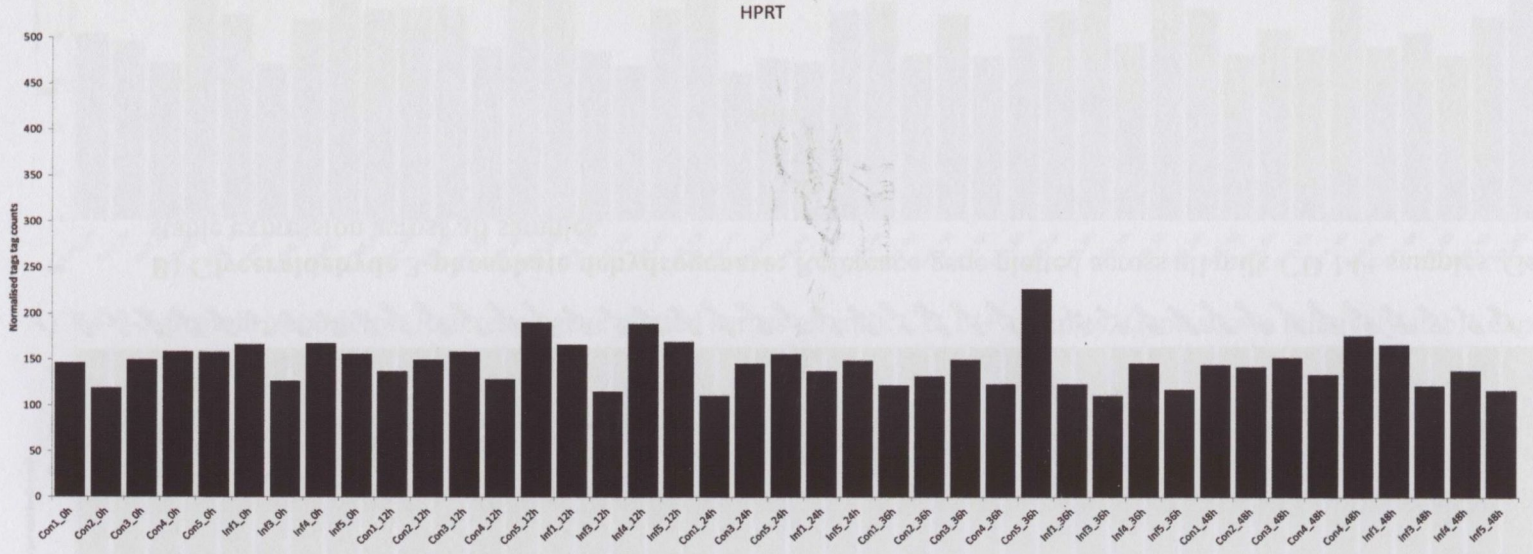
B) Glyceraldehyde 3-phosphate dehydrogenase: Reference gene plotted across all milk CD 14+ samples. Gene shows relatively stable expression across all samples.



C) Phosphoglycerate kinase 1: Reference gene plotted across all milk CD 14+ samples. Gene shows relatively stable expression across all samples with the exception of Infected animal 3 at 12 hpi.

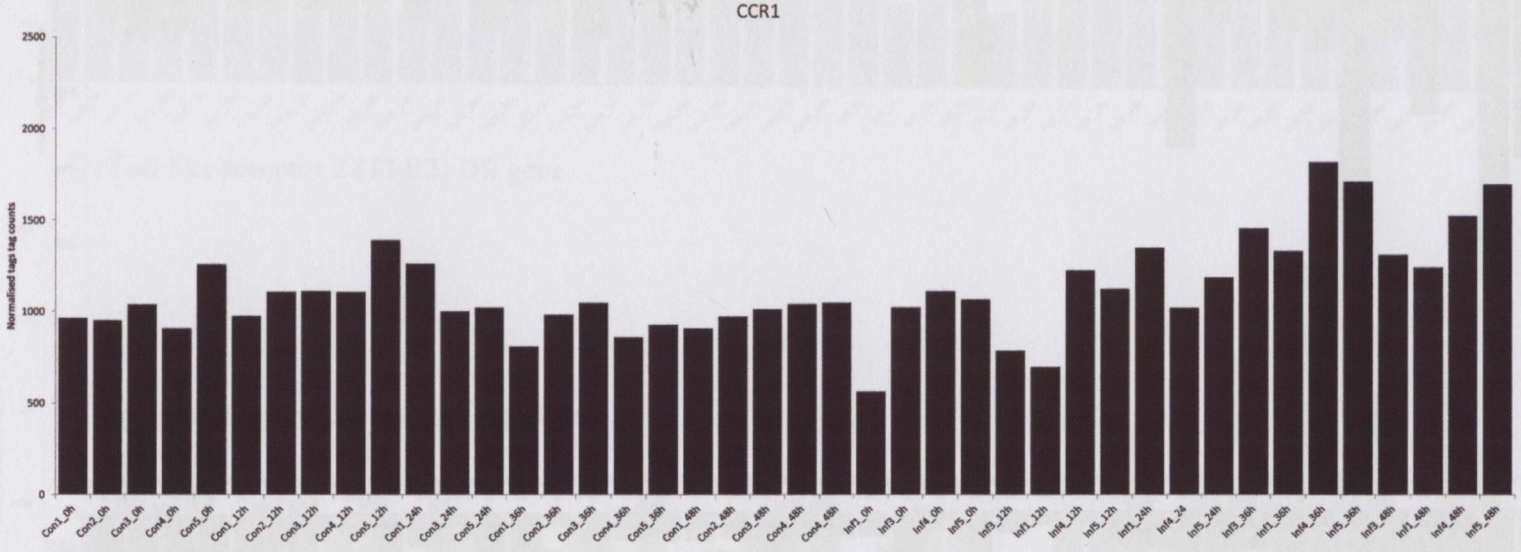


D) Succinate dehydrogenase complex, subunit A: Reference gene plotted across all milk CD 14+ samples. Gene shows relatively stable expression across all samples.

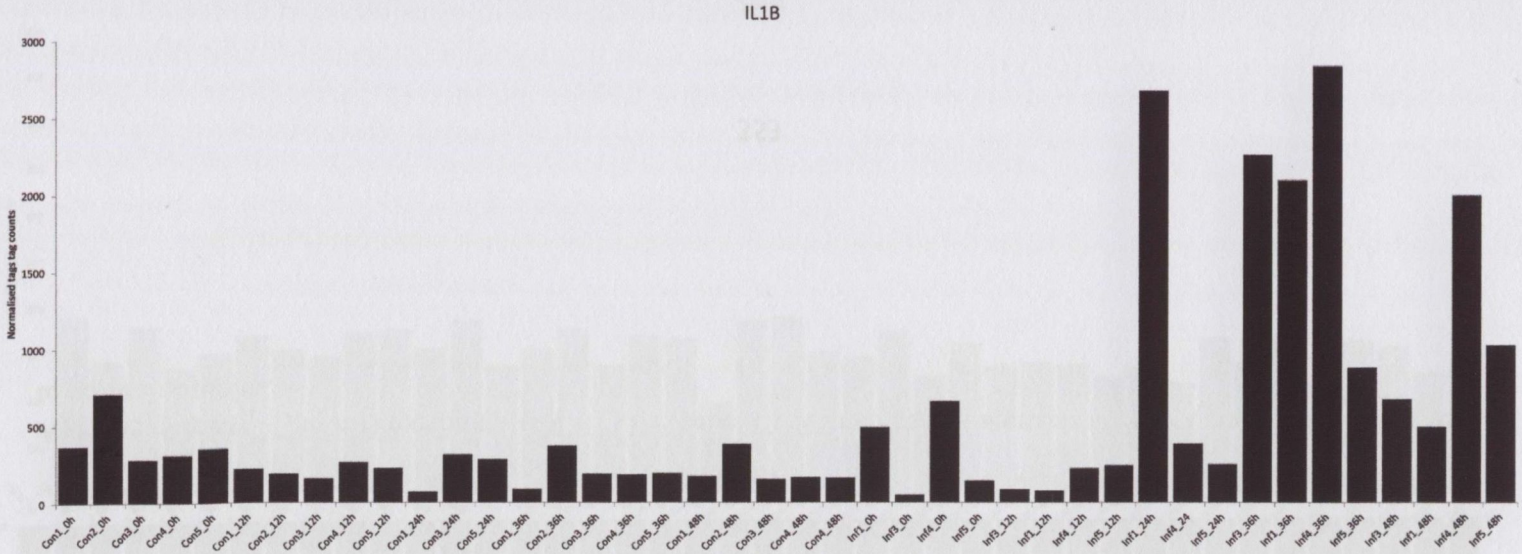


E) Hypoxanthine-guanine phosphoribosyl transferase: Reference gene plotted across all milk CD 14+ samples. Gene shows relatively stable expression across all samples.

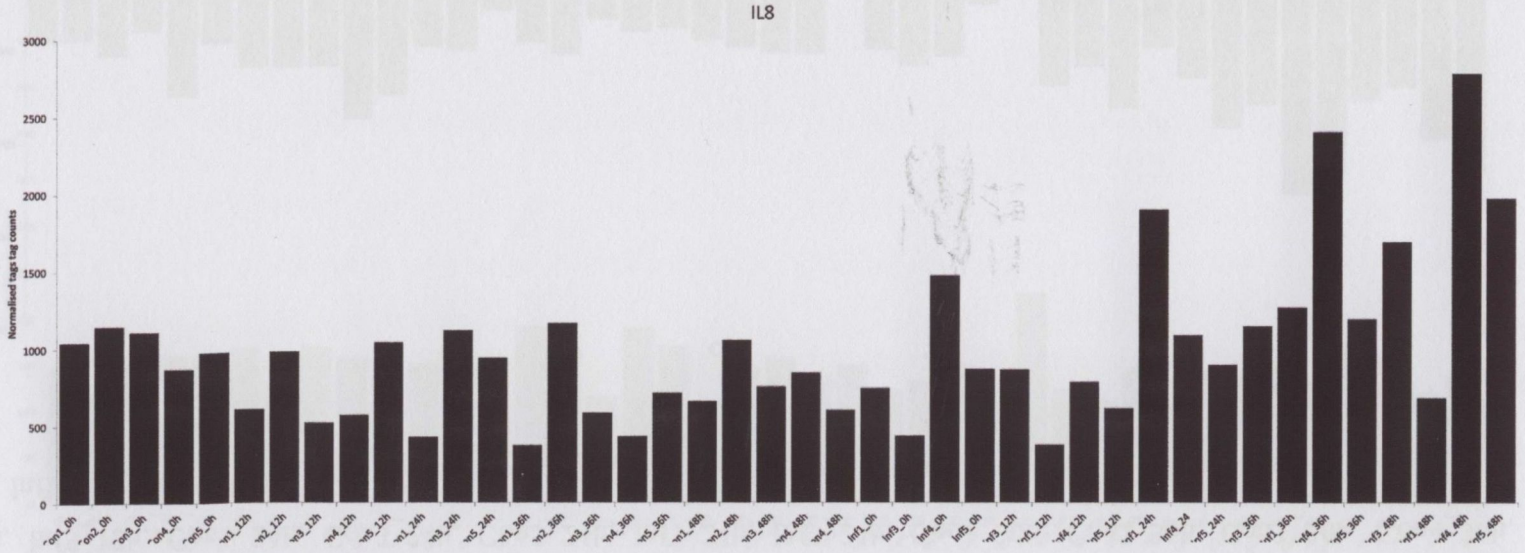
course on normalised data sets as a quality control measure. The expression pattern of each immune gene across the dataset was plotted over the course of the infection model. Each animal displayed a unique infection profile when examined over the 48 hour time course. Samples are ordered in the following order from left to right Con1_0h, Con2_0h, Con3_0h, Con4_0h, Con5_0h, Inf1_0h, Inf3_0h, Inf4_0h, Inf5_0h, Con1_12h, Con2_12h, Con3_12h, Con4_12h, Con5_12h, Inf1_12h, Inf3_12h, Inf4_12h, Inf5_12h, Con1_24h, Con3_24h, Con5_24h, Inf1_24h, Inf5_24h, Con1_36h, Con2_36h, Con3_36h, Con4_36h, Con5_36h, Inf1_36h, Inf3_36h, Inf4_36h, Inf5_36h, Con1_48h, Con2_48h, Con3_48h, Con4_48h, Con4_48h, Inf1_48h, Inf3_48h, Inf4_48h, Inf5_48h.



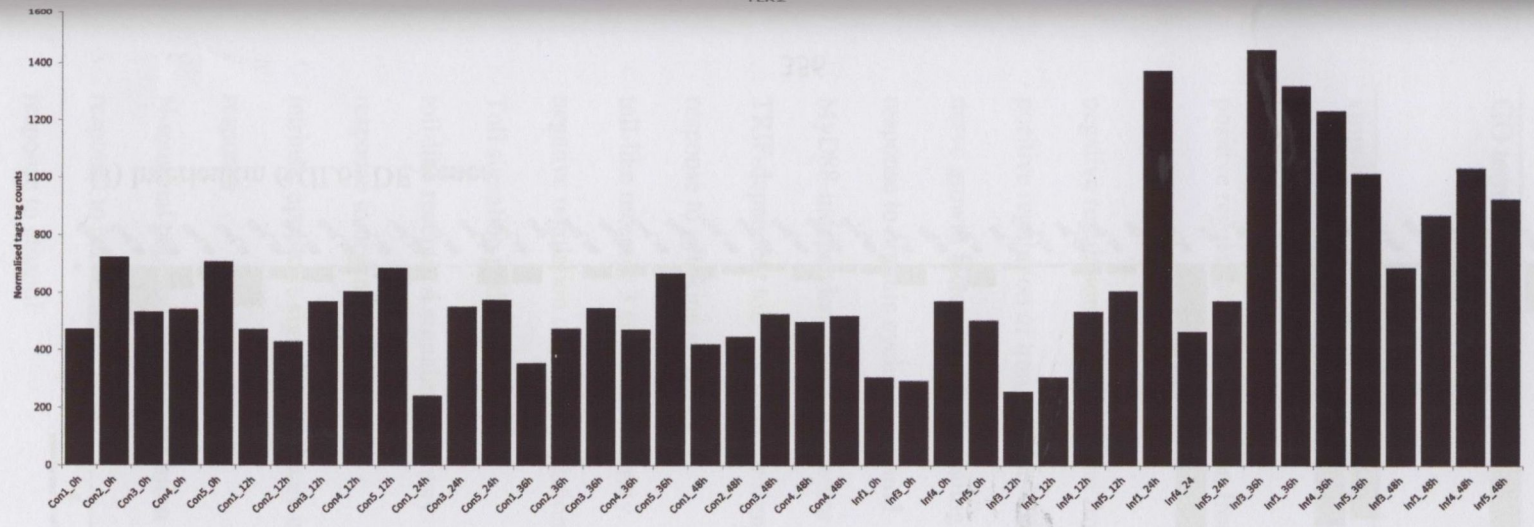
A) Chemokine (C-C motif) receptor 1 (CCR1) DE gene: CCR1 expression increase at 36 hpi and 48 hpi compared to control samples.



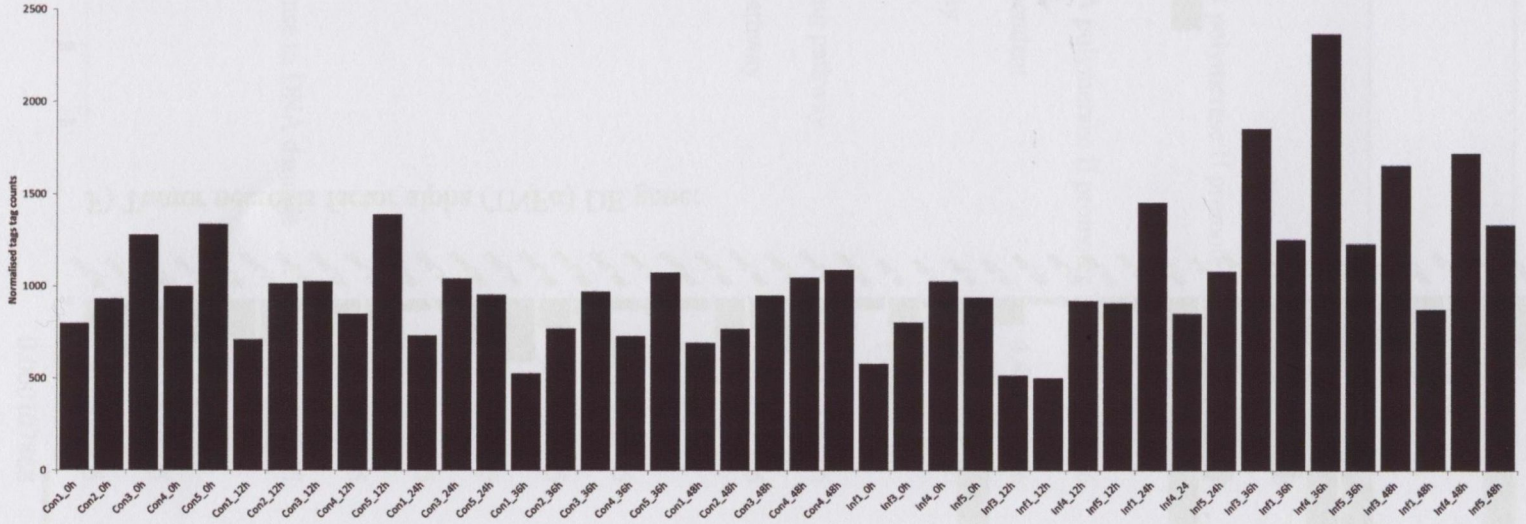
B) Interleukin 1, beta (IL1B) DE gene:



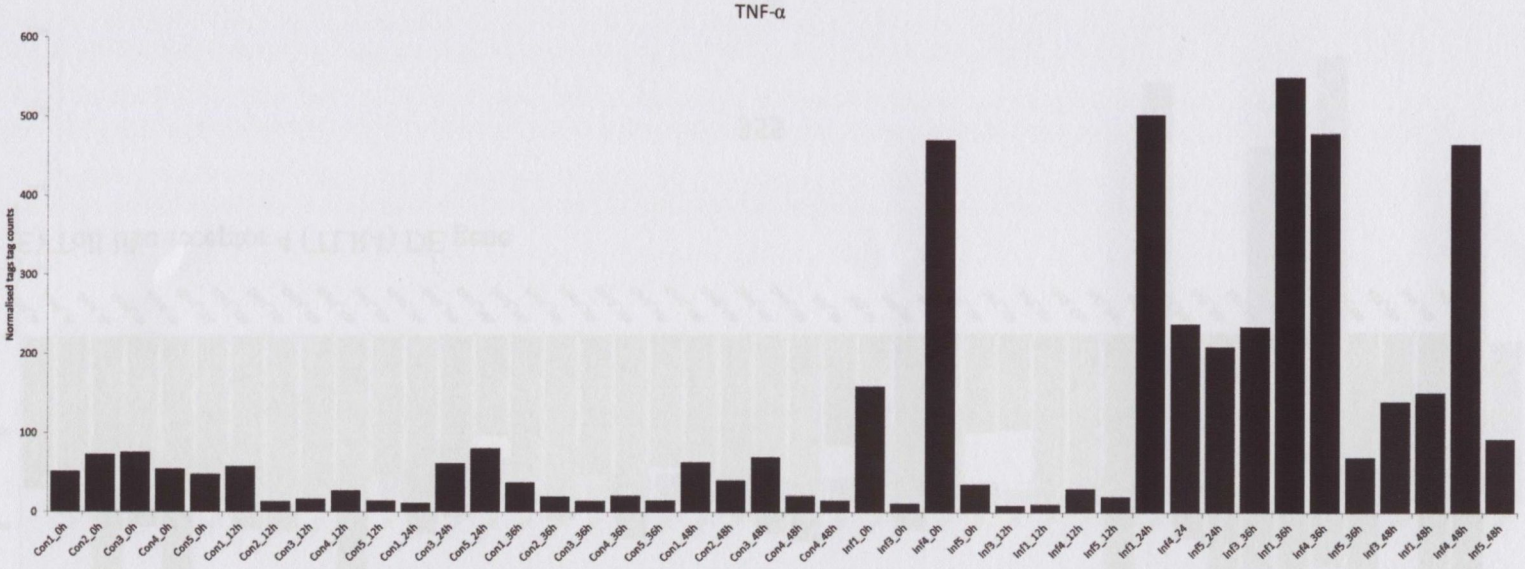
C) Interleukin 8 (IL8) DE gene:



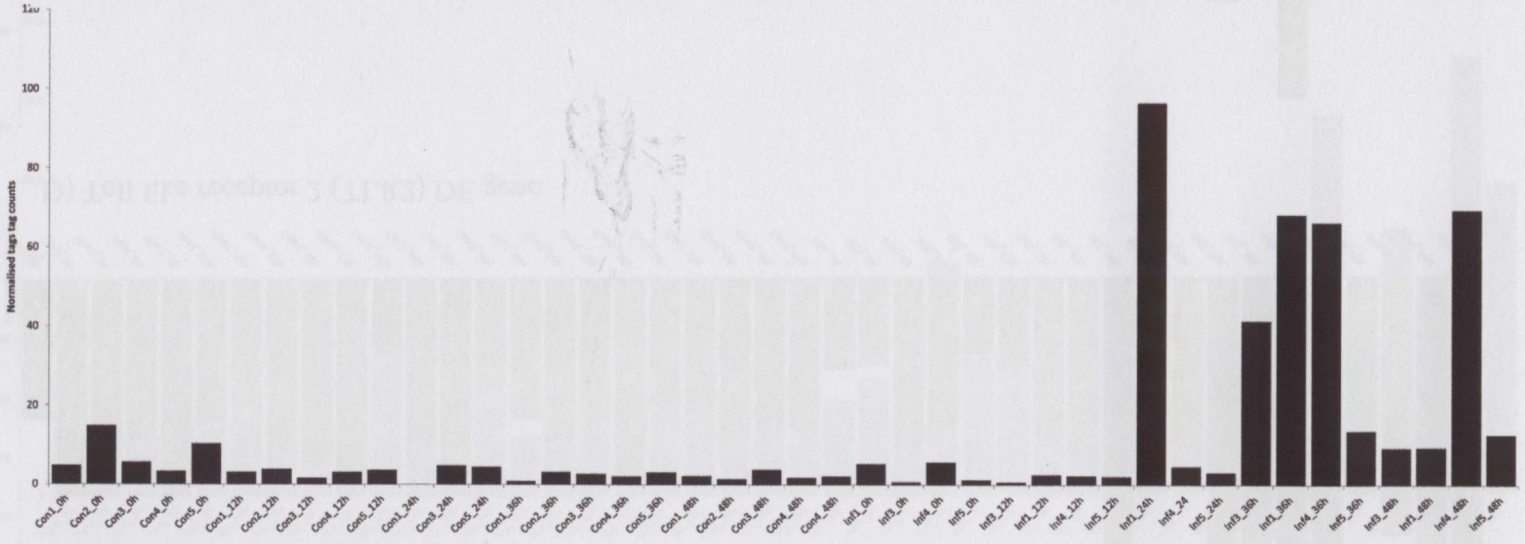
D) Toll like receptor 2 (TLR2) DE gene



E) Toll like receptor 4 (TLR4) DE gene



F) Tumor necrosis factor alpha (TNFα) DE gene:



G) Interleukin 6 (IL6) DE gene:

Appendix 17: The top 20 network hubs identified using CytoHubba and enriched Gene Ontology terms among hubs.

