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Development of a high-throughput platform for evaluation of chicken immune responses

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Author's declaration

I declare that the work in this dissertation is original except where indicated by special reference in the text and no part of the dissertation has been submitted for any other degree.

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Abstract

The poultry industry has successfully applied breeding and production programmes to meet growing consumer demands for chicken meat and eggs. Over the last four decades, poultry breeders have selected birds not only for productivity, but also for improved health, welfare, fitness and environmental robustness. Intensive production settings contribute to faster spread of diseases and greater losses in production due to increased morbidity and mortality of the flock. Traditional methods of disease treatment and prevention have played a critical role in control of disease. However, growing resistance of pathogens to therapeutic measures and consumer concerns led to the withdrawal of antibiotics as growth promoting additives in chicken feed. In addition, some vaccines have been overcome by increasing variation and virulence of pathogens and are no longer successful in disease prevention. The emergence of virulent and drug resistant pathogens have emphasised the need to focus on other solutions to disease, particularly natural genetic resistance. Genetic loci or gene expression patterns associated with the differential resistance of lines to specific pathogens have been identified, providing valuable markers for selective breeding. However, to date relatively few of these have been successfully incorporated into commercial lines. An ability to suppress or resist multiple pathogens, by selection for improved innate immune robustness has also been studied but it has not been introduced in commercial production, partly as the phenotype is ill-defined. Previous studies that focused on pro-inflammatory cytokines and their mRNA levels expressed by innate immune effector cells (heterophils and macrophages) identified differences between resistant and susceptible chicken lines, with the former producing stronger responses, supporting efforts to select poultry with an efficient early innate response. Here, small-scale qPCR screening and cellular techniques were evaluated with the conclusion that a more rapid, cheaper and reproducible method needs to be applied.

The main objective of this project was therefore to design and validate a diagnostic tool that could be used to phenotype the immune responses of chickens at the level of innate immunity. For this purpose, a panel of 89 genes was selected based on previously published infection studies and on RNA-seq results obtained from stimulation of heterophils, macrophages and dendritic cells with lipopolysaccharide (LPS). Target genes were cloned and sequenced to optimise the design of qPCR reactions and primers. A multiplex qPCR platform, the Fluidigm 96.96 Dynamic Array, was selected as the tool of choice with the capacity to measure transcription of 96 genes of interest in 96 samples simultaneously. The preamplification reaction was optimised and the platform validated using a commercial line of chickens housed in clean or pathogen-challenged environments. Lymphoid tissues, including bursa of Fabricius, spleen, ileum with Peyer's patches, caecal tonsils, and blood leukocytes were isolated and transcript levels for immune-related genes defined between organs, birds and farms. For qPCR analysis, a panel of reference genes was normalised and TBP, ACTB and GAPDH genes were selected and validated as the most stable. The high-throughput qPCR analysis identified peripheral blood leukocytes as a potentially reliable indicator of immune responses among all the tissues tested with the highest number of genes significantly differentially expressed between birds housed in varying hygienic environments.

The research described here could potentially aid the selection of poultry for improved immune robustness. The technical optimisation and validation of a new tool to simultaneously quantify expression of tens of relevant immune-related genes will prime research in many areas of avian biology, especially to define baseline immune gene expression for selection, the basis of differential resistance, and host responses to infection, vaccination or immuno-modulatory substances.

Lay summary

Over the last four decades poultry breeders have selected birds not only for productivity, but also for improved health, welfare, fitness and environmental robustness. However, with the intensification of production some traits have been compromised. Traditional methods of disease treatment and prevention have played a critical role in control of disease. However, growing resistance of pathogens to therapeutic measures and consumer concerns led to the withdrawal of antibiotics as growth promoting additives in chicken feed. The emergence of virulent and drug resistant pathogens have emphasised the need to focus on other solutions to disease, particularly natural genetic resistance.

Previous attempts to describe immune robustness in poultry have focused on early responses in chicken immune cells (heterophils and macrophages) and found stronger responses in resistant lines compared to susceptible ones. These experiments suggest that selection of chickens that are characterised with early and efficient immune responses is possible. Popular techniques that are commonly used to characterise immune responses at the level of gene expression were evaluated in this thesis with a conclusion that more rapid, reproducible and cheaper methods need to be applied.

The main objective of this thesis was to design and test a tool that would allow us to assess the levels of expression of many immune-related genes in many samples at once. This would allow for easier and faster characterisation of the ability of chickens to mount immune responses. A group of 89 genes was selected based on previous studies and analysis of genes induced in chicken immune cells upon stimulation with a bacterial agonist of early responses. To confirm the existence of selected genes in chicken genome they were cloned and sequenced. The Fluidigm 96.96 Dynamic Array platform was chosen as the diagnostic tool. This platform can run 9,216 reactions at once. Chicken organs involved in immune responses (spleen, bursa of Fabricius, caecal tonsils, ileum and blood) were sampled from birds raised on two farms that differed in levels of hygiene. To normalise the results from

Fluidigm platform a set of reference genes that show stable expression across a range of conditions was selected. Among all the different tissues tested, blood cells showed the highest number of genes that were differentially expressed between birds from clean and pathogen-challenged farms, suggesting that blood tests to analyse expression of immune-related genes may be useful. The research described here could potentially aid the selection of poultry for improved immune robustness. The technical optimisation and validation of a new tool to simultaneously quantify expression of tens of relevant immune-related genes will prime research in many areas of avian biology, especially to define baseline immune gene expression for selection, the basis of differential resistance, and host responses to infection, vaccination or immuno-modulatory substances.

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Appendix 1

Abbreviations and acronyms

% - Percentage

°C - Degrees Celsius

µg - Microgram

µm - Micromolar

ADOL - Avian Disease and Oncology Laboratory

AIV - Avian Influenza Virus

AM - Arithmetic Mean

APC - Antigen-Presenting Cell

APEC - Avian Pathogenic *Escherichia coli*

BCR – B-cell Receptor

BKI - Best Keeper Index

BLAST - Basic Local Alignment Software Tool

BMDC - Bone Marrow-derived Dendritic Cells

BMDM - Bone Marrow-derived Macrophages

cDNA - Complementary DNA

CEF - Chicken Embryo Fibroblast

CLR - C-type Lectin Receptor

CNV - Copy Number Variation

COS-7 - CV-1 (simian) in Origin, carrying the SV40 genetic material

CS - Chicken Serum

CSF - Colony Stimulating Factor

Cq - Quantification Cycle

CV - Coefficient of Variance

DC - Dendritic Cell

DCFH-DA - Dichloro-dihydro-fluorescein diacetate

DE - Differential Expression

DEAE - Diethylaminoethyl

DMEM - Dulbecco's Modified Eagle's Medium

DMSO - Dimethyl sulphoxide

dsDNA - Double stranded DNA

EDTA - Ethylenediaminetetraacetic acid

eQTL - Expression QTL

Exo I - Exonuclease I

FAM - 5-carboxyfluorescein

FCS - Foetal Calf Serum

FM - Full-text Minute-space

FPKM - Fragments Per Kilobase per Million mapped reads

GM - Geometric Mean

GOI - Gene of Interest

HBSS - Hanks Balanced Salt Solution

HET - Heterophil Extracellular Trap

IBDV - Infectious Bursal Disease Virus

IBV - Infectious Bronchitis Virus

IFC - Integrated Fluidic Circuit

IFN - Interferon

Ig - Immunoglobulin

IL - Interleukin

ILTV - Infectious Laryngotracheitis Virus

IPTG - Isopropyl β-D-1-Thiogalactopyranoside

LPS - Lipopolysaccharide

LRR - Leucine-Rich Repeats

MAS - Marker-Assisted Selection

MDV - Marek's Disease Virus

MHC - Major Histocompatibility Complex

MLN - Mesenteric Lymph Nodes

MNC - Mononuclear Cell

NDV - Newcastle Disease Virus

NE - Necrotic Enteritis

NLR - NOD-Like Receptor

NO - Nitric Oxide

NOD - Nucleotide-binding Oligomerisation Domain

PAMP - Pathogen-Associated Molecular Pattern

PBL - Peripheral Blood Leukocytes

PBS - Phosphate Buffered Saline

PCR - Polymerase Chain Reaction

PMA - Phorbol A-Myristate 13-Acetate

PRR - Pattern Recognition Receptor

qPCR - Quantitative Polymerase Chain Reaction

QTL - Quantitative Trait Locus

RFU - Relative Fluorescent Unit

RIG-I - Retinoic acid-Inducible Gene-1

RLR - Retinoic acid-inducible gene-1 (RIG-I) Like Receptor

RNA - ribonucleic acid

RNA-seq - RNA Sequencing

rRNA - Ribosomal RNA

ROS - Reactive Oxygen Species

RPMI - Roswell Park Memorial Institute

RT - Reverse Transcription

RT-PCR - Reverse Transcription Polymerase Chain Reaction

RT-qPCR – Reverse Transcription quantitative Polymerase Chain Reaction

SAL1 - Salmonellosis Resistance Locus 1

SD - Standard Deviation

SNP - Single Nucleotide Polymorphism

TAE - Tris Acetate-EDTA

TAMRA - Tetramethylrhodamine

TCR – T-cell Receptor

TE - Tris EDTA

TGF - Transforming Growth Factor

Th - T helper

TIR - Toll/interleukin (IL)-1R homology domain

TLR- Toll-Like Receptor

TRT - Turkey Rhinotracheitis

x-gal - 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Chapter 1 Introduction

1.1 Background

Poultry are reared in most parts of the world and are vital to global food security. Since domestication around 4000 years ago, chickens were first bred in Europe by the Romans around 500 B.C. (Parkhurst and Mountney, 2012; page 3). Nowadays the poultry industry is the dominant and fastest growing supplier of high quality and inexpensive animal protein worldwide. Many aspects have contributed to the intensification of chicken farming, for example affordable feed, efficiency of feed conversion relative to other farmed animals, changes in housing and better disease control. In the period of 12 years, between 2000 and 2012, the number of slaughtered chickens increased from 40,635 million to 59,861 million, and the meat weight per bird increased from 1.44kg to 1.55kg (Global Poultry Trends, 2014). It is estimated that these numbers will increase substantially due to global population growth and rising affluence. By the end of this decade, 124 million tons of poultry will be consumed worldwide, with Asia and Africa as the leaders (Meat Atlas; Heinrich Böell Foundation and Friends of the Earth).

To sustain market requirements, most poultry are raised in large operations, which can lead to rapid transmission of diseases and zoonotic pathogens among the flock. Intensive rearing conditions may also lead to stress and injury, making birds more vulnerable to diseases. The chicken immune system is challenged by diverse viral, prokaryotic and eukaryotic pathogens. Some of these microbes can infect both chickens and humans, for example, Gram-negative bacteria such as *Salmonella* and *Campylobacter* and avian influenza. Such agents are sometimes carried in the absence of symptoms, making carriers difficult to identify and enabling agents to spread undetected in flocks and through the food chain. The spread of pathogens is affected not only by host factors but environment and housing. Changes from caged to enriched cages and non-cage system housing regulations for laying hens in EU countries were implemented in 2012. Reports from countries that introduced new housing systems earlier showed an increase in the incidence of bacterial infections (Fossum *et al.,* 2009; Kaufmann-Bart and Hoop, 2009). Conventional battery cages

limited the contact of hens with their faeces and provided less opportunity for stress or injury associated with formation of a social hierarchy.

The most prevalent foodborne pathogens are *Campylobacter* and *Salmonella* species. According to a UK Government report on zoonotic diseases during 2013, *Salmonella* was responsible for 8,459 laboratory-confirmed cases of gastrointestinal infections in humans, whereas *Campylobacter* caused 66,575 cases in United Kingdom in 2013. The increase in the number of reported enteric and non-enteric infections related to *Campylobacter* in part can be attributed to better outbreak surveillance systems, which have resulted in infections being more readily identified. National-scale genotyping was used to quantify the contributions of different sources of human *Campylobacter* infection. The study revealed that the main source of campylobacteriosis in Scotland in 2005-2006 was contaminated chicken meat (Sheppard *et al.,* 2009). Also many *Salmonella* serovars, such as Heidelberg, Enteritidis, Infantis, Typhimurium, linked to human infections have been isolated from poultry, eggs and egg-containing products (Dutil *et al.,* 2010; Yim *et al.,* 2010; Cloeckaert *et al.,* 2007). These pathogens are the main bacterial agents causing food-borne illnesses in developed countries.

Losses in poultry production can be caused by diverse pathogens, some of which cause serious outbreaks with high mortality. In some cases it is necessary to slaughter entire flocks to prevent the infectious agents from spreading. Low pathogenic avian influenza virus (AIV), high pathogenic AIV and Newcastle Disease Virus (NDV) are the most common viruses, which have reservoirs in wild birds, and hence are difficult to control. Despite Infectious Bronchitis Virus (IBV) not having other hosts apart from the chicken, it can cause significant losses in production due to poor weight gain, mortality and reduced egg production. Avian pathogenic *Escherichia coli* (APEC) causes diverse respiratory and systemic diseases (collectively termed colibacillosis) in chickens and other avian species. Infected birds often show lower growth rates and feed conversion efficiency and have inflammation associated with one or more visceral organs. Colibacillosis also leads to higher

mortality causing losses to the industry. Outbreaks of parasitic infections with *Eimeria* species can cause high morbidity and mortality and can persist in chicken's environment in faeces and litter as oocysts. Infected birds often exhibit increased intestinal colonisation by *Clostridium perfringens* leading to necrotic enteritis and with *Salmonella* species, which intensifies the risk to food security and spread of foodborne pathogens (Qin *et al.,* 1996; Collier *et al.,* 2008).

Good husbandry practices together with support from vaccines and coccidiostats make poultry production on industrial scale achievable (Blake and Tomley, 2014). There are vaccines available against many pathogens, for example NDV, IBV, AIV, infectious bursal disease virus (IBDV), Marek's Disease Virus (MDV), fowl pox, Infectious Laryngotracheitis (ILTV), *Salmonella* Enteritidis, *S.* Typhimurium and *Eimeria* species. However, such vaccines can be expensive and time-consuming to administer and in some cases require updating to cover circulating strains.

In the past, the role of genetic disease resistance was limited due to extensive application of antibiotics (Zekarias *et al.,* 2002). The prophylactic administration of antibiotics, as well as the use of in-feed antibiotics at sub-therapeutic doses as growth-promoters, was prohibited in the European Union from 2006 (reviewed in Castanon, 2007) in the light of increasing antibiotic resistance of many pathogens and understandable concerns from consumers. Therapeutic use is still allowed, but is increasingly hindered by transmissible drug resistance and restrictions on antibiotic residues entering the food chain. Despite the availability of vaccines for most of the common poultry diseases, there are still regular breaks where disease can cause devastating problems for the commercial production flock. Numerous factors can contribute to these disease outbreaks. The factors include failure of vaccines to protect against a new or highly virulent form of the pathogen. A recent report by Read *et al.* (2015) established that imperfect viral vaccines that do not result in clearance might select for the occurrence of escape mutants and more virulent strains by allowing a longer period for virus to mutate in the host. In addition, loss of vaccine function due to incorrect storage or use, or the

disease challenge itself may be so high that it overwhelms vaccine-induced immunity (Fulton, 2004). Although recovery after such outbreaks can be observed, production efficiency rarely achieves the levels before the disease occurred.

Given the diversity of infectious threats to poultry health and challenge of preventing and treating diseases, improved immune function would be advantageous for chicken breeders. In this context, selection of more resistant chicken lines offers much potential. The first selection experiments happened in 1935 and were aimed to decrease disease occurrence, which was mainly caused by *Salmonella enterica* serovars Pullorum and Gallinarum that cause severe systemic disease in birds (reviewed in Jie and Liu, 2011). For many decades, poultry breeders focused their breeding schemes on phenotypic traits, which led to production of chickens with increased body weight. The last 60 years of selective breeding in broilers led to decrease in days required to reach processing weight. Nowadays it takes 42 days compared to 84 days in the 1950s. In 1957, a 42-day-old broiler weighed on average 591 g (Havenstein *et al.,* 2003) whereas the avergage slaughter weight of modern broilers is approximately 2672 g. Breeding programs directed towards accelerated growth and feed conversion efficiency may unintentionally reduce the responsiveness to the plethora of immune challenges (Swaggerty *et al.,* 2009).

While it has been possible to improve poultry by genetic selection for resistance to specific diseases (Star *et al.*, 2008), achieving a general increase in immunological competence is considered challenging because of low heritability and the difficulty of measuring this trait. In addition, progress in vaccination and use of antibiotics repressed the requirement to generate immune protection through breeding programs. Although the heritability estimates of disease resistance are reported to be low, phenotypic variation is high in populations and genetic selection can be used to complement improvements in vaccination practices to support poultry in the “arms race” between pathogens and their host (reviewed in Hocking, 2010). The selection based on immune function has no effect on growth promotion,

therefore it could be possible to select for immune responsiveness without causing a decline in weight gain abilities (van der Most *et al.,* 2011). Selection for host responses to individual pathogens is achievable and has been used in breeding programmes (Zekarias *et al.,* 2002) but it is not desirable.

Few lines selected for resistance to specific pathogen have been tested for susceptibility to other pathogens. Hartmann *et al.* (1984) showed correlation of ALV resistance with MD resistance in two pairs of strain, where a third pair presented lower MD mortality rate. This demonstrates that the susceptibility spectrum to pathogens other than the one used for selection has different outcomes in different populations. Approaches to directly select for a single trait by creating divergent populations have been performed in the past. Multi-determinant and non-pathogenic antigen – sheep red blood cells (SRBC), was first used in mice (Biozzi *et al.,* 1979) and resulted in great differences in the magnitude of antibody responses. The application of selection for SRBC responses in chickens led to a long-term experiment using a White Leghorn population. The offspring of divergent lines (high antibody – HA, low antibody – LA) differed in antibody titer to Newcastle Disease Virus, mites, *Mycoplasma gallisepticum, Eimeria necatrix and* splenomeglia virus, with HA chickens characterised by better humoral responses. On the other hand, LA chickens were shown to be less susceptible to *Escherichia coli* and *Staphylococcus aureus* (Gross *et al.,* 1980; Dunnington *et al.,* 1991). Resistance of high antibody chickens to parasitic and viral pathogens was confirmed in experiments with *Eimeria tenella* (Martin *et al.,* 1986) and Marek's Disease (Dunnington *et al.,* 1986). The susceptibility of the HA line to bacterial infections may lie in differential genetic regulation and negative correlation between immune responses to wide spectra of pathogens (Lamont *et al.,* 2003).

Genes involved in adaptive immune responses control resistance to specific pathogens. Therefore, selection based on adaptive immunity may be highly specific against one particular pathogen and unlikely to give resistance to even closely related pathogens. In addition, it can lead to susceptibility to other pathogens.

Breeding chickens for growth traits revealed its negative association with some aspects of immune system performance. Another problem associated with this approach is the difficulty to predict the specific types of microbes that may be present in the commercial poultry environment and thus genetic selection for resistance to a particular pathogen will not ensure infection-free chicken stocks (Lamont *et al.,* 2008).

A well-developed immune system that responds adequately to pathogens is necessary to reduce disease occurrence. The generation of more robust lines of birds with improved liveability would prevent major economic losses or contamination of food products (Swaggerty *et al.,* 2009). In order to do this there is a demand for diagnostic tools that could rapidly and precisely evaluate avian immune responses associated with innate immunity and disease resistance. The innate immune response directs the adaptive response. Before this discovery, immunological research had been focused on clonal expansion of T and B cells by specific antigens (Parish and O'Neill, 1997). Since then many laboratories focused their research on evaluating various aspects of the innate immune response in poultry (Ferro *et al.*, 2004; Wigley *et al.*, 2006; Swaggerty *et al.*, 2008). Kramer *et al.* (2003) performed experiments with various breeds of chickens and their abilities to fight the infection with innate or adaptive immune responses. The study showed that Old Dutch breeds were characterised by higher production of nitric oxide (NO), lower *Salmonella* survival in splenic leukocytes and higher total IgM and IgY antibody concentrations compared to a commercial broiler group. These experiments concluded that many aspects of the immune system should be included in the selective breeding program. Focusing on only one branch of immune system does not reliably indicate general responsiveness or immunocompetence. Therefore, better understanding of avian immunology is crucial in determining disease robustness in chickens.

1.2 The immune system

All living organisms have evolved defence mechanisms against invading pathogens. Apart from commensal microflora that reaches homeostasis, the immune system is constantly challenged with a broad spectrum of microorganisms. Similar to mammalian species, chicken have developed two arms of defence: innate and adaptive immune responses (Figure 1.1).

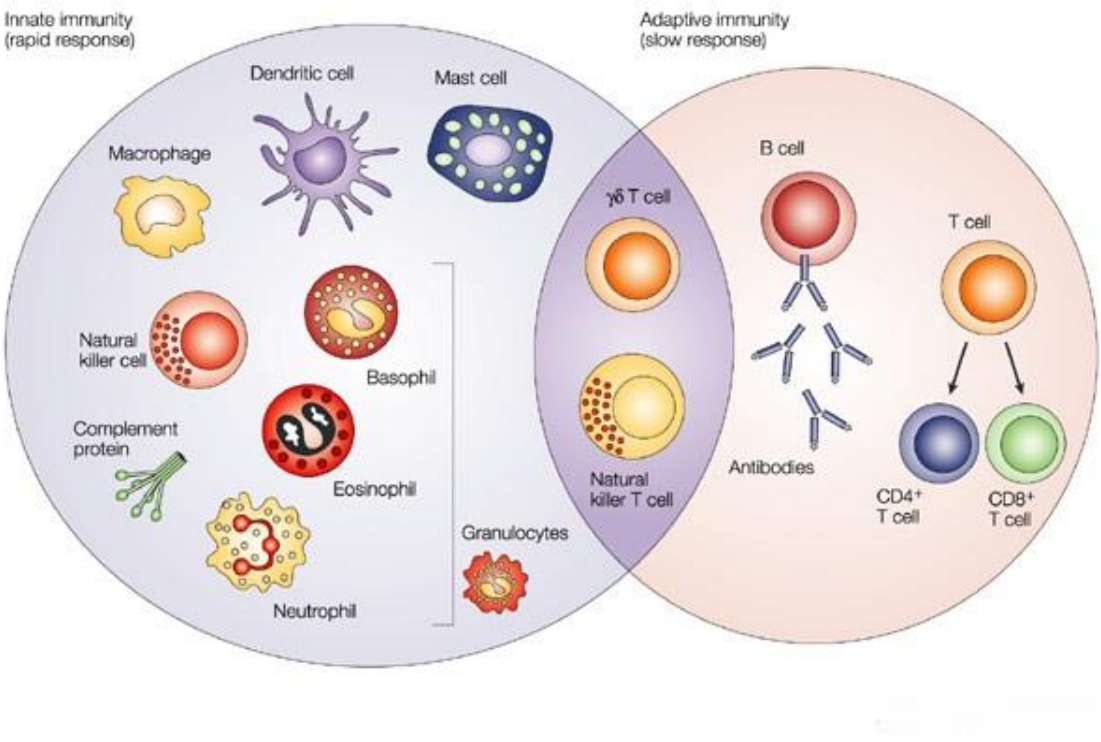


Figure 1.1. The components of innate and adaptive immune system in mammals. The cells (granulocytes, dendritic cells, macrophages, natural killer cells, mast cells) and soluble molecules (complement proteins) of the innate immune response act as the first line of defence against infection. The adaptive immune response is triggered by innate components, and it is highly characterised with high antigenic specificity and memory. The components of adaptive immune system include antibodies, B cells, and CD4+ and CD8+ T lymphocytes. Natural killer T cells and γδ T cells are cytotoxic lymphocytes that interact and bridge the innate and adaptive immunity (Adapted from Dranoff, 2004).

The innate immune component generates responses immediately after the recognition of pathogen-associated molecular patterns (PAMPs). Adaptive immunity starts as a second line of defence and is highly specific to the invading organism(s). It requires time to design responses against invaders and to avoid

damaging of host tissues. The establishment of immunological memory by the adaptive branch of the immune system allows responses to be generated faster when the infection reoccurs. An important aspect of activation of adaptive immune responses is the presentation of foreign antigen to the T cell or B cell receptor by cells from the innate immune system that encountered pathogens via phagocytosis. The Major Histocompatibility Complex (MHC) is essential for such antigen presentation. The genes involved in MHC encode for glycoproteins that, after antigen processing and degradation, present small peptides to the cell surface and present them to the T or B lymphocytes. Naïve B cells can be activated by follicular dendritic cells (FDCs) after presentation of unprocessed antigen in secondary lymphoid organs (Batista and Harwood, 2009). B and T cells have major roles in the development and organisation of adaptive immunity, respectively producing antibody or targeting infected cells for killing. In the adaptive immune response, antigen is recognised by two distinct sets of highly variable receptors, the B cell receptor (BCR) and the T cell receptor (TCR). To protect against pathogens, the host needs to generate a diverse pool of BCR that will recognise a broad range of antigens and initiate the antibody production. TCR diversity has evolved as a result of the arms race with emerging pathogens to cover most of the antigen diversity (Nikolich-Zugich *et al.,* 2004). In the chicken, mechanisms of somatic DNA recombination to create variability in the TCR are identical as in mammals (Jung *et al.,* 2006). A vital part of immunity and control of infections with extracellular and intracellular pathogens are the T helper 1 (Th1) and T helper 2 (Th2) cells. The components of Th1 and Th2 responses required in mammals, including signature cytokines and transcription factors, have been identified in the chicken genome (Kaiser *et al.,* 2005).

The avian immune system differs from those of mammalian species at the genetic, molecular, cellular and organ level. Birds have different repertoires of Toll-like receptors (TLRs) involved in pattern recognition, as well as different antibodies. They also lack draining lymph nodes and the sites of antigen uptake, processing and immunological priming are relatively poorly defined (reviewed in Kaiser, 2010).

Birds have an avian-specific primary lymphoid organ, the bursa of Fabricius, which is the site of development of the B-cell repertoire (Glick *et al.*, 1956). At the cellular level, the avian functional equivalents of mammalian neutrophils are heterophils (Kogut *et al.*, 2005). The chicken eosinophils appear to be non-functional (Maxwell, 1987) and the components that control migration of eosinophils, for example eotaxins, eotaxin receptor and chemokines, are missing in the chicken genome (Kaiser, 2012). Similar to eosinophils, the numbers of basophils and mast cells, all typical cells of Th2 responses, are much lower compared to mammalian species (Schijns *et al.,* 2014). The chicken Major Histocompatibility Complex (MHC) genes differ in the structure, function and architecture from the mammalian MHC (Kaufman *et al.*, 2013). The chicken MHC is more compact, containing only 19 genes within the 92 kb region of the B locus on the chromosome 16. The 20-fold smaller size compared to human MHC has been termed "the minimal essential" MHC (Kaufman *et al.*, 1995).

1.2.1 Avian Pattern Recognition Receptors (PRRs)

Similar to mammals, the chicken innate immune system depends on the recognition of pathogens or pathogen-associated molecular patterns through Pattern Recognition Receptors (PRRs) including TLRs, retinoic acid-inducible gene-1 (RIG-I) like receptors (RLRs), nucleotide-binding oligomerisation domain (NOD)-like receptors (NLRs) and C-type lectin receptors (CLRs) family members. A RIG-I orthologue is present in both the duck and goose genomes (Barber *et al.,* 2010; Sun *et al.*, 2013) but it has not been identified in the chicken (Karpala *et al.,* 2011). The most studied family are TLRs. This family consists of transmembrane glycoproteins, expressed on the cell membrane or intracellularly. The extracellular domain consists of leucine-rich repeats (LRR) responsible for pathogen recognition. The transmembrane domain and intracellular region containing Toll/interleukin (IL)-1R homology domains (TIR) recruits adaptor proteins, which then activate signal transduction cascades. A core signalling pathway is engaged by surface TLRs and leads to activation of transcription factors involved in pro-inflammatory gene expression (O'Neill and Bowie, 2007).

Avian TLR family members comprise mammalian orthologues and avian specific TLRs and their ligands, where known, are listed in (Table 1.1). The mammalian TLR1 family can form heterodimers with TLR2, which increases the capacity to detect PAMPs (Ozinsky *et al.,* 2000). Tandemly duplicated genes, TLR1A, TLR1B and TLR2A, TLR2B represent the chicken TLR1 family (Boyd *et al.,* 2001; Fukui *et al.,* 2001). Similar to mammalian TLR2, chicken TLR1 and TLR2 families can form heterodimers and detect PAMPs (Higuchi *et al.,* 2008). Orthologues for TLR3 (Schwarz *et al.*, 2007), TLR4 (Leveque *et al.,* 2003), TLR5 (Iqbal *et al.,* 2005) and TLR7 (Philbin *et al.,* 2005) are present in the chicken genome. TLR4 detects lipopolysaccharide and is associated with early regulation of *Salmonella* infection (Leveque *et al.,* 2003). TLR5 detects flagellated *Salmonella* serovars (Iqbal *et al.,* 2005). Detection of viral RNA is mediated by intracellular TLR3 and TLR7 (Schwarz *et al.,* 2007; Philbin *et al.*, 2005). Chicken TLR8 is a pseudogene disrupted by a chicken repeat-1 (CR1) retrovirus-like element (Philbin *et al.,* 2005). Although TLR9 gene is absent from the chicken genome, chickens are able to detect CpG motifs via TLR21 (Brownlie *et al.*, 2009; Keestra *et al.,* 2010). Avian-specific TLR15 have been shown to detect range of PAMPs from various pathogens, for example *Salmonella* Typhimurium, *S.* Enteritidis, *Escherichia coli,* B- and C-type CpG oligonucleotides (ODN), tripalmitoylated lipopeptide (PAM3CSK4), LPS, virulence-associated fungal and bacterial proteases, yeast-derived agonist and *Eimeria tenella* sporozoites (Higgs *et al.,* 2006; Shaughnessy *et al.,* 2009; Nerren *et al.,* 2010; Ciraci and Lamont, 2011; De Zoete *et al.*, 2011, Boyd *et al.,* 2013, Zhou *et al.,* 2013).

Table 1.1. TLR family members and their agonists in humans and chickens

Human	Chicken	Agonists	Pathogen
TLR1/6/10	TLR1A TLR1B	Lipoprotein	Mycobacteria
TLR2	TLR2A TLR2B	Peptidoglycan	Bacteria
TLR3	Present	dsRNA	Viruses
TLR4	Present	LPS	Gram- bacteria
TLR5	Present	Flagella	Gram- bacteria
TLR7	Present	ssRNA	Viruses
TLR8	Pseudogene	ssRNA	Viruses
TLR9	TLR21	CpG	Bacteria and viruses
Absent	TLR15	LPS Lipoprotein CpG	Gram+/- bacteria, viruses fungi

1.2.2 Non-cellular components of innate immune system

Cytokines and chemokines play an essential role in the immune response. These small regulatory molecules act as extracellular signals between cells during the course of an immune response. Knowledge of the cytokine repertoire in the chicken radically changed after the chicken genome sequence became available (Hiller *et al.,* 2004). Due to very low (25-35%) identity with their mammalian orthologues there are only few cross-reactive monoclonal antibodies or bioassays (reviewed in Kaiser and Staeheli, 2008). The repertoire of chicken cytokines includes interferons (IFNs), interleukins (ILs), transforming growth factors (TGFs), tumour necrosis factors (TNFs), colony stimulating factors (CSFs) and chemokines (Kaiser *et al.*, 2005). Many studies focus now on expression of pro-inflammatory cytokines in effector cells upon stimulation with TLR agonists. Pro-inflammatory cytokines and chemokines are involved in responses to various pathogens challenges and increased resistance against disease have been shown to be associated with strong pro-inflammatory cytokine and chemokine responses (Coussens *et al.*, 2004, Ferro *et al.*, 2004).

1.2.3 **Role of the effector cells**

Various cells of both arms of the immune system have been described in the chicken. The activity of dendritic cells, macrophages, heterophils, natural killer cells (NKT), γδ T cells against invading microbes is considered a part of the innate immune response. In the context of this study, dendritic cells (DCs), macrophages and heterophils will be reviewed. Dendritic cells and macrophages phagocytose the encountered pathogen and present degraded peptides in the context of MHC molecules. MHC molecules play crucial role as a restriction element presenting antigens to T cells. MHC class I and II molecules have similar functions, both present the antigens to T cells, CD8+ and CD4+, respectively. The difference lies in the origin of the antigen. Generally, MHC class I molecules present peptides originated from intracellular pathogens whereas MHC class II molecules present exogenous peptides (reviewed in Vyas *et al.,* 2008). In some circumstances, extracellular-derived antigens can be presented via MHC class I molecules to CD8+ T cells. The phenomenon of cross presentation has been described 30 years ago (Bevan, 1976) and is still a "hot topic" as it is not yet fully elucidated. The migratory CD103+ DCs have been shown to be most efficient in cross-presentation in mammals (Joffre *et al.,* 2012). It is now known that the immune system uses this mechanism to monitor tissues and phagocytic cells for the presence of antigens.