GO term Name	GO term p-value (corrected)
virus-host interaction	1.66457E-10
innate immune response	1.70887E-08
positive regulation of transcription from RNA polymerase II promoter	1.48971E-07
response to drug	2.8519E-07
negative regulation of transcription from RNA polymerase II promoter	2.98004E-06
positive regulation of transcription, DNA-dependent	4.45696E-06
nerve growth factor receptor signaling pathway	5.28246E-06
response to organic cyclic compound	1.4926E-05
MyD88-independent toll-like receptor signaling pathway	1.88497E-05
TRIF-dependent toll-like receptor signaling pathway	2.30368E-05
response to cytokine stimulus	2.3348E-05
toll-like receptor 3 signaling pathway	2.36909E-05
negative regulation of phosphorylation	3.96217E-05
Toll signaling pathway	3.80524E-05
toll-like receptor 4 signaling pathway	3.80524E-05
response to organic substance	5.13237E-05
intrinsic apoptotic signaling pathway in response to DNA damage response	5.15769E-05
N-terminal peptidyl-lysine acetylation	7.50364E-05
response to bacterium	7.51967E-05
response to ethanol	0.000107868

Appendix 18: The top contextual hubs identified using the CHAT software

ENS	name	prb	BH	bon	alls	sigs	allInteractors	actualSig Interactors
ENSG00000168685	IL7R	2.77E-39	2.22E-36	2.22E-36	18760	1780	114	67
ENSG00000115415	STAT1	6.05E-32	2.42E-29	4.84E-29	18760	1780	173	75
ENSG00000096717	SIRT1	9.74E-32	2.60E-29	7.80E-29	18760	1780	157	71
ENSG00000173039	RELA	4.83E-31	9.67E-29	3.87E-28	18760	1780	303	101
ENSG00000177885	GRB2	2.26E-27	3.63E-25	1.81E-24	18760	1780	515	132
ENSG00000196230	TUBB	1.77E-26	2.03E-24	1.42E-23	18760	1780	81	46
ENSG00000141480	ARRB2	1.77E-26	2.03E-24	1.42E-23	18760	1780	81	46
ENSG00000148400	NOTCH1	1.20E-24	1.20E-22	9.59E-22	18760	1780	45	33
ENSG00000162924	REL	2.45E-24	2.18E-22	1.96E-21	18760	1780	92	47
ENSG00000168610	STAT3	1.38E-23	1.11E-21	1.11E-20	18760	1780	198	70
ENSG00000007171	NOS2	2.49E-23	1.82E-21	2.00E-20	18760	1780	32	27
ENSG00000012048	BRCA1	6.43E-23	4.29E-21	5.15E-20	18760	1780	218	73
ENSG00000162434	JAK1	3.77E-22	2.32E-20	3.02E-19	18760	1780	60	36
ENSG00000005339	CREBBP	4.81E-22	2.67E-20	3.85E-19	18760	1780	268	81
ENSG00000125347	IRF1	5.00E-22	2.67E-20	4.00E-19	18760	1780	136	55
ENSG00000100393	EP300	6.50E-22	3.25E-20	5.20E-19	18760	1780	423	107
ENSG00000100985	MMP9	5.08E-21	2.39E-19	4.07E-18	18760	1780	26	23
ENSG00000109320	NFKB1	5.85E-20	2.55E-18	4.69E-17	18760	1780	220	69
ENSG00000168394	TAP1	6.04E-20	2.55E-18	4.84E-17	18760	1780	21	20
ENSG00000213928	IRF9	9.30E-20	3.73E-18	7.45E-17	18760	1780	41	28
ENSG00000170312	CDK1	2.95E-19	1.13E-17	2.37E-16	18760	1780	115	47
ENSG00000197122	SRC	3.47E-19	1.26E-17	2.78E-16	18760	1780	313	84
ENSG00000120738	EGR1	9.00E-19	3.14E-17	7.21E-16	18760	1780	564	122
ENSG00000188906	LRRK2	1.38E-18	4.42E-17	1.11E-15	18760	1780	27	22
ENSG00000125826	RBCK1	1.38E-18	4.42E-17	1.11E-15	18760	1780	27	22
ENSG00000232810	TNF	1.99E-17	6.14E-16	1.60E-14	18760	1780	85	38
ENSG00000125538	IL1B	2.19E-17	6.50E-16	1.76E-14	18760	1780	29	22
ENSG00000124762	CDKN1A	2.84E-17	7.93E-16	2.28E-14	18760	1780	227	66
ENSG00000166888	STAT6	2.87E-17	7.93E-16	2.30E-14	18760	1780	58	31
ENSG00000177606	JUN	7.03E-17	1.88E-15	5.63E-14	18760	1780	254	70
ENSG00000100906	NFKBIA	1.23E-16	3.18E-15	9.85E-14	18760	1780	121	45
ENSG00000131323	TRAF3	1.71E-16	4.28E-15	1.37E-13	18760	1780	94	39
ENSG00000105647	PIK3R2	2.44E-16	5.92E-15	1.95E-13	18760	1780	37	24
ENSG00000068024	HDAC4	3.43E-16	8.08E-15	2.75E-13	18760	1780	119	44
ENSG00000094631	HDAC6	2.43E-15	5.57E-14	1.95E-12	18760	1780	78	34
ENSG00000161570	CCL5	4.04E-15	8.98E-14	3.23E-12	18760	1780	37	23
ENSG00000127191	TRAF2	5.17E-15	1.11E-13	4.14E-12	18760	1780	232	63
ENSG00000044574	HSPA5	5.32E-15	1.11E-13	4.26E-12	18760	1780	117	42
ENSG00000110330	BIRC2	5.39E-15	1.11E-13	4.32E-12	18760	1780	67	31
ENSG00000104365	IKBKB	6.99E-14	1.40E-12	5.60E-11	18760	1780	146	46
ENSG00000105397	TYK2	7.68E-14	1.50E-12	6.15E-11	18760	1780	34	21
ENSG00000023445	BIRC3	8.63E-14	1.65E-12	6.91E-11	18760	1780	56	27

ENSG00000096968	JAK2	1.50E-13	2.79E-12	1.20E-10	18760	1780	83	33
ENSG00000075624	ACTB	1.66E-13	3.03E-12	1.33E-10	18760	1780	133	43
ENSG00000085733	CTTN	1.74E-13	3.10E-12	1.39E-10	18760	1780	35	21
ENSG00000140105	WARS	5.15E-13	8.96E-12	4.12E-10	18760	1780	12	12
ENSG00000140464	PML	1.06E-12	1.80E-11	8.47E-10	18760	1780	113	38
ENSG00000159840	ZYX	1.12E-12	1.84E-11	9.01E-10	18760	1780	19	15
ENSG00000104856	RELB	1.13E-12	1.84E-11	9.02E-10	18760	1780	103	36
ENSG00000181222	POLR2A	2.28E-12	3.65E-11	1.82E-09	18760	1780	137	42
ENSG00000137275	RIPK1	2.65E-12	4.16E-11	2.12E-09	18760	1780	81	31
ENSG00000010810	FYN	3.83E-12	5.89E-11	3.06E-09	18760	1780	266	63
ENSG00000118785	SPP1	4.18E-12	6.32E-11	3.35E-09	18760	1780	15	13
ENSG00000105355	PLIN3	5.46E-12	8.09E-11	4.37E-09	18760	1780	11	11
ENSG00000198742	SMURF1	9.20E-12	1.34E-10	7.37E-09	18760	1780	115	37
ENSG00000146232	NFKBIE	1.10E-11	1.55E-10	8.83E-09	18760	1780	53	24
ENSG00000104833	TUBB4A	1.10E-11	1.55E-10	8.83E-09	18760	1780	53	24
ENSG00000011422	PLAUR	1.29E-11	1.78E-10	1.04E-08	18760	1780	21	15
ENSG00000111679	PTPN6	1.54E-11	2.10E-10	1.24E-08	18760	1780	101	34
ENSG00000170581	STAT2	2.83E-11	3.71E-10	2.26E-08	18760	1780	35	19
ENSG00000105639	JAK3	2.83E-11	3.71E-10	2.26E-08	18760	1780	35	19
ENSG00000113916	BCL6	3.24E-11	4.18E-10	2.59E-08	18760	1780	125	38
ENSG00000145386	CCNA2	5.32E-11	6.76E-10	4.26E-08	18760	1780	52	23
ENSG00000117118	SDHB	5.78E-11	7.12E-10	4.63E-08	18760	1780	10	10
ENSG00000167552	TUBA1A	5.86E-11	7.12E-10	4.70E-08	18760	1780	75	28
ENSG00000141968	VAV1	5.86E-11	7.12E-10	4.70E-08	18760	1780	75	28
ENSG00000124207	CSE1L	6.06E-11	7.24E-10	4.85E-08	18760	1780	29	17
ENSG00000101336	HCK	7.18E-11	8.45E-10	5.75E-08	18760	1780	57	24
ENSG00000124788	ATXN1	7.35E-11	8.53E-10	5.89E-08	18760	1780	175	46
ENSG00000136238	RAC1	7.64E-11	8.75E-10	6.12E-08	18760	1780	134	39
ENSG00000119048	UBE2B	8.02E-11	9.05E-10	6.43E-08	18760	1780	26	16
ENSG00000067182	TNFRSF1A	9.09E-11	1.01E-09	7.28E-08	18760	1780	158	43
ENSG00000167815	PRDX2	9.58E-11	1.05E-09	7.67E-08	18760	1780	23	15
ENSG00000196498	NCOR2	1.15E-10	1.25E-09	9.24E-08	18760	1780	92	31
ENSG00000066336	SPI1	1.20E-10	1.28E-09	9.62E-08	18760	1780	87	30
ENSG00000106462	EZH2	1.22E-10	1.28E-09	9.75E-08	18760	1780	82	29
ENSG00000100650	SRSF5	2.54E-10	2.64E-09	2.03E-07	18760	1780	18	13
ENSG00000143799	PARP1	2.63E-10	2.70E-09	2.11E-07	18760	1780	151	41
ENSG00000087586	AURKA	2.88E-10	2.92E-09	2.31E-07	18760	1780	47	21
ENSG00000143466	IKBKE	3.09E-10	3.10E-09	2.48E-07	18760	1780	357	72
ENSG00000118503	TNFAIP3	3.22E-10	3.18E-09	2.58E-07	18760	1780	56	23
ENSG00000135679	MDM2	3.89E-10	3.80E-09	3.11E-07	18760	1780	275	60
ENSG00000077150	NFKB2	3.97E-10	3.83E-09	3.18E-07	18760	1780	165	43
ENSG00000154229	PRKCA	4.59E-10	4.38E-09	3.68E-07	18760	1780	81	28
ENSG00000015285	WAS	5.65E-10	5.30E-09	4.53E-07	18760	1780	62	24
ENSG00000173846	PLK3	5.76E-10	5.30E-09	4.61E-07	18760	1780	11	10

ENSG00000173894	CBX2	5.76E-10	5.30E-09	4.61E-07	18760	1780	11	10
ENSG00000077809	GTF2I	6.09E-10	5.54E-09	4.88E-07	18760	1780	53	22
ENSG00000197943	PLCG2	7.62E-10	6.86E-09	6.10E-07	18760	1780	29	16
ENSG00000254087	LYN	9.93E-10	8.84E-09	7.96E-07	18760	1780	94	30
ENSG00000048052	HDAC9	1.37E-09	1.20E-08	1.10E-06	18760	1780	46	20
ENSG00000095585	BLNK	1.48E-09	1.26E-08	1.18E-06	18760	1780	14	11
ENSG00000153071	DAB2	1.48E-09	1.26E-08	1.18E-06	18760	1780	30	16
ENSG00000123609	NMI	1.48E-09	1.26E-08	1.18E-06	18760	1780	30	16
ENSG00000056558	TRAF1	1.59E-09	1.34E-08	1.27E-06	18760	1780	85	28
ENSG00000175899	A2M	1.69E-09	1.41E-08	1.35E-06	18760	1780	80	27
ENSG00000079246	XRCC5	1.72E-09	1.42E-08	1.38E-06	18760	1780	107	32
ENSG00000169429	IL8	2.10E-09	1.71E-08	1.68E-06	18760	1780	56	22
ENSG00000170345	FOS	2.22E-09	1.79E-08	1.77E-06	18760	1780	206	48
ENSG00000178999	AURKB	2.53E-09	2.03E-08	2.03E-06	18760	1780	43	19
ENSG00000186350	RXRA	2.65E-09	2.10E-08	2.12E-06	18760	1780	126	35
ENSG00000185338	SOCS1	2.69E-09	2.11E-08	2.15E-06	18760	1780	52	21
ENSG00000124181	PLCG1	2.72E-09	2.11E-08	2.18E-06	18760	1780	144	38
ENSG00000139842	CUL4A	2.85E-09	2.19E-08	2.28E-06	18760	1780	87	28
ENSG00000117399	CDC20	3.44E-09	2.62E-08	2.76E-06	18760	1780	72	25
ENSG00000178952	TUFM	4.47E-09	3.38E-08	3.58E-06	18760	1780	21	13
ENSG00000168310	IRF2	4.52E-09	3.38E-08	3.62E-06	18760	1780	58	22
ENSG00000078018	MAP2	5.01E-09	3.72E-08	4.01E-06	18760	1780	15	11
ENSG00000173757	STAT5B	5.13E-09	3.77E-08	4.11E-06	18760	1780	49	20
ENSG00000137393	RNF144B	5.54E-09	4.04E-08	4.44E-06	18760	1780	10	9
ENSG00000185507	IRF7	6.77E-09	4.88E-08	5.42E-06	18760	1780	64	23
ENSG00000113368	LMNB1	8.37E-09	5.99E-08	6.70E-06	18760	1780	29	15
ENSG00000137193	PIM1	8.82E-09	6.25E-08	7.06E-06	18760	1780	33	16
ENSG00000197971	MBP	9.91E-09	6.96E-08	7.93E-06	18760	1780	22	13
ENSG00000069399	BCL3	1.19E-08	8.22E-08	9.51E-06	18760	1780	42	18
ENSG00000104312	RIPK2	1.19E-08	8.22E-08	9.53E-06	18760	1780	76	25
ENSG00000154127	UBASH3B	1.23E-08	8.40E-08	9.83E-06	18760	1780	13	10
ENSG00000168884	TNIP2	1.30E-08	8.80E-08	1.04E-05	18760	1780	19	12
ENSG00000149554	CHEK1	1.48E-08	9.98E-08	1.19E-05	18760	1780	47	19
ENSG00000114315	HES1	1.51E-08	1.01E-07	1.21E-05	18760	1780	34	16
ENSG00000070950	RAD18	2.06E-08	1.37E-07	1.65E-05	18760	1780	23	13
ENSG00000055208	TAB2	2.13E-08	1.40E-07	1.71E-05	18760	1780	78	25
ENSG00000141556	TBCD	2.76E-08	1.80E-07	2.21E-05	18760	1780	11	9
ENSG00000090339	ICAM1	2.94E-08	1.90E-07	2.35E-05	18760	1780	20	12
ENSG00000003402	CFLAR	3.29E-08	2.11E-07	2.64E-05	18760	1780	49	19
ENSG00000084207	GSTP1	3.73E-08	2.37E-07	2.98E-05	18760	1780	17	11
ENSG00000108654	DDX5	4.17E-08	2.63E-07	3.34E-05	18760	1780	75	24
ENSG00000113141	IK	5.28E-08	3.30E-07	4.23E-05	18760	1780	9	8
ENSG00000028137	TNFRSF1B	5.34E-08	3.32E-07	4.28E-05	18760	1780	116	31
ENSG00000198959	TGM2	6.37E-08	3.92E-07	5.10E-05	18760	1780	46	18

ENSG00000197442	MAP3K5	7.05E-08	4.31E-07	5.64E-05	18760	1780	88	26
ENSG00000114166	KAT2B	7.62E-08	4.62E-07	6.10E-05	18760	1780	130	33
ENSG00000123416	TUBA1B	7.69E-08	4.63E-07	6.16E-05	18760	1780	25	13
ENSG00000126561	STAT5A	8.15E-08	4.87E-07	6.53E-05	18760	1780	72	23
ENSG00000120885	CLU	8.22E-08	4.88E-07	6.58E-05	18760	1780	42	17
ENSG00000175063	UBE2C	8.68E-08	5.11E-07	6.95E-05	18760	1780	18	11
ENSG00000163932	PRKCD	9.91E-08	5.76E-07	7.94E-05	18760	1780	52	19
ENSG00000050820	BCAR1	9.96E-08	5.76E-07	7.98E-05	18760	1780	57	20
ENSG00000164056	SPRY1	1.00E-07	5.76E-07	8.01E-05	18760	1780	12	9
ENSG00000139112	GABARAPL1	1.30E-07	7.47E-07	1.05E-04	18760	1780	63	21
ENSG00000172936	MYD88	1.36E-07	7.70E-07	1.09E-04	18760	1780	85	25
ENSG00000020633	RUNX3	1.37E-07	7.70E-07	1.09E-04	18760	1780	30	14
ENSG00000111276	CDKN1B	1.42E-07	7.94E-07	1.14E-04	18760	1780	74	23
ENSG00000198900	TOP1	2.11E-07	1.17E-06	1.69E-04	18760	1780	70	22
ENSG00000117450	PRDX1	2.26E-07	1.24E-06	1.81E-04	18760	1780	31	14
ENSG00000168918	INPP5D	2.26E-07	1.24E-06	1.81E-04	18760	1780	31	14
ENSG00000149257	SERPINH1	2.34E-07	1.28E-06	1.87E-04	18760	1780	23	12
ENSG00000142657	PGD	2.39E-07	1.29E-06	1.91E-04	18760	1780	10	8
ENSG00000088325	TPX2	2.55E-07	1.36E-06	2.04E-04	18760	1780	16	10
ENSG00000133246	PRAM1	2.55E-07	1.36E-06	2.04E-04	18760	1780	16	10
ENSG00000171223	JUNB	2.56E-07	1.36E-06	2.05E-04	18760	1780	60	20
ENSG00000105173	CCNE1	2.65E-07	1.39E-06	2.12E-04	18760	1780	45	17
ENSG00000111424	VDR	2.92E-07	1.53E-06	2.34E-04	18760	1780	106	28
ENSG00000131023	LATS1	2.94E-07	1.53E-06	2.36E-04	18760	1780	13	9
ENSG00000115306	SPTBN1	3.53E-07	1.82E-06	2.83E-04	18760	1780	41	16
ENSG00000134460	IL2RA	3.76E-07	1.92E-06	3.01E-04	18760	1780	20	11
ENSG00000178209	PLEC	3.76E-07	1.92E-06	3.01E-04	18760	1780	20	11
ENSG00000167207	NOD2	4.11E-07	2.09E-06	3.30E-04	18760	1780	28	13
ENSG00000069956	MAPK6	4.29E-07	2.16E-06	3.44E-04	18760	1780	166	37
ENSG00000033800	PIAS1	4.62E-07	2.31E-06	3.70E-04	18760	1780	62	20
ENSG00000164136	IL15	4.96E-07	2.47E-06	3.97E-04	18760	1780	8	7
ENSG00000100644	HIF1A	5.12E-07	2.53E-06	4.10E-04	18760	1780	115	29
ENSG00000100201	DDX17	5.39E-07	2.65E-06	4.32E-04	18760	1780	47	17
ENSG00000132170	PPARG	5.56E-07	2.72E-06	4.46E-04	18760	1780	97	26
ENSG00000107968	MAP3K8	5.71E-07	2.77E-06	4.58E-04	18760	1780	33	14
ENSG00000120690	ELF1	6.85E-07	3.31E-06	5.49E-04	18760	1780	38	15
ENSG00000176890	TYMS	7.24E-07	3.47E-06	5.80E-04	18760	1780	6	6
ENSG00000184557	SOCS3	7.31E-07	3.47E-06	5.85E-04	18760	1780	53	18
ENSG00000101210	EEF1A2	7.38E-07	3.47E-06	5.91E-04	18760	1780	25	12
ENSG00000198517	MAFK	7.38E-07	3.47E-06	5.91E-04	18760	1780	25	12
ENSG00000107796	ACTA2	7.46E-07	3.47E-06	5.98E-04	18760	1780	14	9
ENSG00000104921	FCER2	7.46E-07	3.47E-06	5.98E-04	18760	1780	14	9
ENSG00000123358	NR4A1	8.20E-07	3.80E-06	6.57E-04	18760	1780	105	27
ENSG00000162772	ATF3	1.01E-06	4.61E-06	8.08E-04	18760	1780	39	15

ENSG00000150455	TIRAP	1.01E-06	4.61E-06	8.08E-04	18760	1780	39	15
ENSG00000111725	PRKAB1	1.02E-06	4.64E-06	8.17E-04	18760	1780	165	36
ENSG00000166501	PRKCB	1.08E-06	4.89E-06	8.65E-04	18760	1780	30	13
ENSG00000137285	TUBB2B	1.24E-06	5.56E-06	9.95E-04	18760	1780	26	12
ENSG00000135605	TEC	1.24E-06	5.56E-06	9.95E-04	18760	1780	26	12
ENSG00000113407	TARS	1.29E-06	5.76E-06	1.04E-03	18760	1780	22	11
ENSG00000184216	IRAK1	1.31E-06	5.79E-06	1.05E-03	18760	1780	89	24
ENSG00000100385	IL2RB	1.33E-06	5.81E-06	1.06E-03	18760	1780	35	14
ENSG00000084754	HADHA	1.33E-06	5.81E-06	1.06E-03	18760	1780	35	14
ENSG00000134057	CCNB1	1.37E-06	5.98E-06	1.10E-03	18760	1780	66	20
ENSG00000055332	EIF2AK2	1.50E-06	6.49E-06	1.20E-03	18760	1780	72	21
ENSG00000187837	HIST1H1C	1.68E-06	7.25E-06	1.35E-03	18760	1780	31	13
ENSG00000136754	ABI1	1.78E-06	7.64E-06	1.43E-03	18760	1780	56	18
ENSG00000083093	PALB2	2.02E-06	8.61E-06	1.62E-03	18760	1780	9	7
ENSG00000124813	RUNX2	2.07E-06	8.77E-06	1.66E-03	18760	1780	46	16
ENSG00000182199	SHMT2	2.19E-06	9.22E-06	1.75E-03	18760	1780	19	10
ENSG00000159110	IFNAR2	2.25E-06	9.32E-06	1.80E-03	18760	1780	23	11
ENSG00000140379	BCL2A1	2.25E-06	9.32E-06	1.80E-03	18760	1780	23	11
ENSG00000039650	PNKP	2.25E-06	9.32E-06	1.80E-03	18760	1780	23	11
ENSG00000165806	CASP7	2.87E-06	1.18E-05	2.30E-03	18760	1780	37	14
ENSG00000158517	NCF1	3.21E-06	1.32E-05	2.57E-03	18760	1780	28	12
ENSG00000167900	TK1	3.59E-06	1.47E-05	2.87E-03	18760	1780	167	35
ENSG00000115594	IL1R1	3.75E-06	1.53E-05	3.01E-03	18760	1780	24	11
ENSG00000132383	RPA1	3.80E-06	1.54E-05	3.04E-03	18760	1780	76	21
ENSG00000130203	APOE	3.84E-06	1.54E-05	3.07E-03	18760	1780	33	13
ENSG00000196586	MYO6	3.96E-06	1.59E-05	3.17E-03	18760	1780	20	10
ENSG00000160803	UBQLN4	4.10E-06	1.64E-05	3.29E-03	18760	1780	161	34
ENSG00000110619	CARS	4.59E-06	1.79E-05	3.68E-03	18760	1780	7	6
ENSG00000119912	IDE	4.59E-06	1.79E-05	3.68E-03	18760	1780	7	6
ENSG00000163110	PDLIM5	4.59E-06	1.79E-05	3.68E-03	18760	1780	7	6
ENSG00000163517	HDAC11	4.59E-06	1.79E-05	3.68E-03	18760	1780	7	6
ENSG00000101017	CD40	4.64E-06	1.80E-05	3.72E-03	18760	1780	54	17
ENSG00000099942	CRKL	4.65E-06	1.80E-05	3.72E-03	18760	1780	71	20
ENSG00000175793	SFN	4.68E-06	1.80E-05	3.75E-03	18760	1780	155	33
ENSG00000171105	INSR	4.73E-06	1.81E-05	3.79E-03	18760	1780	77	21
ENSG00000114423	CBLB	4.96E-06	1.86E-05	3.97E-03	18760	1780	29	12
ENSG00000213658	LAT	4.96E-06	1.86E-05	3.97E-03	18760	1780	29	12
ENSG00000136824	SMC2	4.96E-06	1.86E-05	3.97E-03	18760	1780	29	12
ENSG00000102554	KLF5	4.96E-06	1.86E-05	3.97E-03	18760	1780	29	12
ENSG00000160949	TONSL	5.07E-06	1.89E-05	4.06E-03	18760	1780	13	8
ENSG00000130707	ASS1	5.07E-06	1.89E-05	4.06E-03	18760	1780	13	8
ENSG00000127824	TUBA4A	5.63E-06	2.09E-05	4.51E-03	18760	1780	34	13
ENSG00000189060	H1F0	6.10E-06	2.25E-05	4.89E-03	18760	1780	10	7
ENSG00000095002	MSH2	6.92E-06	2.54E-05	5.54E-03	18760	1780	50	16

ENSG00000086730	LAT2	7.65E-06	2.80E-05	6.13E-03	18760	1780	5	5
ENSG00000136560	TANK	7.91E-06	2.88E-05	6.34E-03	18760	1780	56	17
ENSG00000137267	TUBB2A	8.11E-06	2.94E-05	6.49E-03	18760	1780	35	13
ENSG00000135655	USP15	9.13E-06	3.29E-05	7.31E-03	18760	1780	51	16
ENSG00000123685	BATF3	9.53E-06	3.41E-05	7.63E-03	18760	1780	26	11
ENSG00000125753	VASP	9.53E-06	3.41E-05	7.63E-03	18760	1780	26	11
ENSG00000198668	CALM1	1.04E-05	3.69E-05	8.29E-03	18760	1780	168	34
ENSG00000151725	MLF1IP	1.07E-05	3.77E-05	8.59E-03	18760	1780	14	8
ENSG00000206560	ANKRD28	1.07E-05	3.77E-05	8.59E-03	18760	1780	14	8
ENSG00000100084	HIRA	1.07E-05	3.77E-05	8.59E-03	18760	1780	14	8
ENSG00000166579	NDEL1	1.11E-05	3.89E-05	8.91E-03	18760	1780	41	14
ENSG00000102755	FLT1	1.22E-05	4.22E-05	9.76E-03	18760	1780	18	9
ENSG00000147168	IL2RG	1.22E-05	4.22E-05	9.76E-03	18760	1780	18	9
ENSG00000072571	HMMR	1.52E-05	5.13E-05	1.22E-02	18760	1780	11	7
ENSG00000158717	RNF166	1.52E-05	5.13E-05	1.22E-02	18760	1780	11	7
ENSG00000185722	ANKFY1	1.52E-05	5.13E-05	1.22E-02	18760	1780	11	7
ENSG00000165030	NFIL3	1.52E-05	5.13E-05	1.22E-02	18760	1780	11	7
ENSG00000169220	RGS14	1.52E-05	5.13E-05	1.22E-02	18760	1780	11	7
ENSG00000158864	NDUFS2	1.52E-05	5.13E-05	1.22E-02	18760	1780	11	7
ENSG00000129465	RIPK3	1.54E-05	5.17E-05	1.23E-02	18760	1780	102	24
ENSG00000106399	RPA3	1.60E-05	5.37E-05	1.28E-02	18760	1780	32	12
ENSG00000091483	FH	1.66E-05	5.48E-05	1.33E-02	18760	1780	8	6
ENSG00000123975	CKS2	1.66E-05	5.48E-05	1.33E-02	18760	1780	8	6
ENSG00000031698	SARS	1.66E-05	5.48E-05	1.33E-02	18760	1780	8	6
ENSG00000125735	TNFSF14	1.66E-05	5.48E-05	1.33E-02	18760	1780	8	6
ENSG00000107263	RAPGEF1	2.08E-05	6.80E-05	1.67E-02	18760	1780	15	8
ENSG00000109805	NCAPG	2.08E-05	6.80E-05	1.67E-02	18760	1780	15	8
ENSG00000175866	BAIAP2	2.10E-05	6.82E-05	1.68E-02	18760	1780	19	9
ENSG00000072501	SMC1A	2.10E-05	6.82E-05	1.69E-02	18760	1780	60	17
ENSG00000154310	TNIK	2.25E-05	7.26E-05	1.80E-02	18760	1780	111	25
ENSG00000144554	FANCD2	2.28E-05	7.33E-05	1.83E-02	18760	1780	33	12
ENSG00000112118	MCM3	2.64E-05	8.47E-05	2.12E-02	18760	1780	61	17
ENSG00000167513	CDT1	2.70E-05	8.61E-05	2.16E-02	18760	1780	44	14
ENSG00000113302	IL12B	2.83E-05	8.92E-05	2.26E-02	18760	1780	24	10
ENSG00000113758	DBN1	2.83E-05	8.92E-05	2.26E-02	18760	1780	24	10
ENSG00000104814	MAP4K1	2.83E-05	8.92E-05	2.26E-02	18760	1780	24	10
ENSG00000157557	ETS2	3.00E-05	9.39E-05	2.40E-02	18760	1780	39	13
ENSG00000082512	TRAF5	3.00E-05	9.39E-05	2.40E-02	18760	1780	39	13
ENSG00000141456	PELP1	3.17E-05	9.87E-05	2.54E-02	18760	1780	29	11
ENSG00000175197	DDIT3	3.23E-05	1.00E-04	2.59E-02	18760	1780	56	16
ENSG00000163655	GMPS	3.30E-05	1.01E-04	2.64E-02	18760	1780	12	7
ENSG00000090487	SPG21	3.30E-05	1.01E-04	2.64E-02	18760	1780	12	7
ENSG00000198791	CNOT7	3.30E-05	1.01E-04	2.64E-02	18760	1780	12	7
ENSG00000087266	SH3BP2	3.45E-05	1.05E-04	2.76E-02	18760	1780	20	9

ENSG00000107201	DDX58	3.55E-05	1.08E-04	2.84E-02	18760	1780	45	14
ENSG00000092621	PHGDH	3.77E-05	1.14E-04	3.02E-02	18760	1780	16	8
ENSG00000151693	ASAP2	3.77E-05	1.14E-04	3.02E-02	18760	1780	16	8
ENSG00000043462	LCP2	4.03E-05	1.21E-04	3.22E-02	18760	1780	40	13
ENSG00000064393	HIPK2	4.03E-05	1.21E-04	3.22E-02	18760	1780	40	13
ENSG00000120899	PTK2B	4.07E-05	1.22E-04	3.26E-02	18760	1780	57	16
ENSG00000167460	TPM4	4.16E-05	1.23E-04	3.33E-02	18760	1780	6	5
ENSG00000204264	PSMB8	4.16E-05	1.23E-04	3.33E-02	18760	1780	6	5
ENSG00000163918	RFC4	4.27E-05	1.26E-04	3.42E-02	18760	1780	25	10
ENSG00000184897	H1FX	4.51E-05	1.31E-04	3.62E-02	18760	1780	9	6
ENSG00000139579	NABP2	4.51E-05	1.31E-04	3.62E-02	18760	1780	9	6
ENSG00000171456	ASXL1	4.51E-05	1.31E-04	3.62E-02	18760	1780	9	6
ENSG00000112182	BACH2	4.51E-05	1.31E-04	3.62E-02	18760	1780	9	6
ENSG00000112033	PPARD	4.53E-05	1.31E-04	3.63E-02	18760	1780	30	11
ENSG00000101945	SUV39H1	4.62E-05	1.33E-04	3.70E-02	18760	1780	46	14
ENSG00000078043	PIAS2	4.62E-05	1.33E-04	3.70E-02	18760	1780	46	14
ENSG00000172845	SP3	4.75E-05	1.36E-04	3.80E-02	18760	1780	130	27
ENSG00000187741	FANCA	4.88E-05	1.39E-04	3.91E-02	18760	1780	70	18
ENSG00000184678	HIST2H2BE	4.96E-05	1.41E-04	3.97E-02	18760	1780	52	15
ENSG00000180182	MED14	4.96E-05	1.41E-04	3.97E-02	18760	1780	52	15
ENSG00000026508	CD44	5.34E-05	1.51E-04	4.28E-02	18760	1780	41	13
ENSG00000169047	IRS1	5.95E-05	1.68E-04	4.77E-02	18760	1780	47	14
ENSG00000113522	RAD50	5.97E-05	1.68E-04	4.78E-02	18760	1780	36	12
ENSG00000131759	RARA	6.25E-05	1.75E-04	5.00E-02	18760	1780	125	26
ENSG00000140525	FANCI	6.28E-05	1.75E-04	5.03E-02	18760	1780	26	10
ENSG00000122861	PLAU	6.36E-05	1.77E-04	5.09E-02	18760	1780	31	11
ENSG00000178921	PFAS	6.44E-05	1.79E-04	5.16E-02	18760	1780	17	8
ENSG00000159335	PTMS	6.48E-05	1.79E-04	5.19E-02	18760	1780	13	7
ENSG00000068028	RASSF1	7.00E-05	1.93E-04	5.61E-02	18760	1780	42	13
ENSG00000112658	SRF	7.14E-05	1.96E-04	5.72E-02	18760	1780	72	18
ENSG00000128272	ATF4	7.55E-05	2.06E-04	6.04E-02	18760	1780	66	17
ENSG00000118271	TTR	7.61E-05	2.07E-04	6.10E-02	18760	1780	48	14
ENSG00000115524	SF3B1	7.85E-05	2.13E-04	6.29E-02	18760	1780	54	15
ENSG00000182481	KPNA2	7.99E-05	2.14E-04	6.40E-02	18760	1780	99	22
ENSG00000134070	IRAK2	8.00E-05	2.14E-04	6.41E-02	18760	1780	37	12
ENSG00000171132	PRKCE	8.00E-05	2.14E-04	6.41E-02	18760	1780	37	12
ENSG00000083799	CYLD	8.00E-05	2.14E-04	6.41E-02	18760	1780	37	12
ENSG00000168564	CDKN2AIP	8.08E-05	2.16E-04	6.47E-02	18760	1780	4	4
ENSG00000196083	IL1RAP	8.38E-05	2.23E-04	6.71E-02	18760	1780	22	9
ENSG00000112208	BAG2	8.77E-05	2.33E-04	7.03E-02	18760	1780	32	11
ENSG00000090621	PABPC4	9.03E-05	2.38E-04	7.23E-02	18760	1780	27	10
ENSG00000197063	MAFG	9.03E-05	2.38E-04	7.23E-02	18760	1780	27	10
ENSG00000198911	SREBF2	9.16E-05	2.41E-04	7.34E-02	18760	1780	67	17
ENSG00000008441	NFIX	1.02E-04	2.67E-04	8.19E-02	18760	1780	10	6

ENSG00000086200	IPO11	1.02E-04	2.67E-04	8.19E-02	18760	1780	10	6
ENSG00000140319	SRP14	1.05E-04	2.71E-04	8.41E-02	18760	1780	18	8
ENSG00000135218	CD36	1.05E-04	2.71E-04	8.41E-02	18760	1780	18	8
ENSG00000077238	IL4R	1.05E-04	2.71E-04	8.41E-02	18760	1780	18	8
ENSG00000073111	MCM2	1.17E-04	2.99E-04	9.37E-02	18760	1780	62	16
ENSG00000171867	PRNP	1.17E-04	2.99E-04	9.37E-02	18760	1780	62	16
ENSG00000173207	CKS1B	1.17E-04	2.99E-04	9.39E-02	18760	1780	14	7
ENSG00000012779	ALOX5	1.17E-04	2.99E-04	9.39E-02	18760	1780	14	7
ENSG00000148737	TCF7L2	1.19E-04	3.03E-04	9.55E-02	18760	1780	33	11
ENSG00000215301	DDX3X	1.21E-04	3.07E-04	9.69E-02	18760	1780	56	15
ENSG00000076003	MCM6	1.21E-04	3.07E-04	9.72E-02	18760	1780	50	14
ENSG00000020129	NCDN	1.32E-04	3.28E-04	1.05E-01	18760	1780	7	5
ENSG00000138092	CENPO	1.32E-04	3.28E-04	1.05E-01	18760	1780	7	5
ENSG00000150961	SEC24D	1.32E-04	3.28E-04	1.05E-01	18760	1780	7	5
ENSG00000198056	PRIM1	1.32E-04	3.28E-04	1.05E-01	18760	1780	7	5
ENSG00000132530	XAF1	1.32E-04	3.28E-04	1.05E-01	18760	1780	7	5
ENSG00000080815	PSEN1	1.38E-04	3.41E-04	1.10E-01	18760	1780	89	20
ENSG00000131462	TUBG1	1.39E-04	3.43E-04	1.11E-01	18760	1780	39	12
ENSG00000165392	WRN	1.60E-04	3.93E-04	1.28E-01	18760	1780	34	11
ENSG00000121858	TNFSF10	1.64E-04	4.02E-04	1.32E-01	18760	1780	19	8
ENSG00000138378	STAT4	1.64E-04	4.02E-04	1.32E-01	18760	1780	19	8
ENSG00000123124	WWP1	1.79E-04	4.36E-04	1.44E-01	18760	1780	40	12
ENSG00000150907	FOXO1	1.79E-04	4.36E-04	1.44E-01	18760	1780	40	12
ENSG00000142166	IFNAR1	1.81E-04	4.37E-04	1.45E-01	18760	1780	24	9
ENSG00000073050	XRCC1	1.81E-04	4.37E-04	1.45E-01	18760	1780	24	9
ENSG00000104221	BRF2	1.82E-04	4.38E-04	1.45E-01	18760	1780	58	15
ENSG00000139618	BRCA2	1.88E-04	4.49E-04	1.50E-01	18760	1780	52	14
ENSG00000131408	NR1H2	1.88E-04	4.49E-04	1.50E-01	18760	1780	52	14
ENSG00000179583	CIITA	1.88E-04	4.49E-04	1.50E-01	18760	1780	46	13
ENSG00000125740	FOSB	1.99E-04	4.73E-04	1.59E-01	18760	1780	15	7
ENSG00000102024	PLS3	1.99E-04	4.73E-04	1.59E-01	18760	1780	15	7
ENSG00000138160	KIF11	2.04E-04	4.80E-04	1.63E-01	18760	1780	11	6
ENSG00000198901	PRC1	2.04E-04	4.80E-04	1.63E-01	18760	1780	11	6
ENSG00000152778	IFIT5	2.04E-04	4.80E-04	1.63E-01	18760	1780	11	6
ENSG00000100297	MCM5	2.11E-04	4.95E-04	1.69E-01	18760	1780	35	11
ENSG00000204209	DAXX	2.24E-04	5.26E-04	1.80E-01	18760	1780	72	17
ENSG00000134686	PHC2	2.39E-04	5.58E-04	1.91E-01	18760	1780	30	10
ENSG00000136826	KLF4	2.48E-04	5.69E-04	1.98E-01	18760	1780	20	8
ENSG00000156299	TIAM1	2.48E-04	5.69E-04	1.98E-01	18760	1780	20	8
ENSG00000000938	FGR	2.48E-04	5.69E-04	1.98E-01	18760	1780	20	8
ENSG00000204103	MAFB	2.48E-04	5.69E-04	1.98E-01	18760	1780	20	8
ENSG00000134107	BHLHE40	2.48E-04	5.69E-04	1.98E-01	18760	1780	20	8
ENSG00000156127	BATF	2.48E-04	5.69E-04	1.98E-01	18760	1780	20	8
ENSG00000196923	PDLIM7	2.55E-04	5.85E-04	2.05E-01	18760	1780	25	9

ENSG00000186716	BCR	2.83E-04	6.43E-04	2.26E-01	18760	1780	54	14
ENSG00000119917	IFIT3	2.83E-04	6.43E-04	2.26E-01	18760	1780	54	14
ENSG00000089159	PXN	2.86E-04	6.49E-04	2.29E-01	18760	1780	168	30
ENSG00000132155	RAF1	2.95E-04	6.68E-04	2.36E-01	18760	1780	123	24
ENSG00000117090	SLAMF1	3.18E-04	7.07E-04	2.55E-01	18760	1780	8	5
ENSG00000172037	LAMB2	3.18E-04	7.07E-04	2.55E-01	18760	1780	8	5
ENSG00000023228	NDUFS1	3.18E-04	7.07E-04	2.55E-01	18760	1780	8	5
ENSG00000078401	EDN1	3.18E-04	7.07E-04	2.55E-01	18760	1780	8	5
ENSG00000173110	HSPA6	3.19E-04	7.07E-04	2.56E-01	18760	1780	31	10
ENSG00000165029	ABCA1	3.19E-04	7.07E-04	2.56E-01	18760	1780	31	10
ENSG00000108771	DHX58	3.20E-04	7.07E-04	2.57E-01	18760	1780	16	7
ENSG00000117632	STMN1	3.20E-04	7.07E-04	2.57E-01	18760	1780	16	7
ENSG00000165672	PRDX3	3.20E-04	7.07E-04	2.57E-01	18760	1780	16	7
ENSG00000089280	FUS	3.43E-04	7.55E-04	2.75E-01	18760	1780	55	14
ENSG00000121060	TRIM25	3.54E-04	7.73E-04	2.83E-01	18760	1780	26	9
ENSG00000065675	PRKCQ	3.54E-04	7.73E-04	2.83E-01	18760	1780	26	9
ENSG00000089685	BIRC5	3.54E-04	7.73E-04	2.84E-01	18760	1780	37	11
ENSG00000136634	IL10	3.62E-04	7.83E-04	2.90E-01	18760	1780	21	8
ENSG00000075426	FOSL2	3.62E-04	7.83E-04	2.90E-01	18760	1780	21	8
ENSG00000049541	RFC2	3.62E-04	7.83E-04	2.90E-01	18760	1780	21	8
ENSG00000175216	CKAP5	3.62E-04	7.83E-04	2.90E-01	18760	1780	21	8
ENSG00000153044	CENPH	3.66E-04	7.85E-04	2.93E-01	18760	1780	5	4
ENSG00000126777	KTN1	3.69E-04	7.85E-04	2.95E-01	18760	1780	12	6
ENSG00000140740	UQCRC2	3.69E-04	7.85E-04	2.95E-01	18760	1780	12	6
ENSG00000197329	PELI1	3.69E-04	7.85E-04	2.95E-01	18760	1780	12	6
ENSG00000146263	MMS22L	3.69E-04	7.85E-04	2.95E-01	18760	1780	12	6
ENSG00000169057	MECP2	4.20E-04	8.93E-04	3.37E-01	18760	1780	32	10
ENSG00000125952	MAX	4.26E-04	9.02E-04	3.41E-01	18760	1780	76	17
ENSG00000094804	CDC6	4.51E-04	9.51E-04	3.61E-01	18760	1780	38	11
ENSG00000112531	QKI	4.51E-04	9.51E-04	3.61E-01	18760	1780	38	11
ENSG00000123240	OPTN	4.55E-04	9.57E-04	3.64E-01	18760	1780	44	12
ENSG00000131711	MAP1B	4.80E-04	1.01E-03	3.85E-01	18760	1780	27	9
ENSG00000112312	GMNN	4.93E-04	1.03E-03	3.95E-01	18760	1780	17	7
ENSG00000143553	SNAPIN	5.46E-04	1.13E-03	4.37E-01	18760	1780	33	10
ENSG00000106976	DNM1	5.46E-04	1.13E-03	4.37E-01	18760	1780	33	10
ENSG00000159459	UBR1	5.46E-04	1.13E-03	4.37E-01	18760	1780	33	10
ENSG00000101665	SMAD7	5.73E-04	1.19E-03	4.59E-01	18760	1780	78	17
ENSG00000175354	PTPN2	6.20E-04	1.27E-03	4.96E-01	18760	1780	13	6
ENSG00000135899	SP110	6.20E-04	1.27E-03	4.96E-01	18760	1780	13	6
ENSG00000105281	SLC1A5	6.20E-04	1.27E-03	4.96E-01	18760	1780	13	6
ENSG00000126351	THRA	6.32E-04	1.29E-03	5.07E-01	18760	1780	65	15
ENSG00000185950	IRS2	6.41E-04	1.29E-03	5.14E-01	18760	1780	28	9
ENSG00000186153	WWOX	6.41E-04	1.29E-03	5.14E-01	18760	1780	28	9
ENSG00000109103	UNC119	6.45E-04	1.29E-03	5.17E-01	18760	1780	93	19

ENSG00000121966	CXCR4	6.46E-04	1.29E-03	5.18E-01	18760	1780	52	13
ENSG00000162909	CAPN2	6.48E-04	1.29E-03	5.19E-01	18760	1780	9	5
ENSG00000178695	KCTD12	6.48E-04	1.29E-03	5.19E-01	18760	1780	9	5
ENSG00000019582	CD74	6.48E-04	1.29E-03	5.19E-01	18760	1780	9	5
ENSG00000158186	MRAS	6.48E-04	1.29E-03	5.19E-01	18760	1780	9	5
ENSG00000137992	DBT	6.48E-04	1.29E-03	5.19E-01	18760	1780	9	5
ENSG00000023330	ALAS1	6.48E-04	1.29E-03	5.19E-01	18760	1780	9	5
ENSG00000164885	CDK5	7.01E-04	1.40E-03	5.61E-01	18760	1780	34	10
ENSG00000155363	MOV10	7.16E-04	1.42E-03	5.73E-01	18760	1780	23	8
ENSG00000017797	RALBP1	7.16E-04	1.42E-03	5.73E-01	18760	1780	23	8
ENSG00000161956	SENP3	7.16E-04	1.42E-03	5.73E-01	18760	1780	23	8
ENSG00000136244	IL6	7.31E-04	1.43E-03	5.85E-01	18760	1780	18	7
ENSG00000111321	LTBR	7.31E-04	1.43E-03	5.85E-01	18760	1780	18	7
ENSG00000178035	IMPDH2	7.31E-04	1.43E-03	5.85E-01	18760	1780	18	7
ENSG00000180353	HCLS1	7.31E-04	1.43E-03	5.85E-01	18760	1780	18	7
ENSG00000083312	TNPO1	7.31E-04	1.43E-03	5.85E-01	18760	1780	18	7
ENSG00000196396	PTPN1	7.58E-04	1.48E-03	6.07E-01	18760	1780	73	16
ENSG00000130222	GADD45G	7.75E-04	1.51E-03	6.21E-01	18760	1780	53	13
ENSG00000160791	CCR5	8.42E-04	1.62E-03	6.74E-01	18760	1780	29	9
ENSG00000198919	DZIP3	8.42E-04	1.62E-03	6.74E-01	18760	1780	29	9
ENSG00000153234	NR4A2	8.42E-04	1.62E-03	6.74E-01	18760	1780	29	9
ENSG00000080986	NDC80	8.42E-04	1.62E-03	6.74E-01	18760	1780	29	9
ENSG00000132780	NASP	8.42E-04	1.62E-03	6.74E-01	18760	1780	29	9
ENSG00000026103	FAS	8.76E-04	1.68E-03	7.02E-01	18760	1780	74	16
ENSG00000157933	SKI	8.88E-04	1.69E-03	7.11E-01	18760	1780	35	10
ENSG00000162231	NXF1	8.88E-04	1.69E-03	7.11E-01	18760	1780	35	10
ENSG00000082781	ITGB5	9.72E-04	1.85E-03	7.79E-01	18760	1780	24	8
ENSG00000105851	PIK3CG	9.72E-04	1.85E-03	7.79E-01	18760	1780	24	8
ENSG00000137101	CD72	9.93E-04	1.86E-03	7.96E-01	18760	1780	6	4
ENSG00000173369	C1QB	9.93E-04	1.86E-03	7.96E-01	18760	1780	6	4
ENSG00000183049	CAMK1D	9.93E-04	1.86E-03	7.96E-01	18760	1780	6	4
ENSG00000079739	PGM1	9.93E-04	1.86E-03	7.96E-01	18760	1780	6	4
ENSG00000144746	ARL6IP5	9.93E-04	1.86E-03	7.96E-01	18760	1780	6	4
ENSG00000103657	HERC1	9.93E-04	1.86E-03	7.96E-01	18760	1780	6	4
ENSG00000168811	IL12A	1.05E-03	1.94E-03	8.39E-01	18760	1780	19	7
ENSG00000118971	CCND2	1.05E-03	1.94E-03	8.39E-01	18760	1780	19	7
ENSG00000164818	HEATR2	1.05E-03	1.94E-03	8.39E-01	18760	1780	19	7
ENSG00000139083	ETV6	1.05E-03	1.94E-03	8.39E-01	18760	1780	19	7
ENSG00000112096	SOD2	1.08E-03	2.00E-03	8.64E-01	18760	1780	42	11
ENSG00000051180	RAD51	1.10E-03	2.02E-03	8.78E-01	18760	1780	55	13
ENSG00000137947	GTF2B	1.16E-03	2.14E-03	9.31E-01	18760	1780	69	15
ENSG00000162512	SDC3	1.17E-03	2.14E-03	9.39E-01	18760	1780	10	5
ENSG00000120949	TNFRSF8	1.17E-03	2.14E-03	9.39E-01	18760	1780	10	5
ENSG00000198931	APRT	1.17E-03	2.14E-03	9.39E-01	18760	1780	10	5

ENSG00000127481	UBR4	1.17E-03	2.14E-03	9.39E-01	18760	1780	10	5
ENSG00000078967	UBE2D4	1.29E-03	2.34E-03	1.00E+00	18760	1780	84	17
ENSG00000130164	LDLR	1.29E-03	2.35E-03	1.00E+00	18760	1780	25	8
ENSG00000168214	RBPJ	1.39E-03	2.51E-03	1.00E+00	18760	1780	31	9
ENSG00000198563	DDX39B	1.39E-03	2.51E-03	1.00E+00	18760	1780	31	9
ENSG00000153094	BCL2L11	1.39E-03	2.51E-03	1.00E+00	18760	1780	31	9
ENSG00000144021	CIAO1	1.44E-03	2.60E-03	1.00E+00	18760	1780	50	12
ENSG00000049449	RCN1	1.48E-03	2.64E-03	1.00E+00	18760	1780	15	6
ENSG00000160703	NLRX1	1.48E-03	2.64E-03	1.00E+00	18760	1780	15	6
ENSG00000138646	HERC5	1.48E-03	2.64E-03	1.00E+00	18760	1780	15	6
ENSG00000010803	SCMH1	1.48E-03	2.64E-03	1.00E+00	18760	1780	15	6
ENSG00000148180	GSN	1.69E-03	3.00E-03	1.00E+00	18760	1780	26	8
ENSG00000108510	MED13	1.69E-03	3.00E-03	1.00E+00	18760	1780	26	8
ENSG00000075413	MARK3	1.70E-03	3.00E-03	1.00E+00	18760	1780	38	10
ENSG00000073756	PTGS2	1.70E-03	3.00E-03	1.00E+00	18760	1780	38	10
ENSG00000114030	KPNA1	1.71E-03	3.02E-03	1.00E+00	18760	1780	51	12
ENSG00000120802	TMPO	1.75E-03	3.07E-03	1.00E+00	18760	1780	32	9
ENSG00000124216	SNAI1	1.75E-03	3.07E-03	1.00E+00	18760	1780	32	9
ENSG00000204580	DDR1	1.95E-03	3.38E-03	1.00E+00	18760	1780	11	5
ENSG00000115355	CCDC88A	1.95E-03	3.38E-03	1.00E+00	18760	1780	11	5
ENSG00000163874	ZC3H12A	1.95E-03	3.38E-03	1.00E+00	18760	1780	11	5
ENSG00000137843	PAK6	1.95E-03	3.38E-03	1.00E+00	18760	1780	11	5
ENSG00000163661	PTX3	1.95E-03	3.38E-03	1.00E+00	18760	1780	11	5
ENSG00000069966	GNB5	1.98E-03	3.44E-03	1.00E+00	18760	1780	21	7
ENSG00000172819	RARG	2.07E-03	3.58E-03	1.00E+00	18760	1780	39	10
ENSG00000078725	DBC1	2.10E-03	3.58E-03	1.00E+00	18760	1780	7	4
ENSG00000143155	TIPRL	2.10E-03	3.58E-03	1.00E+00	18760	1780	7	4
ENSG00000160710	ADAR	2.10E-03	3.58E-03	1.00E+00	18760	1780	7	4
ENSG00000085872	CHERP	2.10E-03	3.58E-03	1.00E+00	18760	1780	7	4
ENSG00000152689	RASGRP3	2.10E-03	3.58E-03	1.00E+00	18760	1780	7	4
ENSG00000111224	PARP11	2.10E-03	3.58E-03	1.00E+00	18760	1780	7	4
ENSG00000141068	KSR1	2.10E-03	3.58E-03	1.00E+00	18760	1780	7	4
ENSG00000132109	TRIM21	2.18E-03	3.70E-03	1.00E+00	18760	1780	27	8
ENSG00000158169	FANCC	2.18E-03	3.70E-03	1.00E+00	18760	1780	33	9
ENSG00000197448	GSTK1	2.19E-03	3.70E-03	1.00E+00	18760	1780	96	18
ENSG00000156970	BUB1B	2.26E-03	3.81E-03	1.00E+00	18760	1780	46	11
ENSG00000185745	IFIT1	2.26E-03	3.81E-03	1.00E+00	18760	1780	46	11
ENSG00000165527	ARF6	2.37E-03	3.98E-03	1.00E+00	18760	1780	128	22
ENSG00000131747	TOP2A	2.50E-03	4.19E-03	1.00E+00	18760	1780	40	10
ENSG00000198947	DMD	2.63E-03	4.39E-03	1.00E+00	18760	1780	22	7
ENSG00000071794	HLTF	2.63E-03	4.39E-03	1.00E+00	18760	1780	22	7
ENSG00000083168	KAT6A	2.63E-03	4.39E-03	1.00E+00	18760	1780	22	7
ENSG00000065328	MCM10	2.68E-03	4.47E-03	1.00E+00	18760	1780	34	9
ENSG00000004975	DVL2	2.74E-03	4.55E-03	1.00E+00	18760	1780	61	13