Since Metchnikoff first used the "macrophage" term to describe phagocytic leukocytes in 1884, the knowledge about these cells has grown hugely. Their role in innate and adaptive immunity, inflammation as well as in tissues homeostasis is well established in mammals (reviewed in Gordon, 2003). The macrophage repertoire consists of heterogeneous cells located in different tissues throughout the body in both vertebrates and invertebrates (Gordon and Taylor, 2005). They function as phagocytes and stimulate immune responses of other cells by expression of cytokines and chemokines, and therefore play primary roles in both innate and adaptive immunity. Carrell and Ebeling first described the isolation of macrophages from chicken blood in 1922. The adherence abilities of macrophages have been used to develop a protocol of selecting monocyte-derived macrophages

from peripheral blood (Peck *et al.*, 1982). It is now possible to culture chicken bone marrow-derived macrophages with colony-stimulating factor 1 (Garceau *et al.*, 2010). Mammalian dendritic cells (DCs) have been identified over 30 years ago (Steinman *et al.*, 1975) and have become the basis of immune responses studies in mammalian species since. Unlike macrophages or B cells, DCs are much more effective at inducing a primary immune response in resting naive T lymphocytes, and are called professional antigen-presenting cells (APCs) (Nakayama, 2015). Apart from phagocytosis and antigen processing, macrophages and dendritic cells also produce cytokines and chemokines and express MHC Class II molecules on their surface after recognition of PAMPs. The level of knowledge and availability of diagnostic tools to study avian effector cells is limited compared to the mammalian field. The populations of both types of effector cells are heterogeneous. Several different subtypes of dendritic cells have been identified and characterised in the chicken – bursal secretory DCs, follicular DCs, thymic DCs, Langerhans cells and bone marrow-derived DCs (Igyarto *et al.*, 2006; Igyarto *et al.*, 2007; del Cacho *et al.*, 2008; Wu *et al.*, 2010, Olah and Nagy, 2013). Recent studies have characterised bone marrow-derived DC during viral infections with AIV and IBDV (Vervelde *et al.*, 2013; Liang *et al.*, 2015). The advent of transgenic chickens in which all cells of the myeloid lineage express a fluorescent protein under the control of the CSF-1 receptor promoter has begun to shed light on the spatial organisation and function of macrophages in the chicken (Garceau *et al.*, 2015).

Heterophils, the avian equivalent of mammalian neutrophils, have not been reported as APCs. However, several genes involved in the MHC class II system (CD80, MHC II β chain, c-KIT) were reported to be upregulated in *Salmonella* Enteritidis-stimulated heterophils derived from a resistant line of chickens (Chiang *et al.*, 2008). Heterophils are useful biomarkers for measuring the innate immune response as they act early (within an hour) to engulf and destroy pathogens via phagocytosis (Swaggerty *et al.*, 2003). Pathogens are then entrapped inside phagosomes that begin to fuse with cytoplasmic granules with microbicidal substances. Similarly to neutrophils, heterophils have evolved specific tools to

prevent pathogens from spreading. They are capable of producing reactive oxygen species (ROS) but the process of oxidative burst differs from the process observed in human neutrophils. The lack of myeloperoxidase, catalase and alkaline phosphatase enzymes distinguish them from neutrophils (Brune *et al.*, 1972).

Apart from intracellular phagocytic killing, heterophils are equipped with extracellular killing mechanisms. Heterophils have granules whose contents are released into the external environment upon contact with pathogens. The release of networked extracellular fibres made up of DNA, histones and granular enzymes has been shown in chicken heterophils stimulated with pathogens or their products. These heterophil extracellular traps (HET) are independent of phagocytosis, making heterophils the most equipped effector cells (Chuammitri *et al.*, 2008). Association of HET production with variation at the locus associated with salmonellosis and *SLC11A1* gene has been revealed in studies on two intercross lines of chickens challenged with *S.* Enteritidis (Redmond *et al.*, 2011), indicating that it may be under genetic control. Antimicrobial proteins, peptides, enzymes and adhesion molecules are among the potentially toxic substances found in chicken heterophil granules. An antimicrobial cationic peptide, cathelicidin-2, has been shown to be released from heterophil granules upon stimulation with *S.* Enteritidis (van Dijk *et al.*, 2009). Heterophils are able to release the granule contents at the infection site in a controlled manner to avoid damaging surrounding tissues (Genovese *et al.*, 2013). Phagocytosis has been shown to be associated with degranulation processes. Similar to phagocytosis, various microbial substances were found to stimulate degranulation (Kogut *et al.*, 2005).

1.2.4 **Adaptive immune response**

The adaptive immune response is activated after the presentation of antigen to lymphocytes (Medzhitov and Janeway, 1998), in the context of the MHC, by antigen-presenting cells, especially DCs. CD8+ T cells and/or CD4+ T cells are stimulated and activated depending on the nature of the pathogen. Stimulation of naive T helper cells, expressing CD4 molecules, with IFN-γ, IL-12 or IL-18 secreted by

innate immune system effector cells initiates the differentiation of Th1 cells. In contrast, IL-4 and IL-13 trigger the differentiation of Th2 cells (Figure 1.2).

Th1 cells enhance cell-mediated immunity against intracellular pathogens, whereas Th2 cells are important for control of extracellular pathogens via humoral responses. They are also responsible for activation of B cells (Lebman and Coffman, 1988). Markers of these Th cell subsets are the transcription factor Tbet, the cell surface marker TRANCE and the signature cytokine IFN-γ for Th1 cells and, for Th2 cells, GATA-3, Tim1 and IL-4 and IL-13 (reviewed by Zhu and Paul, 2008).

Until recently, the Th1/Th2 paradigm (Mosmann and Coffman, 1989) has been used to describe the different adaptive responses triggered by various pathogens. Evidences of Th1/Th2 polarisation in the chicken have been shown based on infection studies with viruses and helminths (Eldaghayes *et al.,* 2006; Degen *et al.,* 2005). The Th1/Th2 paradigm does not explain many complicated pathological situations. The discovery of a third subset of Th cells (Th17) has expanded the Th1/Th2 paradigm (Ouyang *et al.*, 2008). The primary function of Th17 cells is to handle pathogens that have not been cleared by Th1 or Th2 cells. Differentiation of Th17 cells is triggered by a combination of TGF-β, IL-6 and IL-23 (reviewed in Korn *et al.*, 2009). Th17 cells express the transcription factor RORγt, the receptor IL-23R on their surface and the signature cytokines IL-17A and IL-17F (Ivanov *et al.*, 2006). The chicken IL-17 family consists of five members that have been identified in the genome: IL-17A, IL-17B, IL-17C, IL-17D and IL-17F (Kaiser *et al.,* 2005). After successful clearance of the pathogen, some T cells, which previously encountered antigen, remain in the system as memory cells (reviewed in Korn *et al.*, 2009). The immune system developed a mechanism that control the damage caused by immune responses. Regulatory T cells (Treg) are a subset of T cells that specialises in immune suppression. The disruption of regulatory function of immune system causes the autoimmune diseases. One of the unique markers of Treg is Foxp3 (Li *et al.,* 2008b) which has not yet been identified in the chicken. The Treg family consists of Th3 cells, $CD8^{+}Foxp3^{+}$ cells, γδT cells, NKT cells and $CD4^{-}CD8^{-}$

TCRαβ$^+$ cells. Chicken CD4$^+$CD25$^+$ cells have been shown to have similar suppressive functions as mammalian Treg cells (Shanmugasundaram and Selvaraj, 2011).

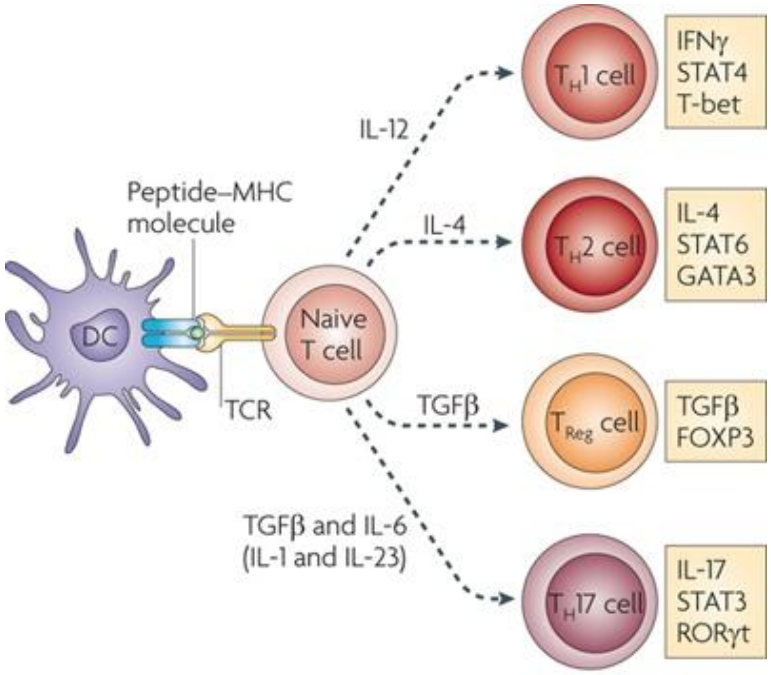


Figure 1.2. Differentiation of helper T cell subset in mammals. Antigen –activated dendritic cells (DCs) interact with naïve CD4+ T cells which leads to polarisation into different effector T cell subsets — T helper 1 (Th1), Th2, Th17 and regulatory T (Treg) cells.Distinct sets of transcription factors control differentiation of each effect T cell type. Naïve T cells can differentiate into Th17 cells in the presence of interleukin-6 (IL-6) and transforming growth factor-β (TGFβ). Th17 cells express the transcription factors retinoic acid receptor-related orphan receptor-γt (RORγt) and signal transducer and activator of transcription 3 (STAT3). FOXP3 - forkhead box P3; GATA3 - GATA-binding protein 3; IFNγ - interferon-γ; TCR - T cell receptor (Adapted from Zou and Restifo, 2010).

The humoral response is a part of the adaptive branch of immunity and involves activity of B lymphocytes. In mammals B cells develop in bone marrow from pluripotent haematopoietic stem cells that give rise to common lymphoid progenitor cells. Progenitor B cells undergo rearrangement of the immunoglobulin (Ig)-heavy chain and light chain genes. This process results in immature B cell population that express the membrane-bound IgM. B cells migrate to lymphoid organs as naïve cells, meaning they have not yet been exposed to antigen. Activated B cells differentiate to plasma cells, express and secrete different classes of Ig. The switch between IgG, IgA, IgD, IgE and IgM is orchestrated by cytokines. In contrast to bone marrow B cell development in mammals, chickens unique lymphoid organ,

the Bursa of Fabricius is the site where B cells originate and differentiate (Barnes, 2001; Glick, 1988). The B cells mature inside the bursa's follicles and develop antigenic diversity after exposure to antigens by APCs (Glick, 1977; Ratcliffe, 1989). Humoral responses are maintained by matured B cells that migrate from bursa to spleen and lymphoid tissues (Pope, 2001).

Avian immunoglobulins are classified into three groups: IgA, IgG and IgM (Hodek and Stiborova, 2003). Because of the structural differences between mammalian IgG and avian IgG, it was suggested to rename it to IgY (from yolk) (Leslie and Clem, 1969). Activation of mature B cells occurs when it recognises antigen in conjunction with signals from Th2 cells, which triggers proliferation and differentiation of B cells into a plasma cells. Plasma cells can be classified as antibody-producing cells or memory cells that survive for many years with the ability to produce antibody against specific antigen. Immunoglobulins consist of dimerised heavy and light chain with constant (C) and variable (V) regions. Somatic recombination of families of gene segments – V and joining (J) genes, produce light chains, and diversity (D) genes in case of heavy chain. The V(D)J recombination leads to great diversity in antigen recognition. Further changes in antigen recognition are manipulated by mutations occurring in the V, D, J genes, called somatic hypermutation. The Ig genes in avian species undergo rearrangement like their mammalian counterparts, but antibody diversity is primarily generated by gene conversion, which usually occurs after initial recombination of the single VJ or VH genes and surface expression of the B cell receptor. Single copies of V_L and J_L genes encode light chain and V_H and J_H genes encode heavy chain, which limits the diversity and the V(D)J rearrangements occurrence (Ratcliffe and Jacobsen, 1994; Reynaud *et al.*, 1985). The recombination with clusters of pseudogenes upstream of the heavy and light chains loci increases the diversity of the Ig V region (Reynaud *et al.*, 1987; Reynaud *et al.*, 1989).

1.3 Chicken breeding for improved immune robustness

Improvements in the poultry industry can be achieved by development of chicken lines with better immune responsiveness. To accomplish this, improved understanding of chicken immunology is necessary. Every aspect of the acquired immune response has its beginning in the innate response of effector cells. Stronger and more rapid innate responses to infection lead to higher resistance in particular chicken lines (Swaggerty *et al.*, 2009). Over the years, many studies have shown that commercial broiler lines have distinct immune function parameters, due to differences in their genetic make-up, which results in disease resistance and/or susceptibility. Poultry breeders and producers should co-select for both immune competence and growth traits (Cheema *et al.*, 2003). Disease resistance is generally a polygenic trait (Cavero *et al.*, 2009). Before the chicken genome became available, the genetics of complex traits had been studied without identifying the genes involved. The basis for selection was estimated breeding values calculated from phenotypic records and pedigrees, and knowledge of the heritability of each trait. However, this process is slow and, in the case of disease resistance, measuring the trait is expensive. Identification of genes controlling this trait would be advantageous in selection of animals carrying the desirable alleles (Goddard and Hayes, 2009). Early studies of immunological traits were limited to measurements of antibody levels to a defined antigen. These biomarkers are still relevant because they are easy to assess and relatively cheap. However, over the past 20 years, the methods available for identification of DNA variation have changed and costs of these assays decreased (reviewed in Lamont *et al.*, 2008). The early methods used to discover the genes were a candidate gene approach and gene mapping to a chromosomal location (reviewed in Jie and Liu, 2011).

1.3.1 Inbred lines

Toward the genetic improvement of poultry, a number of inbred lines of varying phenotypes have been created in the chicken from a selection of breed stocks (Crawford, 1990). First experiments with inbreeding started in 1939 at the Regional Poultry Disease Laboratory, now known as the Avian Disease and Oncology

Laboratory (ADOL). The natural variability inherent in a chicken population was explored with the use of individual cages, artificial insemination and brother-sister mating. Families with inherent high resistance or susceptibility to avian leukosis were formed with carefully selected source stocks. This resulted in production of several specific lines (Stone, 1975). Using inbred lines to identify sources of variation is advantageous, as the level of biological variance of the trait is very low compared to outbred lines. The phenotypic variance decreases in backcross populations. This allows the mapping of genomic regions associated with the trait of interest, termed quantitative trait loci (QTL) by association of the genotype of the backcross progeny with the phenotype under study. In addition, backcrossed populations maintain the non-random association of alleles at different loci, therefore the power to identify a QTL is maintained (Soller *et al.,* 2006). The identification of QTL associated with a trait could lead to greater examination in order to find the causative gene or linked marker that can be used for marker-assisted selection (MAS) in breeding programmes.

1.3.2 Microsatellites markers and QTL mapping

With the development of the polymerase chain reaction, amplification of DNA in a rapid inexpensive manner became available to detect variation. PCR techniques were used to identify short repetitive sequence length variations – microsatellites. Their many characteristics, such as codominance, high polymorphism and multiallele dispersion in the genome were used as DNA-based markers for breeding selection. This approach produced microsatellite-based genomic maps, which were then applied in studies on experimental crosses for identification of genomic regions influencing commercially important traits. With the use of genomic maps of the location of specific microsatellites, the identification of genomic regions responsible for quantitative traits became possible.

Many of the studies utilised the inbred lines and their crosses characterised with resistance and susceptibility to certain pathogens in identification of QTL. Instead of reviewing all literature on how immune function has been implicated in

genomic studies on heritable resistance to infection I will focus on selected pathogens as examples. As of August 2015, the animal genome QTL database reported 224 publications identifying more than 4,676 QTL for 319 traits, including those for specific disease resistance (www.animalgenome.org). The QTLs associated with MDV resistance have been studied in resistant and susceptible inbred lines, which were developed in ADOL. Several potential QTL have been determined from backcrosses between these lines. Vallejo *et al.* (1998) used inbred lines that differ in their susceptibility to Marek's Disease Virus (MDV) to produce intercross progeny in order to map QTL affecting MDV susceptibility. With the use of 78 microsatellites markers, six QTL were mapped with significant and suggestive association with Marek's Disease (MD) traits. Following these experiments Yonash *et al.* (1999) genotyped all birds used in the previous study and added 49 new microsatellites markers. McElroy *et al.* (2005) reported identification of the same QTL for MD resistance in commercial layers, showing that the experimental inbred lines and their crosses are useful tool for commercial populations. In addition, Heifetz *et al.* (2007), using microsatellites markers, confirmed localisation of previously reported QTL significantly associated with resistance to MD in commercial lines.

Mariani *et al.* (2001) used microsatellite markers in low density QTL mapping of a substantial component of resistance to systemic salmonellosis (SAL1) to chicken chromosome 5. These experiments were performed on a highly susceptible inbred line and highly resistant inbred line to *Salmonella* infection and their progeny from the backcross with the parental line. Separately, Beaumont *et al.* (2003) demonstrated the importance of genomic region carrying gene SLC11A1 in *Salmonella* resistance. Populations derived from inbred lines were used in the identification of several QTL for resistance to carrier state using selective genotyping approach (Tilquin *et al.,* 2005). The bacteria count of *S.* Enteritidis and *S.* Typhimurium from cloacal swabs in backcross and F2 progeny determined which individuals were characterised by extreme phenotype and selected birds were genotyped. Studies on *Salmonella* carrier state QTL in commercial lines confirmed that the locus carrying the SLC11A1 (Nramp1) candidate gene is significantly

associated with carrier state resistance variations in different chicken lines. In addition, a QTL associated with *Salmonella* gut colonisation has been identified on chromosome 2 (Calegne *et al.*, 2009).

1.3.3 High-density single nucleotide polymorphism (SNP) panels

Together with the first chicken genome assembly in 2004 a 2,8 million single nucleotide polymorphism (SNP) map was produced (International Chicken Polymorphism Map Consortium, 2004) which further allowed identification of many QTL and determined many disease-related genes, e.g. MHC, SLC11A1 (Nramp1), IFN, TLRs, IRAK-4, MyD88, NFκB, TNFSF, using various molecular technologies (reviewed in Kaiser et al., 2008).

The SNP number and density has been increasing since the first published SNP map (Wong *et al.,* 2004). Re-sequencing of divergent chicken lines provides additional SNP (Rubin *et al.,* 2010). With the use of high-density SNP panel, Fife *et al.* (2009) refined the location of SAL1 locus and two potential candidate genes were identified (SIVA1 and AKT1). The SNP panel was used in studies on identification of *Salmonella* colonisation QTL. The four QTL, mapped to chromosome 2, 3, 12 and 15, are significant at either the genome-wide or the chromosome-wide level (Fife *et al.,* 2010). However, to identify causative genes higher density SNP panels are needed. Genomic analysis of advanced intercross lines responses to *Salmonella* has supported the importance of the SAL1 QTL that contains AKT1 and SIVA1 genes as candidates that control resistance to systemic salmonellosis (Redmond *et al.*, 2011). The fine-mapping of QTLs is limited by poor precision in gathering phenotypic data and reliability of these assessments. The development of new technologies such as RNA-seq, microarray expression analyses and high density SNP genotyping should expedite the search for mechanisms of genetic resistance (reviewed in Calenge *et al.*, 2010).

Other causes of genetic variation, in addition to SNPs, are emerging and can be associated with disease resistance. Copy number variations (CNVs) are difficult for most mapping approaches and for sequence assembly and were therefore

largely ignored in the search for QTL (reviewed in Dodgson *et al.*, 2011). CNVs are a type of genomic structural variation involving deletions, insertions and duplications and range from kilobase to megabases. Now it is clear that CNVs are involved in phenotypic differences such as late feathering (Elferink *et al.*, 2008). The number of nucleotides affected by CNVs has been shown to exceed the number of SNPs (Conrad *et al.*, 2010). Wang *et al.* (2010) observed 96 CNVs in 10 sampled birds. Among those CNVs, fifteen involved functional genes. Recently the number of CNVs and CNV regions (CNVRs) increased to 130 with the use of an Agilent 400k array CGH (comparative genomic hybridisation) platform (Wang *et al.*, 2012).

1.3.4 Combination of gene expression and genomic markers

By considering gene expression as a quantitative trait or phenotype and combining with other genetic markers, the genomic location(s) that control variation can be revealed (Cogburn *et al.,* 2007). The combination of gene expression studies and marker genotypes has a great promise for studies of complex traits. By comparing gene expression data with the location of QTL previously associated with MDV traits in inbred lines, the IRG1 gene was identified as having a potential role in MD susceptibility (Smith *et al.,* 2011). Heams *et al.* (2011) used gene expression with comparison with mapped QTL region to study *Eimeria tenella* infections. This approach highlighted potential candidate genes that are positioned within significant QTL for *Eimeria* lesions. The QTL associated with variation can be either *cis* or *trans* to the gene of interest. The *cis*-eQTL could be interpreted as the sequence flanking gene, hence regulating its expression. The *trans*-acting eQTL could modulate the expression by transcription factors. The former are more difficult to identify, even with a complete genome sequence (Cogburn *et al.,* 2007).

1.4 Studies on differences in gene expression between chicken lines

As stated in the previous section, over the years, genetic resistance to diseases in chickens has been studied in context of various pathogens. In this

section, examples of mRNA gene expression and functional studies will be reviewed in context of heritable natural genetic resistance.

Divergent chicken lines were used to study immune responses to *Salmonella* species, *Eimeria* species, and *Campylobacter jejuni*. Studies by Wigley *et al.* (2002) on resistant inbred birds showed lower mortality and morbidity when infected with *Salmonella* Gallinarum. Subsequent *in vitro* comparison of macrophages from resistant and susceptible lines revealed that innate immunity played a role in responsiveness to *S.* Gallinarum with better clearance of bacteria in the former line. Increased expression of pro-inflammatory cytokines in the resistant line suggested efficient initiation of innate and adaptive immune responses that is pivotal in immunity to systemic salmonellosis (Beal *et al.*, 2004). The results were confirmed in studies on the same lines monocyte-derived macrophages stimulated with *S.* Gallinarum and *S.* Typhimurium (Wigley *et al.*, 2006) where higher and more rapid mRNA gene expression of cytokines and chemokines was observed in macrophages from resistant line.

In addition to macrophages, heterophils from resistant and susceptible lines have been shown to respond differently to infection with *S.* Enteritidis (Swaggerty *et al.*, 2003). Commercial lines and their reciprocal crosses were evaluated based on heterophil degranulation, phagocytosis and oxidative burst responses when challenged with *Salmonella*. The results showed that the ability of heterophils to efficiently react to infection is genetically transferred to the progeny. A study on systemic infection with *S.* Enteritidis administered into the abdomen of day-old chickens from the parental line and reciprocal crosses showed heterophil influx to the site of infection to be much higher in the resistant line and the progeny compared to susceptible line (Swaggerty *et al.*, 2005). These studies were supplemented with experiments on mRNA gene expression after stimulation with *S.* Enteritidis in the same commercial lines and their reciprocal crosses (Ferro *et al.*, 2004; Swaggerty *et al.*, 2004). The upregulation of pro-inflammatory cytokines was observed in heterophils isolated from the resistant chickens and their F1 progeny.

Similar responses in more resistant lines were observed when birds were challenged with the Gram-positive bacterium *Enterococcus* (Swaggerty *et al.*, 2005).

Swaggerty *et al.* (2008) continued the evaluation of commercial broiler line immune responses where mRNA expression of pro-inflammatory cytokines and chemokines from sires and generated progeny were assessed. The RNA from peripheral blood leukocytes (PBL) was used in quantitative PCR reactions where expression of IL-1β, IL-6, CXCLi2 and CCLi2 was tested. Gene expression profiles of pro-inflammatory cytokines and chemokines in leukocytes from high expression sires led to higher expression of the selected genes in generated progeny. In addition, the results showed that progeny from sires characterised with low gene expression had also lower immune performance. The highest values of gene expression from those birds were much lower compared to the lowest values from high expression sires progeny. This approach allowed for identification of sires with higher/lower than average expression of proinflammatory cytokines and chemokines. The progeny produced from the selected sires carried similar immune responsiveness without losing the desired growth qualities.

The same lines, parental and F1 reciprocal crosses were challenged with *Campylobacter jejuni* and evaluation of cloacal swabs revealed that again, resistant lines and their sires progeny had significantly fewer *C. jejuni* colonies. These results indicated that paternal effects influenced the resistance to bacterial colonisation (Li *et al.*, 2008c). The continuation of testing commercial lines and their F1 crosses confirmed that resistant lines and paternal progeny managed to enhance the responses to coccidial infections accompanied by higher gain weight (Swaggerty *et al.*, 2011; Swaggerty *et al.*, 2015), proving that the efficient innate responsiveness guards against not only bacteria but broader range of pathogens, including parasites. Transcriptome analysis of splenic gene expression in parental lines challenged with *Campylobacter* revealed differences in molecular regulations during infection. Moreover, different defence mechanisms were involved in the *Campylobacter* resistant line where genes involved in apoptosis and cytochrome c

release from mitochondria were activated compared to susceptible line (Li *et al.*, 2012).

Schokker *et al.* (2012) studied immune responses of three commercial broiler lines after infection of newly hatched birds with *S.* Enteritidis. The results showed that different lines differed in their response to systemic spread of bacteria possibly due to variation in intestinal development. Another study on responses to *S.* Enteritidis in broiler, layer and Fayoumi lines showed differences in mRNA gene expression in the spleen which suggest that divergent genetic lines use different components of immune responses in the response to *Salmonella* infection (Coble *et al.*, 2011). Redmond *et al.* (2009), obtained similar results with a Fayoumi native line showing higher expression of selected innate immunity-related genes in heterophils stimulated with *S.* Enteritidis. Wang *et al.*, (2014) performed RNA-seq analysis on lungs from Leghorn and Fayoumi chickens samples and showed the latter to be more resistant to AIV infection with haemoglobin family genes playing a pivotal protective role. The studies where immune responses of indigenous Fayoumi chickens were compared to those of commercial broilers and layers show that native lines, without genetic selection, may provide biodiversity to improve breeding programmes for the innate immunity in commercial birds.

1.5 Transcriptomics tools for immune gene expression analysis

Gene expression triggered by infection is a trait that can be measured in both *in vitro* and *in vivo* studies on varied tissues and cells in the organism. This approach leads to understanding the regulation of genes and pathways during the disease and the discovery of biomarkers. To study gene expression, transcriptomics methods need to be applied. The transcriptome comprises the complete set of transcripts in a cell. Transcriptome profiles in response to biological stimuli, i.e. pathogen invasion, provide data to interpret functional elements of the genome and understand disease processes (Wang *et al.*, 2011). The transcription process is the first step of gene expression. Not every transcript will be translated into functioning protein, therefore gene expression cannot be interpreted as

corresponding protein levels. In chicken studies, where availability of tools and reagents lags behind the mammalian field, experiments with the use of transcriptomics methods offer relatively inexpensive way to identify eQTL and biomarkers for selection.

1.5.1 Microarrays

Microarrays have been used as a genomic tool since 1990 and rapidly became the platform of choice in transcriptomics studies in model organisms, including the chicken. The principle is based on comparison of transcript levels between two samples. Data are generated based on the cDNA reverse transcribed from the purified mRNA transcriptome of a sample (Murphy, 2002). The cDNA generated from test and control samples are separately labelled with different fluorescent dyes and hybridised to an array containing DNA probes for different genes. The intensity of the fluorescent signal resulting from hybridisation to a specific probe depends on the amount of hybridised cDNA from a given sample, and in turn reflects the abundance of the transcript from which it was reverse transcribed. The differences in the signals from both samples are analysed and interpreted as differential expression. This technology has been useful in many aspects of science, including potential biomarkers discovery or drug targets. More than a dozen microarrays, either cDNA- or oligonucleotide-based have been developed for chicken gene expression (reviewed in Cogburn *et al.*, 2003).

A commercial microarray for analysis of the chicken transcriptome with 44k probes (Li *et al.,* 2008a) is available from Agilent (http://www.genomic*S.* agilent.com). This new whole genome microarray was designed based on the 2004 chicken (*Gallus gallus*) v1.0 draft assembly and it has been widely used to measure mRNA levels. For example, Chiang *et al.* (2008) used this technique to profile differential gene expression in heterophils from two genetically distinct lines infected with *Salmonella* Enteritidis. It has been recently used to evaluate gene expression in liver of *S.* Enteritidis infected broilers (Coble *et al.*, 2013) and in leukocytes from APEC infected broilers (Sandford *et al.*, 2012). Other chicken arrays

were customised for measurement of gene expression in specific cell types (Bliss *et al.*, 2005) or gene sets (Smith *et al.*, 2006). The global view of gene expression produced by microarrays can identify candidate genes or pathways that are associated with differences in phenotype between the test and control systems (or animal lines) studied. As the probe set on the microarray is set for known cDNA sequences, this technology does not allow the detection of novel transcripts or sequence variants (Mortazavi *et al.*, 2008).

1.5.2 RNA sequencing

An alternative to microarrays is the use of next generation sequencing of cDNA derived by reverse transcription from mRNA (also termed RNA-seq). In this technique, the RNA population is converted to a library of cDNA fragments with adaptors attached to one or both ends. After amplification, each molecule is subjected to massively-parallel sequencing to obtain short sequences of 30-400 bp. The reads of sequenced fragments are then aligned to a reference genome (Wang *et al.*, 2009). This identifies the transcripts present, whereas the abundance of the sequence reads for a specific gene reflect the relative abundance of the transcript in the sample. The microarray approach is still preeminent for large numbers of samples in regard of costs, but RNA-seq techniques have the advantage that no assumptions are made as to which genes are likely to be transcribed. In addition, RNA-seq is more sensitive due to its massively-parallel 'deep sequencing' nature and it is more accurate because the quantification is based on digital counts of the sequence reads corresponding to each transcript. RNA-seq is competing to replace microarrays for analysing the transcriptome in an unbiased and comprehensive manner (Wang *et al.*, 2011). The ability to measure allele-specific expression (ASE) in heterozygotes using RNA-seq is advantageous over microarrays technique in which the same probe set targets both alleles (Sun, 2012). RNA-seq also provides tools for the discovery of new un-annotated genes of interest (Dodgson *et al.*, 2011).

The advantages of RNA-seq over microarrays have advanced progress of transcriptomics field producing great amount of data. Since 2008, when RNA-seq technology became available, more than a thousand research articles on gene expression were published in the PubMed database, including chicken studies. This includes published work on necrotic enteritis (NE) and differential gene expression (DEG) in inbred lines (Truong *et al.,* 2015), AIV infection in Fayoumi and leghorn lines (Wang *et al.,* 2014), and caecal tissues responses to *Campylobacter jejuni* (Connell *et al.,* 2012).

1.5.3 Quantitative polymerase chain reaction (qPCR)

In addition to high-throughput transcriptome analysis tools, quantitative PCR (qPCR) has been used for more than two decades to quantify transcription of specific genes. It is a technique where amplification and simultaneous quantification of targeted DNA molecules is possible. Transcripts are first reverse transcribed to cDNA, for example using oligo dT targeting polyA tails and random hexamers. The abundance of cDNAs is assumed to mirror that of template transcripts. Specific cDNAs can then be detected in PCR reactions using primers that anneal to the target sequence. Two main approaches exist to detect amplification. The first uses a labelled nucleotide that is incorporated into the cDNA, thus the amount of labelled product reflects the abundance of the target transcript. The second uses a probe that hybridises to the target cDNA between the primer annealing sites and which has a 5' fluorophore and 3' quencher. During PCR with flanking primers, the fluorophore is removed by the 5'-3' exonuclease activity of advancing polymerase. The quencher no longer inhibits fluorescence of the released fluorophore and the fluorescence intensity proportional to the amount of product made in exponential phase of PCR (threshold value). Comparison of number of amplification cycles that reached particular quantification threshold fluorescence signal allows the initial amounts of cDNA template to be quantified. Therefore, fewer PCR cycles are needed for the detection if more copies of cDNA molecules are present at the beginning of the reaction. Compared to previously described transcriptome platforms, qPCR is useful in studies where gene expression of only a subset of

transcripts of interest is evaluated during infection or stimulation. This type of targeted expression is beneficial when genes involved are known to be responsible for different outcomes and it has been widely used in chicken immune responses studies (Kaiser *et al.,* 2006; Abasht *et al.,* 2009; He *et al.,* 2012). QPCR analysis does not involve complex bioinformatics analysis of the output and is considered straight forward relative to RNA-seq. After the cautious selection of primers and optimisation of reaction, qPCR delivers results within hours.