ENSG00000057657	PRDM1	2.74E-03	4.55E-03	1.00E+00	18760	1780	54	12
ENSG00000095794	CREM	2.76E-03	4.57E-03	1.00E+00	18760	1780	28	8
ENSG00000101868	POLA1	3.00E-03	4.89E-03	1.00E+00	18760	1780	17	6
ENSG00000112640	PPP2R5D	3.00E-03	4.89E-03	1.00E+00	18760	1780	17	6
ENSG00000149532	CPSF7	3.00E-03	4.89E-03	1.00E+00	18760	1780	17	6
ENSG00000138778	CENPE	3.00E-03	4.89E-03	1.00E+00	18760	1780	17	6
ENSG00000139546	TARBP2	3.00E-03	4.89E-03	1.00E+00	18760	1780	17	6
ENSG00000166128	RAB8B	3.00E-03	4.89E-03	1.00E+00	18760	1780	17	6
ENSG00000196712	NF1	3.00E-03	4.89E-03	1.00E+00	18760	1780	17	6
ENSG00000071054	MAP4K4	3.00E-03	4.89E-03	1.00E+00	18760	1780	17	6
ENSG00000146701	MDH2	3.02E-03	4.90E-03	1.00E+00	18760	1780	12	5
ENSG00000151150	ANK3	3.02E-03	4.90E-03	1.00E+00	18760	1780	12	5
ENSG00000196781	TLE1	3.21E-03	5.20E-03	1.00E+00	18760	1780	92	17
ENSG00000115310	RTN4	3.27E-03	5.27E-03	1.00E+00	18760	1780	35	9
ENSG00000116062	MSH6	3.27E-03	5.27E-03	1.00E+00	18760	1780	35	9
ENSG00000123405	NFE2	3.43E-03	5.51E-03	1.00E+00	18760	1780	23	7
ENSG00000100097	LGALS1	3.45E-03	5.54E-03	1.00E+00	18760	1780	29	8
ENSG00000104228	TRIM35	3.80E-03	6.06E-03	1.00E+00	18760	1780	8	4
ENSG00000151729	SLC25A4	3.80E-03	6.06E-03	1.00E+00	18760	1780	8	4
ENSG00000137842	TMEM62	3.80E-03	6.06E-03	1.00E+00	18760	1780	8	4
ENSG00000070087	PFN2	3.95E-03	6.29E-03	1.00E+00	18760	1780	36	9
ENSG00000114978	MOB1A	4.08E-03	6.38E-03	1.00E+00	18760	1780	18	6
ENSG00000173230	GOLGB1	4.08E-03	6.38E-03	1.00E+00	18760	1780	18	6
ENSG00000128340	RAC2	4.08E-03	6.38E-03	1.00E+00	18760	1780	18	6
ENSG00000038427	VCAN	4.08E-03	6.38E-03	1.00E+00	18760	1780	18	6
ENSG00000070501	POLB	4.08E-03	6.38E-03	1.00E+00	18760	1780	18	6
ENSG00000116830	TTF2	4.08E-03	6.38E-03	1.00E+00	18760	1780	18	6
ENSG00000072364	AFF4	4.08E-03	6.38E-03	1.00E+00	18760	1780	18	6
ENSG00000073614	KDM5A	4.08E-03	6.38E-03	1.00E+00	18760	1780	18	6
ENSG00000163602	RYBP	4.08E-03	6.38E-03	1.00E+00	18760	1780	18	6
ENSG00000104825	NFKBIB	4.10E-03	6.40E-03	1.00E+00	18760	1780	79	15
ENSG00000081237	PTPRC	4.20E-03	6.54E-03	1.00E+00	18760	1780	57	12
ENSG00000116604	MEF2D	4.26E-03	6.63E-03	1.00E+00	18760	1780	30	8
ENSG00000151332	MBIP	4.38E-03	6.78E-03	1.00E+00	18760	1780	24	7
ENSG00000119535	CSF3R	4.38E-03	6.78E-03	1.00E+00	18760	1780	24	7
ENSG00000184702	Sep-05	4.44E-03	6.81E-03	1.00E+00	18760	1780	13	5
ENSG00000115884	SDC1	4.44E-03	6.81E-03	1.00E+00	18760	1780	13	5
ENSG00000121691	CAT	4.44E-03	6.81E-03	1.00E+00	18760	1780	13	5
ENSG00000076864	RAP1GAP	4.44E-03	6.81E-03	1.00E+00	18760	1780	13	5
ENSG00000162616	DNAJB4	4.44E-03	6.81E-03	1.00E+00	18760	1780	13	5
ENSG00000087152	ATXN7L3	4.44E-03	6.81E-03	1.00E+00	18760	1780	13	5
ENSG00000079805	DNM2	4.79E-03	7.33E-03	1.00E+00	18760	1780	58	12
ENSG00000119922	IFIT2	4.91E-03	7.49E-03	1.00E+00	18760	1780	44	10
ENSG00000080839	RBL1	5.08E-03	7.73E-03	1.00E+00	18760	1780	81	15

ENSG00000170458	CD14	5.40E-03	8.17E-03	1.00E+00	18760	1780	19	6
ENSG00000057935	MTA3	5.40E-03	8.17E-03	1.00E+00	18760	1780	19	6
ENSG00000163399	ATP1A1	5.40E-03	8.17E-03	1.00E+00	18760	1780	19	6
ENSG00000149923	PPP4C	5.45E-03	8.23E-03	1.00E+00	18760	1780	59	12
ENSG00000051108	HERPUD1	5.51E-03	8.30E-03	1.00E+00	18760	1780	25	7
ENSG00000149177	PTPRJ	5.60E-03	8.44E-03	1.00E+00	18760	1780	38	9
ENSG00000166228	PCBD1	6.07E-03	9.13E-03	1.00E+00	18760	1780	75	14
ENSG00000133639	BTG1	6.19E-03	9.27E-03	1.00E+00	18760	1780	9	4
ENSG00000141655	TNFRSF11A	6.19E-03	9.27E-03	1.00E+00	18760	1780	9	4
ENSG00000176658	MYO1D	6.26E-03	9.30E-03	1.00E+00	18760	1780	14	5
ENSG00000060566	CREB3L3	6.26E-03	9.30E-03	1.00E+00	18760	1780	14	5
ENSG00000077152	UBE2T	6.26E-03	9.30E-03	1.00E+00	18760	1780	14	5
ENSG00000133835	HSD17B4	6.26E-03	9.30E-03	1.00E+00	18760	1780	14	5
ENSG00000161800	RACGAP1	6.82E-03	1.01E-02	1.00E+00	18760	1780	26	7
ENSG00000175895	PLEKHF2	6.82E-03	1.01E-02	1.00E+00	18760	1780	26	7
ENSG00000092847	EIF2C1	6.82E-03	1.01E-02	1.00E+00	18760	1780	26	7
ENSG00000049540	ELN	6.98E-03	1.01E-02	1.00E+00	18760	1780	20	6
ENSG00000135723	FHOD1	6.98E-03	1.01E-02	1.00E+00	18760	1780	20	6
ENSG00000148773	MKI67	6.98E-03	1.01E-02	1.00E+00	18760	1780	20	6
ENSG00000143228	NUF2	6.98E-03	1.01E-02	1.00E+00	18760	1780	20	6
ENSG00000107960	OBFC1	6.98E-03	1.01E-02	1.00E+00	18760	1780	20	6
ENSG00000032219	ARID4A	6.98E-03	1.01E-02	1.00E+00	18760	1780	20	6
ENSG00000034152	MAP2K3	6.98E-03	1.01E-02	1.00E+00	18760	1780	20	6
ENSG00000059728	MXD1	6.98E-03	1.01E-02	1.00E+00	18760	1780	20	6
ENSG00000164104	HMGB2	6.98E-03	1.01E-02	1.00E+00	18760	1780	20	6
ENSG00000136536	Mar-07	6.99E-03	1.01E-02	1.00E+00	18760	1780	5	3
ENSG00000101109	STK4	7.51E-03	1.08E-02	1.00E+00	18760	1780	33	8
ENSG00000127884	ECHS1	7.51E-03	1.08E-02	1.00E+00	18760	1780	33	8
ENSG00000069667	RORA	7.51E-03	1.08E-02	1.00E+00	18760	1780	33	8
ENSG00000108469	RECQL5	7.51E-03	1.08E-02	1.00E+00	18760	1780	33	8
ENSG00000137936	BCAR3	7.61E-03	1.09E-02	1.00E+00	18760	1780	47	10
ENSG00000129315	CCNT1	7.61E-03	1.09E-02	1.00E+00	18760	1780	47	10
ENSG00000015475	BID	7.61E-03	1.09E-02	1.00E+00	18760	1780	47	10
ENSG00000131788	PIAS3	7.70E-03	1.10E-02	1.00E+00	18760	1780	40	9
ENSG00000118260	CREB1	7.98E-03	1.14E-02	1.00E+00	18760	1780	241	33
ENSG00000071539	TRIP13	8.29E-03	1.18E-02	1.00E+00	18760	1780	55	11
ENSG00000170889	RPS9	8.34E-03	1.18E-02	1.00E+00	18760	1780	27	7
ENSG00000157514	TSC22D3	8.34E-03	1.18E-02	1.00E+00	18760	1780	27	7
ENSG00000068305	MEF2A	8.34E-03	1.18E-02	1.00E+00	18760	1780	27	7
ENSG00000102908	NFAT5	8.50E-03	1.19E-02	1.00E+00	18760	1780	15	5
ENSG00000023909	GCLM	8.50E-03	1.19E-02	1.00E+00	18760	1780	15	5
ENSG00000168411	RFWD3	8.50E-03	1.19E-02	1.00E+00	18760	1780	15	5
ENSG00000144283	PKP4	8.50E-03	1.19E-02	1.00E+00	18760	1780	15	5
ENSG00000109654	TRIM2	8.50E-03	1.19E-02	1.00E+00	18760	1780	15	5

ENSG00000130638	ATXN10	8.50E-03	1.19E-02	1.00E+00	18760	1780	15	5
ENSG00000177374	HIC1	8.85E-03	1.23E-02	1.00E+00	18760	1780	21	6
ENSG00000105245	NUMBL	8.85E-03	1.23E-02	1.00E+00	18760	1780	21	6
ENSG00000128016	ZFP36	8.85E-03	1.23E-02	1.00E+00	18760	1780	21	6
ENSG00000157456	CCNB2	8.85E-03	1.23E-02	1.00E+00	18760	1780	21	6
ENSG00000146729	GBAS	8.89E-03	1.24E-02	1.00E+00	18760	1780	34	8
ENSG00000095951	HIVEP1	8.94E-03	1.24E-02	1.00E+00	18760	1780	41	9
ENSG00000110876	SELPLG	9.34E-03	1.29E-02	1.00E+00	18760	1780	10	4
ENSG00000050405	LIMA1	9.34E-03	1.29E-02	1.00E+00	18760	1780	10	4
ENSG00000240065	PSMB9	9.34E-03	1.29E-02	1.00E+00	18760	1780	10	4
ENSG00000167721	TSR1	9.34E-03	1.29E-02	1.00E+00	18760	1780	10	4
ENSG00000110713	NUP98	1.01E-02	1.38E-02	1.00E+00	18760	1780	28	7
ENSG00000136653	RASSF5	1.01E-02	1.38E-02	1.00E+00	18760	1780	28	7
ENSG00000085978	ATG16L1	1.01E-02	1.38E-02	1.00E+00	18760	1780	28	7
ENSG00000075618	FSCN1	1.03E-02	1.41E-02	1.00E+00	18760	1780	42	9
ENSG00000148498	PARD3	1.04E-02	1.43E-02	1.00E+00	18760	1780	35	8
ENSG00000158710	TAGLN2	1.10E-02	1.50E-02	1.00E+00	18760	1780	22	6
ENSG00000133119	RFC3	1.12E-02	1.52E-02	1.00E+00	18760	1780	16	5
ENSG00000114739	ACVR2B	1.12E-02	1.52E-02	1.00E+00	18760	1780	16	5
ENSG00000197019	SERTAD1	1.20E-02	1.63E-02	1.00E+00	18760	1780	29	7
ENSG00000013810	TACC3	1.33E-02	1.79E-02	1.00E+00	18760	1780	11	4
ENSG00000100442	FKBP3	1.33E-02	1.79E-02	1.00E+00	18760	1780	11	4
ENSG00000108700	CCL8	1.33E-02	1.79E-02	1.00E+00	18760	1780	11	4
ENSG00000117289	TXNIP	1.33E-02	1.79E-02	1.00E+00	18760	1780	11	4
ENSG00000165168	CYBB	1.35E-02	1.81E-02	1.00E+00	18760	1780	23	6
ENSG00000158402	CDC25C	1.35E-02	1.81E-02	1.00E+00	18760	1780	23	6
ENSG00000129219	PLD2	1.35E-02	1.81E-02	1.00E+00	18760	1780	23	6
ENSG00000113810	SMC4	1.44E-02	1.92E-02	1.00E+00	18760	1780	17	5
ENSG00000167978	SRRM2	1.53E-02	2.05E-02	1.00E+00	18760	1780	128	19
ENSG00000116455	WDR77	1.61E-02	2.14E-02	1.00E+00	18760	1780	38	8
ENSG00000112715	VEGFA	1.61E-02	2.14E-02	1.00E+00	18760	1780	38	8
ENSG00000100804	PSMB5	1.66E-02	2.21E-02	1.00E+00	18760	1780	31	7
ENSG00000100697	DICER1	1.80E-02	2.38E-02	1.00E+00	18760	1780	18	5
ENSG00000132376	INPP5K	1.80E-02	2.38E-02	1.00E+00	18760	1780	18	5
ENSG00000117528	ABCD3	1.80E-02	2.38E-02	1.00E+00	18760	1780	18	5
ENSG00000147465	STAR	1.80E-02	2.38E-02	1.00E+00	18760	1780	12	4
ENSG00000186834	HEXIM1	1.80E-02	2.38E-02	1.00E+00	18760	1780	12	4
ENSG00000119408	NEK6	1.91E-02	2.52E-02	1.00E+00	18760	1780	47	9
ENSG00000100368	CSF2RB	1.94E-02	2.54E-02	1.00E+00	18760	1780	25	6
ENSG00000173020	ADRBK1	1.94E-02	2.54E-02	1.00E+00	18760	1780	25	6
ENSG00000136869	TLR4	1.94E-02	2.54E-02	1.00E+00	18760	1780	55	10
ENSG00000110944	IL23A	2.00E-02	2.62E-02	1.00E+00	18760	1780	7	3
ENSG00000157764	BRAF	2.07E-02	2.70E-02	1.00E+00	18760	1780	40	8
ENSG00000072518	MARK2	2.07E-02	2.70E-02	1.00E+00	18760	1780	40	8

ENSG00000112851	ERBB2IP	2.11E-02	2.74E-02	1.00E+00	18760	1780	64	11
ENSG00000164045	CDC25A	2.14E-02	2.78E-02	1.00E+00	18760	1780	56	10
ENSG00000132356	PRKAA1	2.15E-02	2.79E-02	1.00E+00	18760	1780	81	13
ENSG00000146278	PNRC1	2.21E-02	2.87E-02	1.00E+00	18760	1780	19	5
ENSG00000055483	USP36	2.28E-02	2.95E-02	1.00E+00	18760	1780	26	6
ENSG00000157601	MX1	2.36E-02	3.05E-02	1.00E+00	18760	1780	13	4
ENSG00000151491	EPS8	2.37E-02	3.05E-02	1.00E+00	18760	1780	49	9
ENSG00000131981	LGALS3	2.37E-02	3.05E-02	1.00E+00	18760	1780	49	9
ENSG00000104375	STK3	2.52E-02	3.23E-02	1.00E+00	18760	1780	34	7
ENSG00000156587	UBE2L6	2.52E-02	3.23E-02	1.00E+00	18760	1780	34	7
ENSG00000156709	AIFM1	2.61E-02	3.34E-02	1.00E+00	18760	1780	42	8
ENSG00000137462	TLR2	2.61E-02	3.34E-02	1.00E+00	18760	1780	50	9
ENSG00000087095	NLK	2.66E-02	3.39E-02	1.00E+00	18760	1780	27	6
ENSG00000178104	PDE4DIP	2.66E-02	3.39E-02	1.00E+00	18760	1780	27	6
ENSG00000112511	PHF1	2.67E-02	3.39E-02	1.00E+00	18760	1780	20	5
ENSG00000204304	PBX2	2.67E-02	3.39E-02	1.00E+00	18760	1780	20	5
ENSG00000105011	ASF1B	2.85E-02	3.62E-02	1.00E+00	18760	1780	35	7
ENSG00000109062	SLC9A3R1	2.87E-02	3.64E-02	1.00E+00	18760	1780	51	9
ENSG00000168062	BATF2	2.90E-02	3.67E-02	1.00E+00	18760	1780	8	3
ENSG00000129810	SGOL1	2.99E-02	3.77E-02	1.00E+00	18760	1780	14	4
ENSG00000070081	NUCB2	2.99E-02	3.77E-02	1.00E+00	18760	1780	14	4
ENSG00000123136	DDX39A	2.99E-02	3.77E-02	1.00E+00	18760	1780	14	4
ENSG00000156273	BACH1	3.06E-02	3.83E-02	1.00E+00	18760	1780	28	6
ENSG00000128050	PAICS	3.06E-02	3.83E-02	1.00E+00	18760	1780	28	6
ENSG00000138029	HADHB	3.06E-02	3.83E-02	1.00E+00	18760	1780	28	6
ENSG00000161638	ITGA5	3.06E-02	3.83E-02	1.00E+00	18760	1780	28	6
ENSG00000106346	USP42	3.06E-02	3.83E-02	1.00E+00	18760	1780	28	6
ENSG00000172175	MALT1	3.17E-02	3.93E-02	1.00E+00	18760	1780	21	5
ENSG00000079277	MKNK1	3.17E-02	3.93E-02	1.00E+00	18760	1780	21	5
ENSG00000213722	DDAH2	3.17E-02	3.93E-02	1.00E+00	18760	1780	21	5
ENSG00000099940	SNAP29	3.17E-02	3.93E-02	1.00E+00	18760	1780	21	5
ENSG00000090376	IRAK3	3.17E-02	3.93E-02	1.00E+00	18760	1780	21	5
ENSG00000204287	HLA-DRA	3.20E-02	3.95E-02	1.00E+00	18760	1780	36	7
ENSG00000143207	RFWD2	3.20E-02	3.95E-02	1.00E+00	18760	1780	36	7
ENSG00000112664	NUDT3	3.20E-02	3.95E-02	1.00E+00	18760	1780	36	7
ENSG00000123200	ZC3H13	3.20E-02	3.95E-02	1.00E+00	18760	1780	36	7
ENSG00000163435	ELF3	3.21E-02	3.95E-02	1.00E+00	18760	1780	44	8
ENSG00000106100	NOD1	3.49E-02	4.29E-02	1.00E+00	18760	1780	29	6
ENSG00000148356	LRSAM1	3.49E-02	4.29E-02	1.00E+00	18760	1780	29	6
ENSG00000103932	RPAP1	3.57E-02	4.38E-02	1.00E+00	18760	1780	37	7
ENSG00000167325	RRM1	3.69E-02	4.48E-02	1.00E+00	18760	1780	15	4
ENSG00000115267	IFIH1	3.69E-02	4.48E-02	1.00E+00	18760	1780	15	4
ENSG00000160307	S100B	3.69E-02	4.48E-02	1.00E+00	18760	1780	15	4
ENSG00000151320	AKAP6	3.69E-02	4.48E-02	1.00E+00	18760	1780	15	4

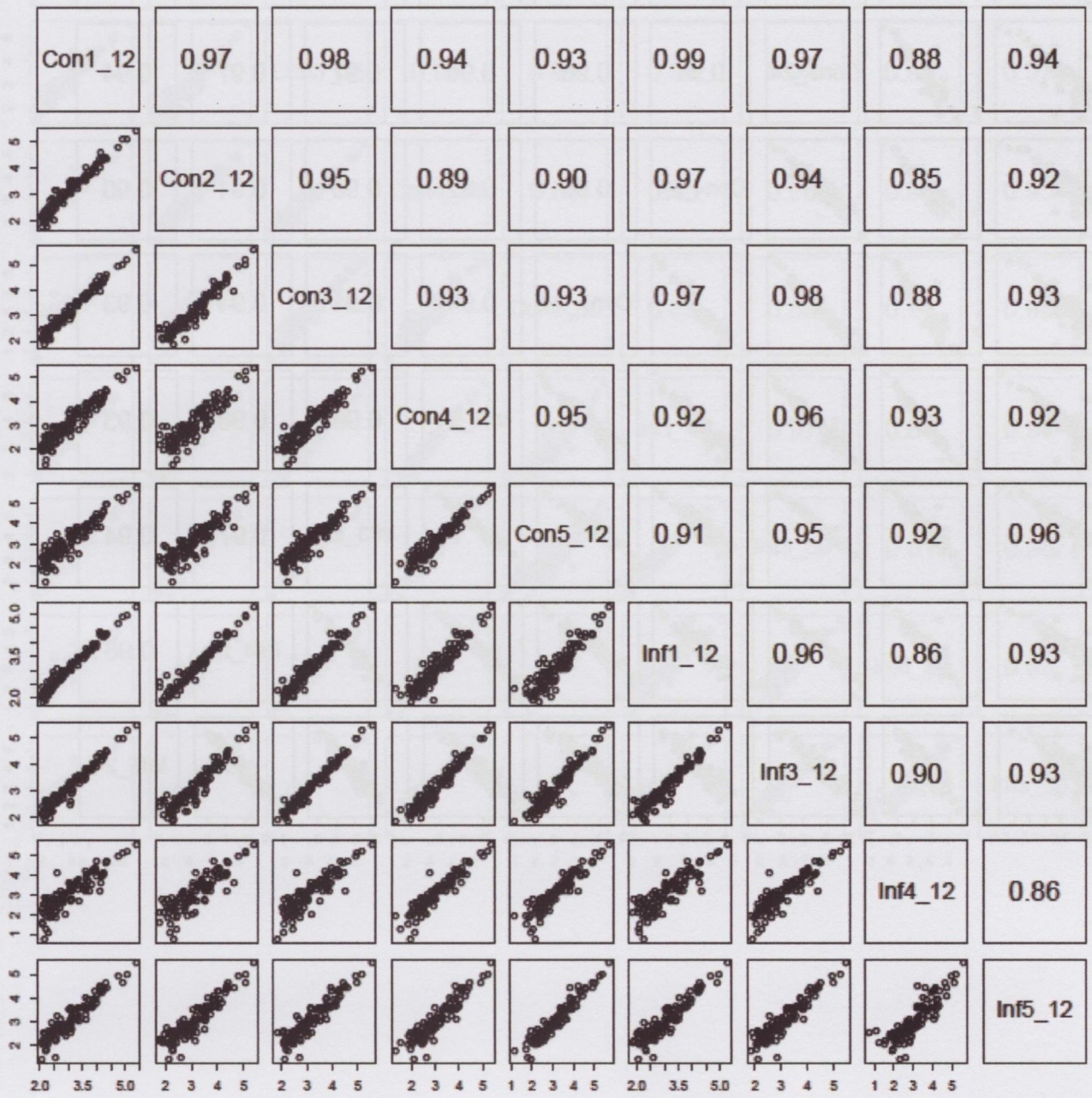
ENSG00000005961	ITGA2B	3.69E-02	4.48E-02	1.00E+00	18760	1780	15	4
ENSG00000213672	NCKIPSD	3.69E-02	4.48E-02	1.00E+00	18760	1780	15	4
ENSG00000120896	SORBS3	3.71E-02	4.49E-02	1.00E+00	18760	1780	22	5
ENSG00000085117	CD82	3.71E-02	4.49E-02	1.00E+00	18760	1780	22	5
ENSG00000122877	EGR2	3.71E-02	4.49E-02	1.00E+00	18760	1780	22	5
ENSG00000179222	MAGED1	3.83E-02	4.62E-02	1.00E+00	18760	1780	63	10
ENSG00000132589	FLOT2	3.94E-02	4.75E-02	1.00E+00	18760	1780	9	3
ENSG00000106366	SERPINE1	3.95E-02	4.75E-02	1.00E+00	18760	1780	30	6
ENSG00000175213	ZNF408	3.95E-02	4.75E-02	1.00E+00	18760	1780	30	6
ENSG00000101400	SNTA1	4.11E-02	4.92E-02	1.00E+00	18760	1780	64	10
ENSG00000168291	PDHB	4.30E-02	5.14E-02	1.00E+00	18760	1780	23	5
ENSG00000184634	MED12	4.35E-02	5.20E-02	1.00E+00	18760	1780	56	9
ENSG00000171793	CTPS1	4.46E-02	5.30E-02	1.00E+00	18760	1780	16	4
ENSG00000204305	AGER	4.46E-02	5.30E-02	1.00E+00	18760	1780	16	4
ENSG00000140374	ETFA	4.46E-02	5.30E-02	1.00E+00	18760	1780	16	4
ENSG00000183283	DAZAP2	4.68E-02	5.56E-02	1.00E+00	18760	1780	57	9
ENSG00000152894	PTPRK	4.91E-02	5.80E-02	1.00E+00	18760	1780	24	5
ENSG00000092094	OSGEP	4.91E-02	5.80E-02	1.00E+00	18760	1780	24	5
ENSG00000139626	ITGB7	4.91E-02	5.80E-02	1.00E+00	18760	1780	24	5
ENSG00000137575	SDCBP	4.91E-02	5.80E-02	1.00E+00	18760	1780	24	5
ENSG00000099917	MED15	5.24E-02	6.18E-02	1.00E+00	18760	1780	41	7
ENSG00000130396	MLLT4	5.24E-02	6.18E-02	1.00E+00	18760	1780	41	7
ENSG00000006468	ETV1	5.27E-02	6.18E-02	1.00E+00	18760	1780	17	4
ENSG00000133818	RRAS2	5.27E-02	6.18E-02	1.00E+00	18760	1780	17	4
ENSG00000102103	PQBP1	5.27E-02	6.18E-02	1.00E+00	18760	1780	17	4
ENSG00000111328	CDK2AP1	5.27E-02	6.18E-02	1.00E+00	18760	1780	17	4
ENSG00000116741	RGS2	5.35E-02	6.26E-02	1.00E+00	18760	1780	59	9
ENSG00000164758	MED30	5.47E-02	6.39E-02	1.00E+00	18760	1780	33	6
ENSG00000186951	PPARA	5.70E-02	6.63E-02	1.00E+00	18760	1780	42	7
ENSG00000105404	RABAC1	5.70E-02	6.63E-02	1.00E+00	18760	1780	42	7
ENSG00000130066	SAT1	6.05E-02	7.04E-02	1.00E+00	18760	1780	61	9
ENSG00000135801	TAF5L	6.14E-02	7.10E-02	1.00E+00	18760	1780	18	4
ENSG00000082074	FYB	6.14E-02	7.10E-02	1.00E+00	18760	1780	18	4
ENSG00000185043	CIB1	6.14E-02	7.10E-02	1.00E+00	18760	1780	18	4
ENSG00000116584	ARHGEF2	6.14E-02	7.10E-02	1.00E+00	18760	1780	18	4
ENSG00000162711	NLRP3	6.35E-02	7.32E-02	1.00E+00	18760	1780	11	3
ENSG00000163635	ATXN7	6.56E-02	7.56E-02	1.00E+00	18760	1780	72	10
ENSG00000164164	OTUD4	6.57E-02	7.56E-02	1.00E+00	18760	1780	35	6
ENSG00000116288	PARK7	6.63E-02	7.62E-02	1.00E+00	18760	1780	44	7
ENSG00000162889	MAPKAPK2	7.04E-02	8.03E-02	1.00E+00	18760	1780	19	4
ENSG00000130254	SAFB2	7.04E-02	8.03E-02	1.00E+00	18760	1780	19	4
ENSG00000092853	CLSPN	7.04E-02	8.03E-02	1.00E+00	18760	1780	19	4
ENSG00000132694	ARHGEF11	7.04E-02	8.03E-02	1.00E+00	18760	1780	19	4
ENSG00000178184	PARD6G	7.04E-02	8.03E-02	1.00E+00	18760	1780	19	4