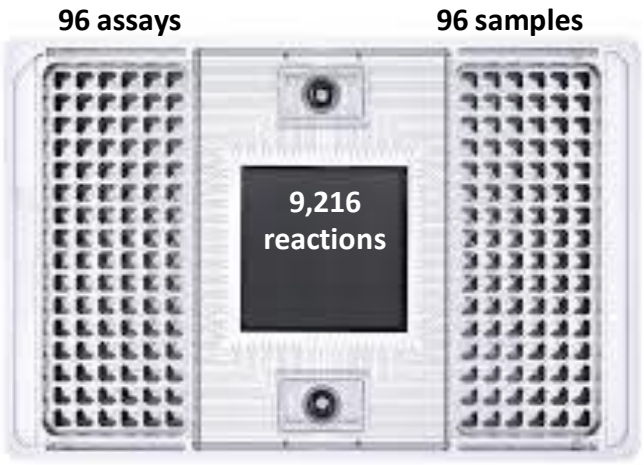
In recent years, emphasis has been placed on selection of reference genes for qPCR to permit comparison between samples that may differ in composition and integrity. The lack of consensus in reagents, protocols and analysis methods used for qPCR, and the high-profile retractions of manuscripts, for example from *Science* (Bohlenius *et al.,* 2007), have forced qPCR experts to provide guidelines for authors, reviewers and editors with specification for the minimum information that must be included in the manuscript methods. As discussed in Chapter 5, the selection of reference genes needs to be more stringent and properly normalised before their use in qPCR reactions (Bustin *et al.,* 2009).

1.5.4 The BioMark System

The BioMark system from Fluidigm is a platform to run multiple qPCR reactions against multiple RNA samples at once. It relies on microfluidics to perform qPCR reactions on a nanolitre scale and was made possible by advances in micro- and nanofabrication. There are four high-throughput qPCR platforms that use microfluidics currently on the market: the BioMark system, the OpenArray from Life Technologies, the LightCyclerTM 1536 from Roche and SmartchipTM from Wafergen, that allow for parallel gene expression analysis. Only the BioMark System is relevant for this project, therefore other platforms will not be discussed here. The Dynamic Array Integrated Fluid Controller is a microfluidic chip for the BioMark system that allows for amplification of single molecule in the microfluidics chip that consists of matrixes of chambers and valves (Figure 1.3). The dynamic array chip exists in two formats: 48.48 and 96.96. The 48 (or 96) samples are simultaneously

used in qPCR with 48 (or 96) assays which results in 2,304 (or 9,216) reactions from one run. The use of a dynamic array greatly reduces the volume of reagents and pipetting and is a reliable and rapid method for high-throughput gene expression analysis (Spurgeon *et al.,* 2008).

a)



b)

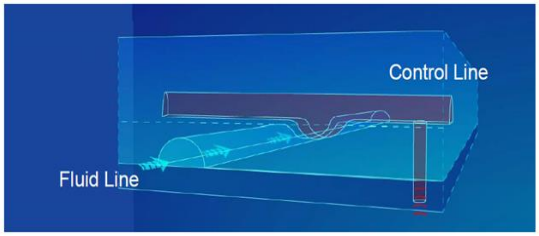


Figure 1.3. Structure of 96.96 Dynamic Array IFC for gene expression. a) 96 assays and 96 samples with qPCR master mix are loaded in different dimensions. The IFC controller mix both samples and assays in 9,216 contained chambers, each with volume of 6,7 nl; b) The mixing of assays and samples is possible with the use of network of control lines and fluid lines that transfer liquids into the reaction chambers where qPCR reaction occurs.

The statistical power of an experiment increases when in a single run greater number of replicated reactions can be performed (Weaver *et al.,* 2010). Therefore, from biomarker screening perspective, high-throughput qPCR is beneficial with additional sensitivity, reproducibility and possible detection of very low abundant targets.

1.6 **Aims and objectives**

For decades, breeding selection has focused on overall livability and phenotypic traits, such as weight gain and feed conversion efficiency, rather than targeting the immune system directly. The widespread use of vaccines and antibiotics has meant that relatively little emphasis has been placed on selection for disease resistance. A danger exists that breeding for resistance to particular pathogen may increase susceptibility to other pathogens. In this context, selection for birds with elevated innate immune function is desirable given the non-specific nature of the response. Selection of lines with improved immune robustness could solve the problems of greater variation of pathogens and emergence of new resistant strains and reduce the need for antibiotic use.

The overarching hypothesis for this study is that disease resistant birds are inherently primed to produce a stronger innate immune response, and hence control invading pathogens better by killing and mounting stronger adaptive immune response. This hypothesis is supported by previous studies, which showed that effector cell function and pro-inflammatory gene expression was stronger and more rapid in resistant lines compared to susceptible lines. I therefore aimed to:

1. Evaluate existing cellular and molecular assays as tools for swift and accurate determination of immune robustness in the chicken, with emphasis on analysis of heterophil, macrophage and dendritic cell phenotypes.

2. Generate a list consisting of approximately 100 genes of interest that are involved in immune responses to a plethora of infections caused by bacteria and bacterial components, viruses and parasites. The gene list will be created based on published challenge studies in chicken and mammalian species as well as an RNA-seq analysis of chicken effector cells (bone marrow-derived dendritic cells (BMDC), bone marrow-derived macrophages (BMDM) and heterophils) stimulated with lipopolysaccharide (LPS). For novel genes identified by this approach I aimed to clone and sequence the genes to aid the design of assays of gene expression.

3. Select a platform for gene expression analysis, optimise and validate it with the use of an Aviagen broiler line housed in low biosecurity, resembling commercial settings (sibling test farm) and high biosecurity (pedigree farm) environments. Primers will be designed for selected genes and used in high-throughput qPCR reactions with tissues and blood RNA as template.

The ultimate goal was to evaluate if peripheral leukocytes isolated from blood could be used to detect changes in the level of immune robustness between chickens raised in different environments and within the same farm.

Chapter 2 Materials and Methods

2.1 *In silico* materials

2.1.1 Basic Local Alignment Search Tool (BLAST)

Sequences of all cloned DNA fragments ,presented in Chapter 4, were compared with the chicken genome database (Gallus_gallus-4.0) using BLAST software. This heuristic algorithm finds similar sequences by locating short matches between the query and target sequence. This method allows observed and expected sequences to be compared and can identify single nucleotide changes or insertion/deletion events (Altschul *et al.,* 1990).

2.1.2 ClustalX 2.1

ClustalX is a general purpose multiple sequence alignment tool for DNA or proteins. Differences, similarities and identities can be visualised after the best matches are calculated and lined up for the selected sequences. ClustalX uses colour coding where the darkest shading indicates highest conservation (Larkin *et al.,* 2007).

2.1.3 GeneDoc

GeneDoc provides tools for visualising multiple alignments of nucleic acid sequences. GeneDoc displays sequence alignments with different shading modes, that depend on the level of conservation between observed and expected sequences (Nicholas *et al.,* 1997). Both, ClustalX and GeneDoc were used to present alignments of cloned genes (Appendix 2, electronic file).

2.1.4 Bowtie 1.0.0

Bowtie software was used in RNA-seq analysis pipeline (Appendix 1, Figure 1). Bowtie is an ultrafast short-read aligner that employs a Burrows-Wheeler index and full-text minute-space (FM) index. Reverse permutation of the characters in text, as in the Burrows-Wheeler algorithm, is applied in Bowtie to allow large data of text to be searched efficiently while keeping the memory footprint small. FM index is the exact-matching algorithm and Bowtie uses two extensions to match the

sequencing errors or genetic variations. Bowtie input data can be in FASTQ and FASTA formats. Bowtie forms the basis for other tools, including TopHat and Cufflinks (Langmead *et al.,* 2009).

2.1.5 **TopHat 2.0.9**

TopHat is a script that aligns RNA-Seq reads to a genome in order to identify exon-exon splice junctions. Bowtie cannot align reads that span introns. TopHat was created to address this issue of large gaps in alignments. It uses Bowtie as an alignment tool and breaks up reads into smaller pieces called segments. Many of these segments align contiguously which results in build-up of an index of splice junctions (Trapnell *et al.,* 2009).

2.1.6 **Cufflinks 2.1.1**

Cufflinks uses output data from TopHat to assemble aligned RNA-seq reads into transcripts. Multiple splice variants may be present for a given gene, therefore to overcome this issue Cufflinks reports a parsimonious transcriptome assembly of the data. Only few full-length transcripts fragments (transfrags) are defined by Cufflinks to sufficiently explain all the splicing event outcomes in the input data. To derive a likelihood of abundance levels and filter out artificial transfrags Cufflinks uses a statistical model of paired-end sequencing experiments. The software then computes the overall likelihood by multiplying these probabilities. The outcome of properly normalised RNA-seq fragment counts can be used as a measure of relative abundance of transcripts. Cufflinks uses Fragments per Kilobase of exon per Million fragments mapped (FPKM) to present expression of each transcript. Cufflinks contains a program (Cuffcompare) that can support comparison of assembled transfrags and reference annotation. Cufflinks also includes a script called Cuffmerge that allows merging of several Cufflinks assemblies. Differential expression of transcripts can be verified using Cuffdiff 2 that tests the observed log-fold change in gene expression against the null hypothesis of no change. Cuffdiff 2 predicts how much variance is in the number of reads originating from a gene or transcript. It is completed in the form of a table that is keyed by the average reads

across replicates. The table is queried to retrieve the variance from the number of reads. By simulating assignment to a locus and to the splice isoforms for that locus, Cuffdiff 2 accounts for probable errors in gene mapping. At the end of the estimation procedure, Cuffdiff 2 obtains an estimate of the number of reads that originated from each gene and transcript, along with variances in those estimates. The read counts are reported along with FPKM values and their variances. Change in expression is reported as the log fold change in FPKM, and the FPKM variances allow the program to estimate the variance in the log-fold-change itself. Genes with highly variable expression will have highly variable log-fold change between two conditions (Trapnell *et al.,* 2010).

2.1.7 **BestKeeper software**

BestKeeper is an Excel-based programme that is used to compare expression levels of reference genes and target genes in up to one hundred biological samples. BestKeeper input data is in form of raw Cq values which are plotted in an Excel table. Pairwise correlation analysis and the geometric mean determine the 'optimal' reference genes (Pfaffl *et al.,* 2004). The BestKeeper software was used in Chapter 5 to select most stable gene.

2.1.8 **NormFinder**

NormFinder is an algorithm for identifying the optimal reference gene among a set of candidates. It ranks a set of genes according to their expression stability in a given sample. The software uses a model-based approach of gene expression and statistical framework to estimate variation in overall expression and variations between subgroups. NormFinder automatically calculates the stability value for all candidate genes (Andersen *et al.,* 2004). The Normfinder software was used in Chapter 5 to select most stable genes.

2.1.9 **geNorm**

GeNorm is an algorithm that determine the most stable reference genes from a set of tested candidate genes in a given sample panel. A gene expression

normalisation factor can be calculated for each sample based on the geometric mean of a user–defined number of reference genes. Pairwise variation of each gene is determined as standard deviation of the logarithmically transformed expression ratios. An M value is calculated for a particular gene as the arithmetic mean of pairwise variation with all other genes in the same panel. Genes that are stably expressed are characterised by the lowest M value. Stepwise exclusion of the least stable genes results in combination of two constitutively expressed reference genes (Vandesompele *et al.,* 2001). The geNorm software was used in Chapter 5 to select most stable genes.

2.2 Molecular cloning

2.2.1 *E. coli* JM109 competent cells

E. coli JM109 strain {*endA1, recA1, gyrA96, thi, hsdR17* (rk–, mk+), *relA1, supE44, Δ(lac-proAB),* [F´, *traD36, proAB, lacIqZΔM15*]} has a mutation in the *recA* gene to improve plasmid stability and reduce scope for unwanted recombination events. The JM109 strain also carries the *endA1* mutation that inactivates a nuclease that might co-purify with plasmids. This mutation allows purification of higher quality plasmids. JM109 bacterial cells are classified as an *E. coli* K strain based on the presence of the restriction and modification system that functions around *EcoK* I. JM109 cells carry the *hsdR17* (r_K-, m_K+) mutation that inactivates the *EcoK* I restriction enzyme but leaves the methylase intact. Therefore, this strain does not degrade plasmid DNA isolated from K strains but does methylate it.

2.2.2 pGEM-T Easy

pGEM-T Easy is a 3015 bp linear vector used for cloning of PCR products (Figure 2.1). The plasmid contains a single thymidine extension at the 3'-ends that are complementary to the non-template-derived 3'-adenosine residues that are added to double-stranded DNA products by *Taq* DNA polymerase. The 3'-end thymidine also prevents the vector from recircularisation during ligation. This high-copy-number vector contains T7 and SP6 RNA polymerase promoters flanking a multiple cloning site located within a truncated *lacZ* gene that encodes the α-

peptide coding region of the enzyme β-galactosidase. The host *E. coli* strain carries a partial *lacZ* deletion (*lacZΔM15*) which encodes the ω-peptide. Neither, α- or ω-peptide is functional by itself. Transformation of plasmid containing *lacZ* α sequence into the *lacZΔM15* cells causes the formation of functional β-galactosidase enzyme. A molecular mimic of allolactose - Isopropyl β-D-1-thiogalactopyranoside (IPTG) is added to the agar to induce the *lac* promoter that drives α-peptide synthesis. The presence of a functional β-galactosidase is detected by galactose linked to a substitute indole, called X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) added to the agar. X-gal is cleaved by β-galactosidase to formo 5-bromo-4-chloro-indoxyl, that spontaneously forms the insoluble pigment 5,5'-dibromo-4,4'-dichloro-indigo resulting in blue colour in cells containing functional enzyme. The process of rescuing function of β-galactosidase by the α-peptide, called α-complementation, is used in the blue/white screening method. White colour of JM109 colonies transformed by pGEM-T recombinants indicate that formation of an active β-galactosidase was disrupted by the insertion of a gene of interest into *lacZα*.

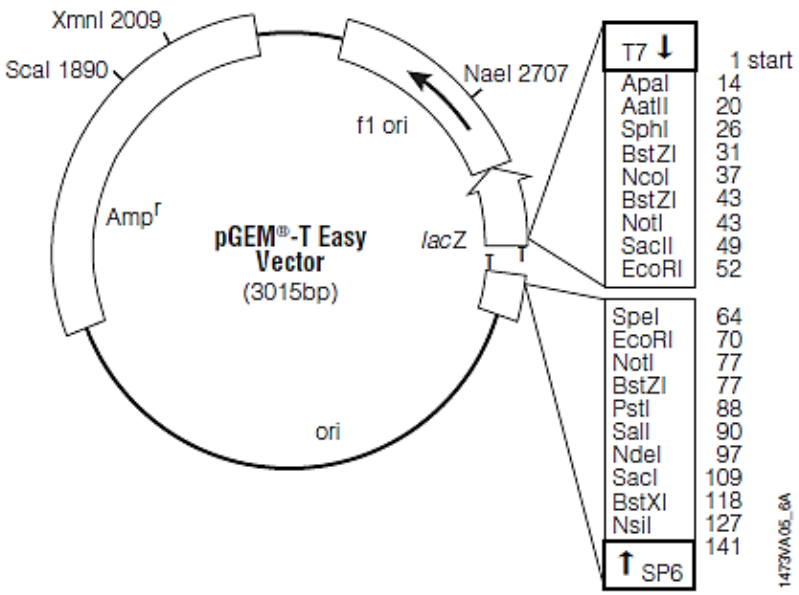


Figure 2.1. Map of pGEM-T EASY vector

2.3 Cell cultures

2.3.1 Resurrection and passage of COS-7 cells.

COS-7 cells were used to produce of recombinant chicken proteins, which were used to generate bone marrow-derived cells, as presented in Chapter 3. COS-7 [**C**V-1 (simian) in **O**rigin, carrying the **S**V40 genetic material] is a fibroblast-like cell line derived from African Green Monkey *Cercopithecus aethiops*. COS-7 cells were removed from liquid nitrogen storage and defrosted in a water-bath at 37°C. Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) heat-inactivated foetal calf serum (FCS), 200 mM L-glutamine, 100X non-essential amino acids, 1 U/ml of penicillin and 1 µg/ml of streptomycin was used to resuspend the cells. After washing and pelleting cells at 1,200 x *g* for 5 min the supernatant was discarded, cells resuspended in 15 ml of complete DMEM in 75 cm^2 culture flask (Thermo Scientific) and incubated at 37°C, 5% CO_2. After 72 hours, cells were 70-80% confluent and ready to passage. The cell layer was washed twice with pre-warmed phosphate-buffered saline (PBS). To lift the cells from the flask 5 ml of PBS containing 10% (w/w) trypsin/versene solution was then added. After 5 min in 5% CO_2, at 37°C the flask was tapped several times to detach any remaining cells from the surface and complete DMEM was added to quench the trypsin enzymatic activity. The cell suspension was pelleted at 1,200 x *g* for 5 min in a 30 ml Universal container. The supernatant was removed and cell pellet resuspended in 10 ml of complete DMEM. Cell number was determined using a haemocytometer and trypan blue, for exclusion of dead cells. Cells were seeded at 7.5 x 10^5 cells/ml in 75 cm^2 culture flasks. All cell culture reagents were sourced from Sigma-Aldrich Ltd (Dorset, UK).

2.4 Transfecting cells with plasmid DNA

2.4.1 DEAE-dextran transient transfection method for COS-7 cells

Transfection mediated by diethylaminoethyl (DEAE)-dextran works very efficiently with the COS-7 cell line. Negatively charged plasmid DNA complexes with positively charged DEAE-dextran to create aggregates, which then bind to negatively charged surface structures on cell membranes and uptake via

endocytosis occurs. Addition of chloroquine prevents endosome acidification and inhibits lysosomal degradation of the DNA.

COS-7 cells were passaged and seeded in complete DMEM media at 2 x 10^6 cells/ml in 25 cm^2 flasks. After twenty-four hours DMEM media were replaced by serum-free media containing chloroquine (0.1 µM), plasmid DNA (37.5 µg), DEAE-dextran (30 µg/ml) and cultures were incubated for 3.5 hours at 37°C, 5% CO_2. After incubation, cells were treated with dimethyl sulphoxide (DMSO) (10% (v/v) in PBS) for 2 min to enhance uptake of adsorbed plasmid DNA by increasing the permeability of cell membranes. DMSO solution was replaced with complete DMEM media and cells were incubated for further a 24 hours at 37°C, 5% CO_2. Complete media was replaced with serum-free media and recombinant protein-containing supernatant was harvested after 72 hours. To remove any cell debris the supernatant was centrifuged at 1,200 x *g* and then stored at 4°C until needed.

2.4.2 Production of recombinant chicken IL-4, GM-CSF and CSF-1

Recombinant chicken IL-4, GM-CSF (CSF-2) and CSF-1 proteins were produced in the COS-7 transient expression system described in section 2.4.1 for generation of bone marrow-derived dendritic cells and macrophages. Plasmids containing CSF-1, IL-4 and GM-CSF inserts were available in *E. coli* strains as glycerol stocks. To reach a single colony bacterial glycerol stocks were streaked out on LB agar plates containing 100 µg/ml of ampicillin (LB_{AMP100}) and incubated overnight in 37°C. Single colonies were placed in 5 ml of LB_{AMP100} Broth and incubated at 37°C, 200 rpm for 24 hours. LB_{AMP100} Broth, in volume of 250 ml, was used to subculture bacteria (2.5 ml) for an additional 18 hours in 37°C, 200 rpm. Bacterial suspensions were centrifuged at 3,000 x *g* for 30 min and pellets were used in a modified plasmid extraction protocol (EndoFree Plasmid Maxi Prep, Qiagen; section 2.8.2).

2.5 Primary cell experiments

2.5.1 Generation of bone marrow-derived dendritic cells (BMDC) and bone marrow-derived macrophages (BMDM)

All the animals used in the work reported in this thesis were handled and killed in accordance with the Animals (Scientific Procedures) Act 1986. The chickens used in the following experiments were Brown Leghorn-J line birds housed by the National Avian Research Facility at The Roslin Institute.

Immediately after death by cervical dislocation, both femurs and tibias were removed aseptically from 4-6 week old birds and placed in phosphate-buffered saline (PBS) on ice until further use. Using sterile scissors both ends of each bone were cut off. Bone marrow was flushed from the bone using a 0.8 x 40 mm diameter needle (21G x 1.5 Terumo) and syringe with 10 ml of PBS. Cells suspensions were passaged through 70 µm nylon mesh strainers (Fisher Scientific) into 50 ml Falcon tubes. Cells were pelleted at 500 x *g* for 10 min at room temperature and resuspended in 10 ml of PBS. Histopaque 1.077 was used to separate mononuclear cells (MNCs) by underlying the bone marrow cell suspension and centrifugation at 1,200 x *g* for 20 min with the brake switched off. The enriched MNC fraction between the plasma and Histopaque layers was carefully removed by aspiration with a Pasteur pipette and placed in 30 ml Universal tube. Cell suspensions were washed in PBS by centrifugation at 500 x *g* for 10 min at 4°C. Cell pellets were resuspended in 10 ml of pre-warmed Roswell Park Memorial Institute (RPMI) 1640 medium. Cell viability was assessed by Trypan blue staining and live cells were counted and adjusted to 1 x 10^6 cells/ml concentration in RPMI medium supplemented with 10% (v/v) chicken serum (CS) (in BMDC cultures) or 2% (v/v) CS + 3% (v/v) foetal calf serum (FCS) (in BMDM), 200 mM L-glutamine, 1U/ml of penicillin and 1 µg/ml of streptomycin. To culture BMDC, cells were placed in 6-well plates (Thermo Scientific) in a volume of 3 ml with appropriate dilutions of recombinant chIL-4 and (1/20) GM-CSF (1/20) (exCOS-7) and incubated at 41°C, 5% CO_2. After two and four days of culture 75% of the medium was replaced with fresh complete RPMI with addition of chIL-4 and chGM-CSF. To culture BMDM cells

concentration was adjusted 1.5 x 10^7/ml in 15 ml complete media with appropriate CSF-1 dilutions, placed in square Petri dish and incubated at 41°C, 5% CO_2. For BMDM culture in 6-well plates, the same concentrations were used as for BMDC. Complete medium with cytokines was replaced at day four of culture.

2.5.2 Optimising LPS concentrations for stimulating BMDC and BMDM

Primary bone marrow cell cultures were used to determine the concentration of lipopolysaccharide (LPS) (*Escherichia coli* 055:B5, Sigma Aldrich) to induce maturation of antigen-presenting cells (APCs). BMDC and BMDM were cultured in 6-well plates, as described in section 2.5.1. Stimulation of cells was performed on day 6 of culture with various amounts of LPS: 20, 200 and 250 ng/ml LPS (20 min, 1 h and 4 h) for BMDM or 20 ng/ml and 200 ng/ml LPS (4 h and 24 h) for BMDC. Cells were removed from wells by pipetting and pelleted for 5 min at 500 x *g*. RLT buffer from RNeasy Mini Kit (QIAGEN) was used to lyse the cells. The lysate was stored at - 20°C until use. Total RNA was purified from each sample following the manufacturer's protocol as described in section 2.6.1. The amount of LPS used in stimulation of heterophils was previously optimised (Farnell *et al.,* 2003) and 10 µg/ml for 1 h was used. LPS stimulated bone marrow-derived cells were used in Chapter 3 (evaluation of cellular methods) and in Chapter 4 (RNA-seq).

2.5.3 Isolation of heterophils from chicken blood

Peripheral blood was collected by cervical dislocation and decapitation of day-old chickens from Hy-Line W36, Aviagen broilers or RI-J line into Spray-coated K_2 ethylenediaminetetraacetic acid (EDTA) tubes (BD Diagnostics, USA). The blood from Novogen breed was collected from embryos at day 20 into Universal tubes containing 5 mM EDTA. Blood from approximately 100 chickens was pooled and mixed 1:1 with 1% (w/v) methylcellulose (Sigma Aldrich, Poole, UK), prepared in RPMI medium. The mix was centrifuged at 25 x *g* for 15 min at 4°C. The supernatant was carefully removed to a fresh 50 ml Falcon tube. Ca^{2+}- and Mg^{2+}-free Hanks balanced salt solution (HBSS, Sigma Aldrich) was added to the supernatant to a final volume of 50 ml. A discontinuous Histopaque gradient (Sigma Aldrich, specific

gravity 1.077/1.119) was prepared by adding 10 ml of Histopaque 1.077 to a Falcon tube and carefully under layering 15 ml of Histopaque 1.119. The supernatant (25 ml) was then layered over the gradient solution and centrifuged at 250 x *g* for 1 h at room temperature. The mononuclear cells appeared between the plasma and gradient phases, whereas heterophils were suspended in the 1.119 gradient phase. Heterophils were collected into a new tube and mixed with RPMI to dilute the cell solution and centrifuged at 425 x *g* for 15 min at 4°C. The cell pellet was then resuspended in RPMI. Cells were counted, diluted to the desired concentration and stored on ice before use. The RNA isolated from heterophils (Novogen, Aviagen, RI-J lines) was used in RT-qPCR reactions, as described in Chapter 3. In addition, RI-J heterophils RNA was used in RNA-seq analysis, as described in Chapter 4.

2.5.4 Isolation of peripheral leukocytes from chicken blood

Blood was collected from three-week-old male Aviagen broilers into tubes containing 5 mM EDTA. Peripheral blood leukocytes were separated from 1 ml of whole blood sample and red blood cells removed, as described above in section 1.5.3. PBS was combined with blood to a total volume of 4 ml. A Histopaque 1.077/1.119 (Sigma Aldrich, Dorset, UK) discontinuous gradient was prepared by underlying 4 ml of Histopaque 1.077 with 4 ml of Histopaque 1.119 in 15 ml Falcon tube and overlying 4 ml of blood/PBS mixture. The gradient mixture was centrifuged at 700 x *g* with brakes off for 30 min at room temperature. Cells were removed from plasma/1.077 Histopaque interface (mononuclear cells) and from the 1.077/1.119 Histopaque interface (heterophils). Cells were combined and washed twice with an equal volume of PBS by centrifugation at 600 x *g* for 10 min at room temperature. Cells were counted and 10^7 cells/ml was pelleted and lysed with buffer RLT with β-mercaptoethanol for further total RNA extraction. The RNA from PBL was used in optimisation of 96.96 Dynamic Array, as decribed in Chapter 6.

2.5.5 Phagocytosis assay

Live *Salmonella* Enteritidis (SE, #97-11771 strain; 10^7 cells/ml) was suspended in normal chicken serum (CS) and opsonised for 30 min at 39°C on a

rotary shaker. Bacterial suspension was washed twice with Ca^{2+}, Mg^{2+} - free HBSS and stored in 4°C until used. Heterophils from W36 Hy-Line were diluted to 5 x 10^6 cells/ml. A combination of 2 ml of heterophils and 2 ml of SE was centrifuged in sterile conical tubes for 15 min at 1,500 x *g* at 4˚C. Heterophils with the SE suspension were then incubated at 39˚C, 5% CO_2 for 1 h. The sample was submerged in an ice bath for 15 min to stop phagocytosis. Cells were washed with ice-cold RPMI and centrifuged for 15 min at 1,500 x *g*, 4˚C. The pellet was resuspended in 2 ml ice-cold gentamicin (100 µg/ml) and diluted in RPMI without phenol red. Cells were incubated for 1 h at 37˚C to kill extracellular bacteria and then centrifuged at 1,500 x *g* for 15 min, at 4˚C. The pellet was washed three times in ice-cold RPMI, for 15 min at 1,500 x *g.* Phagocytosis of SE by heterophils was evaluated microscopically. For each treatment group, five cytospin slides were prepared using 200 µl of cell suspension. Results were recorded as the phagocytosis index: PI = (number of heterophils that contain bacteria x the average number of bacteria per ingesting heterophil) x 100. Results are presented in Chapter 3.

2.5.6 **Oxidative burst**

Production of reactive oxygen species (ROS) by chicken heterophils during oxidative burst was measured by oxidation of 2'7' dichlorofluorescein-diacetate (DCFH-DA) to fluorescent DCF. Chicken heterophils isolated from W36 Hy-Line (900 µl; 1 x 10^7 cells/ml) were incubated with *Salmonella* Enteritidis (#97-11771 strain) (90 µl; 1 x 10^8 cfu/ml) and DCFH-DA (10 µg/ml) for 1 h at 37˚C. Phorbol A-myristate 13-acetate (PMA) (2 µg/ml), a well-known agonist that activates protein kinase C (PKC) was used as a positive control. Aliquots of cell cultures (150 µl) were placed in a black 96-well CoStar flat-bottomed plate and the relative fluorescent units (RFUs) were measured at excitation wavelength 485 nm and emission wavelength 530 nm using a GENios Plus Fluorescence Microplate Reader (TECAN US Inc, Research Triangle Park, NC). Results are presented in Chapter 3.

2.5.7 **Degranulation assay**

Degranulation was monitored by quantifying the levels of β-D-glucuronidase in culture medium following stimulation of heterophils with opsonised *Salmonella* Enteritidis (1 x 10^8 cfu/ml). The heterophils from W36 Hy-Line were adjusted to 1 x 10^7 cells/ml and incubated with stimulants for 1 h, at 39˚C in a 5% CO_2 incubator. The reaction was stopped by transferring the tubes containing the cells to an ice bath for 5-10 min. Cell suspensions were then centrifuged for 10 min at 250 x *g,* at 4˚C. The supernatants were removed from each sample and used for the assay. Each sample (25 µl) was added to non-treated, black CoStar flat-bottomed ELISA plate and incubated with 50 µl of freshly prepared substrate buffer (10 mM 4-methylumbelliferyl-β-D-glucoronide, 0.1% Triton X-100 in 0.1 M sodium acetate buffer) for 4 h at 41˚C. The reaction was stopped by adding 200 µl of stop solution (0.05 M glycine and 5 mM EDTA; pH 10.4). Liberated 4-methylumbelliferone was measured fluorimetrically (excitation wavelength of 355 nm, emission wavelength of 460 nm) with a GENios Plus Fluorescence Microplate Reader (TECAN US Inc, Research Triangle Park, NC). Results are presented in Chapter 3.

2.6 **Purification of nucleic acids**

2.6.1 **Purifying total RNA from chicken cells**

Total RNA from cells (BMDC, BMDM, PBL, heterophils) was extracted using the Qiagen RNeasy Mini Kit following the manufacturer's instructions. The cell lysate was mixed by pipetting with one volume of 70% (v/v) ethanol. The mix was placed in an RNeasy spin column housed in 2 ml collection tube and centrifuged for 15 s at 8,000 x *g.* The flow-through was discarded and 700 µl of RW1 buffer was used once to wash RNA bound to the silica membrane for 15 s at 8,000 x *g* and RPE buffer, diluted in absolute ethanol, was used twice (15 s and 2 min) at 8,000 x *g*. After washing steps, the RNeasy column was placed in a new collection tube and centrifuged at 16,000 x *g* for 1 min to remove any RPE buffer residues. Columns were moved to 1.5 ml collection tubes, 30 µl of RNase-free water was placed onto the membranes and centrifuged for 1 min at 8,000 x *g*. The eluate was re-applied to the silica membrane and centrifugation repeated in order to increase the RNA yield.

2.6.2 Purifying total RNA from chicken tissues

Total RNA from chicken tissues (spleen, bursa, caecal tonsils, and ileum), for experiments in Chapter 5 and Chapter 6, was extracted using an RNeasy Mini Kit (Qiagen) according to manufacturer's instructions. Tissues were stored in RNA*later* (Ambion) solution at 4°C. Stabilised tissues were removed from the reagent using sterile forceps. No more than 30 mg of tissues was used for RNA extraction. FastPrep™ Lysing Matrix tubes containing ~30 mg of tissues were filled with 600 µl of RLT buffer. To disrupt and homogenize tissues the FastPrep® FP120 Cell Disrupter was used. Each sample was homogenized for 45 sec at speed of 6.5 m/sec. The lysate was centrifuged for 3 min at 16,000 x *g* to remove any remaining insoluble material. Cleared lysate was mixed by pipetting with 1 volume of 70% (v/v) ethanol. The mix was placed in an RNeasy spin column placed in a 2 ml collection tube and centrifuged for 15 sec at 8,000 x *g.* The flow-through was discarded and 350 µl of RW1 buffer was used once to wash RNA bound to the silica membrane for 15 sec at 8,000 x *g*. DNase I stock solution was prepared by dissolving the lyophilised DNase I (1500 Kunitz units) in 550 µl of RNase-free water. The DNase I stock solution (10 µl) was mixed with buffer RDD (70 µl) and 80 µl of the mix was placed directly onto the column for 15 min at room temperature. After the incubation, an additional 350 µl of buffer RW1 was added and the column was centrifuged for 15 sec at 8,000 x *g*. RPE buffer, diluted in absolute ethanol, was used twice (15 sec and 2 min) at 8,000 x *g*. After washing steps, the RNeasy column was placed in a new collection tube and centrifuged at 16,000 x *g* for 1 min to remove any RPE buffer residues. Columns were moved to 1.5 collection tubes, 30 µl of RNase-free water was placed onto the membranes and centrifuged for 1 min at 8,000 x *g*. The eluate was re-applied to the silica membrane and centrifugation repeated in order to increase the RNA yield.

2.7 DNA and RNA amplification

2.7.1 Oligonucleotide primer design

Oligonucleotides for cDNA amplification were designed based on predicted sequences available from Ensembl (ensembl.org) or NCBI (http://www.ncbi.nlm.nih.gov/gene) databases. The design of primers for cloning of

full length sequences of target gene (Chapter 4) was performed manually by comparing candidate oligonucleotides melting temperatures and GC content in OligoAnalyzer 3.1 online tool (http://eu.idtdna.com/analyzer/applications/oligoanalyzer/default.aspx).

2.7.2 First-strand reverse transcription using SuperScript III

Complementary DNA (cDNA) was used as a template in gene cloning experiments (Chapter 4). Various mRNA samples were used to generate cDNA panels using SuperScript III (Invitrogen) following the manufacturer's instructions. First strand cDNA synthesis required mixing 10 pg - 5 µg total RNA, 1 µl of oligo$(dT)_{20}$ (50 µM) and 1 µl of 10 mM dNTP Mix with 10 µl of sterile, distilled water. The mixture was incubated at 65°C for 5 min and then placed on ice for 1 min. Next, 4 µl of 4X First-Strand Buffer, 1 µl of dithiothreitol (DTT) reducing agent, 1 µl of RNaseOUT (Promega) and 1 µl of SuperScript III reverse transcriptase were added. The final mixture was incubated at 50°C for 60 min. The reverse transcription reaction was inactivated by heating to 70°C for 15 min. Complementary DNA was ready to use and could be stored at -20°C.

To synthesise template cDNA for qPCR with EvaGreen fluorescent dye for experiments presented in Chapter 5 and Chapter 6, random primers (250 ng) were used instead of oligo$(dT)_{20}$ which modified the second part of reaction. Additional 5 min incubation at 25°C was performed before temperature increased to 55°C for 60 min for the reverse transcription phase. Inactivation of enzymatic reaction was the same.

2.7.3 First strand reverse transcription using High Capacity Reverse Transcription Kit

The High Capacity Reverse Transcription Kit was used to generate cDNA for experiments described in Chapter 6. The reverse transcription kit (Applied Biosystems) contains reagents that when combined form a 2X reverse transcription (RT) master mix (Table 2.1).

Table 2.1 High Capacity Reverse Transcription Master Mix components

Component	Volume [µl]
10X RT Buffer	1
25X dNTP Mix (100 mM)	0.4
10X RT Random Primers	1
MultiScribe™ Reverse Transcriptase	0.5
RNase Inhibitor	0.5
Nuclease-free water	1.6
Total	**5**

The RNA sample was diluted to a concentration of 100 ng/µl and 5 µl of RNA was mixed with 5 µl of master mix. The sample was centrifuged briefly and placed in a thermocycler with set temperatures as follows: 25°C for 10 min, 37°C for 120 min and 85°C for 5 min. Complementary DNA (cDNA) was stored at -20°C until further use.

2.7.4 DNA amplification by PCR

Candidate gene cDNA sequences predicted to reflect transcripts were collected from both Ensembl and NCBI databases. Primers were designed using full-length and/or common regions if there were differences in the predicted sequences between databases or transcripts. Gene list with transcripts accession numbers used to design primers is presented in Appendix 1, Table 4. If needed, more than one primer pair was designed and tested. For longer cDNAs, partial sequences were used to design primers. To clone full or partial sequences, a panel of c DNA was generated by reverse transcription PCR (RT-PCR) of separate RNA samples isolated from caecal tonsils and spleen from a *Campylobacter* trial, bursa from an infectious bursal disease virus (IBDV) trial, spleen from IBDV and Marek's disease virus (MDV) trial, HD11 cells stimulated with LPS, heterophils stimulated with *Salmonella enterica* serovar Enteritidis, or BMDM, BMDC and heterophils stimulated with LPS. Polymerase chain reactions were performed in 20 µl and 50 µl volumes. For each reaction, the following components were added: 1X PCR buffer (-Mg), 0.2 mM of each dNTP, 1.5 mM of $MgCl_2$, 0.5 µM of primers, 1U of Taq DNA Polymerase and

100 ng of template cDNA. All PCRs were performed using an MJ Thermal Cycler (MJ Research).

Thermal cycling conditions (35 cycles):
95°C for 5 min
95°C for 30 sec
X°C/ for 30 sec *
72°C for 1 to 3.5 min **
72°C for 10 min
*Annealing temperatures were dependent on the T_m of the each set of primers and ranged from 50-69°C; **The length of the elongation steps was dependent on the size of the product. For every kb, 1 min was added.

For difficult to amplify regions touchdown PCR was designed with the following cycling conditions:

95°C for 5 min
95°C for 30 sec
X°C/ for 30 sec *
72°C for 1 to 3.5 min ** } 8 cycles
95°C for 30 sec
X°C/ for 30 sec *
72°C for 1 to 3.5 min ** } 8 cycles
95°C for 30 sec
X°C/ for 30 sec *
72°C for 1 to 3.5 min ** } 25 cycles
72°C for 10 min Final extension

*Annealing temperatures were dependent on the T_m of the each set of primers and ranged from 50-69°C.
**The length of the elongation steps was dependent on the size of the product. For every kb, 1 min was added.

2.7.5 Quantitative PCR – hydrolysis probe-based gene expression analysis

Quantitative PCR (qPCR) is a dominant tool for the quantification of gene expression. Both hydrolysis probe(TaqMan)-based RT-qPCR and dye-based qPCR detection methods were used in this project to detect and measure very small amounts of nucleic acids in a range of chicken tissues and cells. In hydrolysis probe-based RT-qPCR, normalisation using r28S ribosomal RNA as a reference was performed to correct the differences between compared samples. This method was

used in Chapter 3. For each gene, a primer pair and hydrolysis probe were designed based on the template target gene sequence using Primer Express software (Applied Biosystems). Primers were synthesised by Sigma Aldrich (Poole, UK) and probes by Eurogentec (Southampton, UK). The 5' end of each probe was labelled with 5-carboxyfluorescein (FAM) fluorophore and the 3' end with tetramethylrhodamine (TAMRA) quencher dye.

Primer design was performed with consideration of general guidelines. The melting temperature of all primers was set between 58°C and 60°C, with probes being 10°C higher. Both probes and primers default length was no longer than 30 nucleotides and GC content in the range of 30-80% where amplicon length did not exceed 150 bp. The last five nucleotides at the 3' end of each primer consisted of ≤ 3 guanines or cytosines and preferably no triplicates of the same base. At least one of the primers or the probe overlapped a predicted intron-exon boundary to increase specificity of reaction.

The 5'-3' exonuclease activity of *Taq* DNA polymerase is the driving force behind the RT-qPCR method. During the RT-qPCR the probe anneals to the target template. Fluorescence from the FAM moiety is quenched by the nearby TAMRA moiety at this stage. If Taq polymerase extends the 3' end of primer annealed to the template it encounters the FAM fluorophore at the 5' end of the probe uses its 5'-3' exonuclease activity to displace the 5' end and degrade the probe. Separation of the fluorophore and quencher at this stage results in emission of fluorescence. The temperature of probe hybridisation is usually set at 8-10°C above the melting point of the primers to ensure attachment to the template and extension is performed at a lower temperature than normal for PCR to guarantee maximum 5'-3' exonuclease activity of the enzyme. These reaction conditions reduce *Taq* polymerase processivity, hence to ensure maximum efficiency short amplicons are designed. The number of cycles at which the fluorescence levels of the probe passes the background of detection is called quantification cycle (Cq) and this value is used to present the raw data. The set of primers used in RT-qPCR were previously

optimised: r28S rRNA, IL-1β, IL-6 (Kaiser *et al.,* 2000); IL-18 (Kaiser *et al.,* 2003); CXCLi1 and CXCLi2 (Kogut *et al.,* 2005). All RT-qPCR reactions were performed using TaqMan FAST Universal PCR Master Mix and One-step RT-PCR MultiScribe (Applied Biosystems). For each sample 10 µl reaction mix consisting of 5 µl 2X Fast Master Mix, 0.5 µl of primer mix, 0.25 µl (125 nM) of probe, 0.25 µl 40X MultiScribe enzyme, 1.5 µl of DEPC treated-H_2O and 2.5 µl of diluted RNA. RNA samples were diluted 1:500 for r28S rRNA analysis and 1:5 for target gene detection. All assays were performed in triplicate wells. For standard curve and slope analysis in r28S rRNA assay RNA from HD11 cells stimulated with LPS (200 ng/ml for 6 h) was used. In IL-1β, IL-6, IL-18, CXCLi2, RNA derived from transfected COS-7 cells was used. The Applied Biosystems 7500 Fast Real-Time PCR System was used to amplify and detect products. The thermal cycles were set for reverse transcription steps: 48°C for 30 min, 95°C for 20 sec followed by PCR steps: 95°C for 3 sec, 60°C for 30 sec repeated for 40 cycles. Primer and probe sequences and concentrations for all target genes tested are given in Appendix 1, Table 2. To calculate levels of expression for all target genes standard curves were created using Cq values of the serially diluted standard RNA for specific gene. The slope of the standard curve (y = mx + c) was used to determine efficiency of the reaction using formula $E = (10^{(-1/slope)})$. To correct for differences between RNA levels in samples within the experiment, the difference factor was calculated by dividing the mean Cq value for r28S rRNA specific product from the same sample. Normalised Cq values were calculated using the formula Cq + (N't – C'q) * S/S', where N't is the mean Cq for r28S rRNA among all samples, C'q is the mean Cq for r28S rRNA in the sample and S and S' are the slopes of the regressions of the standard plots for the cytokine/chemokine mRNA and the r28S rRNA, respectively. Results are expressed as fold-difference from levels in control samples.

2.7.6 **Fluorescent dye-based qPCR**

EvaGreen dye is a green fluorescent nucleic acid dye used in several applications including qPCR. Excitation and emission spectra of EvaGreen are very close to those of fluorescein (FAM) or SYBRGreen I, therefore EvaGreen is already

compatible with instruments equipped with visible light excitation with wavelengths in that region. The dye is nonfluorescent by itself, but becomes highly fluorescent upon binding to DNA. Compared to SYBRGreen, EvaGreen dye is non-mutagenic and non-toxic as it is impermeable to cell membranes. It is also less inhibitory towards qPCR and less likely to cause nonspecific amplification. Similarly to SYBRGreen, EvaGreen binds to double stranded DNA (dsDNA). With every PCR cycle the amount of dsDNA increases which results in fluorescence intensity. The Applied Biosystems 7500 Fast Real-Time PCR System was used to amplify and detect products. The reaction mix was prepared using the following components for each of the samples: 10 µl ABI TaqMan Gene Expression Master Mix (Applied Biosystems), 1 µl 20X EvaGreen (Biotum, VWR-Bie & Berntsen), 2.3 µl 20 µM specific primer pair (forward and reverse) and 4.7 µl nuclease-free water. Each reaction contained 2 µl of cDNA diluted 1:5 in nuclease-free water. The following cycle parameters were used: 2 min at 50°C, 10 min at 95°C, followed by 40 or 30 cycles with denaturing for 15 sec at 95°C and by annealing/elongation for 1 min at 60°C. Melting curves were generated after each run to confirm a single PCR product (from 60°C to 95°C, increasing 1°C/3 sec). The EvaGreen-based qPCR was used in reference gene normalisation experiments (Chapter 5) and in 96.96 Dynamic Array optimisation in Chapter 6, following the sample and assay mix preparation protocols as described in section 2.10.6.

2.7.7 Agarose gel electrophoresis

To determine presence and size of DNA in PCR and qPCR, agarose gel electrophoresis was performed. Agarose gels were prepared by mixing agarose powder at a (w/v)%, which was dependent on the size of expected PCR product, with 1X Tris Acetate-EDTA (TAE) buffer. TAE is a mixture of tris(hydroxymethyl)aminomethane (tris base), glacial acetic acid and EDTA, that works to sequester divalent cations. Compared to TBE buffer, TAE is less stable but double-stranded DNA runs faster through agarose dissolved in TAE buffer. The mix was heated until the agarose powder dissolved completely. An intercalating dye, SYBR Safe® (Invitrogen), was added to the gel solution in order to visualise any DNA

molecules amplified by PCR. The molten agarose was poured into a plastic tray with appropriate comb sizes inserted.

2.7.8 Gel extraction

Agarose gel electrophoresis was used to detect and visualise products of PCRs. To extract products a QIAquick Gel Extraction Kit (QIAGEN) was used according to the manufacturer's instructions, as follows. Agarose gel containing amplified DNA of interest was excised with a clean, sharp scalpel on a UV transilluminator. The gel slice was weighted in a 1.5 ml Eppendorf tube. QG buffer was added at 3 times the volume of the excised gel fragment (300 µl; QG buffer/100 mg of gel) and incubated at 50°C for 10 min with frequent vortexing to liquefy it. The QG buffer contains a pH indicator allowing easy determination of the optimal pH for DNA binding. QG buffer of yellow colour indicated pH is <7.5 which is ideal for DNA adsorption. Sodium acetate (3 M, pH 5.0) was added to the sample when needed to adjust for the optimal pH. To increase the yield of DNA fragments <500 bp, 1 volume of isopropanol was added to the mix. To bind DNA, the sample was placed in the QIAquick column with 2 ml collection tube and centrifuged at 10,000 x *g* for 1 min. The flow-through was discarded and an additional 500 µl of QG buffer was added to remove all traces of agarose and centrifuged for 1 min. The flow-through was discarded and the silica membrane was washed by adding 750 µl of PE buffer to the column and centrifugation for 1 min. The flow-through was discarded and dry spin was applied to remove residual ethanol from PE buffer. The column was then placed in a clean 1.5 ml microcentrifuge tube. DNA was eluted by adding 30 µl of EB buffer (10 mM Tris Cl, pH 8.5) to the centre of the column and incubated at room temperature for 1 min followed by centrifugation at 13,000 x *g* for 1 min. Samples were used immediately in ligation reactions. Remaining samples were stored at -20°C.

2.7.9 Ligation

Ligation is a process to create recombinant DNA molecules by joining DNA fragments together. The DNA ends are joined together by the formation of

phosphodiester bonds between the 3'-hydroxyl of one DNA terminus with the 5'-phosphoryl of another. The ligation reaction buffer contains adenosine triphosphate (ATP), which is required as the energy source for T4 DNA ligase. This enzyme is derived from Enterobacteria phage T4 and its ability to repair nicks in double stranded DNA with 3' OH and 5' phosphate ends is used in connecting DNA fragments with plasmids. Cohesive end ligation was carried out at 16°C to maintain good balance between annealing of ends and activity of the enzyme. All ligation reactions were carried out using 2X ligation buffer (80 mM Tris HCl, 20 mM $MgCl_2$, 20 mM DTT, 2 mM ATP), pGEM-T Easy Vector, T4 DNA ligase (Promega) and nuclease-free water.

2.7.10 **Transformation**

Transformation was performed using high-efficiency chemically competent *E. coli* cells (≥ 1 x 10^8 cfu/µg DNA). *Escherichia coli* JM109 cells (Promega) were removed from -80°C storage and defrosted on ice for 5 min. A 50 µl aliquot of the cell suspension was added to 1.5 ml Eppendorf tube containing 2 µl of ligation reaction, mixed by gently flicking the tube and incubated on ice for 20 min. Sample was then heat-shocked for 50 s at 42°C and immediately placed on ice for additional 2 min. The heat-shock approach creates small holes in the cell wall of the bacteria allowing the uptake of DNA. Super Optimal Broth media (SOB) was added in 950 µl volume and incubated for 1.5 h at 37°C with shaking (~180 rpm) to allow transformants to recover and express plasmid-encoded antibiotic resistance. Cells were pelleted by centrifugation for 10 min at 1,000 x *g* and resuspended in 200 µl of SOB media. LB agar containing 100 µg/ml ampicillin (LB_{AMP100}), 100 µl isopropyl β-D-1-thiogalactopyranoside (IPTG) and 20 µl X-gal (BCIG, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was used to plate out transformation reactions in duplicate (100 µl each).

2.7.11 **Screening bacterial colonies by colony PCR**

In colony PCR, gene-specific primers or plasmid-specific primers flanking the multiple cloning region (Appendix 1, Table 1) were used to examine for presence of

inserted DNA of expected size. Single white colonies were picked using a pipette tip and streaked in numbered areas of an LB_{AMP100} agar plate. The same pipette tip was used to inoculate 50 µl of dH_2O for 1 min. The bacterial suspensions were then heat-shocked for 5 min at 95°C and centrifuged for 10 min at 9,000 x *g.* Supernatant was used as a template DNA in PCR. Amplicons were analysed by agarose gel electrophoresis. The streaked LB_{AMP100} agar plates were incubated for 24 h in 37°C. The positive colonies identified by colony PCR were used to inoculate 5 ml of LB_{AMP100} broth for 24 h in 37°C with shaking at 200 rpm for plasmid purification.

2.8 Plasmid DNA purification

2.8.1 Small scale plasmid purification

To confirm cloning of genes (Chapter 4), recombinant pGEM-T Easy plasmids were sequenced. To prepare samples for sequencing, DNA from *Escherichia coli* pGEM-T Easy–containing cells was purified using QIAprep Spin Miniprep Kit (QIAGEN) as follows. After inoculation of LB_{AMP100} broth with a single colony of a putative recombinant the bacterial suspension was pelleted for 15 min at 3,000 x *g*. Supernatant was discarded and cells were resuspended in 250 µl of P1 buffer with added RNase A for sufficient digestion and LyseBlue as a colour indicator for visual identification of optimum buffer mixing. The sample was moved to a 1.5 ml Eppendorf tube and 250 µl of lysis buffer P2 was added. The solutions were mixed by inverting the tube 4-6 times without vortexing to avoid genomic DNA shearing. To neutralize the lysate and adjust to high-salt binding conditions, 350 µl of N3 buffer was added and mixed by inverting the tube 4-6 times or until the solution became cloudy and without visible localised precipitation. The sample was then centrifuged for 10 min at 17,900 x *g* to pellet denatured proteins, chromosomal DNA and cellular debris. Supernatant containing smaller plasmid DNA was applied to the QIAprep spin column by pipetting and centrifuged for 1 min at 17,900 x *g* to capture DNA in the silica matrix. A brief wash step with 500 µl of PB buffer was performed to efficiently remove endonucleases and prevent plasmid DNA degradation. A second wash step using 750 µl of PE buffer was applied to remove salts. The flow-through was discarded and the column was centrifuged for an

additional 1 min to remove residual wash buffer, which could inhibit subsequent enzymatic reactions. The QIAprep column was placed in a clean 1.5 ml Eppendorf tube and 50 µl of elution buffer EB was added to the centre of the spin column for centrifugation for 1 min followed by 1 min, at 17,900 x *g*. The plasmid DNA yield was examined using NanoDrop™ 1000 Spectrophotometer.

2.8.2 Large scale endotoxin-free plasmid purification

To obtain a high yield of plasmid DNA for transfection and recombinant protein production an EndoFree Plasmid DNA Purification Maxi Kit was used according to the manufacturer's instructions with minor modifications as follows. Previously sequenced plasmids containing genes of interest were stored as glycerol stock of bacterial cells (i.e. JM109, DH5α). Single colonies were used to inoculate 5 ml of LB_{AMP100} broth and samples were incubated at 37°C with shaking at 200 rpm for 12 h. Larger volumes (250 ml) of LB_{AMP100} broth were inoculated with 2.5 ml of the bacterial starter culture and incubated for 24 h at 37°C with shaking at 200 rpm. Bacterial cells were pelleted in 50 ml Falcon tubes for 30 min at 2,465 x *g*. Supernatant was discarded and the pellet resuspended completely by vortexing in 10 ml of P1 buffer containing RNase A and LyseBlue. To lyse the cells 10 ml of buffer P2 was added and mixed by inverting the tube 4-6 times. The lysate was incubated at room temperature for 5 min. During the incubation, a QIAfilter Cartridge was prepared by screwing the cap onto the outlet nozzle of the QIAfilter Maxi Cartridge and placing in a convenient tube. Precipitation was enhanced by adding 10 ml of chilled buffer P3 to the lysate and inverting the tube 4-6 times. Precipitated material (genomic DNA, proteins, cell debris) forms fluffy white material. The lysate was moved immediately into QIAfilter Cartridge and incubated at room temperature for 10 min. After this period, the precipitate floated and formed a layer on top of the solution. The outlet nozzle was opened on the QIAfilter Cartridge and a plunger inserted to filter the lysate into the 50 ml tube. The endotoxin removal (ER) buffer that prevents LPS molecules from binding to the resin in the QIAGEN tips was added in 2.5 ml volume to the filtered lysate and allows purification of DNA. The solution was incubated on ice for 30 min, when the

QIAGEN-tip 500 was being equilibrated by applying 10 ml of buffer QBT and allowing the column to be emptied by gravitational flow. After the incubation on ice the filtered lysate was applied to the QIAGEN-tip and allowed to enter the resin by gravity flow. The tip was washed twice with 30 ml of QC buffer. DNA was eluted using 15 ml of buffer QN and precipitated by addition of 10.5 ml of isopropanol. The solution was mixed and centrifuged immediately at 3,220 x *g* for 1 hour. Supernatant was carefully removed and the pellet was washed with 5 mL of 70% (v/v) ethanol and collected by centrifugation at 3,220 x *g* for 1 h, 4°C. Supernatant was carefully removed without disturbing the pellet. The DNA was left to air-dry for 5-10 min and redissolved in appropriate volume of TE buffer for 5 min at room temperature. The DNA solution was vortexed for several seconds and its concentration was measured using NanoDrop™ 1000 Spectrophotometer.

2.8.3 Sequencing plasmid DNA

To confirm gene cloning, recombinant plasmids were sequenced using plasmid-specific primers and analysed using Sanger ABI 3730xl (GATC Biotech, Germany). Expected and observed sequences were aligned using ClustalX (section 2.1.2).

2.9 RNA-seq

2.9.1 Sample preparation and sequencing

The BMDC, BMDM and heterophils were cultured as described in Chapter 2, section 2.5. For RNA sequencing, BMDM were stimulated with 250 ng/ml of LPS (Escherichia coli O55:B5, Sigma Aldrich) for 4 h, BMDC with 200 ng/ml for 24 h (Wu *et al.,* 2010) and heterophils with 10 µg/ml for 1 h (Farnell *et al.,* 2003). Total RNA was extracted as described in Chapter 2, section 2.6.1 with additional steps. The cells were lysed with 600 µl of buffer RLT with added β-mercaptoethanol (β-ME) and on-column DNase digestion was performed. Total RNA extracted from BMDC, BMDM and heterophils (control and LPS stimulated) was diluted to 100 ng/µl in 20 µl of RNase-free water. The sample preparation was performed by Edinburgh Genomics facility (Roslin Institute, Midlothian, UK) using a Tru-Seq total RNA Sample

Preparation v2 kit as per the manufacturer's protocol. Resulting libraries were quality-checked on an Agilent DNA 1000 Bioanalyzer (Agilent Technologies, South Queensferry, UK) and then clustered onto a paired end flowcell using the Illumina TruSeq® Rapid PE Cluster Kit at a 8 pM concentration. The paired-end sequencing, consisting of 100 cycles, was carried out on the Illumina HiSeq 2500 using an Illumina TruSeq® Rapid SBS Kit (Illumina, Little Chesterford, UK).

2.9.2 Transcriptome alignment and differential expression analysis

The RNA-seq pipeline is presented in Appendix 1, Figure 1. The raw reads were subject to quality control measures, including the removal of remaining sequence adapters. The cleaned, paired-end 100 bp reads were aligned to the chicken reference genome (Galgal4) assembly from the Ensembl database (http://ensembl.org) with TopHat (v2.0.9) splice junction mapper, which aligned reads using Bowtie aligner (v1.0.0). Cufflinks software (v2.1.1) assembled reads into transcripts that were used as input data together with aligned reads in Cuffdiff to determine expression levels by calculating the Fragments per Kilobase per Million mapped reads (FPKM) and the differential expression between conditions using default options.

2.10 Quantitative PCR (qPCR) using 96.96 Dynamic Array Integrated Fluidic Circuits.

The BioMark System 96.96 Dynamic Array is a high-throughput platform that allows combining 96 samples with 96 primer pairs into 9,216 qPCRs in one integrated fluidic circuit (IFC). The system includes optical, thermal cycling and software components to perform quantitative PCR. The Dynamic Array IFC is a nanofluidic network that allows to run 24-fold more reactions compared to 384-well plate. The liquid handling steps, number of pipetting and volumes of reactions are greatly reduced. The high-resolution CCD camera that covers whole chip area images all reactions simultaneously. The quantification cycle (Cq) values from each reaction chamber in the chip are visualised as an easy to analyse heat map. The values behind the heat map can be exported and further analyse using gene expression analysis software.

2.10.1 **Primer design and optimisation**

Primers for qPCR using 96.96 Dynamic Array were designed in the same manner as primers for RT-qPCR outlined in section 2.7.5. At least one primer was designed to overlap intron-exon boundaries of a gene, where possible. Optimisation of primers was performed to assess their specificity in binding to a single template at a working concentration of 1.15 µM. Primer amplification efficiencies and dynamic range were acquired from standard curves constructed from dilution series of highly responding samples. Melting curves were inspected for all primer assays. Agarose gel electrophoresis and sequencing of selected amplicons were performed to ensure primer specificity. To prepare qPCR products for sequencing DNA bands were excised from an agarose gel using sterile scalpel and products were cleaned-up using Nucleo-Spin Gel and PCR Clean-up kit (Macherey-Nagel, UK) following manufacturer's instructions. For every 100 mg of gel fragment, 200 µl of buffer NTI was added and incubated at 50°C for 10 min. Completely dissolved samples was then placed in Nucleo-Spin Gel and PCR Clean-up Column with collection tube and centrifuged for 30 s at 11,000 x *g*. The flow-through was discarded and column was washed with 700 µl of buffer NT3 with added 96-100% ethanol, by centrifugation for 30 s at 11,000 x *g.* The silica membrane was dried by centrifugation for 1 min at 11,000 x *g* to remove buffer NT3 completely. The column was placed into a new 1.5 ml microcentrifuge tube and 30 µl of buffer NE was added. The column was incubated at room temperature for 1 min and centrifuged for 1 min at 11,000 x *g.* Cleaned-up qPCR product was sent for direct sequencing.

2.10.2 **Reference gene normalisation**

The stability of reference genes was examined using BestKeeper, NormFinder and geNorm outlined in sections 2.1.7, 2.1.8 and 2.1.9. More detail is provided in Chapter 5.

2.10.3 RNA extraction and cDNA generation for 96.96 Dynamic Array IFC

RNA for high throughput qPCR was extracted as described in paragraph 2.6.2. The cDNA was reversely transcribed using SuperScript III Kit and High Capacity Reverse Transcription Kit as described in section 2.7.2 and 2.7.3, respectively.

2.10.4 Preamplification

Preamplification was performed using TaqMan PreAmp Master Mix (Applied Biosystems, PN 4391128). A stock of 200 nM primer mix was prepared combining equal concentration of all primers used in the following qPCR. TaqMan PreAmp Master Mix (10 µl) was mixed with 5 µl of 200 nM stock primer mix and 5 µl of cDNA in concentration of 185 ng/µl. Reaction tubes were vortexed and centrifuged briefly before PCR. Samples were incubated at 95°C for 10 min followed by 14 cycles of 95°C for 15 sec and 60°C for 4 min. Preamplified cDNA was stored in -20°C until further use. Steps taken to optimise preamplification are discussed in Chapter 6, section 6.3.3.

2.10.5 Exonuclease I treatment

A clean-up step using Exonuclease I (*E. coli*) (New England Biolabs; M0293S) was performed to remove unincorporated primers from preamplified cDNA. Exonuclease I was diluted to 4 U/µl by mixing 1.4 µl of water, 0.2 µl of Exonuclease I Reaction Buffer and Exonuclease I (at 20 U/µl). For each 5 µl of preamplified cDNA a total volume of 2 µl Exo I reaction solution was added and incubated at 37°C for 30 min. The reaction was stopped by heating at to 80°C for 15 min. Reactions were held at 4°C until storage at -20°C.

2.10.6 Quantitative PCR assay and sample master mix preparation

Assay mix was prepared by mixing 2.5 µl 2X Assay Loading Reagent (Fluidigm, PN 85000736), 2.3 µl of primer pair mix and 0.2 µl low EDTA TE buffer which combined gave 5 µl of assay mix. Sample mix was prepared by mixing 2.5 µl TaqMan Gene Expression Master Mix (Applied Biosystems; PN 4369016), 0.25 µl

20X DNA Binding Dye Sample Loading Reagent (Fluidigm; PN 100-388), 20X EvaGreen DNA binding dye (Biotum; PN 31000) and 2 µl of preamplified cDNA which combined gave 5 µl of sample mix.

2.10.7 **Chip priming**

96.96 Dynamic Array IFC was primed by injecting 150 µl of control line fluid into each accumulator on the chip followed by placing the chip into the Integrated Fluidic Circuit (IFC) controller and running the Chip Prime (138x) script.

2.10.8 **Chip loading**

Assay mix and sample mix solutions (5 µl) were pipetted into the inlets on the chip after priming. Using IFC controller software, Load Mix (138x) script was applied. After loading finished, the chip was removed from IFC controller and any dust particles or debris were cleaned from the chip surface using scotch tape.

2.10.9 **qPCR and data analysis**

Quantitative PCR was performed in the BioMark HD instrument using the Data Collection Software. The loaded chip was placed into the reader. After barcode verification, application was set as Gene Expression (GE), passive reference as ROX, probe as single probe and probe type as EvaGreen. Thermal cycling protocol was chosen for 96.96 chip: GE 96x96 PCR+Melt v1.pcl. Auto Exposure was confirmed and the program was verified.

Thermal conditions for GE 96x96 qPCR:

Thermal mix	50°C for 2 min 70°C for 30 min 25°C for 10 min
Hot start	50°C for 2 min 95°C for 10 min
PCR cycle (x30)	95°C for 15 sec 60°C for 60 sec
Melting	60°C for 3 sec to 95°C

Real-Time PCR Analysis software was used to visualise results. Analysis settings were as follows: quality threshold was set to 0.65, baseline correction to linear (derivative) and Cq threshold method to auto (global). Data from the qPCR were analysed under these settings and a heat map was generated to visualise the results. Sample names and assays symbols were assigned to each row/column using template documents in .xls format. Melting curves and amplification graphs were examined for each gene. Data from the heat map view were exported as .csv file that was used as an input data in GenEx5 software.

2.10.10 GenEx5 pre-processing

Data from 96.96 Dynamic Array runs was examined in Real Time PCR Analysis software and handled in GenEx5 software before statistical analysis. Data exported from heat map views were arranged with the measured genes in columns headed with gene symbols and samples in rows. Additional classification columns were included with labels: #tissue, #bird, #farm. A classification column for #repeats was added automatically by the GenEx5 software. Validation of data in pre-processing included removal of columns/rows with less than 50% values. Gene quality graphs were produced to examine the number of empty values. The missing data were either filled with the mean of replicates or by imputation based on a tissue. Data were corrected for reaction efficiency for each primer assay individually before normalisation with the reference genes: ACTB, GAPDH and TBP as selected in Chapter 5. Normalised dataset repeats were averaged and further normalisation to maximum Cq value for a given gene was performed. Relative quantities were transformed to logarithmic scale (log2) before statistical analysis.

2.10.11 GenEx statistical analysis

Groups for comparison were created using GenEx5 Data Manager. Data were grouped depending on the farm (pedigree, sibling test), bird (1-8), tissue (bursa, spleen, caecal tonsils, ileum, PBL) and tissue/farm (e.g. bursa/sib, bursa/ped). Groups were compared in bar graphs using descriptive statistics. Each comparison generated a table with data values sorted by gene and group. Statistical analysis

was performed using a t-test. For principal component analysis (PCA), a trial version of GenEx Enterprise was used. Gene expression was considered highly significant (p value ≤ 0.0005) and significant (p value ≤ 0.05) for fold change values lower than -1 and higher than 1.