ENSG00000101557	USP14	7.14E-02	8.13E-02	1.00E+00	18760	1780	36	6
ENSG00000089220	PEBP1	7.63E-02	8.68E-02	1.00E+00	18760	1780	28	5
ENSG00000091409	ITGA6	7.66E-02	8.70E-02	1.00E+00	18760	1780	12	3
ENSG00000122257	RBBP6	7.71E-02	8.74E-02	1.00E+00	18760	1780	37	6
ENSG00000143546	S100A8	7.71E-02	8.74E-02	1.00E+00	18760	1780	37	6
ENSG00000162367	TAL1	7.79E-02	8.82E-02	1.00E+00	18760	1780	56	8
ENSG00000007968	E2F2	7.97E-02	8.99E-02	1.00E+00	18760	1780	20	4
ENSG00000116809	ZBTB17	7.97E-02	8.99E-02	1.00E+00	18760	1780	20	4
ENSG00000124214	STAU1	8.07E-02	9.10E-02	1.00E+00	18760	1780	47	7
ENSG00000123908	EIF2C2	8.19E-02	9.21E-02	1.00E+00	18760	1780	98	12
ENSG00000136603	SKIL	8.34E-02	9.36E-02	1.00E+00	18760	1780	88	11
ENSG00000140368	PSTPIP1	8.56E-02	9.60E-02	1.00E+00	18760	1780	48	7
ENSG00000129245	FXR2	8.57E-02	9.60E-02	1.00E+00	18760	1780	68	9
ENSG00000088930	XRN2	8.91E-02	9.96E-02	1.00E+00	18760	1780	21	4
ENSG00000137822	TUBGCP4	9.06E-02	1.01E-01	1.00E+00	18760	1780	30	5
ENSG00000136436	CALCOCO2	9.27E-02	1.03E-01	1.00E+00	18760	1780	70	9
ENSG00000094880	CDC23	9.45E-02	1.05E-01	1.00E+00	18760	1780	40	6
ENSG00000087074	PPP1R15A	9.85E-02	1.10E-01	1.00E+00	18760	1780	22	4
ENSG00000163220	S100A9	1.08E-01	1.20E-01	1.00E+00	18760	1780	23	4
ENSG00000172493	AFF1	1.17E-01	1.30E-01	1.00E+00	18760	1780	24	4
ENSG00000100320	RBFOX2	1.17E-01	1.30E-01	1.00E+00	18760	1780	24	4
ENSG00000132254	ARFIP2	1.18E-01	1.30E-01	1.00E+00	18760	1780	15	3
ENSG00000103510	KAT8	1.19E-01	1.31E-01	1.00E+00	18760	1780	34	5
ENSG00000110651	CD81	1.26E-01	1.39E-01	1.00E+00	18760	1780	25	4
ENSG00000160049	DFFA	1.35E-01	1.49E-01	1.00E+00	18760	1780	26	4
ENSG00000102081	FMR1	1.35E-01	1.49E-01	1.00E+00	18760	1780	26	4
ENSG00000130826	DKC1	1.44E-01	1.58E-01	1.00E+00	18760	1780	17	3
ENSG00000164327	RICTOR	1.48E-01	1.63E-01	1.00E+00	18760	1780	51	6
ENSG00000168002	POLR2G	1.49E-01	1.64E-01	1.00E+00	18760	1780	39	5
ENSG00000135503	ACVR1B	1.52E-01	1.66E-01	1.00E+00	18760	1780	28	4
ENSG00000151090	THRB	1.62E-01	1.77E-01	1.00E+00	18760	1780	56	6
ENSG00000028277	POU2F2	1.66E-01	1.81E-01	1.00E+00	18760	1780	30	4
ENSG00000125166	GOT2	1.66E-01	1.81E-01	1.00E+00	18760	1780	30	4
ENSG00000187239	FNBP1	1.68E-01	1.83E-01	1.00E+00	18760	1780	19	3
ENSG00000067082	KLF6	1.79E-01	1.94E-01	1.00E+00	18760	1780	20	3
ENSG00000130307	USHBP1	2.00E-01	2.16E-01	1.00E+00	18760	1780	37	4
ENSG00000138448	ITGAV	2.06E-01	2.23E-01	1.00E+00	18760	1780	23	3
ENSG00000160271	RALGDS	2.06E-01	2.23E-01	1.00E+00	18760	1780	23	3
ENSG00000240972	MIF	2.32E-01	2.50E-01	1.00E+00	18760	1780	28	3
ENSG00000157227	MMP14	2.75E-01	2.96E-01	1.00E+00	18760	1780	17	2
ENSG00000005810	MYCBP2	2.83E-01	3.04E-01	1.00E+00	18760	1780	19	2
ENSG00000142192	APP	9.99E+02	1.00E+00	1.00E+00	18760	1780	120	319
ENSG00000166803	KIAA0101	9.99E+02	1.00E+00	1.00E+00	18760	1780	3	102
ENSG00000187608	ISG15	9.99E+02	1.00E+00	1.00E+00	18760	1780	37	46

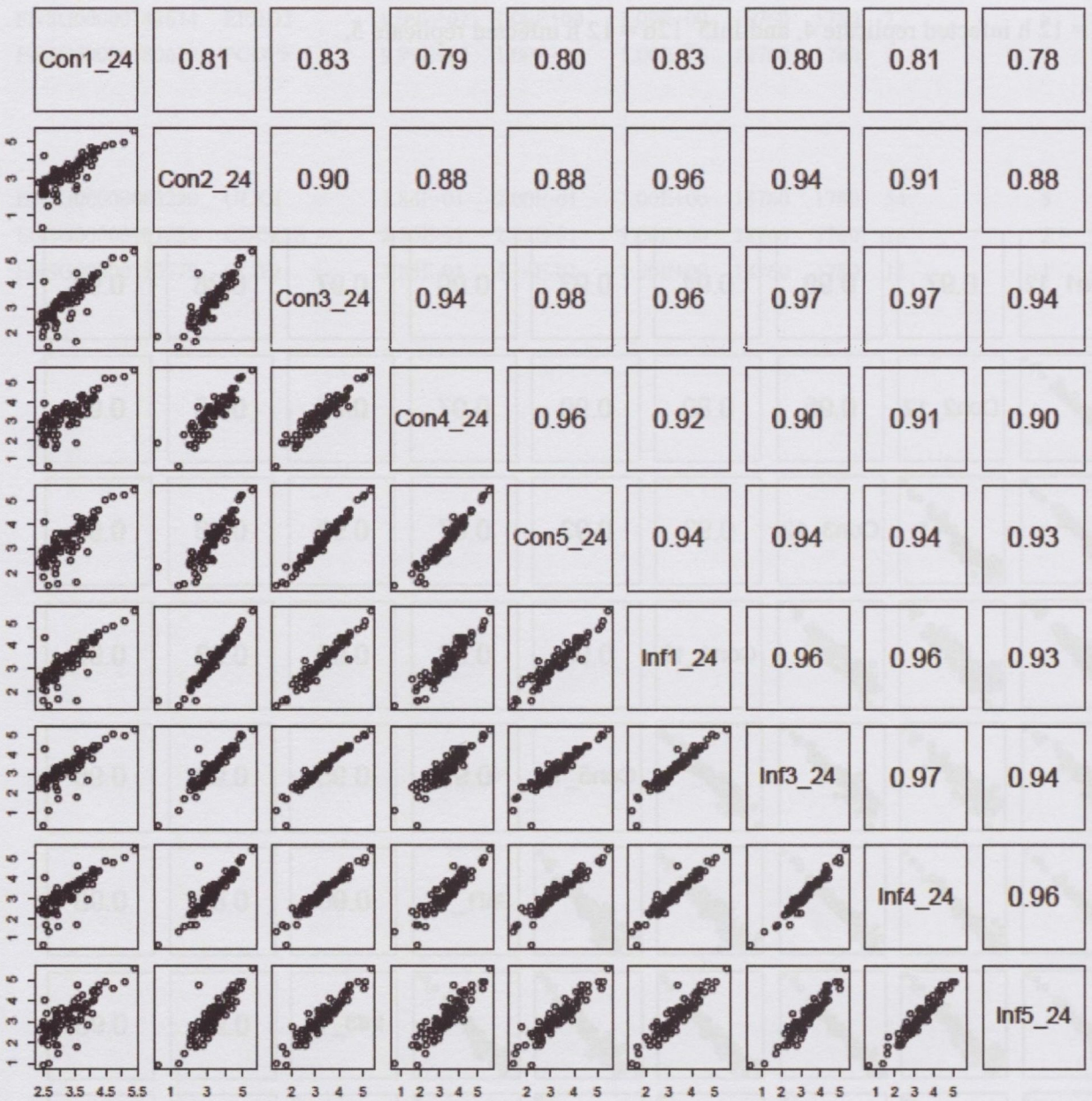
ENSG00000145901	TNIP1	9.99E+02	1.00E+00	1.00E+00	18760	1780	29	30
ENSG00000114346	ECT2	9.99E+02	1.00E+00	1.00E+00	18760	1780	12	29
ENSG00000109685	WHSC1	9.99E+02	1.00E+00	1.00E+00	18760	1780	7	23
ENSG00000163104	SMARCAD1	9.99E+02	1.00E+00	1.00E+00	18760	1780	2	23
ENSG00000174405	LIG4	9.99E+02	1.00E+00	1.00E+00	18760	1780	14	22
ENSG00000090447	TFAP4	9.99E+02	1.00E+00	1.00E+00	18760	1780	7	18
ENSG00000197272	IL27	9.99E+02	1.00E+00	1.00E+00	18760	1780	5	19
ENSG00000185215	TNFAIP2	9.99E+02	1.00E+00	1.00E+00	18760	1780	2	18
ENSG00000171206	TRIM8	9.99E+02	1.00E+00	1.00E+00	18760	1780	11	16
ENSG00000115009	CCL20	9.99E+02	1.00E+00	1.00E+00	18760	1780	9	17
ENSG00000184922	FMNL1	9.99E+02	1.00E+00	1.00E+00	18760	1780	8	16
ENSG00000125868	DSTN	9.99E+02	1.00E+00	1.00E+00	18760	1780	12	16
ENSG00000124523	SIRT5	9.99E+02	1.00E+00	1.00E+00	18760	1780	2	10
ENSG00000176014	TUBB6	9.99E+02	1.00E+00	1.00E+00	18760	1780	8	10
ENSG00000164062	APEH	9.99E+02	1.00E+00	1.00E+00	18760	1780	8	12
ENSG00000140263	SORD	9.99E+02	1.00E+00	1.00E+00	18760	1780	6	9
ENSG00000159363	ATP13A2	9.99E+02	1.00E+00	1.00E+00	18760	1780	1	8
ENSG00000167508	MVD	9.99E+02	1.00E+00	1.00E+00	18760	1780	4	9
ENSG00000139517	LNX2	9.99E+02	1.00E+00	1.00E+00	18760	1780	4	7
ENSG00000258947	TUBB3	9.99E+02	1.00E+00	1.00E+00	18760	1780	2	7
ENSG00000141293	SKAP1	9.99E+02	1.00E+00	1.00E+00	18760	1780	8	9
ENSG00000174547	MRPL11	9.99E+02	1.00E+00	1.00E+00	18760	1780	2	8
ENSG00000087302	C14orf166	9.99E+02	1.00E+00	1.00E+00	18760	1780	6	8
ENSG00000135677	GNS	9.99E+02	1.00E+00	1.00E+00	18760	1780	6	8
ENSG00000117525	F3	9.99E+02	1.00E+00	1.00E+00	18760	1780	6	8
ENSG00000131203	IDO1	9.99E+02	1.00E+00	1.00E+00	18760	1780	7	8
ENSG00000138413	IDH1	9.99E+02	1.00E+00	1.00E+00	18760	1780	2	8
ENSG00000110047	EHD1	9.99E+02	1.00E+00	1.00E+00	18760	1780	7	8
ENSG00000005020	SKAP2	9.99E+02	1.00E+00	1.00E+00	18760	1780	5	7
ENSG00000083720	OXCT1	9.99E+02	1.00E+00	1.00E+00	18760	1780	2	8
ENSG00000135636	DYSF	9.99E+02	1.00E+00	1.00E+00	18760	1780	1	5
ENSG00000115919	KYNU	9.99E+02	1.00E+00	1.00E+00	18760	1780	1	7
ENSG00000119125	GDA	9.99E+02	1.00E+00	1.00E+00	18760	1780	3	6
ENSG00000167792	NDUFV1	9.99E+02	1.00E+00	1.00E+00	18760	1780	3	6
ENSG00000124831	LRRFIP1	9.99E+02	1.00E+00	1.00E+00	18760	1780	4	6
ENSG00000160131	VMA21	9.99E+02	1.00E+00	1.00E+00	18760	1780	4	5
ENSG00000091140	DLD	9.99E+02	1.00E+00	1.00E+00	18760	1780	5	6
ENSG00000107159	CA9	9.99E+02	1.00E+00	1.00E+00	18760	1780	3	6
ENSG00000164904	ALDH7A1	9.99E+02	1.00E+00	1.00E+00	18760	1780	4	6
ENSG00000143436	MRPL9	9.99E+02	1.00E+00	1.00E+00	18760	1780	4	6
ENSG00000115364	MRPL19	9.99E+02	1.00E+00	1.00E+00	18760	1780	4	6
ENSG00000112304	ACOT13	9.99E+02	1.00E+00	1.00E+00	18760	1780	4	5
ENSG00000198554	WDHD1	9.99E+02	1.00E+00	1.00E+00	18760	1780	4	6
ENSG00000104687	GSR	9.99E+02	1.00E+00	1.00E+00	18760	1780	3	4

ENSG00000160193	WDR4	9.99E+02	1.00E+00	1.00E+00	18760	1780	1	4
ENSG00000114686	MRPL3	9.99E+02	1.00E+00	1.00E+00	18760	1780	3	5
ENSG00000155975	VPS37A	9.99E+02	1.00E+00	1.00E+00	18760	1780	4	5
ENSG00000154723	ATP5J	9.99E+02	1.00E+00	1.00E+00	18760	1780	4	5
ENSG00000112972	HMGCS1	9.99E+02	1.00E+00	1.00E+00	18760	1780	2	5
ENSG00000154719	MRPL39	9.99E+02	1.00E+00	1.00E+00	18760	1780	3	5
ENSG00000142634	EFHD2	9.99E+02	1.00E+00	1.00E+00	18760	1780	2	5
ENSG00000180628	PCGF5	9.99E+02	1.00E+00	1.00E+00	18760	1780	3	4
ENSG00000083290	ULK2	1.84E-01	2.00E-01	1.00E+00	18760	1780	54	5
ENSG00000101224	CDC25B	2.55E-01	2.75E-01	1.00E+00	18760	1780	28	2
ENSG00000130779	CLIP1	3.85E-01	4.14E-01	1.00E+00	18760	1780	11	1

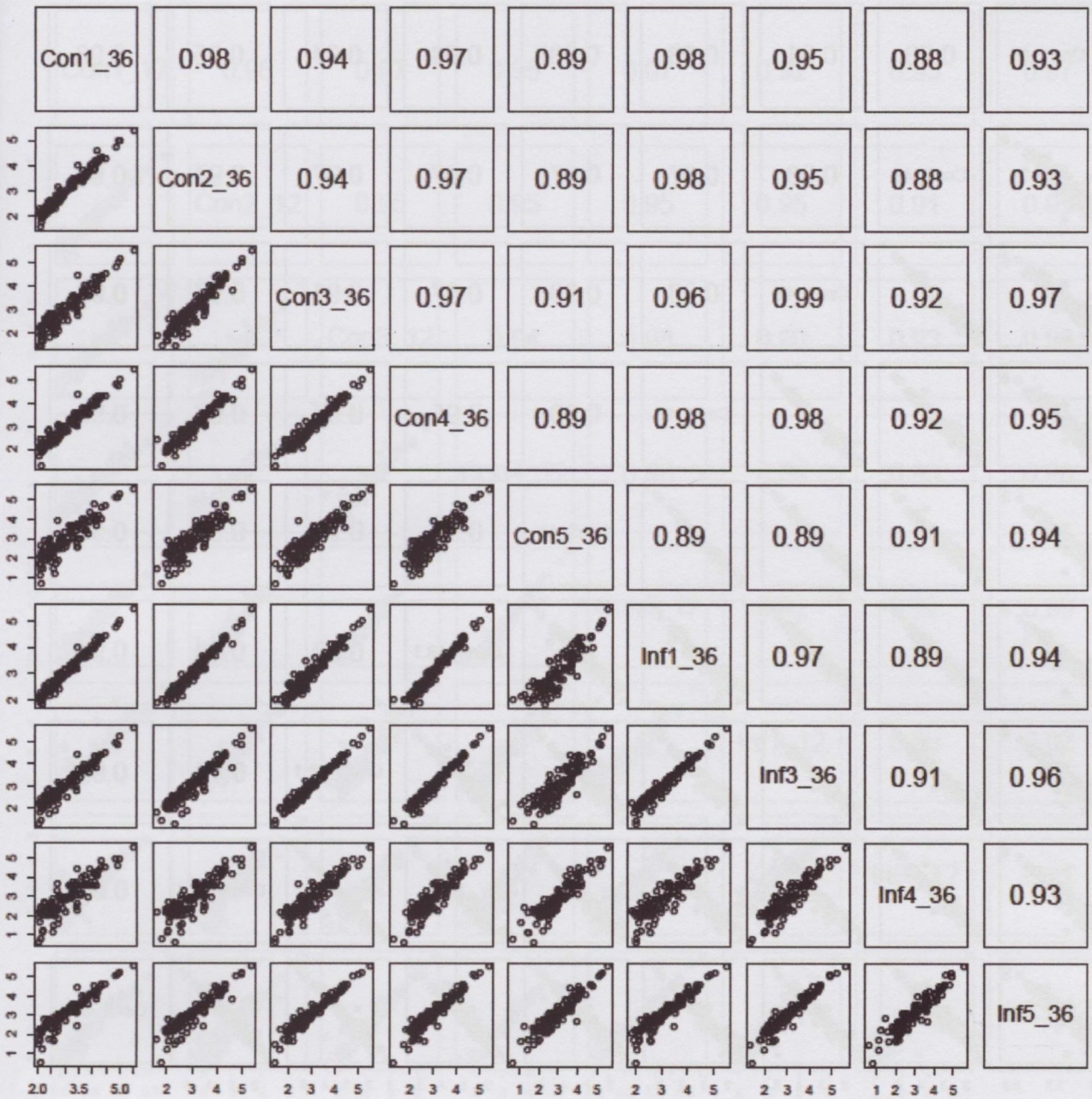
Appendix 19.1: Correlation coefficients of each blood and milk miRNA normalised read count dataset between samples at each time-point for control and infected technical replicates above 10 tpm. All correlation data was generated in R version 3.0.2 The average correlation between each time-point for both control and infected was > 0.93 indicating reproducibility of the data among replicates. **Correlation coefficients data for all 12 h blood samples.** Note Con1_12hr = 12 h control replicate 1, Con2_12hr = 12 h control replicate 2, Con3_12hr = 12 h control replicate 3, Con4_12hr = 12 h control replicate 4, Con5_12hr = 12 h control replicate 5 and Inf1_12h = 12 h infected replicate 1, Inf3_12h = 12 h infected replicate 3, Inf3_12h = 12 h infected replicate 4, and Inf5_12h = 12 h infected replicate 5.



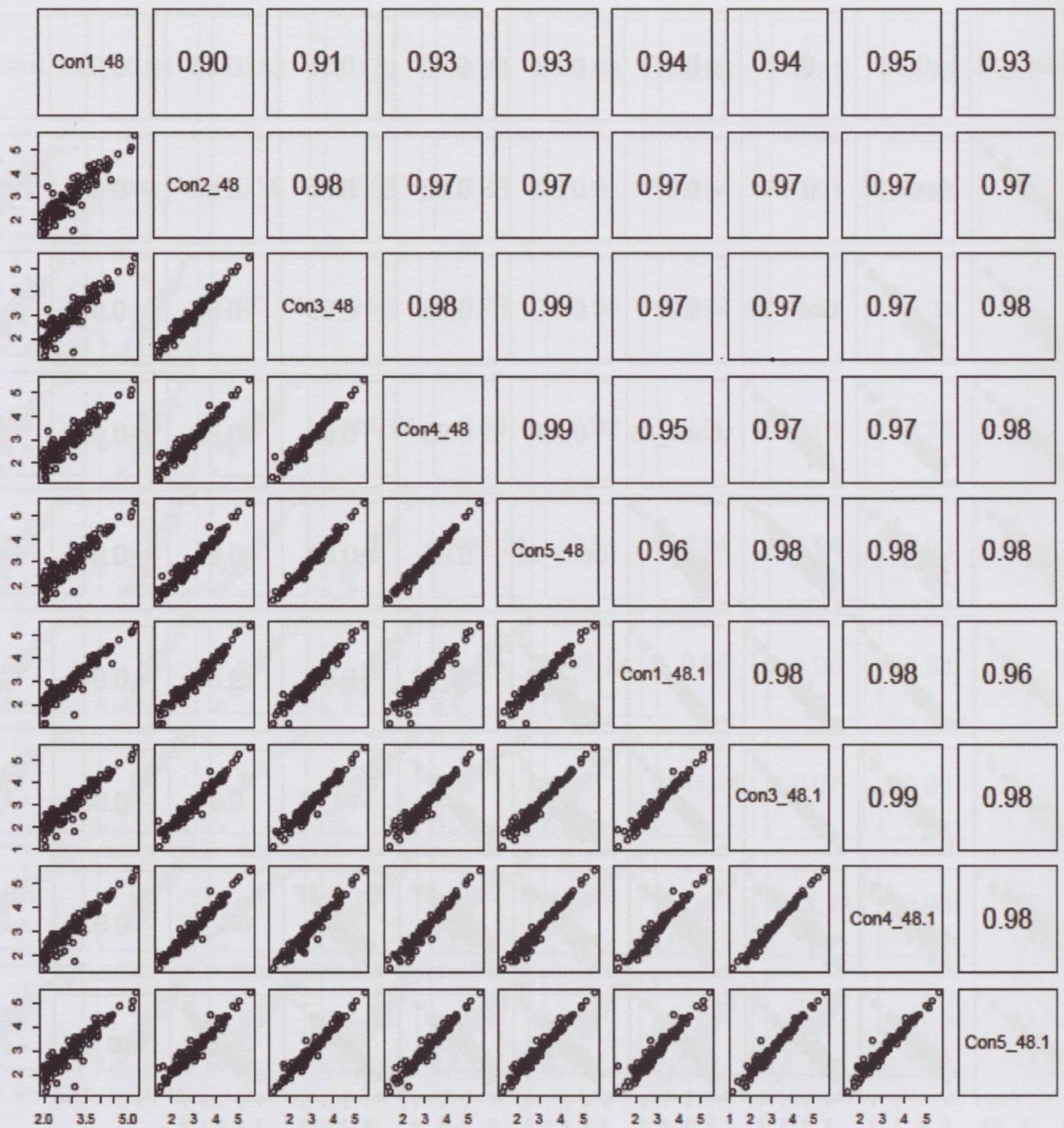
Correlation coefficients data for all blood 24 h samples. Note Con1_24hr = 24 h control replicate 1, Con2_24hr = 24 h control replicate 2, Con3_24hr = 24 h control replicate 3, Con4_24hr = 24 h control replicate 4, Con5_24hr = 24 h control replicate 5 and Inf1_24h = 24 h infected replicate 1, Inf3_24h = 24 h infected replicate 3, Inf4_24h = 24 h infected replicate 4 and Inf5_24h = 24 h infected replicate 5.