2.11 Statistical analysis

Chapter 3 oxidative burst and degranulation data were analysed by the Student's t-test using SigmaStat software (Jandel Scientific, USA) and expressed as the mean ± SD. RT-qPCR data in Chapter 3 were checked for normality and statistical analyses were carried out using Mann Whitney-U in Minitab 16.1.0 (State College, USA). Statistical significance was determined as $p \leq 0.05$ (significant) or $p \leq 0.001$ (highly significant). Mann-Whitney-U test (GenEx5) was used in validation of the arrays expeiment.

Chapter 3 Evaluation of cellular and molecular methods as diagnostic tools for immune robustness

3.1 Introduction

The main aim of this study was to evaluate the practicality of widely used cellular and molecular methods for measuring immune robustness in chickens. This work was completed to answer the question if *in vitro* cultured bone marrow-derived macrophages (BMDM) and dendritic cells (BMDC) and heterophils isolated from blood provide an inexpensive, swift and informative tool for predicting immune robustness that could be used in wider selection programmes. Evaluation was based on BMDM and BMDC from Roslin Institute J line (RI-J) birds and on heterophils isolated from blood samples of RI-J line, Novogen layers and Aviagen broilers. A panel of proinflammatory cytokines and chemokines, known to be differentially expressed during the regulation of immune responses, was examined in effector cells stimulated with a lipopolysaccharide. The phagocytosis, degranulation abilities and production of reactive oxygen species (ROS) was examined in heterophils isolated from W36 Hy-Line and stimulated with opsonised *Salmonella* Enteritidis.

In the animal breeding sector, genetic selection can increase resistance to specific pathogens (Janss and Bolder, 2000) but genetic or phenotypic markers for resistance to the wide spectrum of viral, bacterial and protozoan pathogens are lacking. Disease resistance is often a polygenic trait therefore genetic selection for the immune robustness is complex. It is therefore of interest to explore phenotypes that may be predictive of the response to infection. There have been studies on chicken immune responses to infections which showed that the number and types of effector cells and the timing and magnitude of effector cells of their responses influences resistance and susceptibility to diseases (Chapter 1).

The bone marrow, as the source of the myeloid lineage of white blood cells, can be used to develop functional macrophages and dendritic cells, which can be screened for phenotypes associated with the response to infection. The use of

primordial cells to derive effector cells results in broad representation of the immune repertoire that has not influenced by cytokines and other features of immune system (Sun *et al.,* 2015). Studies on the effector cells and their performance during stimulation with TLR ligands and/or live pathogens suggest that the increased resistance to *Salmonella* correlates with the increased levels of transcripts encoding proinflammatory cytokines and chemokines (Swaggerty *et al.,* 2003; Ferro *et al.,* 2004, Wigley *et al.,* 2006). Such molecules orchestrate the nature, strength and kinetics of later stages of immunity. Hence, stronger and more rapid innate defences can not only influence the first line of defence against pathogens but also trigger more effective adaptive immune responses.

In chickens, the degranulation process of heterophils is closely associated with phagocytosis (Kogut *et al.,* 2001). Not only live bacteria but also various microbial components have been shown to stimulate degranulation (Kogut *et al.,* 2005). The ability of heterophils to degranulate and produce an oxidative burst was examined *in vivo* after CpG ODN treatment or *Salmonella* stimulation (He *et al.,* 2007; Kogut *et al.,* 2005) and *in vitro* in two F1 reciprocal crosses (Swaggerty *et al.,* 2006). Chicken macrophages cell line (HD11), monocyte-derived macrophages and heterophils isolated from blood have been used in many studies where immune gene expression was evaluated upon stimulation with antigens in different breeds (Iqbal *et al.,* 2005; Smith *et al.,* 2005, Lavric *et al.,* 2008; Nerren *et al.,* 2009). The macrophages isolated from a *Salmonella*-resistant inbred line expressed proinflammatory cytokines and chemokines more rapidly, and at greater levels, compared to a susceptible line (Wigley *et al.,* 2006) and could be used as biomarkers. Since the first report on generation of the chicken bone-marrow derived dendritic cells was published (Wu *et al.,* 2010) recent studies have focused on the nature of interactions between BMDC and various antigens/pathogens (Liang *et al.,* 2013; Vervelde *et al.,* 2013; Rajput *et al.,* 2014). The finding of differential responses to the pathogens between resistant and susceptible inbred chicken lines and the studies on the nature of gene expression in effector cells isolated or

differentiated from such lines suggest that it may be feasible to find biomarkers of the innate response that are predictive of resistance to disease.

3.2 Materials and methods

3.2.1 Generation and stimulation of bone marrow-derived dendritic cells and macrophages

Chicken macrophages and dendritic cells can be derived from the bone marrow cells by differentiation driven by recombinant CSF-1 or IL-4 and GM-CSF (CSF-2), respectively. The recombinant chicken proteins were produced by transfecting the COS-7 cells with plasmids containing chicken CSF-1, IL-4, GM-CSF gene inserts, as described in Chapter 2, section 2.4.2. The bone marrow cells from 4-6 week old RI-J line chickens were differentiated into dendritic cells (BMDC) and macrophages (BMDM) after six days of cultures, as described in Chapter 2, section 2.5.1.

3.2.2 Phagocytosis, oxidative burst and degranulation assays

The peripheral blood was used to isolate a heterophil population as described in Chapter 2, section 2.5.3. Live *Salmonella Enteritidis,* opsonised with normal chicken serum was used to stimulate phagocytosis, oxidative burst and degranulation of heterophils after 1 h incubation. All three experiments were performed during a visit to the USDA facility in College Station, Texas using 100 one day old Hy-Line W36 chickens. The protocols are described in Chapter 2, section 2.5.5, 2.5.6 and 2.5.7.

3.2.3 Assessment of cytokine and chemokine expression using reverse transcription quantitative polymerase chain reaction (RT-qPCR)

The total RNA from unstimulated and stimulated BMDM, BMDC and heterophils was used in RT-qPCRs to determine the levels of expression of cytokines and chemokines. Primers and probes used in this experiment are listed in Appendix 1, Table 2. The amplification and detection was carried out using fluorescent hydrolysis probes in the Applied Biosystems 7500 Fast Real-Time PCR System as described in Chapter 2, section 2.7.5.

3.3 Results

The populations of macrophages and dendritic cells in chicken body tissues are generally vast but difficult to isolate, therefore to evaluate their immune gene expression levels, cells were differentiated from bone marrow for analysis of responses to different stimuli *ex vivo*. If such assays prove reliable they could be used to understand the basis of differential resistance and/or as predictive tools for selection of immune robustness.

3.3.1 Bone marrow-derived macrophage and dendritic cell cultures

In this study, addition of CSF-1 and GM-CSF and IL-4 to bone marrow cells generated clumping formation of adherent colonies. On day six, cells were stimulated with LPS to induce immune gene expression. LPS is a strong stimulator of innate immunity. For the purpose of this experiment 250 ng/ml was added to BMDM. Dendritic cells were stimulated with 200 ng/ml of LPS, a concentration previously described as optimal to trigger immune response in BMDC (Wu *et al.,* 2010). The stimulation with LPS triggered changes in the appearance of both BMDM and BMDC + LPS cultures (Figure 3.1b and Figure 3.1c) compared to the unstimulated control cells (Figure 3.1a). In BMDC culture, larger colonies of cells decreased in numbers and single cells became more visible. BMDC that underwent maturation during LPS stimulation displayed long veils or dendritic appearance (Figure 3.1d). The BMDM population after stimulation with LPS was characterised by colonies of spindle shaped strongly adherent cells (Figure 3.1b).

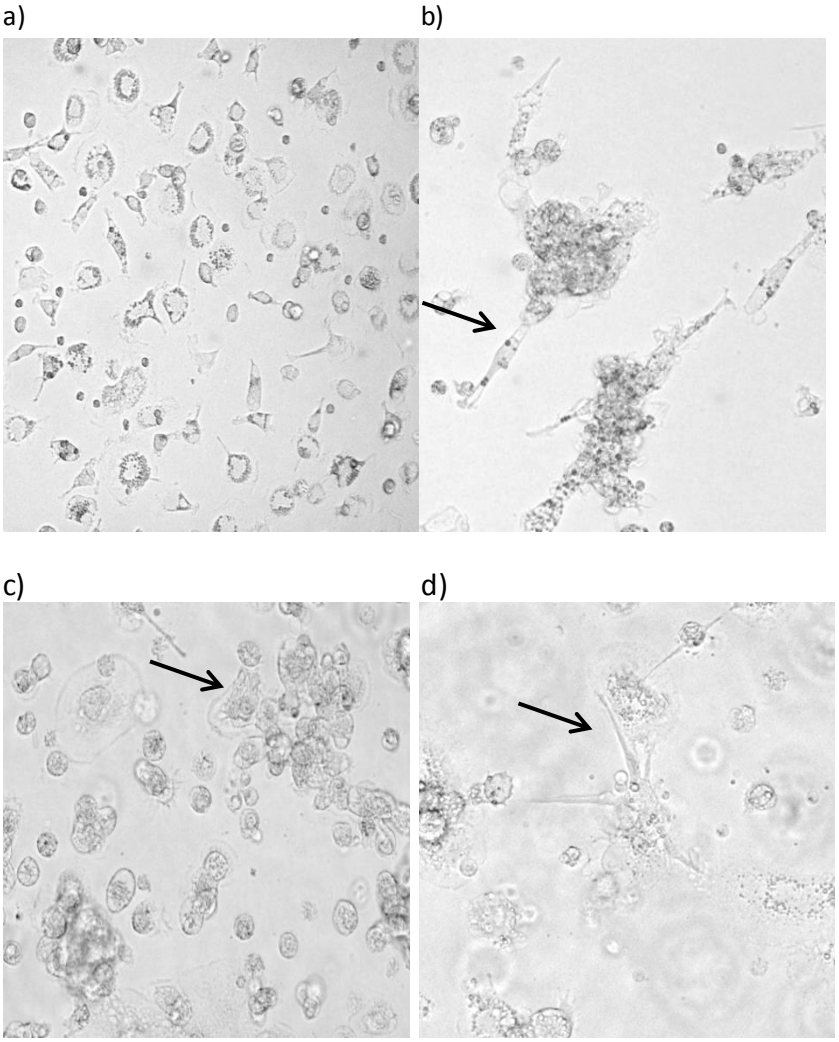


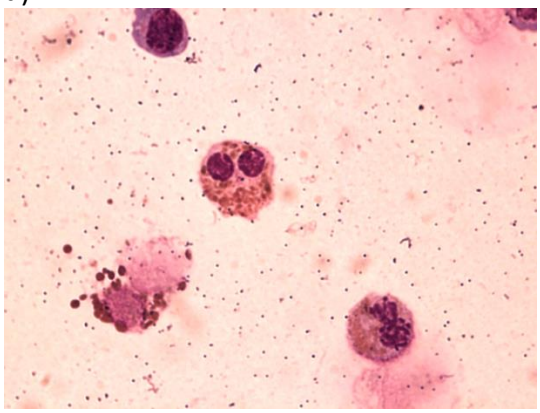
Figure 3.1. Morphology of chicken bone marrow cells from RI-J line**.** a) control cells; magnification 60x; b) cells cultured for 7 days in presence of CSF-1and stimulated with 250 ng/ml LPS for 24 h; magnification 60x; c) cell aggregates at day 7 of culture with presence of GM-CSF and IL-4 and stimulated with 200 ng/ml of LPS; magnification 60x; d) single cell from BMDC culture after stimulation with 200 ng/ml of LPS for 24 h, showing apparent long veils; 100x.

3.3.2 Isolation of heterophils from blood

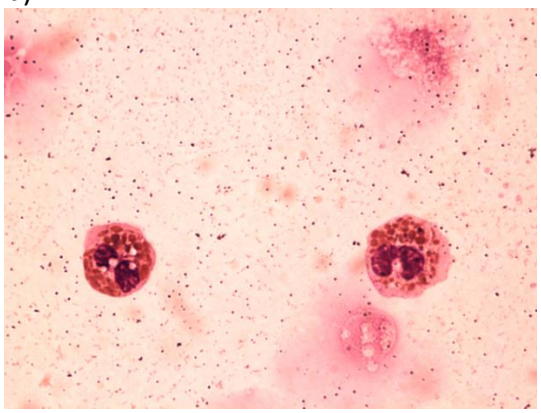
A technique described by Kogut *et al.* (2001) was followed to isolate heterophils, initially using blood from four to eight birds of 3-6 weeks age. The number of circulating heterophils drastically drops from ~70% of total blood cells at the first week to ~25% at the third week of life (reviewed by Maxwell and Robertson, 1998). The age of the chickens and the small amount of blood (~8 ml) used affected the final yield of heterophils. The purity and number of the isolated cells was low (Figure 3.2a and Figure 3.2b). The population consisted of different cells with very few heterophils detected. It can be observed that mature circulating

heterophils are round, although it has been reported that their shape may be distorted because of a polar distribution of the specific cytoplasmic granules or lobulation. They have two nuclear lobes, faintly pink cytoplasmic matrix, brick-red rod-shaped granules with the May-Grunwald and Giemsa combination of stains, as reported by Lucas and Jamroz (1961) (reviewed by Maxwell and Robertson, 1998). To undertake any heterophil functional assays the number of cells has to be at least 1 x 10^6 per ml. The use of ~100 day-old chickens increased the yield and purity of isolated heterophils with approximately 95 % pure cells in 10^7 – 10^8 cells/ml (Figure 3.2c).

a)



b)



c)

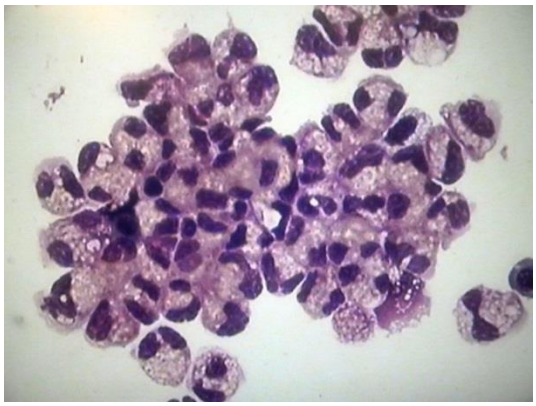


Figure 3.2. Leukocytes isolated from blood using a discontinuous gradient. Cells were cytocentrifuged and stained with May-Grunwald Giemsa stain (a and b) and with Hematology 3-step stain (c); a) and b) heterophils isolated from blood of three-six week old RI-J line chickens; (b) heterophils isolated from blood of W36 Hy-Line day-old chickens, magnification with immersion oil 100x.

3.3.3 Induction of heterophil phagocytosis, degranulation and oxidative burst by *Salmonella* Enteritidis

The ability of live opsonised *Salmonella* Enteritidis to induce phagocytosis, degranulation and oxidative burst was measured in heterophils isolated from blood of day-old W36 Hy-Line chickens after 1 h incubation. Phagocytosis in heterophils was evaluated microscopically (Figure 3.3). Not all heterophils contained bacterial cells but those that did phagocytose *Salmonella*, on average, contained more than two bacterial cells. For every 100 heterophils on a cytospin slide, 63 contained *Salmonella* Enteritidis. Rarely, heterophils were observed with a large number of internalised bacteria. The results are presented as phagocytic index (Chapter 2, section 2.5.5.). Data are expressed as mean ± standard deviation (Table 3.1).

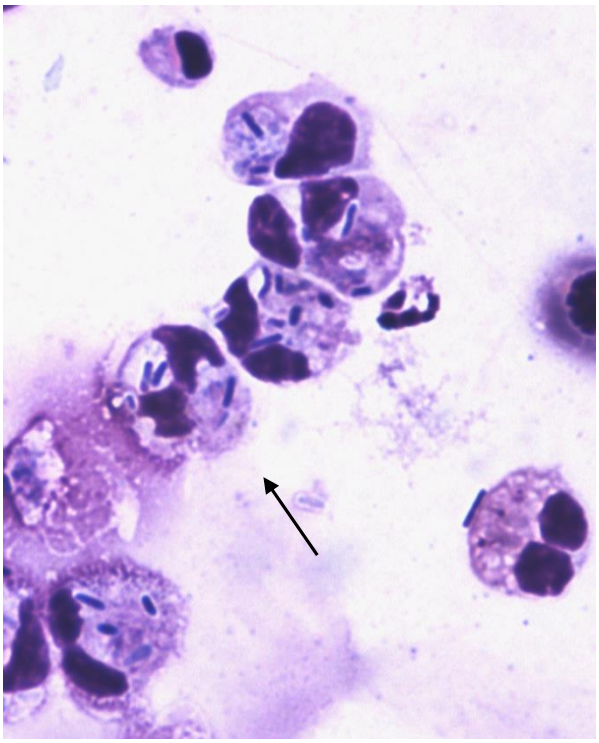


Figure 3.3. Phagocytosis of *S.* Enteritidis by heterophils isolated from day-old chickens. Cytospin smears were stained with Hematology 3-step stain and examined by light microscopy with the oil immersion objective (100x).

Table 3.1. Phagocytic index. Phagocytosis of opsonised *S.* Enteritidis (10^7 cfu/ml) by heterophils (10^6 cells/ml) isolated from day-old chickens. The numbers are from two independent studies, each consisting of five technical replicates.

% heterophils containing *S.* Enteritidis	**Average number of bacteria per heterophil**	**Phagocytic Index (PI)**
63 ± 2.94	2.62 ± 0.38	**165.17 ± 23.10**

Live, opsonised *S.* Enteritidis and phorbol A-myristate 13-acetate (PMA) were used to evaluate production of reactive oxygen species (ROS) by heterophils using an oxidative burst assay. The levels of ROS production were indicated by the amount of fluorescence caused by oxidation of DCFH-DA to DCF. PMA gave the strongest stimulation to the oxidative burst. Although bacteria were a less effective stimulant than PMA, the level of ROS produced by heterophils in the presence of *S.* Enteritidis doubled after 1 h incubation compared to control cells (Figure 3.4).

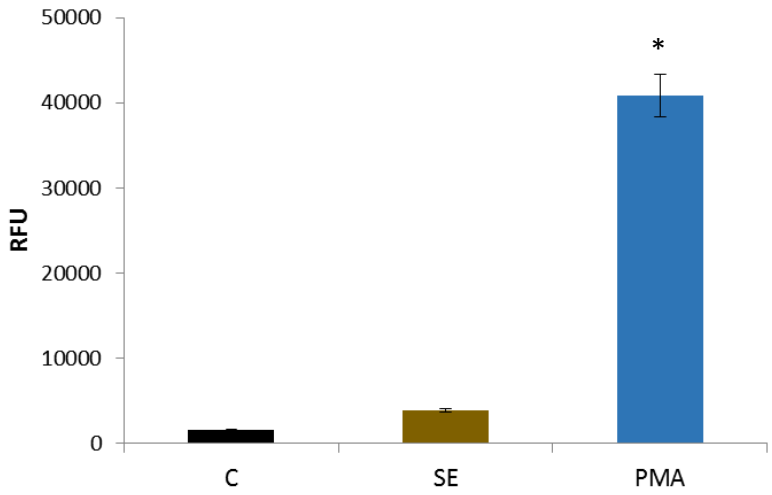


Figure 3.4. Ability of heterophils to generate an oxidative burst response following stimulation with opsonised *S.* Enteritidis and the inflammatory agonist phrorbol A-myristate 13-acetate. Reactions contained 1 x 10^7 cells/ml, 10 µg DCFH-DA/ml and 10^8 cfu/ml of opsonised *S.* Enteritidis. Samples were incubated at 37˚C for 1 h. The control relative fluorescence units (RFU) value was obtained from unstimulated cells at the same time point. Data represent the mean ± standard deviation (SD) of three independent assays. C - control cells, SE - cells stimulated with *S.* Enteritidis, PMA - cells stimulated with . phorbol A-myristate 13-acetate; *$p \leq 0.05$.

The levels of enzymatic processes of degranulation was measured in heterophils incubated with *S.* Enteritidis. The presence of bacteria triggered the release of granule contents, which was detected by quantifying the levels of β-D-

glucuronidase, an enzyme that cleave the glyosidic bond of glucoronide. This is detected owing to the fluorescence of 4-methylumbelliferone liberated from the 4-methylumbelliferyl-b-D-glucuronide complex, which is highly fluorescent in alkaline solution and proportional to the enzyme activity present in a sample. The degranulation ability of heterophils following stimulation with bacteria was compared with that of unstimulated cells. The level of bactericidal intracellular granules released after stimulation of heterophils with live, opsonised *S.* Enteritidis doubled at 1 h timepoint (Figure 3.5).

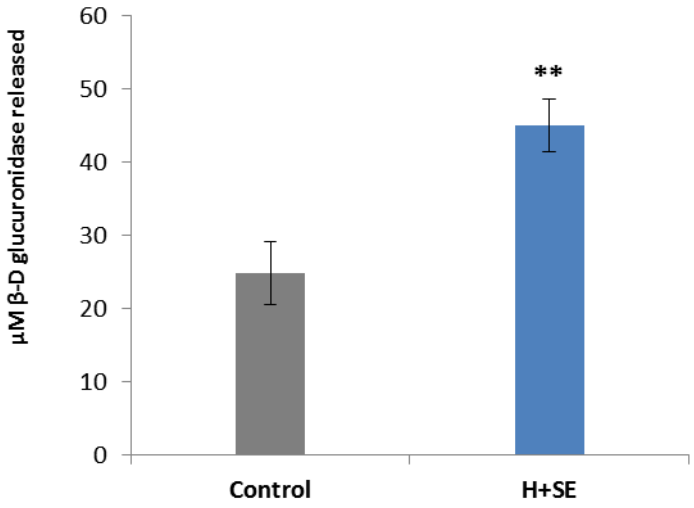


Figure 3.5. Effect of opsonised *S.* Enteritidis on degranulation by heterophils. Degranulation of heterophils (10^7 cell/ml) induced by stimulation with opsonised *S*. Enteritidis (10^8 cfu/ml). Data represent the mean±standard deviation (SD) of two biological replicates, each with four technical replicates; H + SE – heterophils stimulated with *S*. Enteritidis; ** $p \leq 0.001$.

The phagocytosis triggers the release of bactericidal components in a form of degranulation and reactive oxygen species and all three processes often occur simultaneously. Based on the above experiments, heterophils are a good indicator of early immune responses when stimulated with live bacteria or mitogen. The presented assays can be performed within minutes after isolation of heterophils from blood. However, the isolation process itself and the high cell yield required for

the experiments make these methods not useful for screening older and larger populations of birds.

3.3.4 Pro-inflammatory cytokine and chemokine expression upon stimulation with lipopolysaccharide (LPS)

The value of bone marrow-derived cell cultures in evaluating immune robustness was examined by testing BMDC and BMDM responses to LPS as an example. The mRNA levels of the pro-inflammatory cytokines IL-1β and IL-6, and of the Th1-associated cytokine IL-18, were studied in BMDM and BMDC + LPS after six days of culture. Additionally, transcript levels for the pro-inflammatory chemokines CXCLi1 and CXCLi2 were quantified. The BMDC cultures were stimulated with 200 ng/ml of LPS for 24 hours and the BMDM with 250 ng/ml of LPS for 4 hours. The timepoints were selected based on the published data.

The level of transcription varied greatly between cell types. For some transcripts, there was also a large variation observed between replicates. The highest induction of expression was observed for IL-6 transcripts, in both BMDC and BMDM. The IL-1β transcript was upregulated in both cell types with higher levels in BMDM after 4 h stimulation. The Th1-associated cytokine IL-18 was also upregulated in both cell types but the level of expression was much lower in BMDM when compared to IL-1β and IL-6. The mRNA expression of CXCLi1 in BMDC was detected at 24 hours but the levels were much lower compared to BMDM. The mRNA expression of CXCLi2 chemokine in BMDC was upregulated reaching 20 fold change, where in BMDM expression of CXCLi2 was not detected at 4 h timepoint compared to the unstimulated control cells. Data are presented in Figure 3.6.

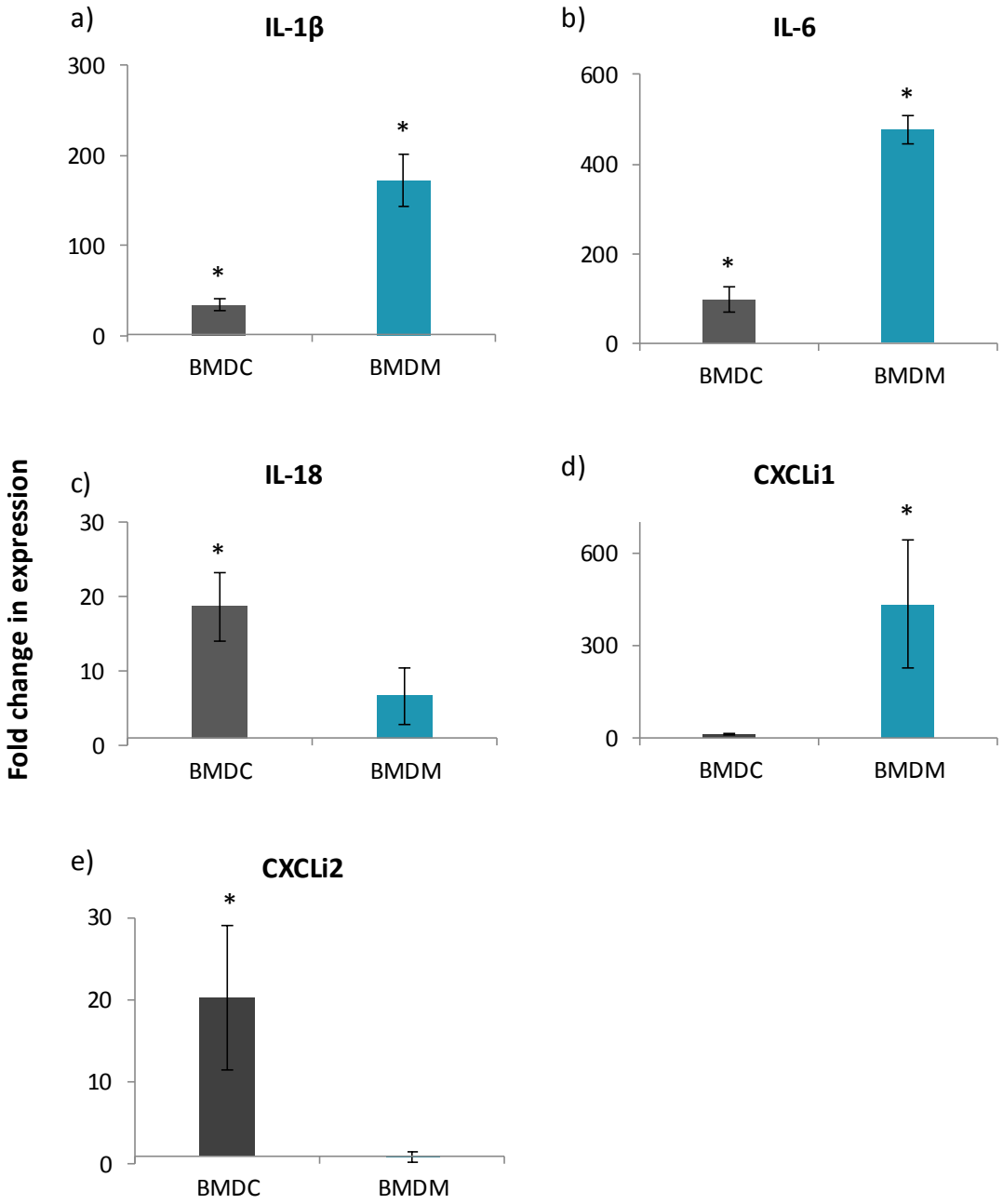


Figure 3.6. Evaluation of expression of proinflammatory cytokine genes in BMDM and BMDC stimulated with LPS a) IL-1β; b) IL-6; c) IL-18 and chemokines d) CXCLi1; e) CXCLi2; in bone marrow-derived dendritic cells (BMDC), stimulated for 24 h with 200 ng/ml of LPS and in bone marrow-derived macrophages (BMDM), stimulated for 4 h with 250 ng/ml of LPS. Data are presented as fold change compared to unstimulated control cells, each bar represent average of three biological replicates ± SD; *p ≤ 0.05.

The transcript levels for proinflammatory cytokines (IL-1β, IL-6 and IL-18), proinflammatory chemokine (CXCLi2) and anti-inflammatory cytokine (TGF-β4) were also examined in heterophils upon LPS stimulation (Figure 3.7). Three different breeds were used to isolate heterophils from blood of day-old chickens. The highest

level of expression of IL-1β was observed in the Novogen line heterophils. In the same line IL-6 mRNA expression was the lowest compared with other lines tested, where the RI-J line showed the highest level of mRNA expression. The IL-18 cytokine was differentially expressed compared to the control cells in all lines tested but the levels were low. In the case of CXCLi2, Aviagen line and RI-J line exhibited similar level of expression with Novogen line demonstrating higher upregulation. The anti-inflammatory cytokine TGF-β4 showed little or no consistent change in expression across the lines studied.

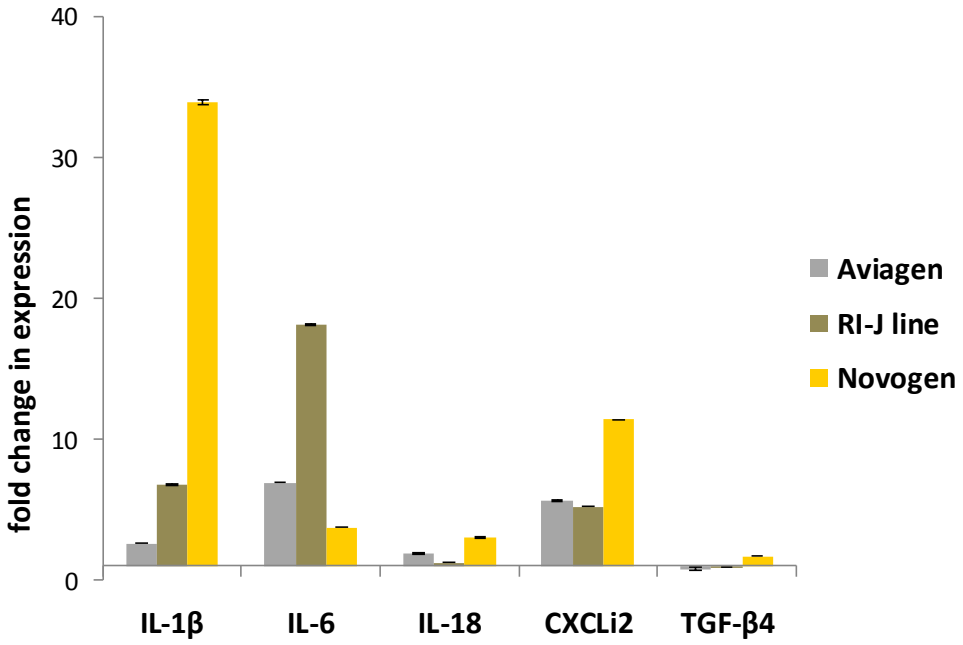


Figure 3.7. Evaluation of proinflammatory gene expression in heterophils as measured by RT-qPCR. Level of mRNA expression of cytokines: IL-1β; IL-6; IL-18; chemokine CXCLi2 and anti-inflammatory cytokine TGF-β4 in heterophils isolated from three breeds; heterophils were stimulated with 10 µg/ml of LPS for 1 h. Data are presented as fold change compared to unstimulated control cells, each bar represent average of three technical replicates ± SEM.

3.4 Discussion

Commercial broiler lines have distinct immune function parameters, due to differences in their genetic make-up, which results in a disease resistance and/or susceptibility (Lakshmanan *et al.,* 1997). Disease resistance is a complex trait, which is influenced by genetics, environment, diet and other factors. Enhancing innate resistance to pathogens in chickens is of increasing interest as an alternative to antimicrobial use. There are widely used laboratory-based assays that could be implemented to test chicken's responsiveness to different stimuli. Their usage in screening chickens, as a diagnostic tool, has been evaluated here in pilot studies. Studies on regulation of innate immune cells are important as effector cells direct the adaptive immune response. Although studies of cytokine expression in primary cells provide comprehensive data, culturing dendritic cells and macrophages from bone marrow is time-consuming and the quality of cell culture will vary depending on breed type and the age of the birds. More rapid, cheaper and reproducible techniques will have to be applied for assessment of differences in immune robustness in chicken lines.

Heterophils act early in response to invading microorganisms and have evolved many mechanisms to clear pathogens. They are therefore useful functional biomarkers for evaluating innate immune competence in poultry (Swaggerty *et al.,* 2003). Recognition of PAMPs of foreign microorganisms stimulates phagocytosis, degranulation and generation of oxidative burst (Kogut *et al.,* 2003; He *et al.,* 2005) that was also shown in current study in which heterophils were stimulated with *S.* Enteritidis. Similar results were obtained by He *et al.* (2003) where *S.* Enteritidis-stimulated heterophils increased production of oxidative burst. The above data and other studies show that functional comparisons of heterophils, using phagocytosis, degranulation and oxidative burst assays, provide comprehensive data on the performance of the early immune response. Heterophils were also used in mRNA expression experiments and similarly to published studies (Swaggerty *et al.,* 2003) there were great differences observed in cytokine expression between different breeds of chickens. The RT-qPCR experiment showed that, although most of the

genes tested were expressed in heterophils stimulated with LPS, there were great differences in level of expression between broiler (Aviagen) and layer (Novogen and RI-J) lines. There have been few reports on differences in immune responses between layers and broilers in studies on LPAI H7N2 strains (Ladman *et al.,* 2008), after IBV infection (Smith *et al.,* 1985) and on cellular and humoral responses to antigens (Koenen *et al.,* 2002; Parmentier *et al.,* 2010).

An alternative method could involve assessment of the number and activity of heterophils and the measurement of differences in levels of proinflammatory responses. It has been shown that the number of heterophils that migrate to the site of infection is associated with increased resistance against systemic *S.* Enteritidis (Swaggerty *et al.,* 2005). Together with the correlation between resistance to *S.* Enteritidis and differences in expression of innate immune genes, these methods could be used to select for increased resistance to other pathogens but their role as diagnostic tool would be limited considering high number of heterophils needed to perform the experiments. Insufficient numbers can be obtained in blood to permit analysis of heterophil function as a screening tool. Without the need for culture, they are able to phagocytose, degranulate and produce ROS, but those functions can be deficient in newly hatched birds (Wells *et al.,* 1998). Although using heterophils to study differences in responses to pathogens is faster than using bone marrow-derived effector cells, the isolation of sufficient number of cells requires bleeding of at least 100 day-old chicks.

Quantitative PCR was performed to determine whether pro-inflammatory cytokines (IL-1β, IL-6), the Th1-associated cytokine IL-18 and pro-inflammatory chemokines (CXCLi1, CXCLi2) were induced upon stimulation of BMDM and BMDC with LPS. Upregulation of IL-1β, IL-6 and CXC chemokines by *Salmonella* has been shown in various tissues, including ileum and liver (Withanage *et al.,* 2004), spleen (Cheeseman *et al.,* 2007), heterophils and macrophages (Kaiser *et al.,* 2006; Kogut *et al.,* 2006; Swaggerty *et al.,* 2008). Bone marrow-derived macrophages and dendritic cells have been proven a good source for studying mRNA gene expression

triggered by pathogens and mitogens (Wigley *et al.,* 2006; Wu *et al.,* 2010; Vervelde *et al.,* 2013). In the current study, LPS stimulation of BMDM and BMDC from outbred, vaccinated chickens has shown the mRNA levels of cytokines markedly different in magnitude compared to unstimulated cells and between biological replicates.

In the current study, the mRNA gene expression varied greatly between cell types, timepoints and LPS concentrations. Depending on the time of stimulation and LPS concentration, some genes may not be detected leading to a conclusion that they are not expressed, but the real reason may be that this particular cell culture has responded and is in homeostasis. As the bone marrow-derived cells have to be in culture for six days, their responses may differ due to differences in developmental stages and maturity at the time of stimulation. Therefore, the selection of stimulant concentration and the timepoints would have to be well thought through as the improper choices may lead to false conclusions.

The cited studies confirm that different types of microbes and different strains trigger varied levels of response of the genes tested in presented experiments not only in *in vitro* studies but also in tissues from infection studies. It is difficult to agree what genes and at what level of expression are perfect signatures of immune responsiveness. The bone-marrow derived macrophages and dendritic cells are not a good choice for determination of immune resistance or susceptibility. As the current study and previous reports show, the differences in the speed and the magnitude of gene expression depend on a time of stimulation, type of antigen/pathogen used and antigen/pathogen load. The use of progenitor cells, their culture outside the body in simulated settings and stimulation with one pathogen or antigen does not mimic the real environment of broilers. The culture of BMDM and BMDC are a standard technique in chicken mRNA gene expression studies where immune responses are evaluated. They may be informative and useful to further characterise cell populations but the selection of birds that supposed to have a robust immune response, based on expression of few genes

produce less information compared to high-throughput techniques. Although a small number of immune-related genes could be practical in distinguishing which line of chickens have better early immune response to particular pathogen, a bigger panel of genes would return a broader view.

Chapter 4 Generation of gene list - selection and cloning of genes involved in early immune responses.

4.1 Introduction

The cellular and molecular assays described in the previous chapter were dismissed as being effective and rapid tools for testing immune robustness in chicken lines. To screen the immune performance of greater number of birds, a new high-throughput qPCR platform, the 96.96 Dynamic Array with BioMark System from Fluidigm, will be tested as alternative (introduced in Chapter 6). This type of qPCR platform allows the expression of up to 96 genes across 96 samples to be tested simultaneously. Therefore, it is necessary to select a panel of ~100 immune-related genes of interest to test their expression in commercial lines of chickens.

There have been many studies performed on various chicken lines, susceptible and resistant to particular pathogens and on cells isolated from chickens and stimulated with antigens and/or pathogens. These types of studies are important to examine the immune response to the particular pathogen of interest but they do not reflect immune responses in the commercial environment where birds are exposed to multiple pathogens and stressors. Many of those microorganisms act simultaneously on the chicken's immune system. As the screening tool should be suitable for testing birds reared under both clean and commercial circumstances, selected genes have to reflect immune responses to different antigens and/or pathogens. A list of genes was compiled from studies using resistant and susceptible chicken lines as well as cell lines and primary cells challenged with a range of pathogens or constituents thereof. Studies with similar agents but using mammalian species were also included to determine if there are conserved immune responses across species to different stimuli. Additionally, RNA-seq analysis was performed on effector cells stimulated with LPS and the results were compared to the panels of genes differentially expressed (DE) in previous studies to increase the number of genes of interest.

The selected genes have their sequences available in the latest chicken genome assembly (Galgal4). With the use of Ensembl and NCBI databases each gene sequence can be compared and used as a template in primer design for future qPCR applications. As the chicken genome sequence is still under development and the published studies based their studies on preceding genome release, there is a need to confirm the sequences using molecular cloning followed by sequencing. This approach will allow the design of qPCR assays for use in Chapter 6 and by the wider research community.

4.2 Materials and methods

4.2.1 Selection criteria for creation of gene list

The articles were collected by searching the NCBI database with the queries 'innate immune response', 'gene expression infection' in both chicken and mammalian species, and by doing searches of cited references in selected articles. The publications used to select the genes had to contain an analysed differential expression (DE) dataset in the body of text and/or in the supplementary data available online. Infection studies on various pathogens and their interactions with the host as well as *in vitro* studies on stimulated primary cells and/or cell lines were included in this collection. Each article's differential expression (DE) dataset was compared with other studies and the genes that were upregulated in two or more studies were considered a good candidate for the gene list.

4.2.2 RNA sequencing of BMDC, BMDM and heterophils

Sample preparation and RNA sequencing methods are described in Chapter 2, section 2.9.1. The analysis of sequencing results was performed as described in Chapter 2, section 2.9.2. The RNA-seq pipeline is shown Appendix 1, Figure 1.

4.2.3 Comparison of RNA-seq data between analyses and published studies for further gene selection

The three lists of significantly DE genes obtained from RNA-seq analysis of BMDC, BMDM and heterophils stimulated with LPS were compared between each other. The genes that were common for two or more lists were selected. The genes with significant differential expression in one cell type in the RNA-seq results were screened against the lists of genes from the articles used in the first selection (Chapter 4, 4.3.1). Genes that were present in two or more lists (RNA-seq results and published studies lists) were placed on the final gene list.

4.2.4 Amplification, cloning and sequencing of candidate gene cDNA

Amplicon lengths, primer sequences and genes IDs are described in Appendix 1, Table 3 and Table 5. PCR was performed as described in Chapter 2, section 2.7.4. PCR products were separated and visualised by agarose gel electrophoresis

(Chapter 2, section 2.7.7) and amplicons of the expected size were excised DNA extracted and ligated into the pGEM-T Easy vector (Chapter 2, section 2.7.8 and 2.7.9). Transformation of highly competent JM109 cells with ligated PCR products was performed as described in Chapter 2 section 2.7.10. Blue/white screening of transformed bacterial cells helped to distinguish putative recombinants, which were then confirmed by dideoxy chain termination (Sanger) sequencing. The results were assembled and visualised using the DNASTAR® SeqMan Pro application. Cloned and reference sequences were aligned by ClustalX (Chapter 2, section 2.1.2). Alignments were visualised by using GeneDoc software (Chapter 2, section 2.1.3). Alignments of all cloned genes and their master sequences are shown in Appendix 2 (electronic file).

4.3 Results

4.3.1 Creation of innate immune gene list

The data from 16 infection studies on different chicken lines and cells and the data from 13 studies on mammalian species were collected. By selecting the upregulated genes from each study, a file consisting of 29 separate lists was created. The results of PubMed search queries: 'innate immune response', 'gene expression infection' in chickens, humans and other mammalian species are shown in Table 4.1.

Table 4.1. Results of search queries for creation of the gene list.

Authors	Studies	Type of platform used
Bliss *et al*., 2005	Chicken PBL-derived macrophages stimulated with LPS	Microarray; GEO accession GSE1794
Chaussabel *et al.,* 2003	Human macrophages and dendritic cells stimulated with parasites	Affymetrix HU95A microarray; no accession number
Chiang *et al*., 2008	Chicken heterophils from two distinct parental meat-type broiler lines stimulated with *S*. Enteritidis	44 K Agilent microarray; GEO accession: GSE9416
Ciraci *et al*., 2010	Chicken HD11 macrophage-like cell line stimulated with *S*. Typhimurium endotoxin	Affymetrix GeneChip; GEO accession GSE23881
Connell *et al*., 2012	Caecal response of Barred Rock chickens to *C. jejuni* infection	RNA-seq; raw data as supplementary file
de Kleijn *et al*., 2012	Human neutrophils stimulated with LPS	Affymetrix Human ST 1.0 exon array; GEO accession GSE35590
Gou *et al*., 2012	Chicken PBMCs response to *S.* Enteritidis infection	qPCR
Guo *et al*., 2012	Chicken bursal response to IBDV infection	Agilent microarray; no accession number
Huang *et al.,* 2001	Human monocyte-derived dendritic cells stimulated with *E. coli, Candida albicans,* influenza virus	Human oligonucleotide microarray, no accession number
Jensen *et al*., 2006	Bovine PBMC stimulated with LPS	5 K bovine macrophage specific cDNA microarray; no accession number
Kapetanovic *et al*., 2012	Pig macrophages stimulated with LPS	Affymetrix GeneChip Porcine Genome Array; GEO accession GSE30956
Killick *et al*., 2011	PBL from *Mycobacterium bovis* infected and non-infected Holstein-Friesian cattle	Affymetrix GeneChip Bovine Genome Array; GEO accession GSE33359
Kim *et al*., 2011	Chicken duodenal response to primary and secondary infections with *Eimeria acervulina*	9.6K avian intestinal intraepithelial lymphocyte cDNA microarray (AVIELA); GEO accession GSE16230
Lavric *et al*., 2008	*In vitro* studies on monocyte-derived macrophages responses to *M. synoviae* and	Avian macrophage microarray (AMM) and the avian innate

Authors	Studies	Type of platform used
	E. coli stimulation	immunity microarray (AIIM); GEO accession GSE1794
Lee *et al.,* 2010	Chicken embryo lung cells infected with ILTV	44 K Agilent custom microarray; GEO accession GPL6413
Li *et al.,* 2010	Caecal response to *C. jejuni* infection in two commercial broiler lines	44K Agilent microarray; GEO accession GSE10257
Martins *et al.,* 2013	Mesenteric lymph nodes (MLN) from piglets infected with *S.* Typhimurium	qPCR
Mellits *et al.,* 2009	Human colonocyte line (HCA-7) incubated with *C. jejuni*	Affymetrix Human Genome U133A array; no accession number
Munir *et al.,* 2004	Chicken embryo cells infected with avian metapneumovirus	cDNA microarray; no accession number
Nau *et al.,* 2002	Human macrophages stimulated with multiple bacteria and bacterial components	Affymetrix Hu6800 GeneChip; no accession number
Reemers *et al.,* 2010	Chickens infected with avian influenza H9N2	*Gallus gallus* Roslin/ARK CoRe Array Ready Oligo Set V1.0; accession numbers: E-TABM-771 and E-TABM-772
Roach *et al.,* 2007	Human peripheral blood-derived mononuclear cells stimulated with LPS	HG-U133 Plus2 GeneChip; GEO accession GSE5504
Ruby *et al.,* 2006	Chickens infected with IBDV	Microarray; accession number E-MEXP-756
Rue *et al.,* 2011	*In vitro* studies on chicken splenocytes responses to NDV stimulation	Agilent microarray; no accession number
Sanz-Santos *et al.,* 2011	Neutrophils from Iberian pigs stimulated with LPS	Affymetrix Porcine GeneChip; no accession number
Schokker *et al.,* 2012	Chicken jejunal response to *S.* Enteritidis infection	ARK-genomics *Gallus gallus* 20 K; GEO accession GSE27069
Schreiber *et al.,* 2006	Human U937 cells stimulated with LPS	custom DNA microarray; accession number E-WMIT-6
Wang *et al.,* 2012	Porcine alveolar macrophage responses to *Haemophilus parasuis*	Affymetrix GeneChip Porcine Genome Array; GEO accession GSE30172
Zaffuto *et al.,* 2008	Chicken embryo cell lines infected with avian influenza virus	Agilent complete chicken genome (42K) microarray;

A list of 12 immune-related genes was generated from the studies on infected chicken cells and tissues by selecting the significantly upregulated genes that were present in two or more separate studies. The same selection method was used when comparing the 16 panels of DE genes from chicken studies with the mammalian gene expression studies. This approach resulted in the additional twenty genes of interest (GOI). The final list consisted of 32 genes upregulated during *in vitro* and *in vivo* infections in mammalian species and in the chicken (Table 4.2).

Table 4.2. A list of genes of interest upregulated upon stimulation with pathogens or agonists based on published studies.

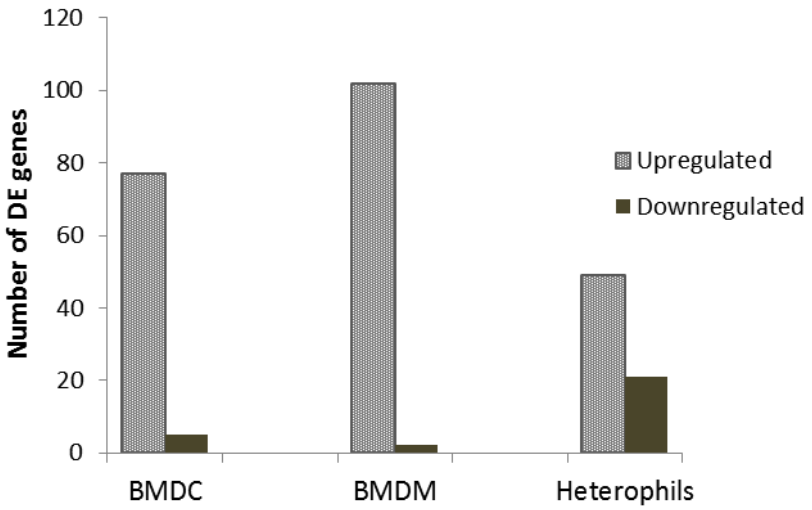
Chicken and mammalian species	Chicken only
CCL20; CCL4;CCL5; CD83; CXCL13; IL12B; IL18; IL1B; IL8; IRAK2; IRF1; MYD88; NFKBIA; NFKBIZ; NLRC5; SOCS1; SOCS3; TIMD4; TLR4; TNFAIP3	CD80; CXCLi1; IFIT5; iNOS; IRF7; IRF10; JUN; LYZ; PPARG; TIRAP; TOLLIP; TRAF3IP2

The accession numbers of genes with different symbols were compared to confirm that the same transcript was differentially expressed in the selected studies. The gene ID's from the chicken studies with known differences in sequences were compared to clarify which transcript was upregulated in a particular study. The chemokine CCL4 (C-C motif ligand 4) was present in five chicken panels (Chiang *et al.,* 2008; Ciraci *et al.,* 2010; Lee *et al.,* 2010; Rue *et al.,* 2011; Guo *et al.,* 2012). The accession numbers for genes that were used to either design the probe for a microarray or that were a result of a RNA-seq alignment analysis differed. The transcript with accession number NM_204720 was chosen, as it was differentially expressed in two studies. This transcript sequence is identical with the sequence of chicken CCLi3. The chemokine CCL5 (C-C motif ligand 5), also known as CCLi4 or ah294, was upregulated in two chicken studies and both of the transcripts had different accession numbers assigned (Lavric *et al.,* 2007; Guo *et al.,* 2012). Guo *et al.* (2012) study used ENSGALT00000001405 Ensembl identification number, which is no longer present in gene set, therefore the sequence under the NM_001045832 number, was used in further studies. The chicken K60 gene, also known as IL-8-like 1 or CXCLi1, was upregulated in two studies where commercial and customised microarrays were used and probes were designed based on the same sequence – NM_205018 (Chiang *et al.,* 2008; Connell *et al.,* 2012). A third study did not include accession numbers for studied genes. Interleukin 8 (IL-8 or CXCLi2) was present in six panels and the same sequence (NM_205498) was used in probe design. One of the studies used RNA-seq platform for DE and the assembled transcripts aligned to region of a genome where IL-8 is annotated (Connell *et al.,* 2012).

4.3.2 **RNA-seq analysis**

The genes selected, based on previous studies, resulted in a small list consisting of 32 genes involved in immune response to pathogens or agonists. To increase the number of the genes of interest, an RNA-seq analysis was performed on RNA from chicken primary cells (BMDC, BMDM and heterophils) stimulated with LPS. The Illumina HiSeq 2500 platform generated 14.1-18.6 million RNA-seq tag pairs per sample (95.8 million in total) each 100 nucleotides in length resulting in 19.7 Gb of data. The Cufflinks program containing Cuffdiff algorithm was used to find significant changes in transcript levels between LPS-treated and control cells. The DE genes were determined by using the fold change >1 (Figure 4.1a). Among the differentially expressed genes, there was only one gene, TGM4, upregulated in all of the three cell types. Between BMDC and BMDM, 30 DE genes were common and each cell type shared only nine DE genes with the heterophils (Figure 4.1b). The DE analysis revealed that many of the significantly expressed genes have not been annotated in chicken genome. For further analysis, only the annotated genes were taken under consideration. The reads for four differentially expressed genes assembled into transcripts that spanned the location of two closely annotated genes. It has resulted in pair of genes assigned to the same genomic location with shared fold change value. Therefore, 24 genes significantly differentially expressed in two or more cell types were chosen as genes of interest.

a)



b)

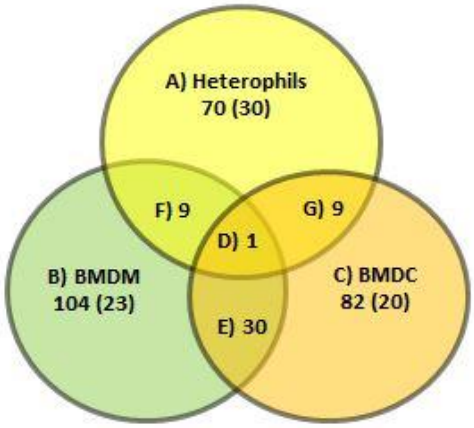


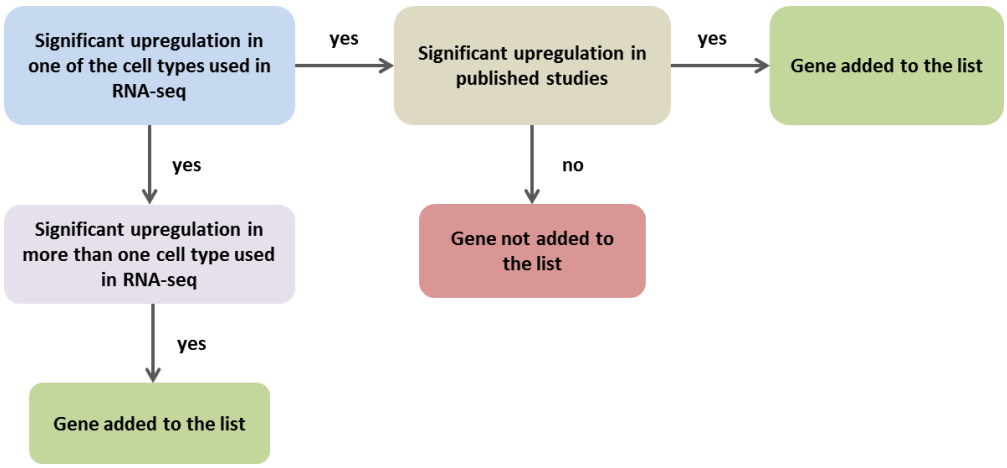
Figure 4.1. RNA-seq results a) Number of significantly differentially expressed genes in chicken primary cells stimulated with LPS. Abbreviations: BMDC, bone marrow-derived dendritic cells; BMDM, bone marrow-derived macrophages; DE- differentially expressed compared to unstimulated cells; b) Venn diagram showing the number of transcripts significantly DE; A) in heterophils; B) in BMDM; C) BMDC; D) common DE gene for three cell types; E) shared between BMDC and BMDM; F) shared between BMDM and heterophils; G) shared between BMDC and heterophils; numbers of unannotated genes are presented in brackets.

4.3.3 Comparison of RNA-seq results with previous studies

The criteria for genes to be ranked as genes of interest were as follows: candidate gene had to be differentially expressed in any of the cell type tested from the RNA-seq analysis and at least once in the previous studies used to generate the initial list of 32 genes. Against this criterion, 17 genes qualified. A further 15 genes were added to the list, based on their overexpression in more than one of the previous studies analysed. From the RNA-seq data, 44 genes were added to the list

as they were differentially expressed in at least two cell types stimulated with LPS. A flow chart visualising the pathway of gene selection is presented in Figure 4.2a. Based on these, the final list consisted of 104 genes of interest (Figure 4.2b). The type of cell and treatment that caused the differential expression of the selected genes are shown in Table 4.3.

a)



b)

Figure 4.2. Comparison of published studies and RNA-seq gene lists ; a) flow chart representing the criteria for selection of GOI based on RNA-seq data and published studies; b) the Venn diagram showing the number of genes selected from different analyses and the final number of genes of interest (GOI).

Table 4.3. Genes of interest and the type of studies with differential expression in mammalian species and the chicken.

Gene symbol	Type of study with differential expression
ABCG2	BMDM and BMDC + LPS RNA-seq
ADM	BMDM and BMDC + LPS RNA-seq; hNEUT + LPS; bMph + LPS; hMph + bacteria and bacterial components
ATF3	HET +LPS RNA-seq; hMph + LPS
BATF3	BMDM and BMDC + LPS RNA-seq; ELC + ILTV; hNEUT + LPS
BCL2A1	HET and BMDM + LPS RNA-seq; hMph and hDCs + parasites; hDCs + *E. coli*, *C. albicans*, influenza virus
C3ORF52	BMDM and BMDC + LPS RNA-seq
CCL19	BMDM + LPS RNA-seq; chicken intestines + *C. jejuni*
CCL20	ELC + ILTV; chicken intestines + *C. jejuni*; chMph + LPS; HCA-7 *C. jejuni*; hNEUT + LPS; bMph + LPS
CCL4	HET + *S.* Enteritidis; chPBMC + *S.* Enteritidis; HD11 + *S.* Typhimurium; ELC + ILTV; Chicken spleen + NDV; hNEUT+ LPS; hMph + LPS; pMph + LPS
CCL5	bursa + IBDV; chMph + APEC; hMph + LPS; bMph + LPS; pMph+ LPS; hMph and DCs + parasites
CD40	BMDM and HET +LPS RNA-seq; bMph + LPS; chPBMC + *S.* Enteritidis
CD72	BMDM and BMDC + LPS RNA-seq; chicken intestines + *C. jejuni*
CD80	Chicken jejunum + *S.* Enteritidis; chPBMC + *S.* Enteritidis; bMph + LPS
CD83	HET + LPS RNA-seq; mMph + LPS; Chickens + IBDV; bMph + LPS; hNEUT + LPS; HD11 + *S.* Typhimurium; chMph + APEC; chicken intestines + *C. jejuni;* hMph + bacteria and bacterial components; hMph and DCs + parasites
CSF1	BMDC RNA-seq ; pMph + LPS
CXCL13	BMDM and BMDC + LPS RNA-seq; Chicken spleen + NDV; pMph + LPS
CXCL13L2	BMDM and BMDC + LPS RNA-seq; chicken intestines + *C. jejuni*
CXCLI1	HET + *S.* Enteritidis; chicken intestines + *C. jejuni;* CE cells infected with avian metapneumovirus
CXORF21	HET and BMDM + LPS RNA-seq
DLG3	BMDM and BMDC + LPS RNA-seq
DTX2	HET and BMDM + LPS RNA-seq; chicken intestines + *C. jejuni*
EAF2	BMDM and BMDC + LPS RNA-seq
EDN1	BMDM + LPS RNA-seq; pMph +LPS; bMph + LPS; hMph and DCs + parasites
EGR1	HET + LPS RNA-seq; mMph + LPS; chicken intestines + *C. jejuni*
ENSGALG00000002955	BMDM and BMDC + LPS RNA-seq
ENSGALG00000005747	BMDM and BMDC + LPS RNA-seq
ENSGALG00000011172	BMDM and BMDC + LPS RNA-seq

ENSGALG00000015395, ENSGALG00000027419	BMDM and BMDC + LPS RNA-seq
ENSGALG00000022324	BMDM and BMDC + LPS RNA-seq
ENSGALG00000025905	BMDM and BMDC + LPS RNA-seq
ENSGALG00000026592	HET and BMDC + LPS RNA-seq
ETS2	BMDM + LPS RNA-seq; bMph + LPS; hMph + LPS; hNEUT + LPS; hMph + bacteria and bacterial components
F3	BMDC + LPS RNA-seq; bMph + LPS
G0S2	HET and BMDC + LPS RNA-seq; hNEUT + LPS; hMph and DCs + parasites
GABRA5	BMDM + LPS RNA-seq ; chicken intestines + *C. jejuni*;
GCH1	BMDM + LPS RNA-seq; HD11 + *S.* Typhimurium; hNEUT + LPS; hMph + bacteria and bacterial components
GLUL	BMDM and BMDC + LPS RNA-seq
HPS5	BMDM and BMDC + LPS RNA-seq
IFIT5	Chicken spleen + NDV; chMph+ APEC; chicken intestines + *C. jejuni;* Chickens + IBDV
IL10RA	HET ; hNEUT + LPS
IL12B	HET + LPS RNA-seq; PMph + LPS; HET + *S.* Enteritidis; chPBMC+ *S.* Enteritidis; hMph + bacteria and bacterial components
IL13RA2	BMDC + LPS RNA-seq; chMph+ APEC
IL17REL	HET and BMDM + LPS RNA-seq; chicken intestines + *C. jejuni*
IL18	Chicken spleen + NDV; Chickens + IBDV; pMph + LPS; hNEUT + LPS; bMph + LPS; Chickens + AIV
IL19	BMDM and BMDC + LPS RNA-seq
IL1B	BMDC + LPS RNA-seq; hNEUT + LPS; bMph + LPS; hMph + LPS; pMph + LPS; chMph+ APEC; HD11 + *S.* Typhimurium; ELC+ ILTV; chPBMC+ *S.* Enteritidis; chMph+ LPS; HET + *S.* Enteritidis; hMph and DCs + parasites; hDCs + *E. coli*, *C. albicans*, influenza virus
IL1R2	HET + LPS RNA-seq; hNEUT + LPS
IL20RA	BMDM + LPS RNA-seq ; chicken intestines + *C. jejuni*
IL4I1	HET and BMDC + LPS RNA-seq
IL6	HET and BMDM + LPS RNA-seq; bMph + LPS; HET + *S.* Enteritidis; ELC+ ILTV; chPBMC+ *S.* Enteritidis; hMph + bacteria and bacterial components; hMph and DCs + parasites; hDCs + E. coli, *C. albicans*, influenza virus
IL8	HD11 + *S.* Typhimurium; ELC+ ILTV; chPBMC+ *S.* Enteritidis; chicken intestines + *C. jejuni*; HET + *S.* Enteritidis; ELC+ ILTV; Chickens + IBDV; hMph + LPS; HCA-7 *C. jejuni*; pMph + LPS; bMph + LPS; hMph + bacteria and bacterial components; hMph and DCs + parasites; hDCs + *E. coli, C. albicans,* influenza virus
iNOS	chMph+ LPS; Chicken spleen + NDV; ELC+ ILTV; chMph+ APEC; Chickens + IBDV
IRAK2	HD11 + *S.* Typhimurium; hNEUT + LPS; bMph + LPS

IRF1	chPBMC+ *S.* Enteritidis; chicken intestines + *C. jejuni*; Chicken spleen + NDV; Chickens + IBDV; hMph + LPS; pMph + LPS; hMph + bacteria and bacterial components; hMph and DCs + parasites; Chickens + AIV
IRF10	Chicken spleen + NDV; chPBMC+ *S.* Enteritidis; Chickens + AIV
IRF7	Chicken spleen + NDV; chicken intestines + *C. jejuni*; chPBMC+ *S.* Enteritidis; hMph + LPS; pMph + LPS; hMph and DCs + parasites
IRG1	HET RNA-seq ; chicken intestines + *C. jejuni*; ELC+ ILTV
JUN	HD11 + *S.* Typhimurium; chPBMC+ *S.* Enteritidis; hMph + bacteria and bacterial components
LYG2	BMDM + LPS RNA-seq; chicken intestines + *C. jejuni*; ELC+ ILTV
LYZ	Chicken spleen + NDV; ELC+ ILTV; chicken intestines + *C. jejuni*
MADPRT	BMDM and BMDC + LPS RNA-seq; chicken intestines + *C. jejuni*
MAFA	BMDM and BMDC + LPS RNA-seq
MAFF	BMDC + LPS RNA-seq; hMph + LPS
MARCKSL1	BMDM and BMDC + LPS RNA-seq; chicken intestines + *C. jejuni*
MYD88	ChPBMC+ *S.* Enteritidis; pMph + LPS; hMph and DCs + parasites; Chickens + AIV
NDNF	BMDM and BMDC + LPS RNA-seq
NFKB2	BMDC + LPS RNA-seq; chPBMC+ *S.* Enteritidis; chicken intestines + *C. jejuni*; hMph + LPS; hNEUT + LPS; hMph and DCs + parasites
NFKBIA	HD11 + *S.* Typhimurium; hNEUT + LPS; bMph + LPS; pMph + LPS; hMph + bacteria and bacterial components; hMph and DCs + parasites
NFKBIZ	chPBMC+ *S.* Enteritidis; ELC+ ILTV; hNEUT + LPS; PMph + LPS
NLRC5	BMDC + LPS RNA-seq ; HD11 + *S.* Typhimurium; hNEUT + LPS
NR4A3	HET + LPS RNA-seq; chicken intestines + *C. jejuni*; bMph + LPS
PFKFB3	BMDM and BMDC + LPS RNA-seq; bMph + LPS
PKD2L1	BMDM and BMDC + LPS RNA-seq
PLA2G5	BMDM + LPS RNA-seq ; chicken intestines + *C. jejuni*
PLK3	BMDM and BMDC + LPS RNA-seq; hNEUT + LPS
PPARG	ELC+ ILTV; HET + *S.* Enteritidis: chicken intestines + *C. jejuni*
PTGS2	BMDM and BMDC + LPS RNA-seq; bMph + LPS; HET + *S.* Enteritidis; hMph and DCs + parasites
RASD1	HET + LPS RNA-seq; chicken intestines + *C. jejuni*; ELC+ ILTV
RNF19B	BMDC + LPS RNA-seq ; chicken intestines + *C. jejuni*
SAAL1	BMDM and BMDC + LPS RNA-seq
SDC4	BMDM + LPS RNA-seq; HCA-7 + *C. jejuni;* hMph + bacteria and bacterial components; Chickens + AIV
SELE	BMDC + LPS RNA-seq; pMph + LPS; chicken intestines + *C. jejuni*
SERPINE2	BMDM and BMDC + LPS RNA-seq
SLC39A8	BMDM + LPS RNA-seq; bMph + LPS

SLCO6A1	BMDM and BMDC + LPS RNA-seq
SNX10	HET + LPS RNA-seq; ELC+ ILTV
SOCS1	BMDC and BMDM + LPS RNA-seq; Chicken spleen + NDV; chPBMC+ *S.* Enteritidis; ELC+ ILTV; pMph + LPS; Chickens + AIV
SOCS3	BMDC + LPS RNA-seq ; Chicken intestines + *C. jejuni*; ELC+ ILTV; chPBMC+ *S.* Enteritidis; bMph + LPS; pMph + LPS; hNEUT + LPS; hMph and DCs + parasites
SPTSSB	BMDM + LPS RNA-seq; chicken intestines + *C. jejuni*
STEAP1	BMDM and BMDC + LPS RNA-seq
STEAP4	HET and BMDC + LPS RNA-seq; pMph + LPS
TGM4	HET, BMDM and BMDC + LPS RNA-seq; chicken intestines + *C. jejuni*; CE cell lines + AIV
TIMD4	HET + *S.* Enteritidis; pMph + LPS
TIRAP	chPBMC+ *S.* Enteritidis; HET + *S.* Enteritidis; bMph + LPS
TLR15	HET + LPS RNA-seq; chPBMC+ *S.* Enteritidis
TLR4	HET + LPS RNA-seq ; ChPBMC+ *S.* Enteritidis; PMph + LPS; BMph + LPS
TNFAIP3	Chicken intestines + *C. jejuni*; HD11 + *S.* Typhimurium; bMph + LPS; HCA-7 *C. jejuni*; hNEUT + LPS; pMph + LPS; hMph + bacteria and bacterial components; hMph and DCs + parasites
TNIP2	BMDM and BMDC + LPS RNA-seq
TOLLIP	ChPBMC+ *S.* Enteritidis; ChPBMC+ *S.* Enteritidis
TP53I11	BMDM + LPS RNA-seq ; hNEUT + LPS
TRAF3IP2	ELC+ ILTV; ChPBMC+ *S.* Enteritidis
UPP1	BMDC RNA-seq ; hNEUT + LPS
WDR24	HET + LPS RNA-seq; chicken intestines + *C. jejuni*

Legend: hNEUT – human neutrophils; bMph – bovine macorphages; chMph – chicken macrophages; hMph – human macrophages; pMph – pig macrophages; chPBMC – chicken peripheral blood mononuclear cells; HET- heterophils; ELC – embryonic lung cells; hDC- human dendritic cells; CE – chicken embryo; + LPS – stimulated with LPS; Genes symbols highlighted in red – genes not cloned in this study; blue – downregulated genes in selected studies

4.3.4 Molecular cloning and sequencing of candidate genes

Among the 32 candidate genes selected based on analysis of published literature (Table 4.1), 12 were cloned and confirmed by other group members. From the 20 remaining genes, 19 were cloned and sequenced in this study. In order to design the primer pairs for molecular cloning, the sequences for the candidate genes were searched in both the Ensembl (www.ensembl.org) and the NCBI (www.ncbi.nlm.nih.gov/) databases. The primer pairs were designed to clone either

full or partial sequence of the gene in cases were more than one transcript was available and either 5' or 3' end sequence differed.