Correlation coefficients data for all blood 36 h samples. Note Con1_36hr = 36 h control replicate 1, Con2_36hr = 36 h control replicate 2, Con3_36hr = 36 h control replicate 3, Con4_36hr = 36 h control replicate 4, Con5_36hr = 36 h control replicate 5 and Inf1_36h = 36 h infected replicate 1, Inf3_36h = 36 h infected replicate 3, Inf4_36h = 36 h infected replicate 4 and Inf5_36h = 36 h infected replicate 5.



Correlation coefficients data for all blood 48 h samples. Note Con1_48hr = 48 h control replicate 1 Con2_48hr = 48 h control replicate 2, Con3_48hr = 48 h control replicate 3, Con4_48hr = 48 h contro replicate 4, Con5_48hr = 48 h control replicate 5 and Inf1_48h = 48 h infected replicate 1, Inf3_48h = 48 infected replicate 3, Inf4_48h = 48 h infected replicate 4 and Inf5_48h = 48 h infected replicate 5.



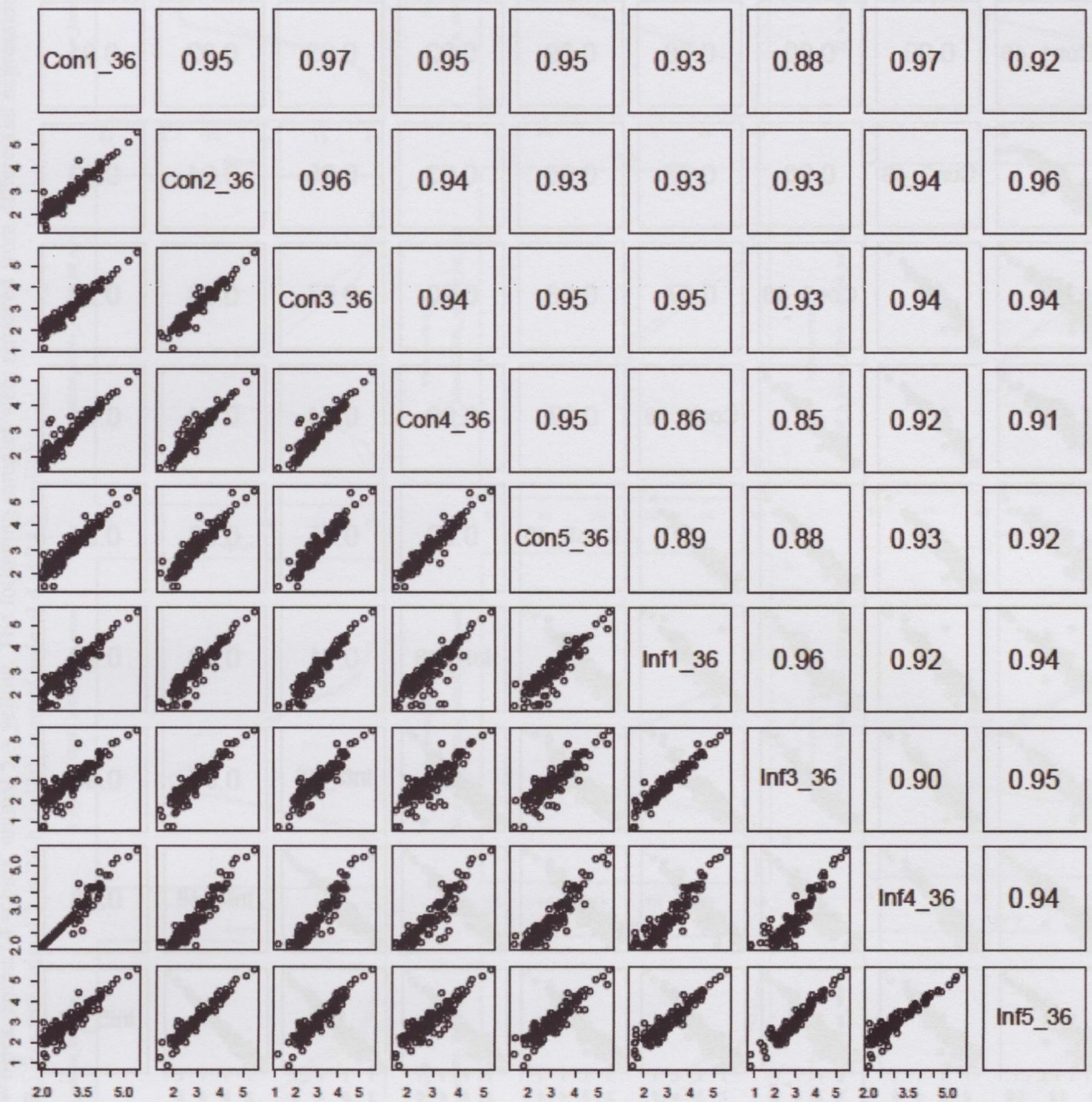
Correlation coefficients data for all 12 h milk samples. Note Con1_12hr = 12 h control replicate 1, Con2_12hr = 12 h control replicate 2, Con3_12hr = 12 h control replicate 3, Con4_12hr = 12 h control replicate 4, Con5_12hr = 12 h control replicate 5 and Inf1_12h = 12 h infected replicate 1, Inf3_12h = 12 h infected replicate 4, and Inf5_12h = 12 h infected replicate 5.



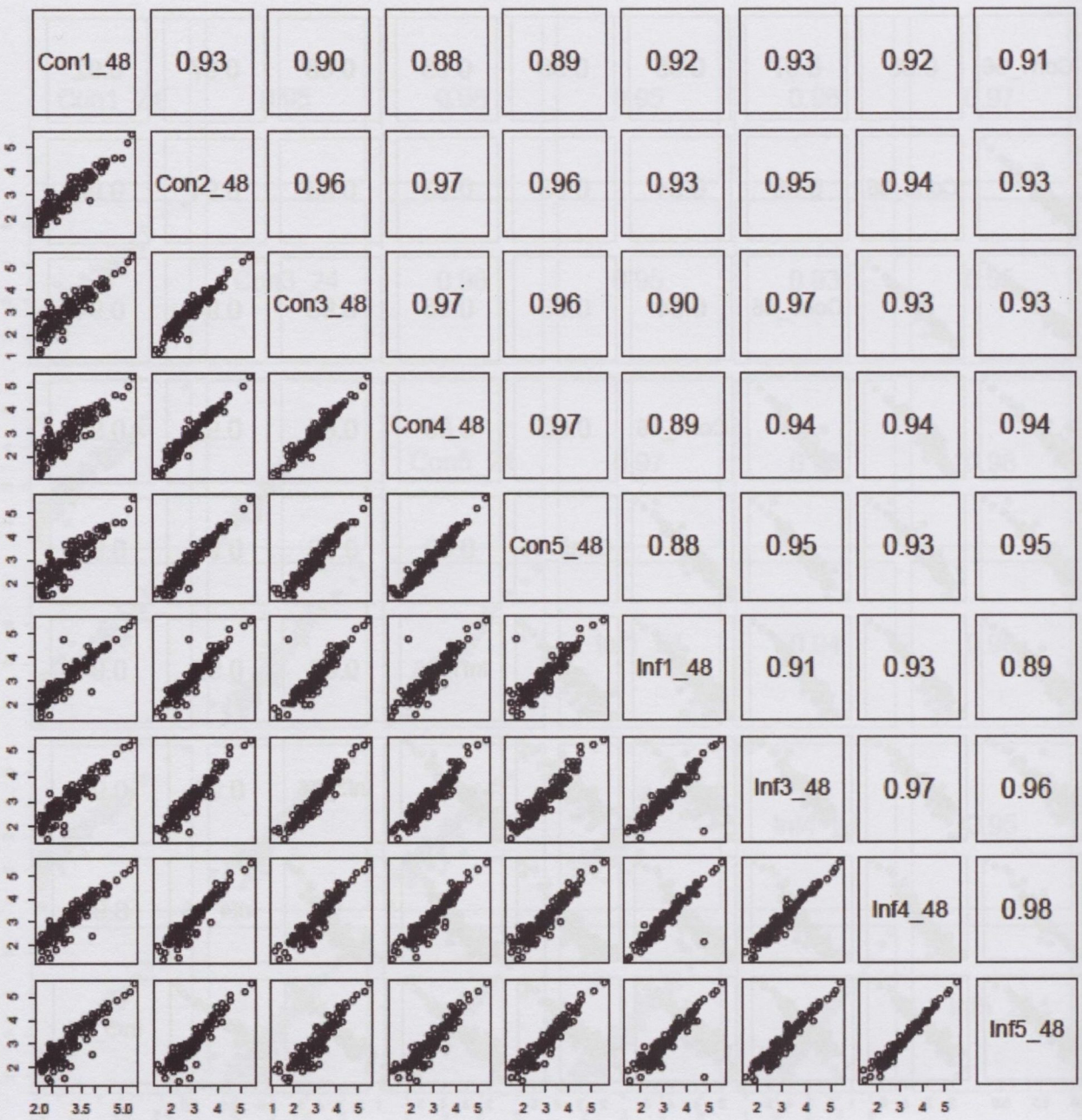
Correlation coefficients data for all milk 24 h samples. Note Con1_24hr = 24 h control replicate 1, Con4_24hr = 24 h control replicate 4, Con5_24hr = 24 h control replicate 5 and Inf1_24h = 24 h infected replicate 1, Inf4_24h = 24 h infected replicate 4 and Inf5_24h = 24 h infected replicate 5.



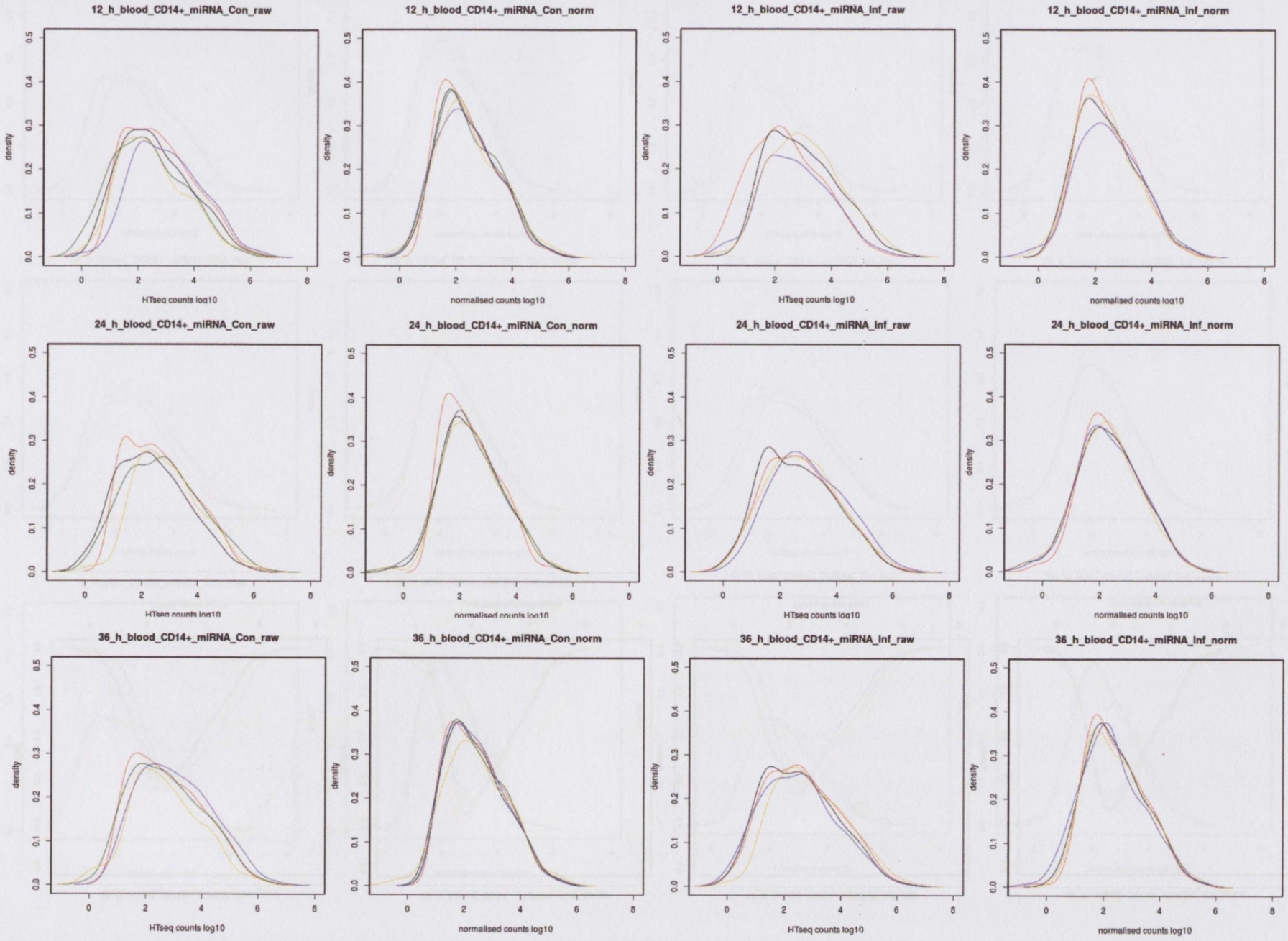
Correlation coefficients data for all milk 36 h samples. Note Con1_36hr = 36 h control replicate 1, Con2_36hr = 36 h control replicate 2, Con3_36hr = 36 h control replicate 3, Con4_36hr = 36 h control replicate 4, Con5_36hr = 36 h control replicate 5 and Inf1_36h = 36 h infected replicate 1, Inf3_36h = 36 h infected replicate 3, Inf4_36h = 36 h infected replicate 4 and Inf5_36h = 36 h infected replicate 5

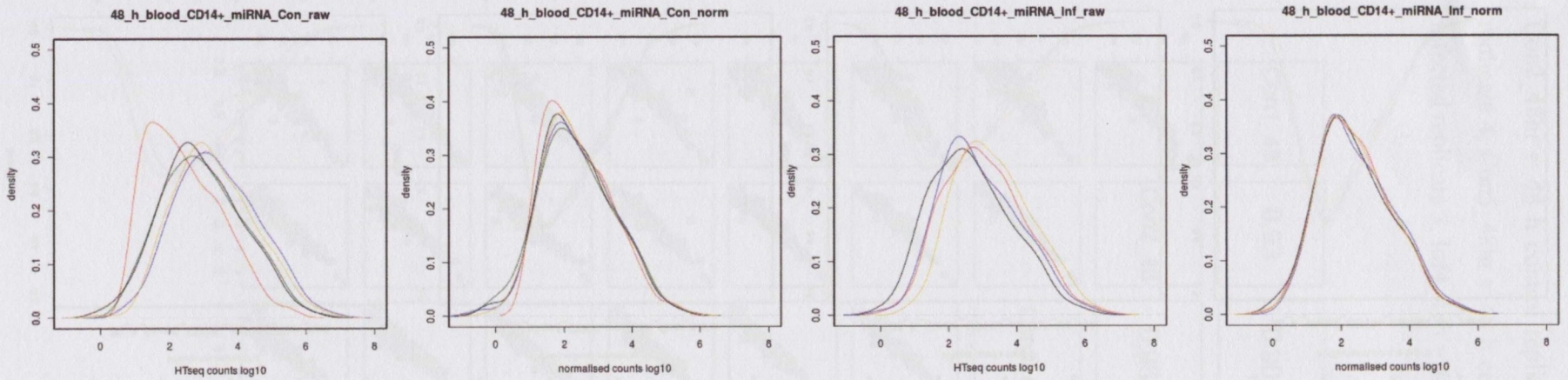


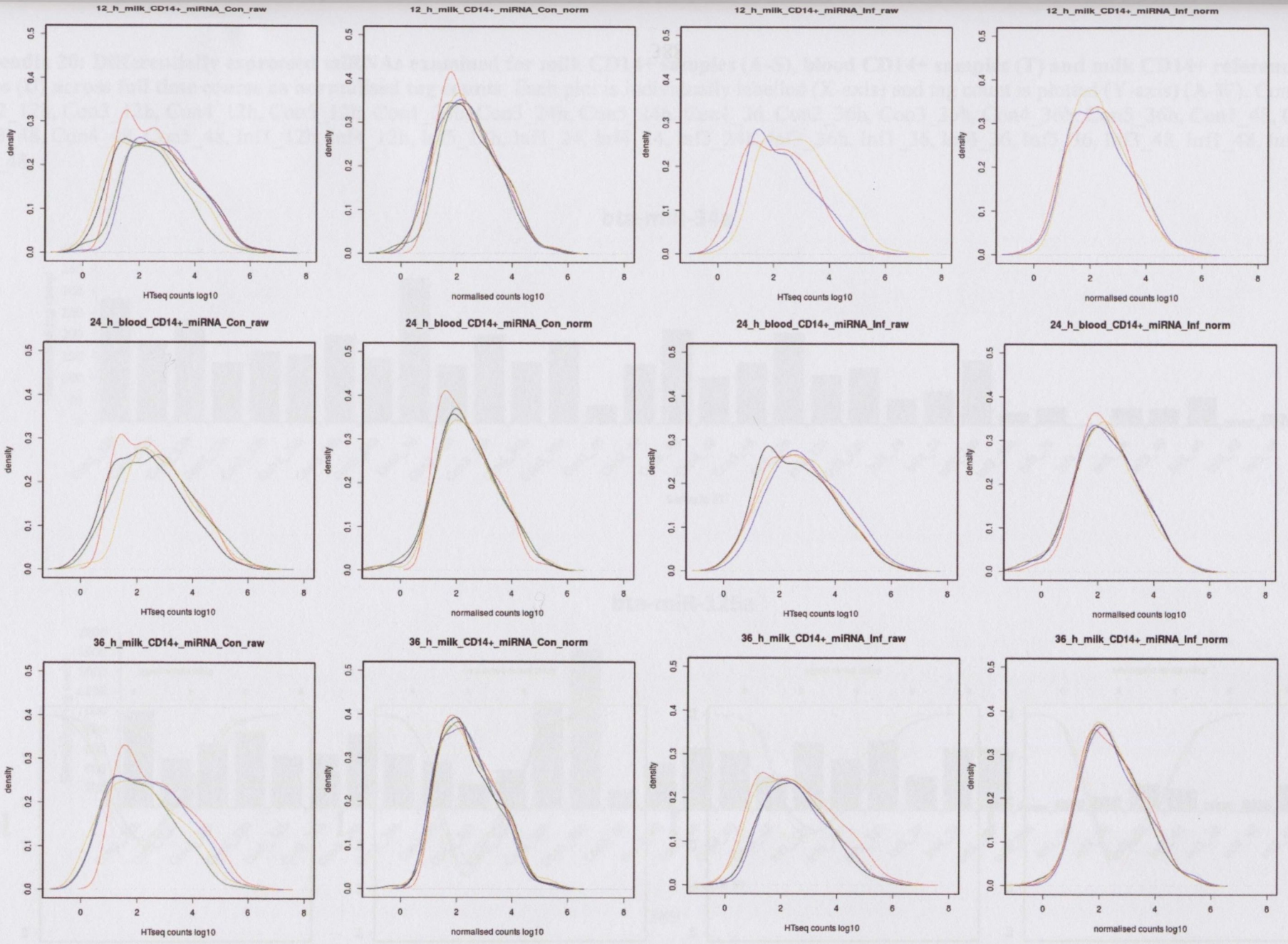
Correlation coefficients data for all milk 48 h samples. Note Con1_48hr = 48 h control replicate 1, Con2_48hr = 48 h control replicate 2, Con3_48hr = 48 h control replicate 3, Con4_48hr = 48 h control replicate 4, Con5_48hr = 48 h control replicate 5 and Inf1_48h = 48 h infected replicate 1, Inf3_48h = 48 h infected replicate 3, Inf4_48h = 48 h infected replicate 4 and Inf5_48h = 48 h infected replicate 5.

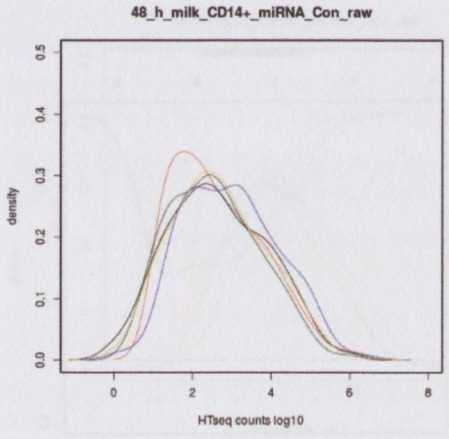


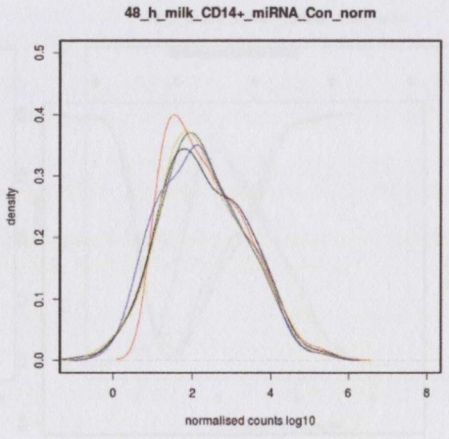
each sample. Each graph is individually labelled for time, tissue type, control/infected, and norm = "normalised reads" or HTseq = "raw reads". For samples/graphs sample animal 1 = red, animal 2 = green, and animal 3 = black, animal 4 = blue, animal 5 = yellow. The following samples were removed from differential expression analysis infected animal 2 all time-points, infected animal 3 at 12 h (milk), control animal 2 at 24 h (milk), control animal 4 at 24 h (milk).

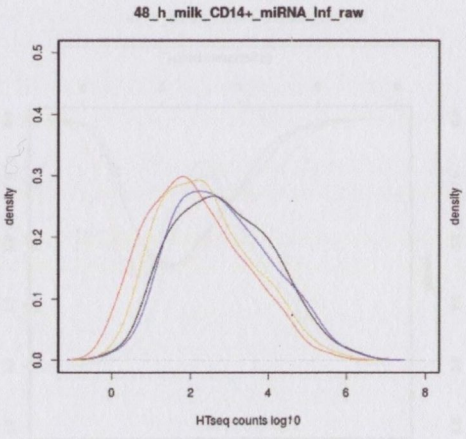


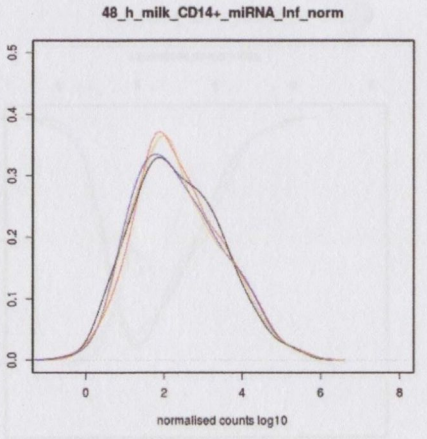

48_h_blood_CD14+_miRNA_Con_raw
density
HTseq counts log10
48_h_blood_CD14+_miRNA_Con_norm
density
normalised counts log10
48_h_blood_CD14+_miRNA_Inf_raw
density
HTseq counts log10
48_h_blood_CD14+_miRNA_Inf_norm
density
normalised counts log10


12_h_milk_CD14+_miRNA_Con_raw
12_h_milk_CD14+_miRNA_Con_norm
12_h_milk_CD14+_miRNA_Inf_raw
12_h_milk_CD14+_miRNA_Inf_norm
24_h_blood_CD14+_miRNA_Con_raw
24_h_blood_CD14+_miRNA_Con_norm
24_h_blood_CD14+_miRNA_Inf_raw
24_h_blood_CD14+_miRNA_Inf_norm
36_h_milk_CD14+_miRNA_Con_raw
36_h_milk_CD14+_miRNA_Con_norm
36_h_milk_CD14+_miRNA_Inf_raw
36_h_milk_CD14+_miRNA_Inf_norm
density
HTseq counts log10
normalised counts log10