The same approach was used to clone the mRNA coding sequences of genes selected based on the RNA-seq results. Out of 104 genes of interest, six were cloned previously by other members of the group. From the remaining number of genes, 72 were cloned in this study and their sequences were confirmed. The cloned sequences for all the genes are shown in Appendix 2 (electronic file). Examples of agarose gel images for selected amplicons are shown in Figure 4.3.

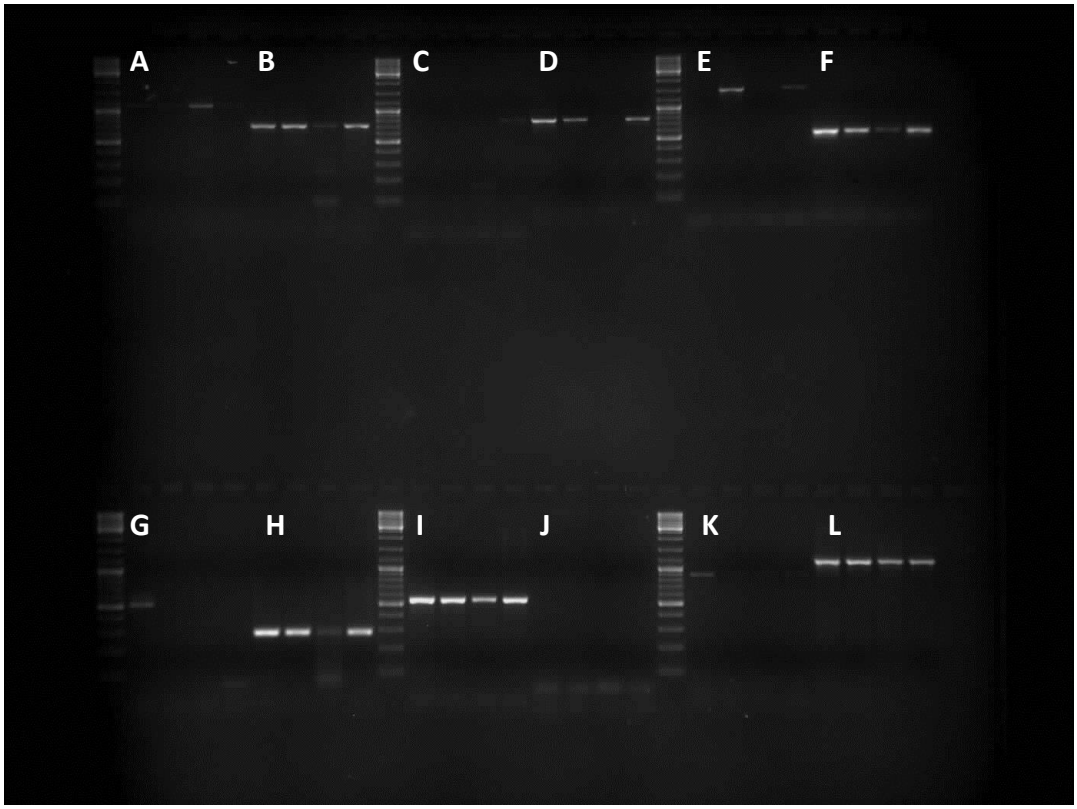


Figure 4.3. Examples of agarose gel images of amplicons of cDNAs for selected genes of interest. Primers for each gene were used in PCR reactions with cDNA from BMDC + LPS, HD11 cell line + LPS and CD40L, heterophils + LPS, BMDM + LPS (left to right for each amplicon A-L). Panel A - ENSGALG00000005747 (1056 bp), B - C3ORF52 (700 bp), C - MADPRT (891 bp), D - STEAP1 (759 bp), E - ETS2 (1440 bp), F - LYG2 (541 bp), G - EDN1 (618 bp), H - BATF3 (292 bp), I - SDC4 (545 bp), J - MARCKL (no band), K - MAFA (861 bp), L - PFKFB3 (1189 bp); Ladder - GeneRuler^{TM} DNA Ladder Mix 10kb.

An example of agarose gel electrophoresis to confirm cloning of amplicons by colony PCR is shown in Figure 4.4. Though in most cases amplicons of the expected size were obtained, rarely amplicons of an aberrant number or size were detected (e.g. amplicons from clones E2, G2, I3 and K1) .

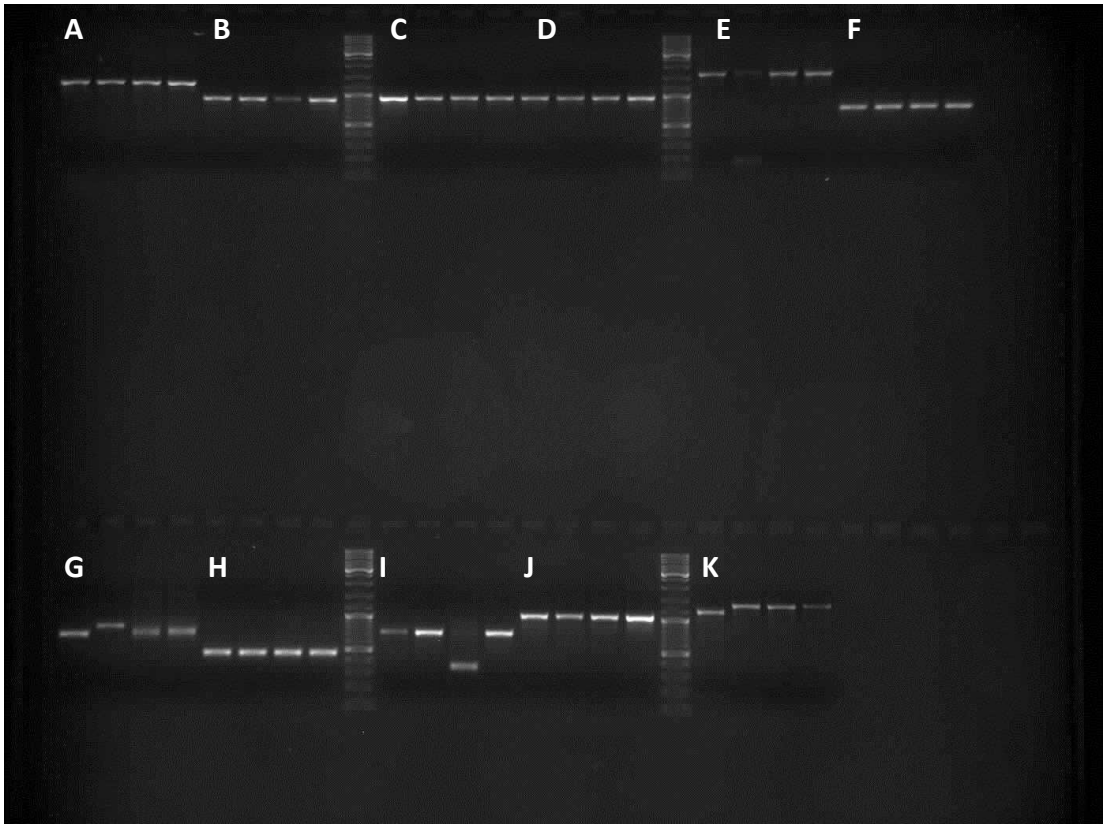


Figure 4.4**.** Examples of agarose gel electrophoresis to validate pGEM-T clones of selected amplicons by PCR. Panel A - ENSGALG00000005747 (1056 bp), B - C3ORF52 (700 bp), C - MADPRT (891 bp), D - STEAP1 (759 bp), E - ETS2 (1440 bp), F - LYG2 (541 bp), G - EDN1 (618 bp), H - BATF3 (292 bp), I - SDC4 (545 bp), J - MAFA (861 bp), K - PFKFB3 (1189 bp); Ladder - GeneRuler^{TM} DNA Ladder Mix 10kb.

4.4 Discussion

The aim of this study was to select ~100 genes involved in innate immune responses in chickens that represent immune robustness/performance. Their expression will be examined using a high-throughput qPCR platform 96.96 Dynamic Array from Fluidigm. Many genes are repeatedly used in qPCR experiments on immune responses, such as proinflammatory cytokines and chemokines. These genes are proven to be important markers in immune-related studies but there are many additional genes which may assist in estimation of the immune robustness. To create a broader view of immune performance, a list of genes of interest was generated by scrutiny of available literature and RNA-seq analysis of primary cells stimulated with bacterial LPS.

The use of whole transcriptome platforms, such as microarrays, or the use of RNA-seq would be more informative as a screening tool but these techniques could not be used for screening a larger number of birds in a rapid and cost-effective manner. In addition, analysis of the sequencing data would be complex and time-consuming. To be included in the list of genes of interest, the corresponding transcript had to be upregulated at least twice in two different studies. This instruction allowed selection of more genes, as many of the DE genes were only characteristic to one of the experimental settings. The published studies were performed with a number of pathogens infecting different organs or cell types. The genes selected from those studies were upregulated in early timepoints suggesting their involvement in the innate immune responses. Addition of the data from RNA-seq studies performed on primary chicken cells stimulated with LPS expanded the list of genes.

Molecular cloning was performed in order to confirm the sequences of selected genes of interest as many of them have few mRNA transcript sequences available in the databases. Therefore, the primer design had to include only the common part of the transcript to avoid difficulties with molecular cloning. As a result, many genes were detected in numerous cDNA samples used as a template, which means that they were involved in immune responses to different

pathogens/antigens used in a particular study. There were also amplicons that were only obtained in one of the cDNA templates used in the cloning panel and few were not obtained at all. The reason behind this may lie in the primers not being optimal for the qPCR reactions but also the design could have been based on an incorrectly assembled or annotated sequence. The first release of the chicken genome draft in 2004 has proven to be an important tool in avian genomic research but since then many changes have been introduced to improve the annotation. The main tools for building predicted annotations were based on the homology with sequences from human and mouse full-length cDNA making this approach successful for protein-coding genes. This method was not effective when translated across to birds which are evolutionary distant from mammals. This has proven to be even less useful for the annotation of rapidly evolving innate immune genes or non-coding genes. The Ensembl gene annotation protocol now incorporates the RNA sequencing data to expand the prediction of chicken genes (Flicek *et al.,* 2014). The gene list was created before the update of Ensembl chicken genome with the RNA-seq results, which could lead to changes in the mRNA sequences of the selected innate immune genes. When genes were selected from previous studies, the gene symbols along with the annotation IDs were examined to ensure that all studies reported upregulation of the same mRNA fragment. Many genes have multiple names but also mammalian nomenclature is often assigned to genes, which cause confusion when used interchangeably with chicken names. By carefully selecting the gene accession numbers and sequences behind them, the final list of cloned genes of interest consists of transcripts that were confirmed to be overexpressed in various studies.

Before high-throughput qPCR assays can be used to evaluate transcription of the immune-related genes selected here, careful consideration of the reference genes for normalisation of data is required. This is the subject of the next Chapter.

Chapter 5 Reference gene normalisation

5.1 Introduction

The aim of this study was to select a set of genes that could serve as internal controls in chicken immune-related tissue mRNA expression studies. The expression levels of 7 reference genes: β-actin (ACTB), β-2-microglobulin (B2M), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-glucuronidase (GUSB), TATA box binding protein (TBP), α-tubulin (TUBAT), r28S ribosomal RNA (r28S) were measured in tissues (spleen, bursa, caecal tonsils, ileum and blood) isolated from Aviagen male broilers reared on pedigree and sibling test farms. In order to identify the most stable reference genes across the tested samples, the expression stabilities of the reference genes were examined using three algorithms: NormFinder, Best Keeper and geNorm. NormFinder and geNorm software recognise the most suitable control genes by determining their stability value whereas BestKeeper uses descriptive statistics and the Pearson correlation coefficient to evaluate the most stable reference gene. The use of three algorithms to calculate reference gene stability will guide the selection of the most reliable reference gene(s) to avoid misinterpretation of expression studies.

Over the decades, different techniques have been applied to study transcriptional regulation in infection studies from northern blotting, cDNA microarrays, *in situ* hybridisation to quantitative PCR (qPCR) (Matulova *et al.,* 2013; Sandford *et al.,* 2012; Bojesen *et al.,* 2004). High sensitivity and potential for high throughput and enhanced specificity makes qPCR the most exploited molecular technique in host-pathogen interaction studies. These characteristics are very important in immunological research where genes of interest frequently have many splice variants and very low expression levels (Huggett *et al.,* 2005). It is therefore an especially useful technique in chicken immunology where not many species-specific antibodies are commercially available (de Boever *et al.,* 2008). Although it is the most relevant method for many experiments, there are still many problems associated with its use, mainly inherent variability of RNA samples and differences in efficiencies of reverse transcription (RT) and PCRs (Bustin, 2002). An efficient

normalisation strategy is needed to collect reproducible and relevant qPCR results corrected for non-biological sample-to-sample variations (Andersen *et al.,* 2004). Several strategies are available to correct experimentally introduced variation, each with their own advantages and considerations (Huggett *et al.,* 2005). Not all the sources of variation can be completely reduced, therefore control along the entire workflow of qPCR analysis is very important. Lack of standardisation of each step and application of normalisation at the final step cannot eliminate errors (Ståhlberg *et al.,* 2003). Similar sample sizes for RNA extraction with DNase treatment and reverse transcription are recommended together with use of standard curves using samples spiked with known quantities of RNA molecules (Gilsbach *et al.,* 2006). These are the methods to indicate the efficiency of reverse transcription and qPCR inhibition. All these aspects were discussed at the 3rd London qPCR Symposium in April 2005 and the agreement was that the reference gene concept is the favoured technique for normalisation of qPCR data. The Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin *et al.,* 2009) describes the necessary information that should be included in evaluation of qPCR experiments and publication of the work. The guidelines emphasise the need to use more than one reference gene and the need to report the method of determination of reference gene stability.

Several commercial companies provide optimised reference gene panels for many species. The idea of using a housekeeping gene as an internal control is attractive as those genes are affected by the same experimental variations as the tested genes but are predicted to show stable constitutive expression. Quantitative PCR results for a gene of interest are then normalised by using the housekeeping reference gene that is measured using the same methods (Vandesompele *et al.,* 2009). To minimise inaccuracies there are several rules that can be followed, for example: uniform sample size, RNA extraction method, reduction of gDNA contamination and internal controls. These guidelines are not mutually exclusive and can be all included in the protocol (Huggett *et al.,* 2005). In theory, mRNA expression levels of any reference gene are stable due to the permanent function

that they play in cellular processes (Zhu *et al.,* 2008). Ideal reference genes show consistent expression in varying experimental and environmental conditions. Expression of genes of interest can then be normalised with internal control genes in samples that vary in qualities and quantities of starting RNA. Differences in enzymatic efficiencies are compensated when reference genes are used as normalisers because they undergo the same preparation steps and are exposed to the same treatments as the gene of interest.

So far an ideal reference gene has not yet been identified (Bär *et al.,* 2009). Many authors suggest that definite or universal internal control gene for every condition in different tissues and cells does not exist (Vandesompele *et al.,* 2002; Coulson *et al.,* 2008; Maltseva *et al.,* 2013). There is an increasing number of studies on widely employed reference genes that prove many of them are not resistant to changes in the experimental environment (Bas *et al.,* 2004; Yin *et al.,* 2011; Sugden *at al.,* 2010; Yang *et al.,* 2013; Schmittgen and Zakrajsek 2000; Lupberger *et al.,* 2002; Dheda *et al.,* 2004; He *at al.,* 2008; Yue *et al.,* 2010). Based on these and many other studies that prove there is no universal internal control for gene expression analyses it has been suggested that determination of appropriate reference genes should be performed for every qPCR experiment (Riemer *et al.,* 2012). Most frequently used reference genes are: ACTB, GAPDH, r18S or r28S. GAPDH is a catalytic enzyme in glycolysis and it participates in DNA repair (Meyer-Siegler *et al.,* 1991). In the early years of qPCR technique, the use of GAPDH as a reference gene was shown to be constant (Edwards and Denhardt 1985; Winer *et al.,* 1999). There have been many reports of GAPDH instability in gene expression induced during the experiment (Lin *et al.,* 2009; Sudgen *et al.,* 2010). An investigation of IL-4 and Toll-like receptors expression in TB patients has shown that normalisation to inappropriate reference gene (GAPDH and ACTB) changed the significance of results (Dheda *et al.,* 2005). Earlier, Suzuki *et al.* (2000), Stűrzenbaum and Kille (2001) established that GAPDH mRNA expression levels could be influenced by experimental conditions, both *in vivo* and *in vitro*. Mozdziak *et al.*

(2003) also established that in chickens, nutritional manipulations can alter transcription of GAPDH and its levels are upregulated with age.

Yin *et al.* (2011) evaluated the stability of expression for 11 genes in mouse and 8 genes in chicken under different experimental conditions. Results have shown that practice of use of popular reference genes without prior stability normalisation could lead to inaccurate and divergent qPCR data. The conventional use of a single reference gene was also demonstrated to introduce large errors (Vandesompele *et al.* 2002). Often the decision which gene should be included as reference in particular study is based on past experiments. The published studies on evaluation of mRNA expression levels in different tissues or cells, under different treatments and from various species use similar reference genes to normalise the data (Banskota *et al.,* 2015; Ji *et al.,* 2014, Xiao *et al.,* 2015). A literature search has also shown that no single gene has been consistently used as reference gene in studies related to chicken immune system (Abasht *et al.,* 2009; Adams *et al.,* 2009, Brisbin *et al.,* 2010).

RNA extraction techniques do not guarantee the total exclusion of genomic DNA (gDNA), which in consequence could give false-positive readings unless reverse transcription is designed to span exon-intron boundaries. Genomic pseudogenes retain introns, thus their size is bigger (Smith *et al.,* 2001). There are also intronless pseudogenes that have arisen by retrotransposition. They resemble target sequences and have 3' polyA tail, therefore, designing primers that span intron-exon boundaries is not applicable as these DNA structures cannot be distinguished from cDNA (Mighell *et al.,* 2000).

Researchers have used many methods to identify reference genes. The most popular strategies are the use of algorithms such as GeNorm (Vandesompele *et al.,* 2002), BestKeeper (Pfaffl *et al.,* 2004) and NormFinder (Andersen *et al.,* 2004). As the statistical methods behind these programs differ in their assumptions, many researchers use more than one program to assess stability of candidate reference genes (Chang *et al.,* 2012; Ledderose *et al.,* 2011; Perez *et al.,* 2008).

Vandesompele *et al.* (2002) suggests using an expression stability index (M value) that would be calculated based on mean pair-wise variations between the gene and the other candidate genes across all samples. The underlying principle for software is that the expression ratio of two reference genes is constant across all samples. geNorm calculates pairwise variation for each gene with all other genes as the standard deviation of the logarithmic transformed expression ratios. Next, the stability value (M) is calculated as the pairwise variation of particular reference gene with all other tested candidate genes. The software performs additional variation analysis of normalisation factors calculated for an increasing number of reference genes to determine the minimal number of reference genes for accurate normalisation (Vandesompele *et al.,* 2009). The NormFinder algorithm, developed by Andersen *et al.* (2004), identifies the most stable gene (pair of genes) with the use of mathematical model supported by a statistical framework. It ranks the candidate genes in a given sample and given experimental design based on their expression stability value. Mathematical, ANOVA-based model uses statistical framework to determine the overall expression variation of the genes but also the variation between subgroups. NormFinder relies on Q values to estimate the stability of each gene. Quantities are first log-transformed and then used in an ANOVA model-based approach to calculate expression variation where intra- and inter-group variations are estimated. The two sources of variation represent systematic error that will occur when the given gene will be used (Andersen *et al.,* 2004). The gene with the lowest M value is the most stable in expression and the gene with the highest M value has the least stable expression. BestKeeper calculates the gene expression variation for the chosen reference genes based on the collected Cq values. The examination consists of two steps: first is the descriptive statistics analysis: geometric mean (GM), arithmetic mean (AM), minimal (Min) and maximal (Max) value, standard deviation (SD) and coefficient of variance (CV). Using CV and SD values, in a second step, pairwise correlation analysis is performed using BestKeeper Index (BKI). The most stable genes have the lowest CV and where the SD value is below 1. Internal controls with SD higher than

1 are regarded as unreliable. All the candidate genes showing stable expression are combined into BKI values for the individual sample using geometric mean of Cq values for each of the reference genes using the Equation 1. BestKeeper also analyses inter-HKG relations by Pearson correlation coefficient (r) and the probability (p) value.

$$BKI = \sqrt[z]{Cq1\ x\ Cq2\ x\ Cq3\ x\ \dots Cqz}$$

Equation 1. BestKeeper Index calculation

The Pearson correlation coefficient (r) and the p-value are then used to describe the correlation between each candidate gene and the index. Comparison of each gene using pair-wise correlation analysis and the BKI gives the optimal reference genes in terms of their stability (Pfaffl *et al.,* 2004).

5.2 Materials and methods

5.2.1 Tissue collection

Samples were collected from broiler chickens reared at an Aviagen high biosecurity pedigree farm and a farm where environment aimed to resemble broader commercial conditions - sibling test farm. The 3-week-old birds (n=10) came from the same hatch. Bursa of Fabricius, spleen, caecal tonsils and part of the gut (ileum) were collected and stored at 4°C in RNA*later* until further use. Blood, in a volume of ~1 ml, was collected into EDTA containing tubes and isolation of white blood cells was performed the same day, as described in Chapter 2, section 2.5.4.

5.2.2 RNA extraction and cDNA synthesis

To perform EvaGreen-based qPCRs for reference gene normalisation, total RNA was isolated from collected tissue and blood samples and reversely transcribed to generate cDNA, which was then used as a qPCR template. Total RNA was isolated using an RNeasy Mini Kit (Qiagen, Crawley, UK), as described in Chapter 2, section 2.6.1 and 2.6.2 with an additional DNase step. The quality and quantity of extracted total RNA was evaluated by spectrophotometry using a NanoDrop™ 1000 instrument. First-strand cDNA synthesis was performed using a SuperScript III reverse transcription kit (Paisley, UK) containing random primers (Sigma-Aldrich, Poole, UK), as described in Chapter 2, section 2.7.2. The same amount of total RNA (1 µg) was used to generate cDNA from the tissues and blood collected. The cDNA was stored in -20°C until further use.

5.2.3 Gene selection and quantitative PCR

Seven genes commonly used as reference genes in quantitative PCR (qPCR) gene expression experiments were selected and are briefly described in Table 5.1. All qPCR primers were designed using Primer Express Software 3.0 (Life Technologies) and synthesised by Sigma Aldrich (Poole, UK). Primer sequences and amplicons length are shown in Appendix 1, Table 6. Quantitative PCR, using 7500 Fast Real-Time PCR System was performed as described in Chapter 2, section 2.7.6.

For each primer pair internal standard curves (serial dilutions of pooled cDNA samples for all tissues) were used to assign relative concentrations to the samples.

Table 5.1. Reference genes selected for evaluation and the function of the encoded products.

Gene symbol	Gene name	Function
ACTB	β-actin	Essential component of the cytoskeleton, critical roles in a wide range of cellular processes, including cell migration and cell division
B2M	β-2-microglobulin	Associated with the heavy chain of MHC class I
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	Catalytic role in the oxidative phosphorylation of glyceraldehyde 3-phosphate to 1,3-bisphospoglycerate during glycolysis and in gluconeogenesis
GUSB	β-glucuronidase	Catalytic role in breakdown of complex carbohydrates
TBP	TATA box binding protein	Subunit of the transcription factor TFIID, first protein to bind to DNA during the formation of the pre-initiation transcription complex of RNA polymerase II (RNA Pol II)
TUBAT	α-tubulin	Part of the microtubule forming system, binds to GTP
r28S	r28S ribosomal RNA	Structural RNA for the large component of eukaryotic cytoplasmic ribosomes

5.2.4 **NormFinder, geNorm and BestKeeper analyses**

To select suitable internal controls, the stability of each gene was statistically analysed with three software tools: GeNorm (Vandesompele *et al.*, 2002; section 2.1.?); NormFinder (Andersen *et al.*, 2004; section 2.1.8) and BestKeeper (Pfaffl *et al.*, 2004; section 2.1.7). All three packages were used according to the manufacturer's instructions.

5.3 Results

5.3.1 Validation of reference gene primers

Primer pairs for seven reference genes were designed against the template using Primer Express software 3.0 and following six strict conditions described in Chapter 2, section 2.7.5. Primers pairs span exon-intron boundaries for the ACTB, B2M, GAPDH, GUSB and TBP gene. The r28S ribosomal RNA does not carry coding sequence and the TUBAT gene primer pair does not cross exon-intron boundaries, as attempts to optimise previously designed oligonucleotides were unsuccessful. Primer pair specificity was tested in qPCR by examining the melting curve performed at the end of the reaction (Figure 5.1a). The single peak with no shouldering suggested the specificity of primer annealing. Primer pairs that produced more than one peak and/or shoulder were tested again and, when needed, redesigned. Amplicons from qPCR reactions were confirmed by agarose gel electrophoresis, where a single band suggested a single amplicon. Results of melting curve analysis are shown in Figure 5.1b.

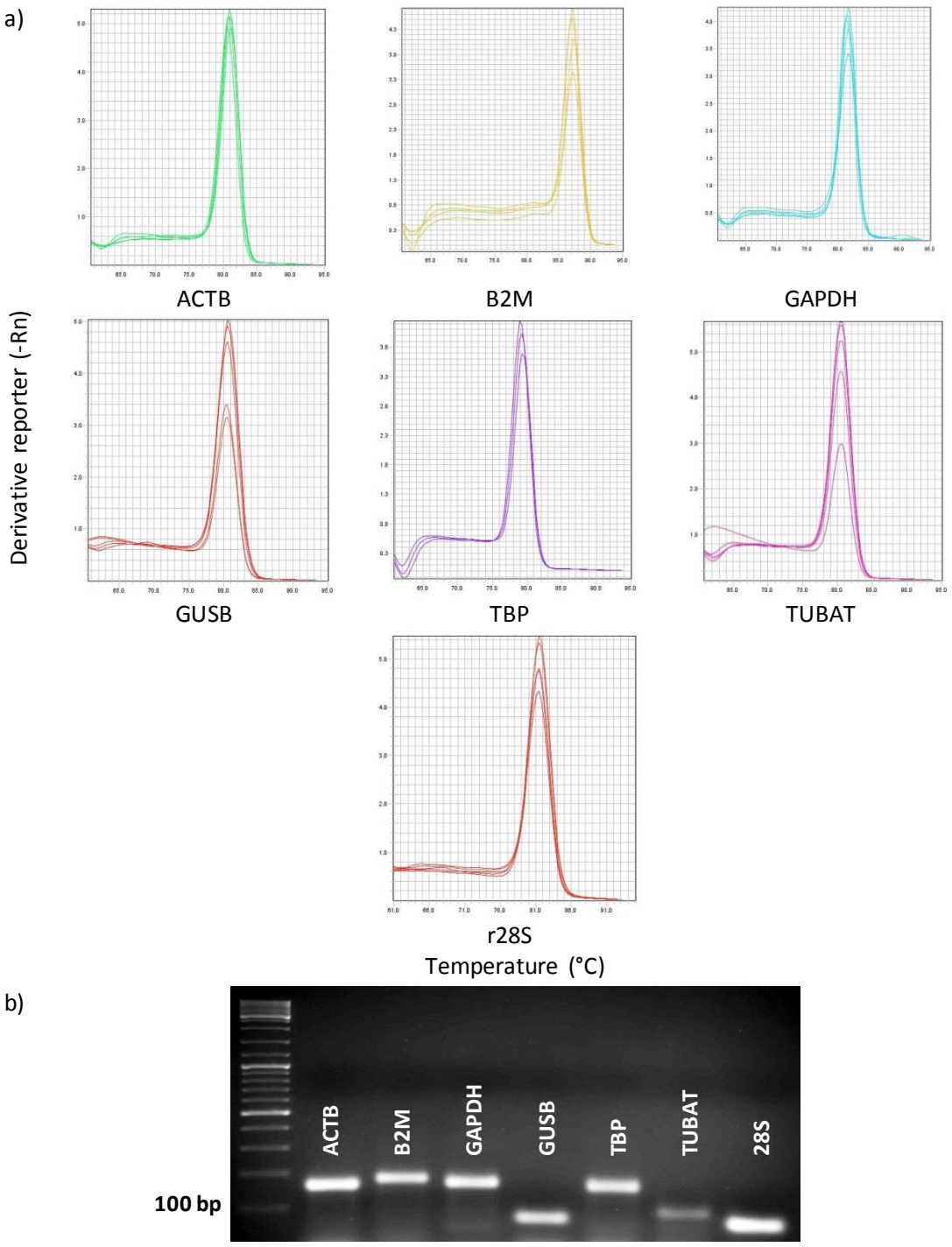


Figure 5.1**.** Specificity of qPCR reaction with reference gene primers ; a) melting curve analysis to define the specificity of reference gene qPCR; b) agarose gel electrophoresis of the corresponding qPCR amplicons.

5.3.2 Expression profiles of reference genes

Expression profiles of each reference gene were examined by calculating quantification cycle (Cq) value, which represents the cycle number at which the fluorescence generated in the reaction crosses the fluorescence threshold above the background signal. It usually is the middle of exponential phase of reaction. The expression levels of the seven reference genes tested varied widely between the genes studied with Cq values ranging from 9 to 38 cycles (Figure 5.2) and most of the Cq values were between 18 and 31 cycles. The r28S gene was most abundantly transcribed with Cq values of less than 17 cycles and ACTB, B2M and GAPDH were moderately expressed with average Cq values between 18 and 23 cycles. GUSB, TBP and TUBAT showed average Cq values between 27 and 31 cycles. The lowest expression level was observed in TUBAT with Cq value as high as 38 cycles. The inspection of raw Cq values alone is not sufficient for determining gene expression stability. The data obtained from qPCR were further analysed using NormFinder, geNorm and BestKeeper. Only the last algorithm allows for input of raw Cq data from qPCR. For the others, data was transformed into relative quantities. The transformation process is described in paragraph 5.3.3.