48_h_milk_CD14+_miRNA_Con_raw
density
HTseq counts log10

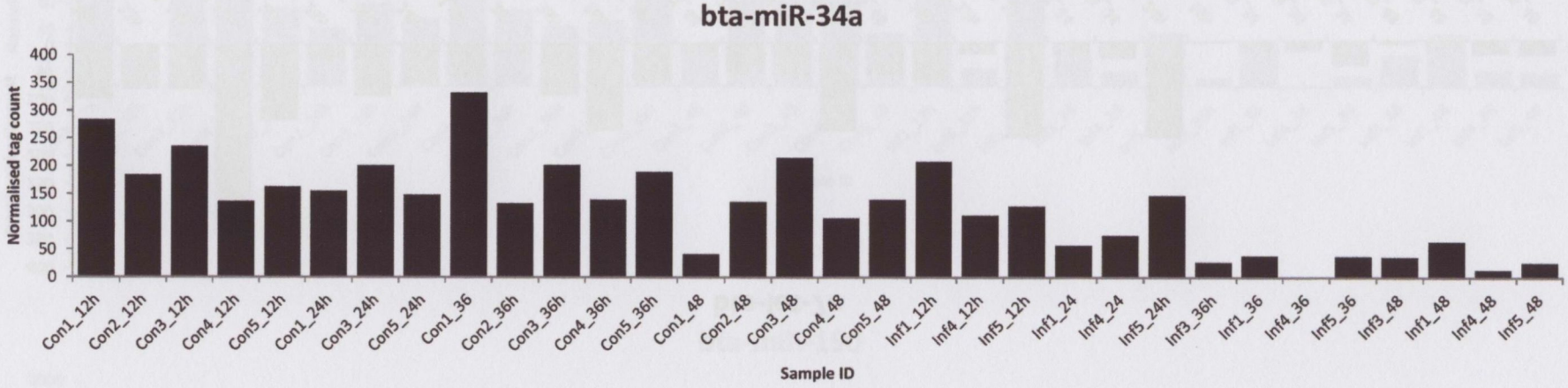

48_h_milk_CD14+_miRNA_Con_norm
density
normalised counts log10


48_h_milk_CD14+_miRNA_Inf_raw
density
HTseq counts log10

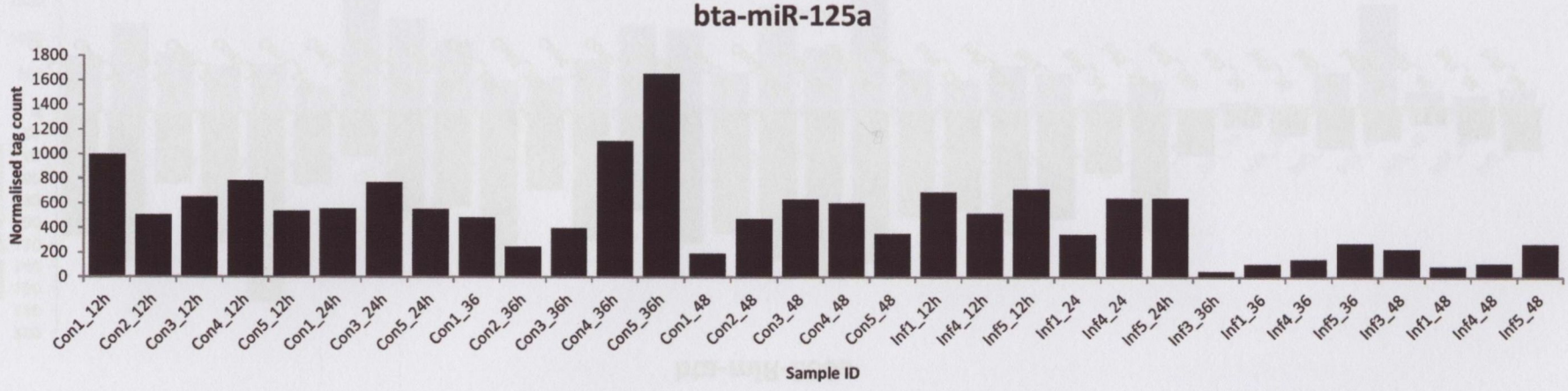

48_h_milk_CD14+_miRNA_Inf_norm
density
normalised counts log10

Appendix 20: Differentially expressed miRNAs examined for milk CD14+ samples (A-S), blood CD14+ samples (T) and milk CD14+ reference miRNA genes (U) across full time course as normalised tag counts. Each plot is individually labelled (X-axis) and tag count is plotted (Y-axis) (A-W). Con1_12h, Con2_12h, Con3_12h, Con4_12h, Con5_12h, Con1_24h, Con3_24h, Con5_24h, Con1_36, Con2_36h, Con3_36h, Con4_36h, Con5_36h, Con1_48, Con2_48, Con3_48, Con4_48, Con5_48, Inf1_12h, Inf4_12h, Inf5_12h, Inf1_24, Inf4_24, Inf5_24h, Inf3_36h, Inf1_36, Inf4_36, Inf5_36, Inf3_48, Inf1_48, Inf4_48, Inf5_48

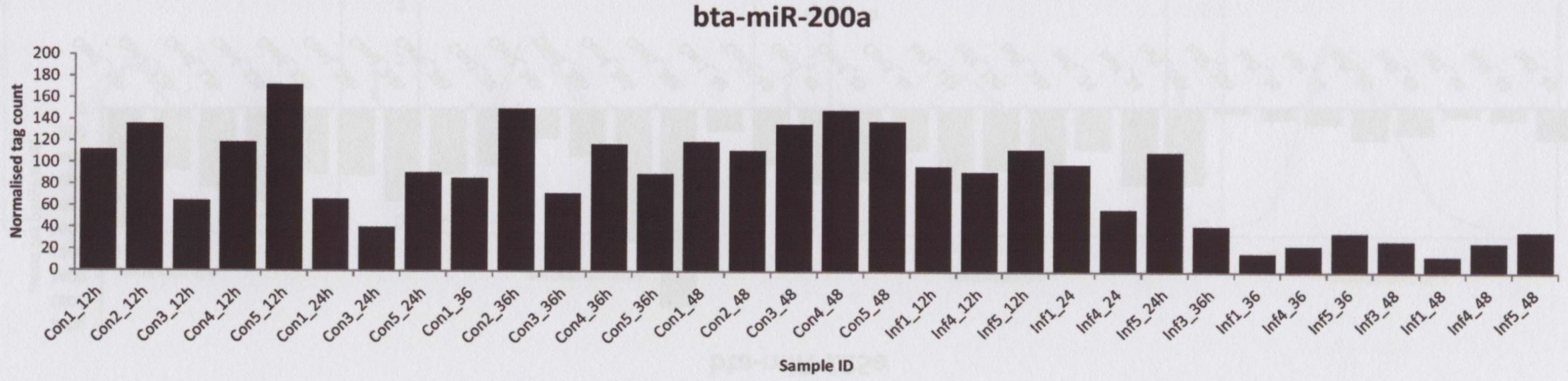
A



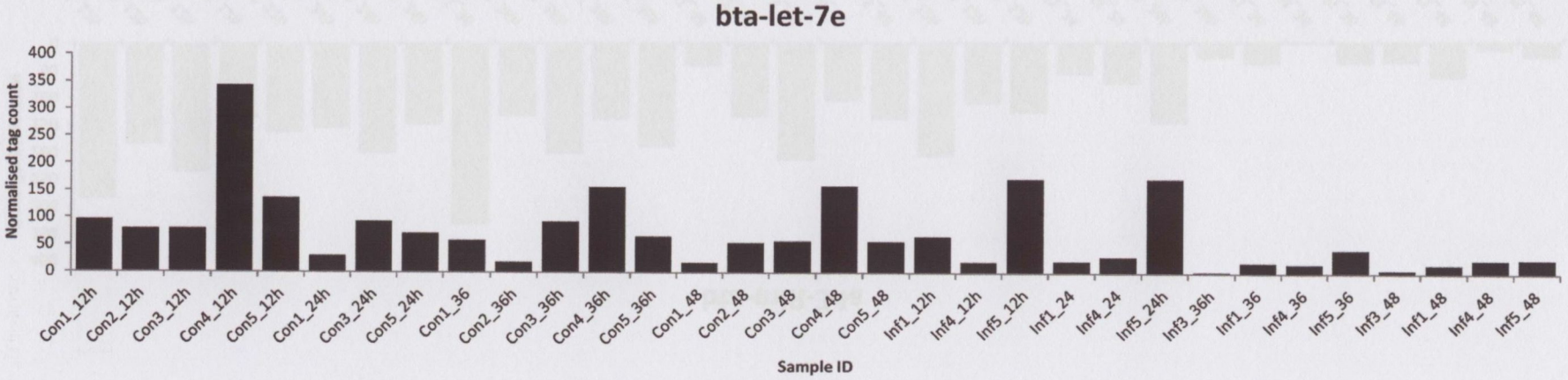
B



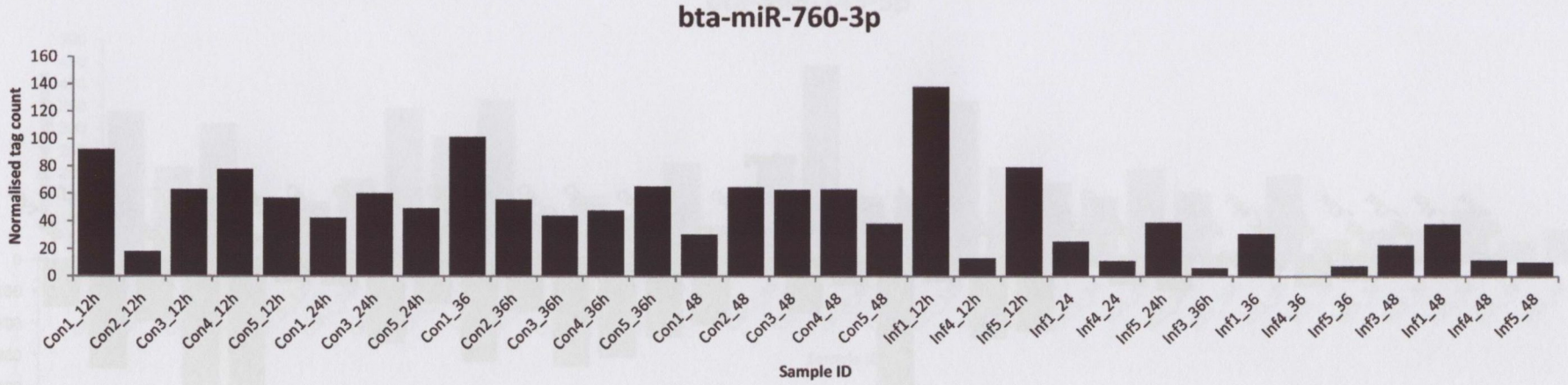
C


bta-miR-200a
Normalised tag count
200
180
160
140
120
100
80
60
40
20
0
Con1_12h
Con2_12h
Con3_12h
Con4_12h
Con5_12h
Con1_24h
Con3_24h
Con5_24h
Con1_36
Con2_36h
Con3_36h
Con4_36h
Con5_36h
Con1_48
Con2_48
Con3_48
Con4_48
Con5_48
Inf1_12h
Inf4_12h
Inf5_12h
Inf1_24
Inf4_24
Inf5_24h
Inf3_36h
Inf1_36
Inf4_36
Inf5_36
Inf3_48
Inf1_48
Inf4_48
Inf5_48
Sample ID

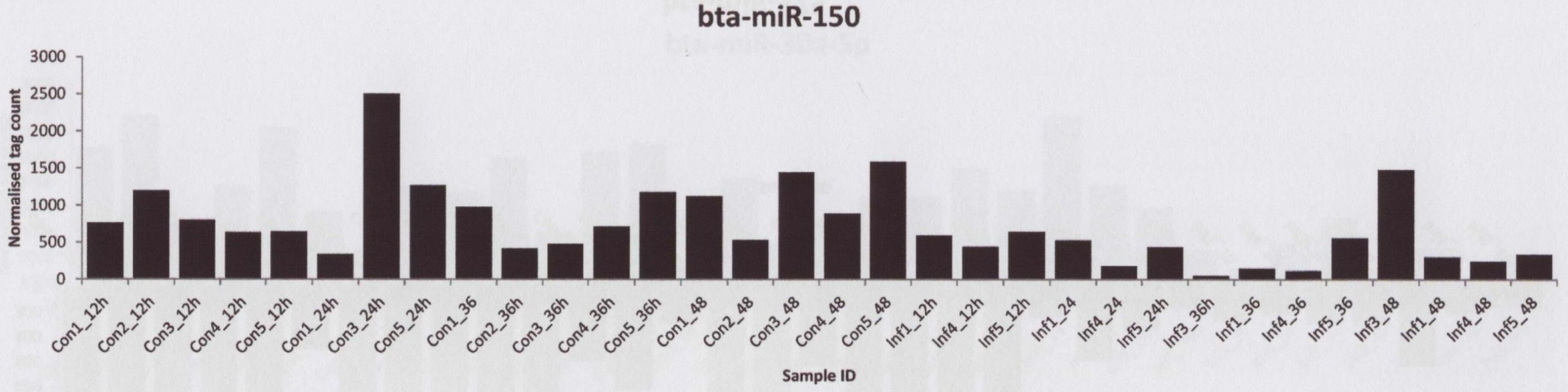
D


bta-let-7e
Normalised tag count
400
350
300
250
200
150
100
50
0
Con1_12h
Con2_12h
Con3_12h
Con4_12h
Con5_12h
Con1_24h
Con3_24h
Con5_24h
Con1_36
Con2_36h
Con3_36h
Con4_36h
Con5_36h
Con1_48
Con2_48
Con3_48
Con4_48
Con5_48
Inf1_12h
Inf4_12h
Inf5_12h
Inf1_24
Inf4_24
Inf5_24h
Inf3_36h
Inf1_36
Inf4_36
Inf5_36
Inf3_48
Inf1_48
Inf4_48
Inf5_48
Sample ID

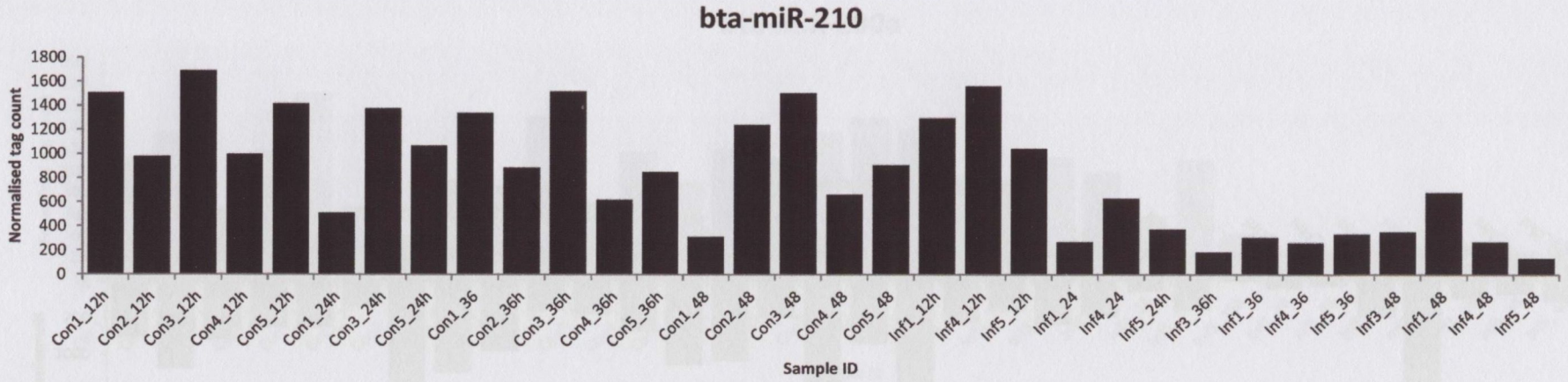
E


bta-miR-760-3p
Normalised tag count
160
140
120
100
80
60
40
20
0
Con1_12h
Con2_12h
Con3_12h
Con4_12h
Con5_12h
Con1_24h
Con3_24h
Con5_24h
Con1_36
Con2_36h
Con3_36h
Con4_36h
Con5_36h
Con1_48
Con2_48
Con3_48
Con4_48
Con5_48
Inf1_12h
Inf4_12h
Inf5_12h
Inf1_24
Inf4_24
Inf5_24h
Inf3_36h
Inf1_36
Inf4_36
Inf5_36
Inf3_48
Inf1_48
Inf4_48
Inf5_48
Sample ID

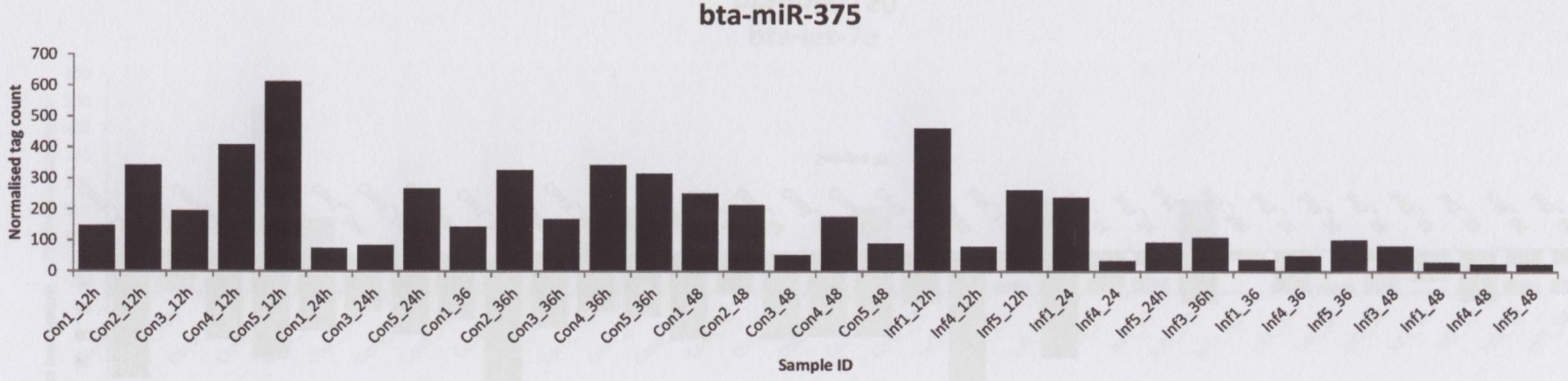
F


bta-miR-150
Normalised tag count
3000
2500
2000
1500
1000
500
0
Con1_12h
Con2_12h
Con3_12h
Con4_12h
Con5_12h
Con1_24h
Con3_24h
Con5_24h
Con1_36
Con2_36h
Con3_36h
Con4_36h
Con5_36h
Con1_48
Con2_48
Con3_48
Con4_48
Con5_48
Inf1_12h
Inf4_12h
Inf5_12h
Inf1_24
Inf4_24
Inf5_24h
Inf3_36h
Inf1_36
Inf4_36
Inf5_36
Inf3_48
Inf1_48
Inf4_48
Inf5_48
Sample ID

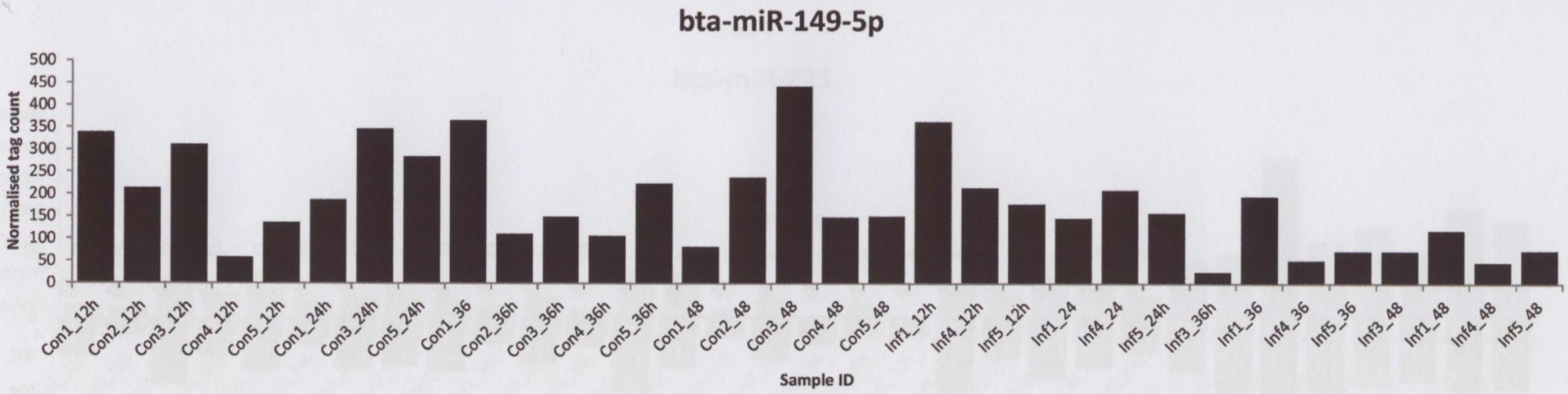
G


bta-miR-210
Normalised tag count
1800
1600
1400
1200
1000
800
600
400
200
0
Con1_12h
Con2_12h
Con3_12h
Con4_12h
Con5_12h
Con1_24h
Con3_24h
Con5_24h
Con1_36
Con2_36h
Con3_36h
Con4_36h
Con5_36h
Con1_48
Con2_48
Con3_48
Con4_48
Con5_48
Inf1_12h
Inf4_12h
Inf5_12h
Inf1_24
Inf4_24
Inf5_24h
Inf3_36h
Inf1_36
Inf4_36
Inf5_36
Inf3_48
Inf1_48
Inf4_48
Inf5_48
Sample ID

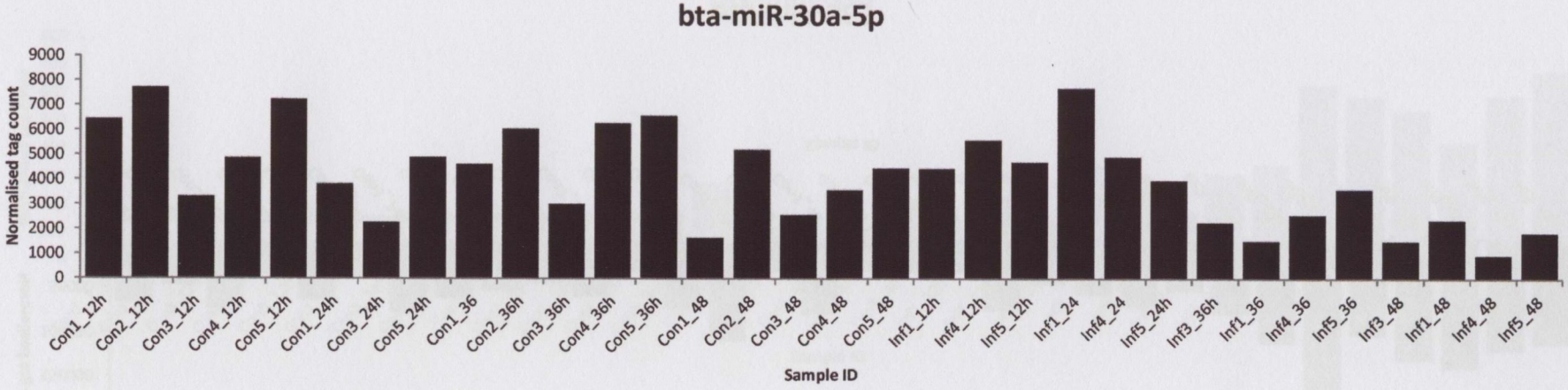
H


bta-miR-375
Normalised tag count
700
600
500
400
300
200
100
0
Con1_12h
Con2_12h
Con3_12h
Con4_12h
Con5_12h
Con1_24h
Con3_24h
Con5_24h
Con1_36
Con2_36h
Con3_36h
Con4_36h
Con5_36h
Con1_48
Con2_48
Con3_48
Con4_48
Con5_48
Inf1_12h
Inf4_12h
Inf5_12h
Inf1_24
Inf4_24
Inf5_24h
Inf3_36h
Inf1_36
Inf4_36
Inf5_36
Inf3_48
Inf1_48
Inf4_48
Inf5_48
Sample ID

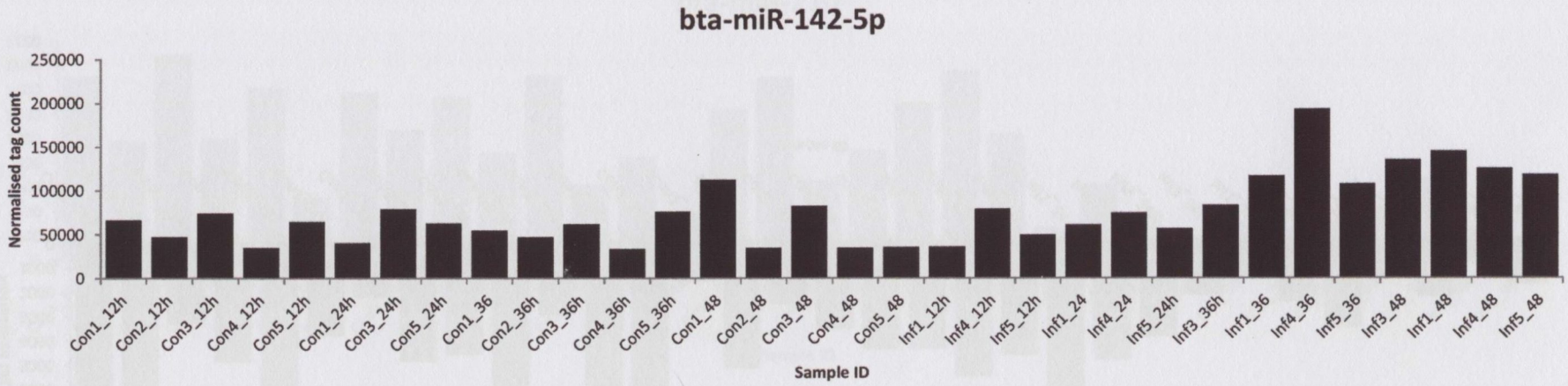
I


bta-miR-149-5p
Normalised tag count
500
450
400
350
300
250
200
150
100
50
0
Con1_12h
Con2_12h
Con3_12h
Con4_12h
Con5_12h
Con1_24h
Con3_24h
Con5_24h
Con1_36
Con2_36h
Con3_36h
Con4_36h
Con5_36h
Con1_48
Con2_48
Con3_48
Con4_48
Con5_48
Inf1_12h
Inf4_12h
Inf5_12h
Inf1_24
Inf4_24
Inf5_24h
Inf3_36h
Inf1_36
Inf4_36
Inf5_36
Inf3_48
Inf1_48
Inf4_48
Inf5_48
Sample ID

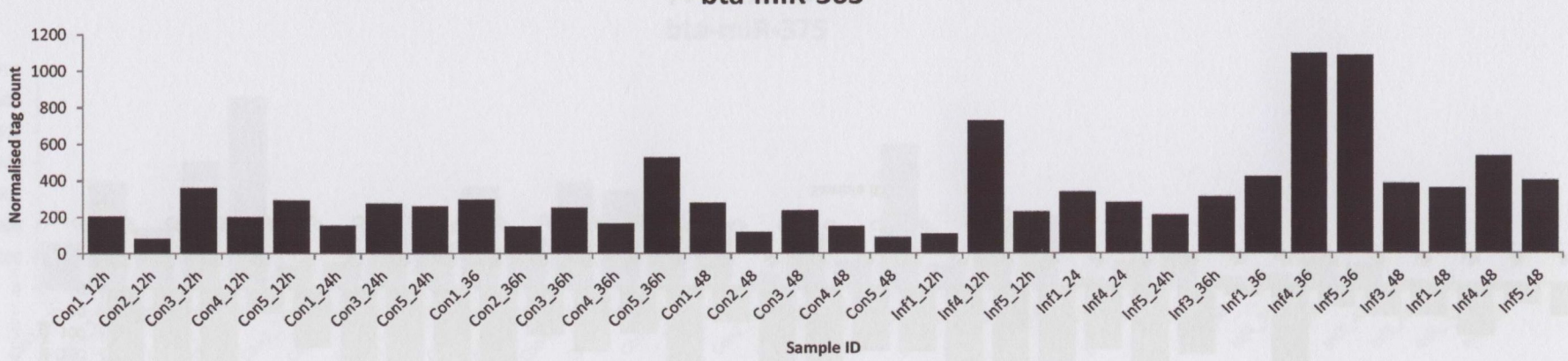
J


bta-miR-30a-5p
Normalised tag count
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
Con1_12h
Con2_12h
Con3_12h
Con4_12h
Con5_12h
Con1_24h
Con3_24h
Con5_24h
Con1_36
Con2_36h
Con3_36h
Con4_36h
Con5_36h
Con1_48
Con2_48
Con3_48
Con4_48
Con5_48
Inf1_12h
Inf4_12h
Inf5_12h
Inf1_24
Inf4_24
Inf5_24h
Inf3_36h
Inf1_36
Inf4_36
Inf5_36
Inf3_48
Inf1_48
Inf4_48
Inf5_48
Sample ID

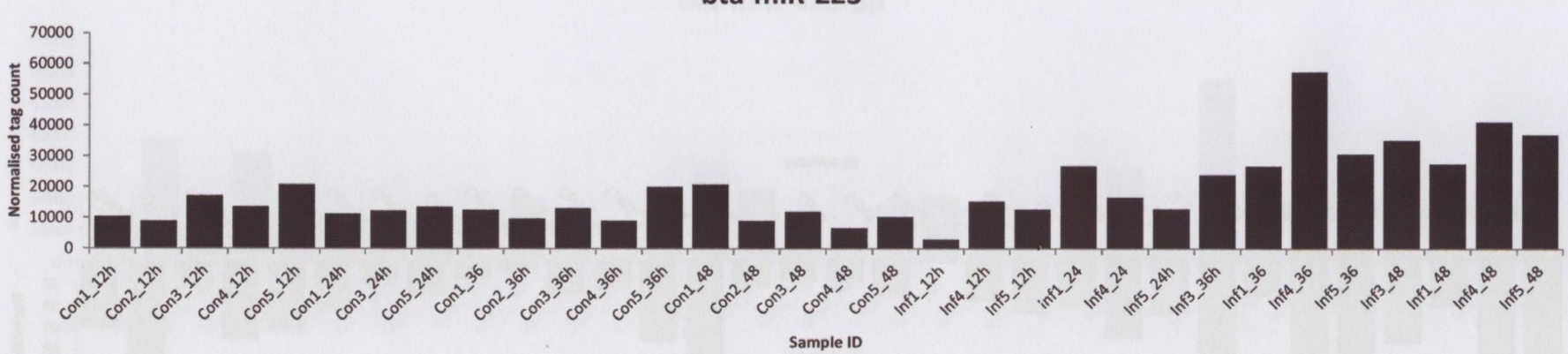
K


bta-miR-142-5p
Normalised tag count
250000
200000
150000
100000
50000
0
Con1_12h
Con2_12h
Con3_12h
Con4_12h
Con5_12h
Con1_24h
Con3_24h
Con5_24h
Con1_36
Con2_36h
Con3_36h
Con4_36h
Con5_36h
Con1_48
Con2_48
Con3_48
Con4_48
Con5_48
Inf1_12h
Inf4_12h
Inf5_12h
Inf1_24
Inf4_24
Inf5_24h
Inf3_36h
Inf1_36
Inf4_36
Inf5_36
Inf3_48
Inf1_48
Inf4_48
Inf5_48
Sample ID

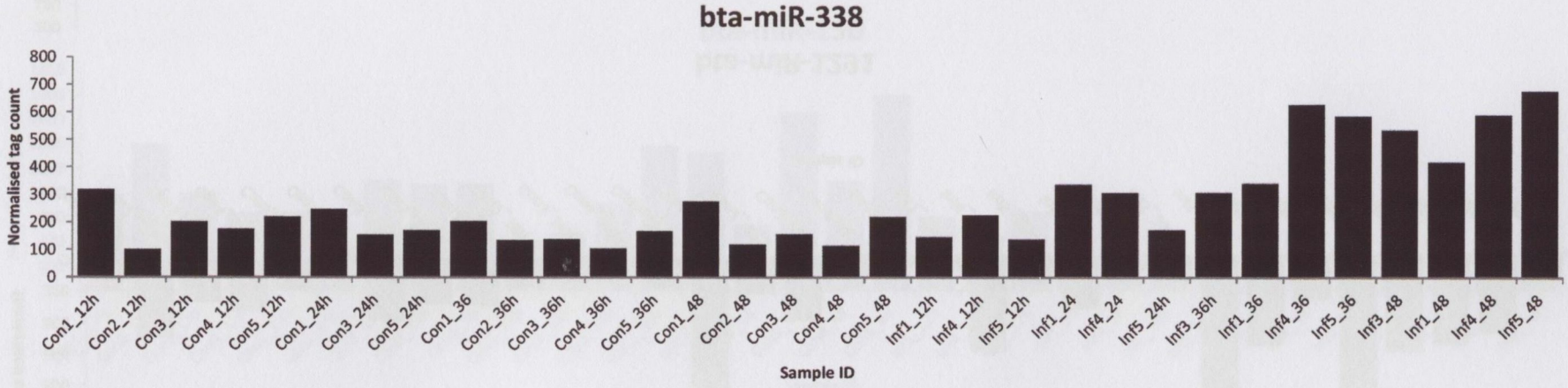
L


bta-miR-363
Normalised tag count
1200
1000
800
600
400
200
0
Con1_12h
Con2_12h
Con3_12h
Con4_12h
Con5_12h
Con1_24h
Con3_24h
Con5_24h
Con1_36
Con2_36h
Con3_36h
Con4_36h
Con5_36h
Con1_48
Con2_48
Con3_48
Con4_48
Con5_48
Inf1_12h
Inf4_12h
Inf5_12h
Inf1_24
Inf4_24
Inf5_24h
Inf3_36h
Inf1_36
Inf4_36
Inf5_36
Inf3_48
Inf1_48
Inf4_48
Inf5_48
Sample ID

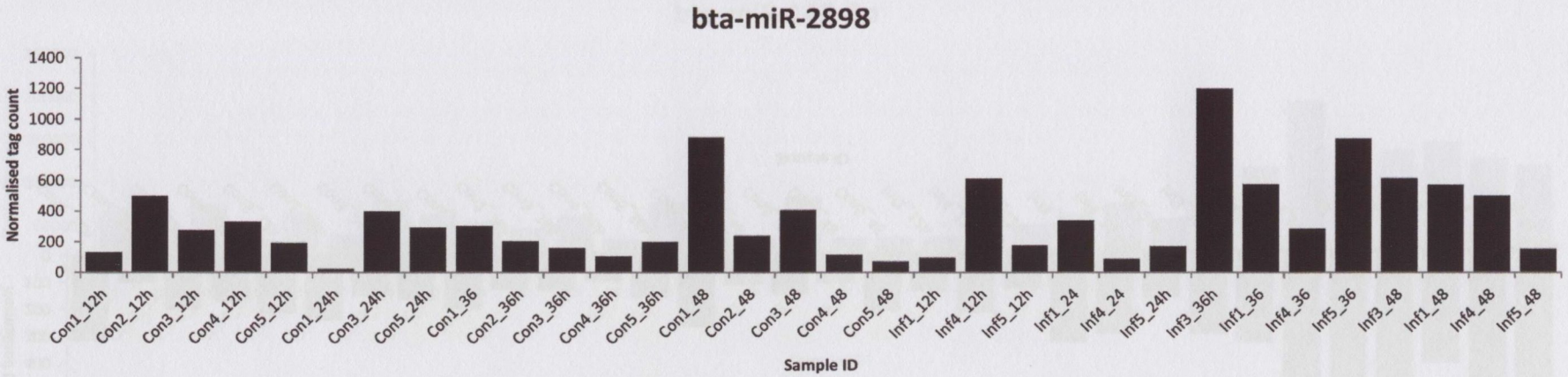
M


bta-miR-223
Normalised tag count
70000
60000
50000
40000
30000
20000
10000
0
Con1_12h
Con2_12h
Con3_12h
Con4_12h
Con5_12h
Con1_24h
Con3_24h
Con5_24h
Con1_36
Con2_36h
Con3_36h
Con4_36h
Con5_36h
Con1_48
Con2_48
Con3_48
Con4_48
Con5_48
Inf1_12h
Inf4_12h
Inf5_12h
inf1_24
Inf4_24
Inf5_24h
Inf3_36h
Inf1_36
Inf4_36
Inf5_36
Inf3_48
Inf1_48
Inf4_48
Inf5_48
Sample ID

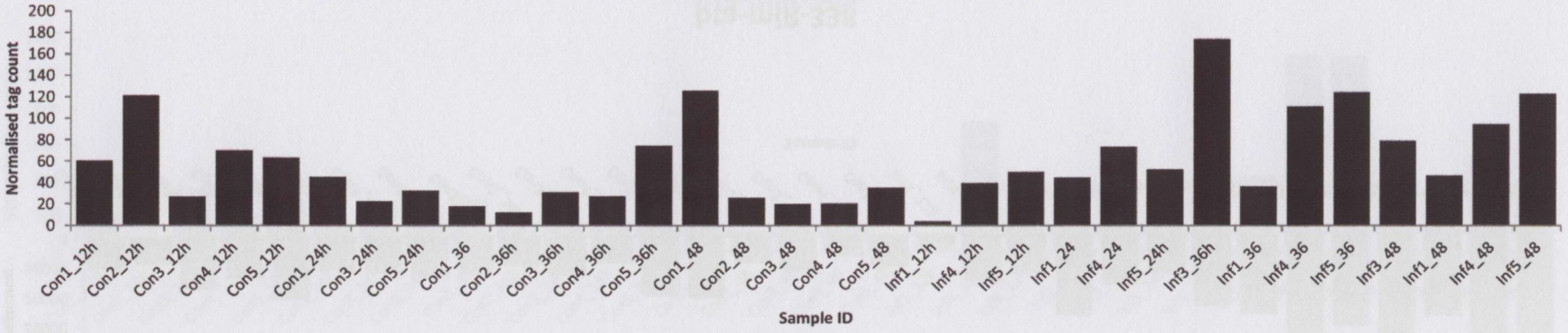
N


bta-miR-338
Normalised tag count
800
700
600
500
400
300
200
100
0
Con1_12h
Con2_12h
Con3_12h
Con4_12h
Con5_12h
Con1_24h
Con3_24h
Con5_24h
Con1_36
Con2_36h
Con3_36h
Con4_36h
Con5_36h
Con1_48
Con2_48
Con3_48
Con4_48
Con5_48
Inf1_12h
Inf4_12h
Inf5_12h
Inf1_24
Inf4_24
Inf5_24h
Inf3_36h
Inf1_36
Inf4_36
Inf5_36
Inf3_48
Inf1_48
Inf4_48
Inf5_48
Sample ID

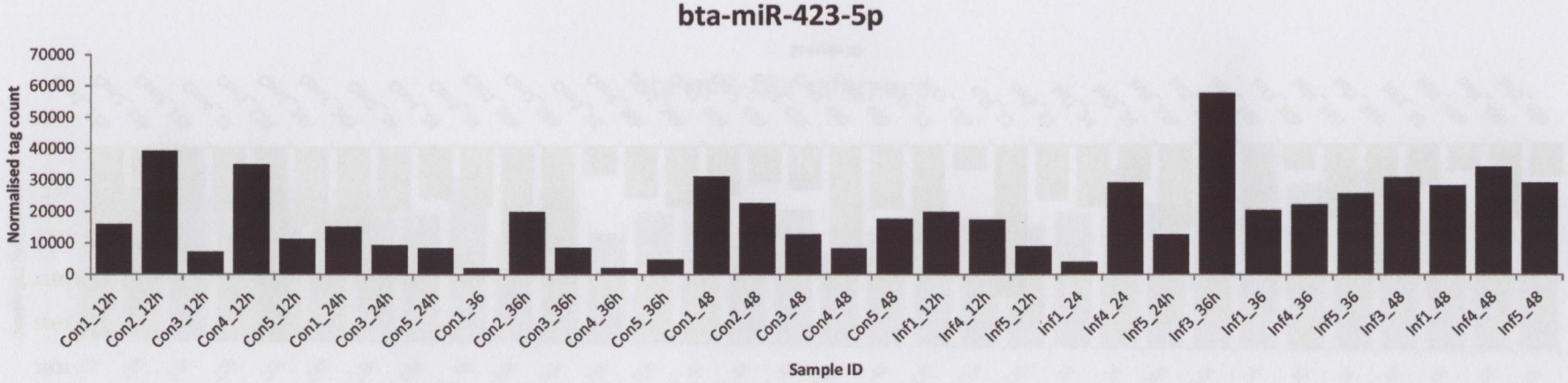
O


bta-miR-2898
Normalised tag count
1400
1200
1000
800
600
400
200
0
Con1_12h
Con2_12h
Con3_12h
Con4_12h
Con5_12h
Con1_24h
Con3_24h
Con5_24h
Con1_36
Con2_36h
Con3_36h
Con4_36h
Con5_36h
Con1_48
Con2_48
Con3_48
Con4_48
Con5_48
Inf1_12h
Inf4_12h
Inf5_12h
Inf1_24
Inf4_24
Inf5_24h
Inf3_36h
Inf1_36
Inf4_36
Inf5_36
Inf3_48
Inf1_48
Inf4_48
Inf5_48
Sample ID

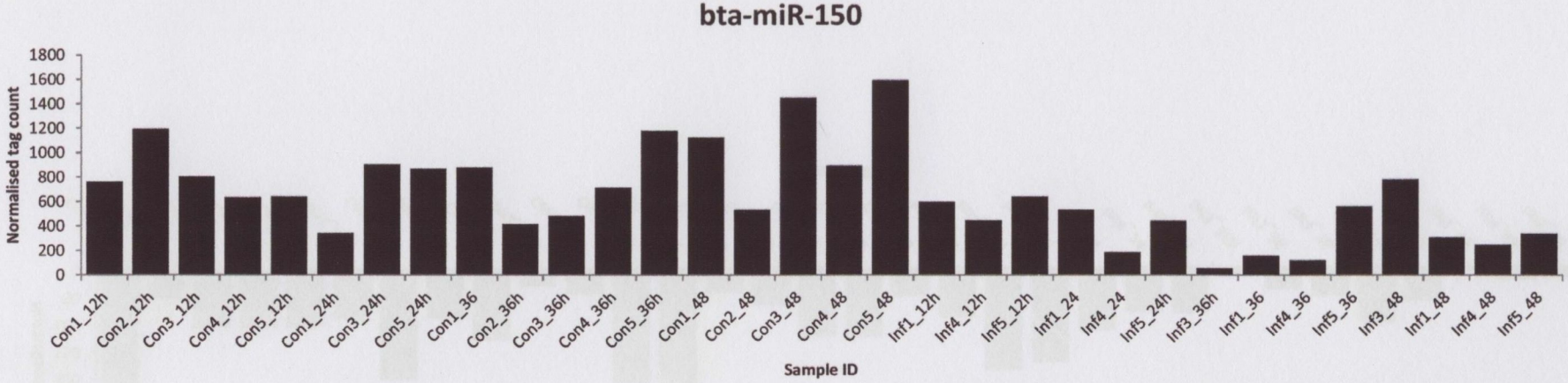
P


bta-miR-1291
Normalised tag count
200
180
160
140
120
100
80
60
40
20
0
Con1_12h
Con2_12h
Con3_12h
Con4_12h
Con5_12h
Con1_24h
Con3_24h
Con5_24h
Con1_36
Con2_36h
Con3_36h
Con4_36h
Con5_36h
Con1_48
Con2_48
Con3_48
Con4_48
Con5_48
Inf1_12h
Inf4_12h
Inf5_12h
Inf1_24
Inf4_24
Inf5_24h
Inf3_36h
Inf1_36
Inf4_36
Inf5_36
Inf3_48
Inf1_48
Inf4_48
Inf5_48
Sample ID

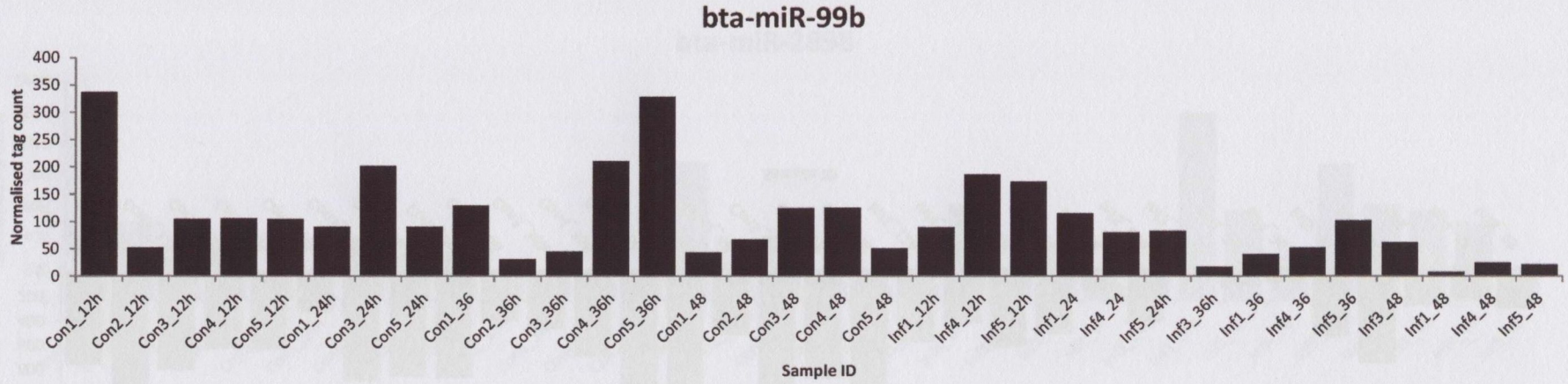
Q


bta-miR-423-5p
Normalised tag count
70000
60000
50000
40000
30000
20000
10000
0
Con1_12h
Con2_12h
Con3_12h
Con4_12h
Con5_12h
Con1_24h
Con3_24h
Con5_24h
Con1_36
Con2_36h
Con3_36h
Con4_36h
Con5_36h
Con1_48
Con2_48
Con3_48
Con4_48
Con5_48
Inf1_12h
Inf4_12h
Inf5_12h
Inf1_24
Inf4_24
Inf5_24h
Inf3_36h
Inf1_36
Inf4_36
Inf5_36
Inf3_48
Inf1_48
Inf4_48
Inf5_48
Sample ID

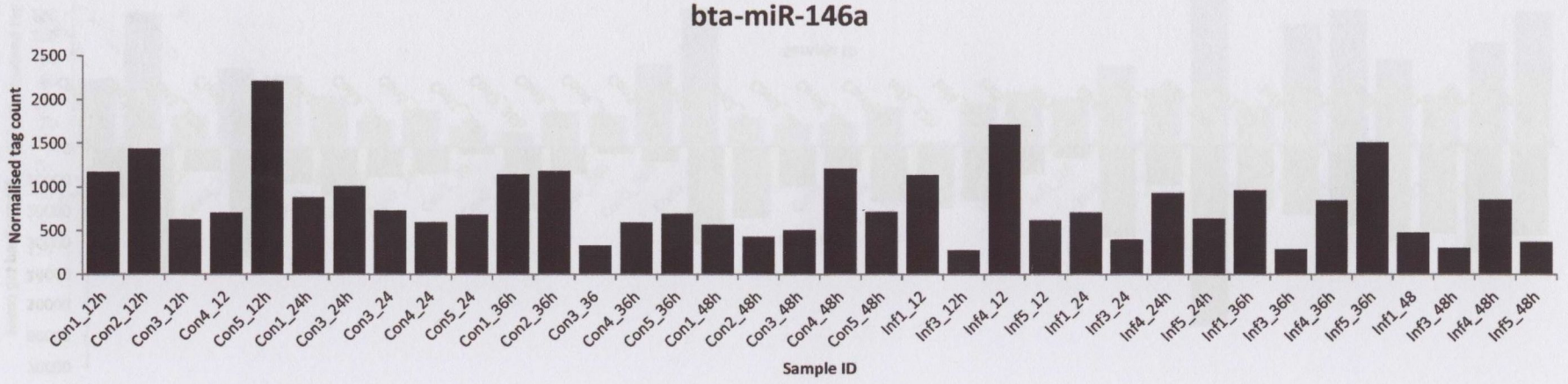
R


bta-miR-150
Normalised tag count
1800
1600
1400
1200
1000
800
600
400
200
0
Con1_12h
Con2_12h
Con3_12h
Con4_12h
Con5_12h
Con1_24h
Con3_24h
Con5_24h
Con1_36
Con2_36h
Con3_36h
Con4_36h
Con5_36h
Con1_48
Con2_48
Con3_48
Con4_48
Con5_48
Inf1_12h
Inf4_12h
Inf5_12h
Inf1_24
Inf4_24
Inf5_24h
Inf3_36h
Inf1_36
Inf4_36
Inf5_36
Inf3_48
Inf1_48
Inf4_48
Inf5_48
Sample ID

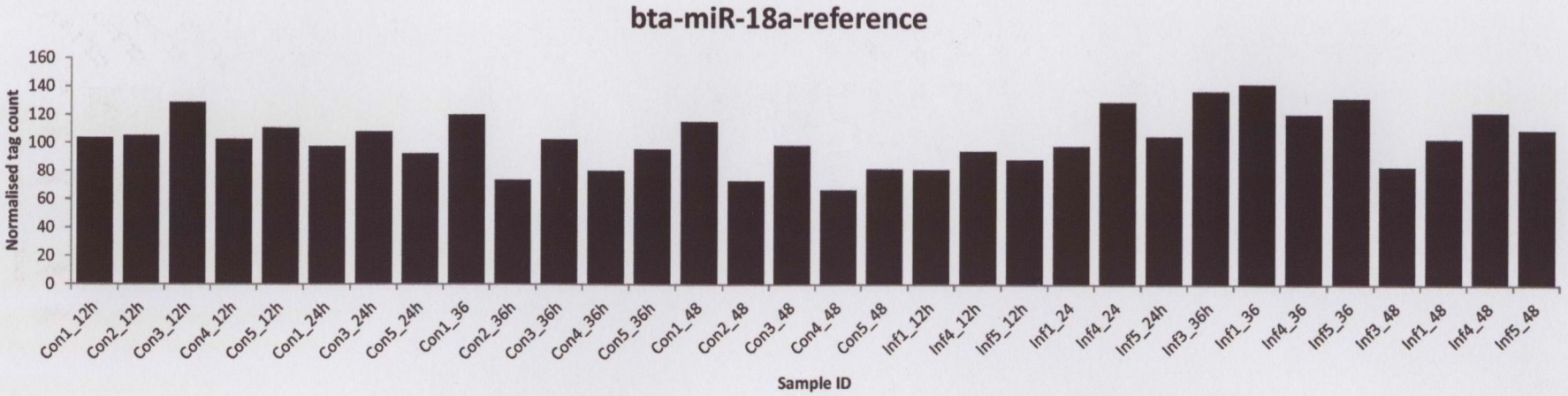
S


bta-miR-99b
Normalised tag count
400
350
300
250
200
150
100
50
0
Con1_12h
Con2_12h
Con3_12h
Con4_12h
Con5_12h
Con1_24h
Con3_24h
Con5_24h
Con1_36
Con2_36h
Con3_36h
Con4_36h
Con5_36h
Con1_48
Con2_48
Con3_48
Con4_48
Con5_48
Inf1_12h
Inf4_12h
Inf5_12h
Inf1_24
Inf4_24
Inf5_24h
Inf3_36h
Inf1_36
Inf4_36
Inf5_36
Inf3_48
Inf1_48
Inf4_48
Inf5_48
Sample ID

T


bta-miR-146a
Normalised tag count
2500
2000
1500
1000
500
0
Con1_12h
Con2_12h
Con3_12h
Con4_12
Con5_12h
Con1_24h
Con3_24h
Con3_24
Con4_24
Con5_24
Con1_36h
Con2_36h
Con3_36
Con4_36h
Con5_36h
Con1_48h
Con2_48h
Con3_48h
Con4_48h
Con5_48h
Inf1_12
Inf3_12h
Inf4_12
Inf5_12
Inf1_24
Inf3_24
Inf4_24h
Inf5_24h
Inf1_36h
Inf3_36h
Inf4_36h
Inf5_36h
Inf1_48
Inf3_48h
Inf4_48h
Inf5_48h
Sample ID

U


bta-miR-18a-reference
Normalised tag count
160
140
120
100
80
60
40
20
0
Con1_12h
Con2_12h
Con3_12h
Con4_12h
Con5_12h
Con1_24h
Con3_24h
Con5_24h
Con1_36
Con2_36h
Con3_36h
Con4_36h
Con5_36h
Con1_48
Con2_48
Con3_48
Con4_48
Con5_48
Inf1_12h
Inf4_12h
Inf5_12h
Inf1_24
Inf4_24
Inf5_24h
Inf3_36h
Inf1_36
Inf4_36
Inf5_36
Inf3_48
Inf1_48
Inf4_48
Inf5_48
Sample ID