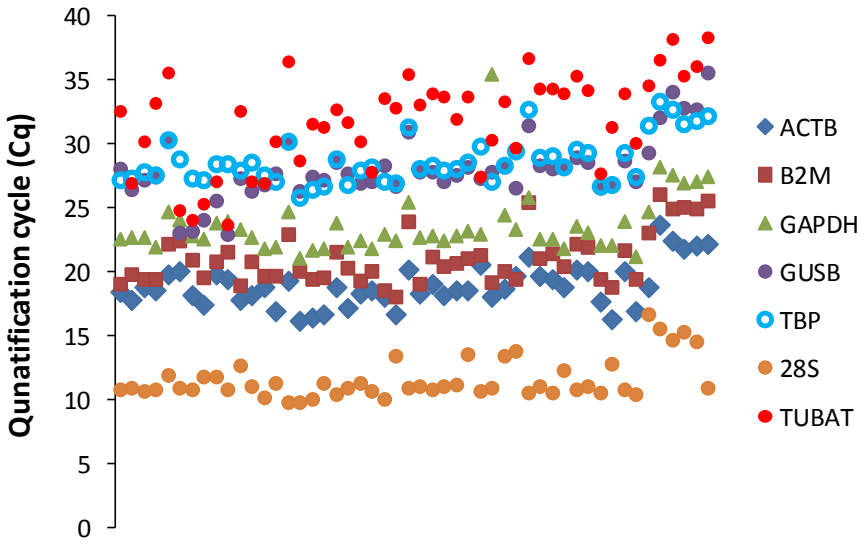


Figure 5.2. Quantification cycles (Cq) values reflecting expression levels of seven reference genes in all tissues tested. Data points represent single sample (n=50, 10 birds, 5 tissues) Cq value for particular reference gene.

5.3.3 Transformation of Cq values to quantities (Q)

Quantification cycle values were generated in qPCRs for each gene tested and in each tissue sample derived from birds reared on the pedigree and sibling test farms. In order to optimise qPCRs for each reference gene, 10-fold serial dilutions of pooled cDNA samples (n=50) were tested on the same plate to avoid run-to-run differences and results were used to generate standard curves. To calculate the efficiency of reaction, the slope value was calculated from serial dilutions for each gene, which was then used to determine the efficiency of reaction (Equation 2). Complete calculations for all reference genes are presented in Appendix 1, Table 7 for the sibling-test farm and Table 8 for the pedigree farm. In order to obtain accurate and reproducible results, reactions should have efficiency close to 2 (100%), which means that the template doubles with each cycle during exponential amplification. A slope of - 3.32 indicates optimal PCR efficiency.

$$\boldsymbol{E = 10^{(-1/slope)}}$$

alternative $$[\%]\ \boldsymbol{E = (10^{(-1/slope)} - 1)\ x\ 100}$$

Equation 2. Calculation of efficiency (E) of reaction

Mean quantification cycle (Cq) values were transformed into quantities (Q) by the deltaCq (ΔCq) method where the Cq value of a particular sample was related to the control/calibrator. In this experiment, the control/calibrator was the sample with the highest expression (lowest Cq value). Relative quantities were generated by the ΔCq formula for transforming Cq values to relative quantities with the highest expression level set to one (Equation 3).

$$\boldsymbol{Q = E^{-\Delta Cq}}$$

Equation 3. Relative quantities formula

The ΔCq method generated raw, non-normalised expression values, which required normalisation if used for gene expression studies, by dividing with the normalisation

factor. The Q values were used as input data in NormFinder and geNorm programmes.

5.3.4 NormFinder analysis

The analysis with NormFinder software identified ACTB and TBP as the best combination of genes for all tissues with a stability value across all tissues of 0.076, where TBP (0.110) was ranked the most stable gene. The TUBAT gene (0.596) was ranked the most unstable gene in tissue panel tested. The stability of the genes varied depending on the tissue tested. Analysis was also performed for each tissue type separately. TBP was ranked the most stable gene in bursa (0.188), spleen (0.052) and caecal tonsils (0.157). In the Peyer's patches region of the ileum, GUSB (0.220) was ranked the most stable. In blood samples, GAPDH (0.137) was selected as the best gene. In spleen and blood TBP was paired with GUSB (0.037 and 0.147), in bursa samples with GUSB (0.171), in caecal tonsils with r28S (0.125) and in ileum with ACTB (0.194) as the best pair of genes. Results showing the most stable genes and their stability values as predicted by NormFinder are shown in Table 5.2.

Table 5.2. NormFinder (NF) analysis of the most stable reference genes and their stability values for all sample tested.

Tissue	Best gene	NF stability value	Combination of two genes	NF stability value
Bursa	TBP	0.188	GUSB, TBP	0.171
Spleen	TBP	0.052	GAPDH, TBP	0.037
Caecal tonsils	TBP	0.157	r28S, TBP	0.125
Ileum	GUSB	0.22	ACTB, TBP	0.194
Blood	GAPDH	0.137	B2M, GAPDH	0.147
All tissues	TBP	0.11	ACTB, TBP	0.076

Legend: ACTB - β-actin; b2m - β-2-microglobulin; GAPDH - glyceraldehyde-3-phosphate dehydrogenase; GUSB - β-glucuronidase; TBP - TATA box binding protein; TUBAT - α-tubulin; r28S - r28S ribosomal RNA.

Overall, the TBP gene was selected as the most stable gene in most of the tissues either as the best gene or paired with a second gene as the best reference gene combination in given tissue panel. When samples from two different farms

were examined separately, TBP was selected as the best gene for tissues from birds raised on the pedigree farm and the best combination of two genes was TBP and GAPDH (0.154). For the sibling test farm GAPDH was paired with GUSB (0.096) and GUSB was ranked the most stable.

5.3.5 geNorm analysis

Reference gene normalisation was also performed using geNorm, a visual basic application (VBA) for Excel. The analysis of all samples from both farms using geNorm identified TBP and B2M (M = 0.73) as the most stable pair of genes (Table 5.3). Similar to analysis with NormFinder, ranking of genes was performed on separate tissues. For both bursa and spleen tissues GAPDH and TBP were selected the best pair of genes with stability M values of 0.4 and 0.34, respectively. Ileum and blood tissues shared B2M as one of the most stable genes that was paired with TBP (M = 0.79) and GAPDH (M = 0.4), respectively. Caecal tonsils analysis resulted in ACTB and r28S (M = 0.42) being the best pair of reference genes. Results are shown in Table 5.3.

Table 5.3. geNorm analysis showing the pairs of most stable reference genes for each tissue sample tested and their stability expression M value.

Tissue	Combination of two genes	geNorm M value
Bursa	GAPDH, TBP	0.4
Spleen	GAPDH, TBP	0.34
Caecal tonsils	ACTB, r28S	0.42
Ileum	B2M, TBP	0.79
Blood	B2M, GAPDH	0.4
All tissues	B2M, TBP	0.73

Legend: ACTB - β-actin; b2m - β-2-microglobulin; GAPDH - glyceraldehyde-3-phosphate dehydrogenase; GUSB - β-glucuronidase; TBP - TATA box binding protein; TUBAT - α-tubulin; r28S - r28S ribosomal RNA.

The geNorm algorithm proposes the optimal number of reference genes in studied samples. The levels of variation in average reference gene stability are

calculated based on the stepwise inclusion of a subsequent gene or genes. The geNorm software calculates normalisation factor (NF) from at least two genes. The pairwise variation (V) between two sequential NFs is determined. The additional NF for reference gene should decrease the variation, preferably below the cut-off point of 0.15 in order to include it in the panel of final reference genes for given samples. In this study, addition of a fourth gene caused the decrease in variation in stability in all types of tissues. For bursa and spleen tissues, ACTB and B2M were included, which decreased variation in stability to 0.13 and 0.14 respectively. For caecal tonsils, TBP and GAPDH addition had similar effect (V = 0.14). In case of ileum ACTB and r28S, decreased variation in stability from 0.25 to 0.22 and in blood ACTB and TBP reduced the variation to 0.17. When all tissues were analysed together, addition of ACTB and GAPDH to the calculation caused the decrease in variation to an almost ideal point of 0.16 (Figure 5.3).

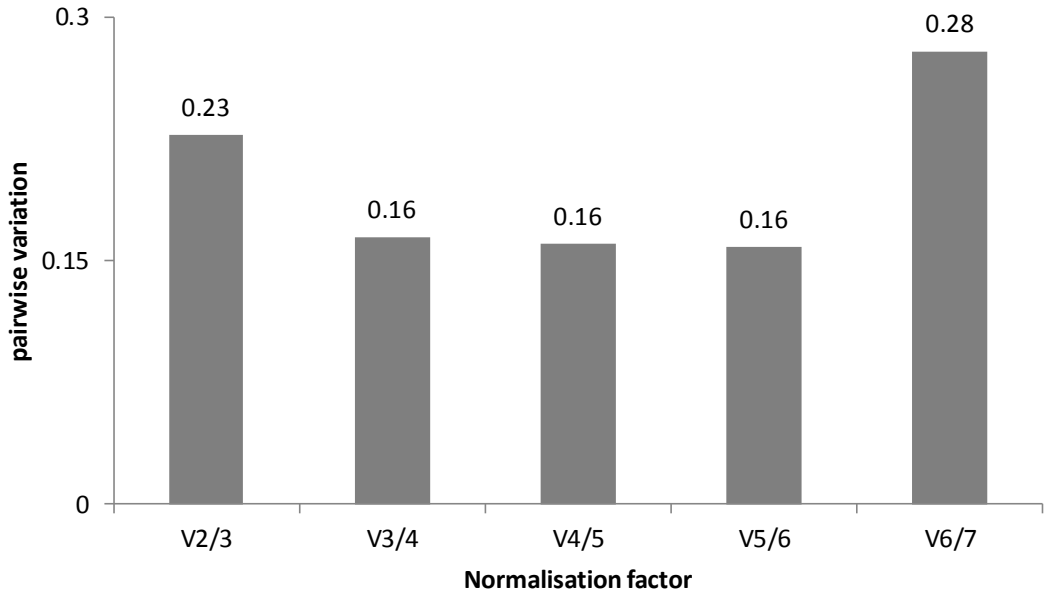


Figure 5.3. The pairwise variation ($V_n/V_n + 1$) between the normalisation factors NF_n and NF_{n+1} to determine the optimal number of reference genes for normalisation.

5.3.6 **BestKeeper analysis**

In this study, Cq values of all tissues for each reference gene tested were combined. The first step of analysis showed that none of the genes passed the ranking based on standard deviation (SD) and coefficient of variance (CV) levels. According to BestKeeper developers, the standard deviation for any given gene should be below value of 1. Genes with this parameter higher than 1 can be considered inconsistent. Standard deviations for all of the analysed genes were higher than 1, with TUBAT SD of 3.03. The lowest SD were calculated for ACTB (1.27) and r28S (1.22). Based on the results from descriptive statistics for all tissues combined, ranking of reference genes using BestKeeper was impossible.

The Cq values for tissues were tested separately. Results are presented in Table 5.4. Standard deviation of Cq values from bursa (Table 5.4a) were below value of 1 for ACTB, GAPDH, TBP and r28S. The lowest CV was calculated for TBP. Based on those rankings B2M, GUSB and TUBAT were excluded from the analysis. BestKeeper Index calculated based on the remaining four reference genes was then correlated with each gene separately. The highest correlation was for ACTB, followed by GAPDH, TBP and r28S.

The same approach was used to analyse Cq data from caecal tonsils (Table 5.4b). Standard deviation for B2M and TUBAT were above 1 and genes were excluded from the analysis. This resulted in high correlations, with p values lower than 0.001, for all remaining genes: r28S, TBP, GUSB, GAPDH and ACTB.

In BestKeeper analysis of ileum tissues (Table 5.4c) three reference genes (B2M, TUBAT and r28S), were excluded from further calculations based on their high SD. Further analysis with four remaining genes showed the highest correlation for TBP, GUSB and ACTB, with GAPDH having the lowest correlation among tested genes.

Based on descriptive statistics calculations performed on Cq values from spleen, TBP and TUBAT did not pass the ranking (Table 5.4d). After exclusion of TBP and TUBAT from the analysis, calculations of correlation showed that ACTB was the most stable gene, followed by GAPDH, B2M, r28S and GUSB.

Table 5.4. BestKeeper analysis results. SD, CV values and pair-wise correlation with BestKeeper Index describing stability for tested reference genes. Genes with SD > 1 (red) were excluded from the pair-wise correlation analysis. The most stable genes for each tissue are highlighted in green.

a) Bursa	ACTB	B2M	GAPDH	GUSB	TBP	TUBAT	28S
Standard deviation [± Cp]	**0.79**	**1.05**	**0.74**	**2.05**	**0.79**	**3.65**	**0.43**
Coefficient of variance[% Cp]	**4.25**	**5.16**	**3.22**	**7.95**	**2.82**	**12.90**	**3.88**
Pair-wise correlation with BestKeeper Index (n = 4)							
BestKeeper vs.	**ACTB**	B2M	GAPDH	GUSB	TBP	TUBAT	28S
Coefficient of correlation [r]	**0.959**	-	**0.929**	-	**0.856**	-	**0.802**
p-value	**0.001**	-	**0.001**	-	**0.002**	-	**0.005**
b) Caecal tonsils	ACTB	**B2M**	GAPDH	GUSB	TBP	**TUBAT**	28S
Standard deviation [± Cp]	**0.51**	**1.22**	**0.58**	**0.79**	**0.71**	**1.59**	**0.49**
Coefficient of variance[% Cp]	**2.78**	**6.07**	**2.53**	**2.85**	**2.51**	**4.88**	**4.45**
Pair-wise correlation with BestKeeper Index (n = 5)							
BestKeeper vs.	ACTB	B2M	GAPDH	GUSB	TBP	TUBAT	**28S**
Coefficient of correlation [r]	**0.906**	-	**0.916**	**0.939**	**0.972**	-	**0.984**
p-value	**0.001**	-	**0.001**	**0.001**	**0.001**	-	**0.001**
c) Ileum	ACTB	**B2M**	GAPDH	GUSB	TBP	**TUBAT**	28S
Standard deviation [± Cp]	**0.69**	**1.23**	**0.85**	**0.76**	**0.93**	**2.29**	**1.27**
Coefficient of variance[% Cp]	**3.54**	**5.82**	**3.63**	**2.69**	**3.18**	**6.96**	**10.99**
Pair-wise correlation with BestKeeper Index (n = 4)							
BestKeeper vs.	ACTB	B2M	GAPDH	GUSB	**TBP**	TUBAT	28S
Coefficient of correlation [r]	**0.825**	-	**0.719**	**0.836**	**0.940**	-	-
p-value	**0.003**	-	**0.019**	**0.003**	**0.001**	-	-
d) Spleen	ACTB	B2M	GAPDH	GUSB	**TBP**	**TUBAT**	28S
Standard deviation [± Cp]	**0.97**	**0.88**	**0.93**	**0.82**	**1.02**	**2.15**	**0.67**
Coefficient of variance[% Cp]	**5.50**	**4.35**	**4.14**	**2.97**	**3.72**	**6.95**	**6.32**
Pair-wise correlation with BestKeeper Index (n = 5)							
BestKeeper vs.	**ACTB**	B2M	GAPDH	GUSB	TBP	TUBAT	28S
Coefficient of correlation [r]	**0.920**	**0.846**	**0.917**	**0.680**	-	-	**0.756**
p-value	**0.001**	**0.002**	**0.001**	**0.031**	-	-	**0.011**

Legend: ACTB - β-actin; B2M - β-2-microglobulin; GAPDH - glyceraldehyde-3-phosphate dehydrogenase; GUSB - β-glucuronidase; TBP - TATA box binding protein; TUBAT - α-tubulin; r28S - r28S ribosomal RNA.

Blood samples Cq values were also tested in BestKeeper. Results were similar to the test performed on all tissues. All reference gene standard deviation values were higher than one, which excluded them from analysis.

5.4 Discussion

The present study simultaneously investigated a panel of seven commonly used reference genes in order to establish their expression stability in varied tissue samples and birds. NormFinder and geNorm results differed slightly. BestKeeper ranking only agreed with NormFinder when caecal tonsil samples were analysed. The results for all tissues combined in one analysis were available only from NormFinder and geNorm. Three out of four most stable genes were common for both analyses: TBP, GAPDH and ACTB. In separate tissues, TBP was the most common gene selected by NormFinder and geNorm. There are no published studies that use TBP as a reference gene in chicken mRNA expression experiments. Recent investigation of five candidate genes in tissues related to growth and fat deposition showed that TBP was the most stably expressed in chicken thigh muscle and second most stable in liver but its expression varied in breast muscle and fat tissue (Bages *et al.,* 2015). TBP was stably expressed together with B2M in bovine granulosa cells subjected to different plating densities, oxygen concentrations and follicle stimulating hormone (FSH) stimulation (Baddela *et al.,* 2014). The geNorm software selected B2M and ACTB as the most optimal reference genes in qPCR studies on human mesenchymal stem cells where other programs did not agree and ranked ACTB, GAPDH and TBP as the most unstable for that type of cells (Li *et al.,* 2015). The variation of expression of a particular candidate gene may be due to its role in the tested tissue which has been shown in case of GAPDH where 72 human cells and tissues where used. The results exposed up to 14-fold difference of expression between some of the samples used (Barber *et al.,* 2005). In this study, GAPDH and ACTB surprisingly were ranked as the second or third stable reference genes in some chicken lymphoid tissues. A different study on human bone marrow-derived mesenchymal stromal cells showed that TBP is the most stably expressed together with TFRC and HPRT1 (Rauh *et al.,* 2015).

There are few published studies on results of reference gene normalisation in chicken tissues or cells but all of the existing results differ in ranking of the genes. Most of the studies focused on chicken embryo fibroblasts (CEFs) as a virus

infection model. Yin *et al.* (2011) indicated ACTB as the most stably expressed gene in CEFs infected with Newcastle disease virus (NDV) and GAPDH with 18S as the least stable genes based on their transcriptional profiles only. Yue *et al.* (2010) used CEFs infected with avian influenza virus (AIV) and showed in cell response studies that YWHAZ is the most stable gene, where in virus replication studies ACTB and RPL4 were most reliable controls according to geNorm calculations. The same software was used to determine the best reference gene in CEFs infected with subgroup J avian leukosis virus (ALV-J). The geNorm ranked RPL30 and SDHA as the best candidates and ACTB and GAPDH as the least stable genes (Yang *et al.,* 2013). Studies by the de Boever *et al.* (2008) on LPS inflammation in chickens resulted in GAPDH and UBC being the best pair of internal controls. In duck and chicken primary lung cells, infected with low pathogenic AIV and high pathogenic AIV, GAPDH was ranked as the second best reference gene after 18S (Kuchipudi *et al.*, 2012). The use of ribosomal RNA as normaliser has been controversial based on its technical limitations and led to exclusion from the analysis (Lu *et al.,* 2013). Nevertheless, ribosomal RNA, including r28S and r18S has been shown to be a stably expressed reference (Wang *et al.,* 2011; Roge *et al.,* 2007). Li *et al.* (2007) reported that r28S was among few genes with stable expression in chicken embryo cells (CEs) infected with infectious bursal disease virus (IBDV) but in the same experiment, B2M and TBP were the least stable according to gene fold changes. In *in vitro* stimulation of human blood cells, TBP was shown to be good reference gene in studies on T lymphocytes, neutrophils and total blood leukocytes (Ledderose *et al.,* 2011). In the current study r28S ranked as the most stable gene in caecal tissues together with TBP according to NormFinder and geNorm. After including additional genes to the best combination in geNorm analysis, r28S was the fourth most stable reference gene in ileum tissue. Although all tissues were normalised with genes selected by analysing values from all samples together it would be advantageous to select different panel for each tissue in separate calculations.

This study is the first report of reference gene normalisation in chicken lymphoid tissues. These results demonstrate the need to carefully select reference genes for immune gene expression studies as the frequently used genes and ribosomal RNA are not always appropriate internal controls. Although this experiment showed that TBP, GAPDH and ACTB are the suitable gene expression normalisers it is strongly recommended to test internal controls as a first experiment in gene expression studies. The selected reference genes will be used in the high-throughput qPCR experiment described in Chapter 6.

Chapter 6 Optimisation and validation of 96.96 Dynamic Array as a diagnostic tool

6.1 Introduction

The main objectives for this chapter were to develop and validate a custom Fluidigm qPCR array for analysis of the transcription of immune-related genes as a tool for rapid screening of immune robustness in chickens. Working with our partners in Aviagen, the aim was to compare gene expression in related birds in high biosecurity clean housing ('pedigree farm') and an environment with lower biosecurity akin to commercial broiler farms ('sibling test farm'), as well as to define the extent of variance between animals in a given environment. A further goal for this study was to compare responses in peripheral blood compared to internal organs as a predictor of the robustness of immune responses in the chicken, as non-lethal methods would allow selective breeding from the live birds screened.

Immune responses to encountered microorganisms are coordinated on cellular and molecular levels. The most exhaustive analysis of tissues and cells can be achieved by studying RNA expression. For many years researchers focused their efforts on nucleic-acid based tools to detect transcripts, with microarrays being the most widely used tool (Germain *et al.,* 2011). Although the levels of mRNA inside the cells do not always correspond with the amount of proteins that will be produced, exploring immune responses at transcript levels is more accessible. Large-scale gene expression analyses are widely used in biological and medical studies (Zhong *et al*., 2008; Nie *et al*., 2010; Zheng-Bradley *et al*., 2010). Depending on the aim of the study, different tools can be implemented, such as RNA sequencing, microarrays or qPCR-based platforms, to analyse changes in the transcriptome (Svec *et al*., 2013). Screening hundreds of targets and samples in parallel qPCR for gene expression is possible with high throughput qPCR tools, for example the 96.96 Dynamic Array from Fluidigm (Spurgeon *et al.,* 2008). This type of gene expression platform supports the effort of broad, unbiased explorations of biological systems in comparison to studying a single component in much detail with previously set hypotheses (Benoist *et al.,* 2006).

The Fluidigm chip has been used to study mRNA and miRNA gene expression in different species, for example in pig lung tissues infected with influenza virus H1N2 (Skovgaard *et al.,* 2013) or bovine mammary epithelial cells from mastitis infections (Sorg *et al.,* 2013). There are also Fluidigm platforms available for single-cell gene expression analysis (Pieprzyk and High, 2009). The Fluidigm 96.96 chip produces data for 96 transcripts in each of 96 total RNA samples (9,216 reactions in total). Optimisation of primers, reverse transcription and preamplification, prior the use of chip is essential in order to obtain reliable data. The integration of results from such a high-throughput qPCR platform with confirmed biological networks is a useful approach for gene expression studies.

In this study, transcription of immune-related genes defined in Chapter 4 was compared in a genotypically similar commercial line of chickens housed separately at two different levels of biosecurity. The organs that were sampled included: gut-associated lymphoid tissues (caecal tonsils, ileum part with Peyer's Patches), bursa, spleen and peripheral blood leukocytes. All of these tissues are well known to play a role in immune responses. Gene expression responses to viral infections have been studied in the bursa (Smith *et al.,* 2015; Rasoli *et al.,* 2015; Smith *et al.,* 2014). Gene expression in caecal tonsils has been studied in response to bacterial infections, mainly with *Salmonella* and *Campylobacter* (Akbari *et al.,* 2008; Shaughnessy *et al.,* 2009) but also to viral (Heidari *et al.,* 2015) and parasitic infections (Wils-Plotz *et al.,* 2013). The spleen is very important in mounting both innate and adaptive immune responses and is crucial for immune regulation, therefore its responses to different stimuli, including parasites and viruses, have been widely studied (Pleidrup *et al.,* 2014; Lian *et al.,* 2012; Hong *et al.,* 2012). The most frequently studied chicken blood cells are lymphocytes but heterophils and peripheral blood leukocytes as a whole population were also examined during infections (Chiang *et al.,* 2008; Sandford *et al.,* 2012).

The high-biosecurity 'pedigree' farm sampled herein resembles a specific-pathogen free setting and the 'sibling-test' farm mimics the commercial farm

environment. The populations received different vaccines (in detail 6.2.1 section). It cannot be said with precision what types of pathogens and microflora were acting on the immune system of birds in the different environments but there were certainly pathogens present on sibling-test farm, including *E. coli* and *Eimeria* species. From a breeder point of view, identifying transcriptional signatures associated with immune robustness in a basal state, or improved responses to exposure to pathogen challenge, would only be valid if birds with desirable characteristics could be further used in breeding programme, hence a blood test would be the ideal tool.

6.2 Materials and methods

6.2.1 Tissue collection and sample preparation

Tissues and blood samples were obtained from eight Aviagen broilers from the same hatch. Four birds were raised in a high-biosecurity environment (the pedigree farm), and four birds were raised in an experimental, pathogen-challenged, sibling-test farm. The pedigree farm birds were vaccinated at hatchery against MDV and IBV then against coccidiosis at day 5, against avian rhinotracheitis (TRT) at day 11 and against IBDV at day 15. Birds housed at the sibling-test farm were vaccinated against TRT, NDV and IBV at hatch and only received IBDV vaccine at day 19. The samples were collected at three weeks of age. Fragments (0.5 x 0.5 cm) of four tissues (bursa, spleen, caecal tonsils and ileum with Peyer's patches) were collected aseptically from the same locations in each bird and stored in RNA*later* until further use. Peripheral blood (1 ml) was collected into tubes containing EDTA. Peripheral blood leukocytes (PBLs) were isolated on the same day, as described in Chapter 2, section 2.5.4. RNA extractions for PBLs and tissues were performed as described in Chapter 2 section 2.6.1 and 2.6.2 with an additional DNase digestion step in the cell extraction protocol.

6.2.2 cDNA synthesis

Reverse transcription was performed using SuperScript III (Invitrogen) as described in Chapter 2, section 2.7.2. In the first strand reaction, one µg of total RNA was used. Synthesised cDNA was diluted 1:5 in nuclease-free water prior to preamplification. Reverse transcription using a High Capacity Reverse Transcription Kit (Applied Biosystems, UK) was performed as described in Chapter 2, section 2.7.3.

6.2.3 Primer design and optimisation

The qPCR primer design is described in Chapter 2, section 2.7.5. Final primer sequences are presented in Appendix 1, Table 5. The primer pairs were tested in qPCR reactions with serial dilutions of pooled cDNA obtained from RNA isolated from tissues and cells from various infection studies. A melting curve step was performed to evaluate the specificity of primer pairs. The selected products were

purified using Nucleo-Spin Gel and a PCR Clean-up kit (Macherey-Nagel, UK) before sequencing. The primer pairs were screened for potential cross-reactivity using the AutoDimer algorithm.

6.2.4 The BioMark System qPCR

The BioMark System with 96.96 Dynamic Array platform allows to simultaneously perform 9,216 reactions, as described in Chapter 1, section 1.5.4 and Chapter 2, section 2.10. A stock of primer mix was prepared by mixing one µl of each primer pair, excluding r28S and ENSGALG00000015395 primers, to the final concentration of 200 nM. Preamplification was performed as described in Chapter 2; section 2.10.4. Unincorporated primers were digested with exonuclease I enzyme, as described in Chapter 2, section 2.10.5. To optimise the preamplification, different volumes of PreAmp Master Mix and different dilutions of template cDNA were used to test optimal concentrations. The qPCR was performed using the Applied Biosystems 7500 Fast Real-Time PCR System. Thermal cycling conditions are described in Chapter 2, section 2.7.6. Sample and assay mixes were prepared as described in Chapter 2, section 2.10.6 and stored in 4°C until further use. The priming and loading of 96.96 chip was performed as described in Chapter 2, section 2.10.7 and 2.10.8. The BioMark HD system Data Collection Software was used to perform the qPCR as described in Chapter 2, section 2.10.9.

6.2.5 Data analysis settings and visualisation

To analyse the data from 96.96 Dynamic Array Real-Time PCR Analysis Software was used as described in Chapter 2, section 2.10.9. The raw Cq values in a .csv file were used as an input data in GenEx5 software. Pre-processing, statistical analysis and visualisation was performed as described in Chapter 2, section 2.10.10 and 2.10.11.

6.3 Results

6.3.1 Primer optimisation

The designed primer pairs were tested in qPCR using serial dilutions of pooled cDNA samples isolated from tissues from various infection studies as a template. The same cDNA pool was used throughout. The melting curve of each primer pair was examined to evaluate their specificity. Examples of melting curves and their interpretation are shown in Figure 6.1. Each primer pair in this study was set to work efficiently at the concentration of 1.15 µM in the final reaction mix to avoid mistakes in the qPCR workflow.

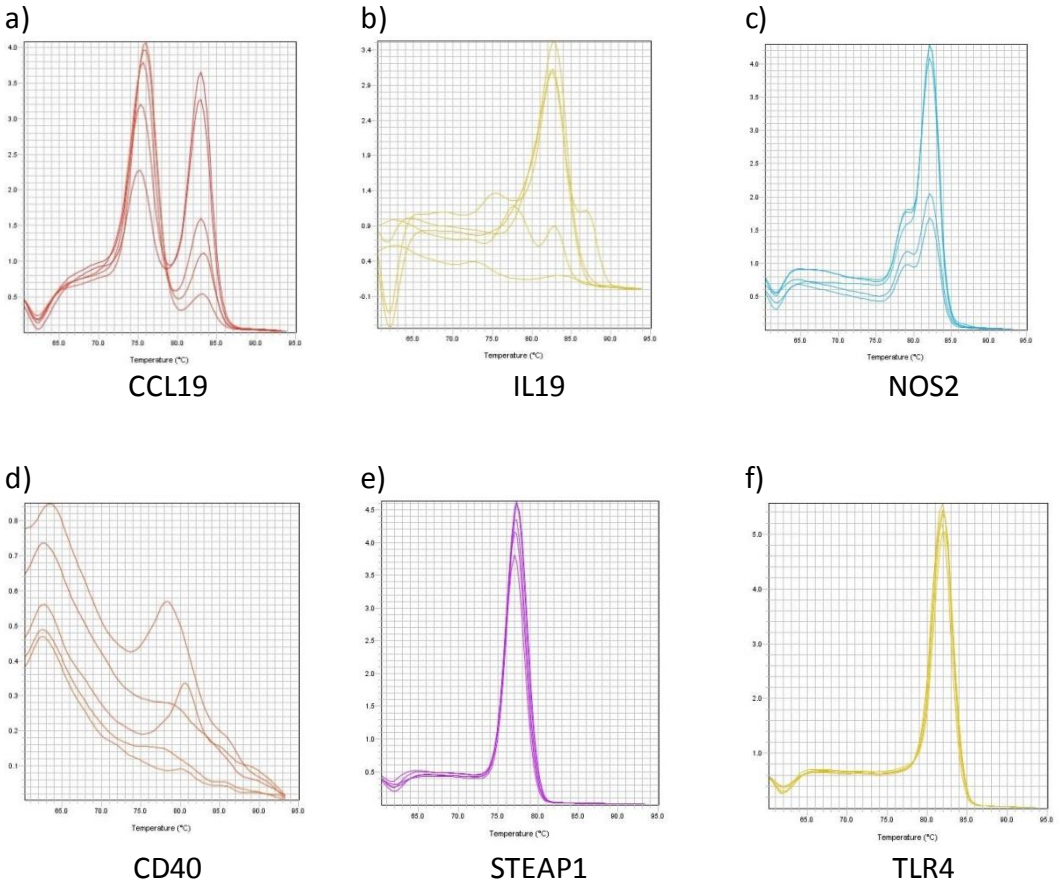


Figure 6.1. Examples of optimisation of primer pairs based on melting curve analysis. The melting curves for each primer pair were investigated; a) two peaks reflects two amplicons; b) one distinctive peak corresponds to a single amplicon and additional peaks to genomic DNA products; c) one peak with a shoulder corresponds to genomic DNA amplification; d) no peak corresponds to no amplification; panels e) and f) show further examples of the desired melting curve.

The use of DNA binding dye – EvaGreen, as an amplification detector, instead of internal oligonucleotide probes, allowed to design and test several primer pairs around the transcript of interest. The design of primer pairs was considered successful once the melting curve analysis showed a single sharp peak with no additional peaks or shoulders and when single band was visualised by agarose gel electrophoresis. The qPCR products with many peaks in the melting curves presented in Figure 6.1 produced multiple species on agarose gels, as shown in Figure 6.2.

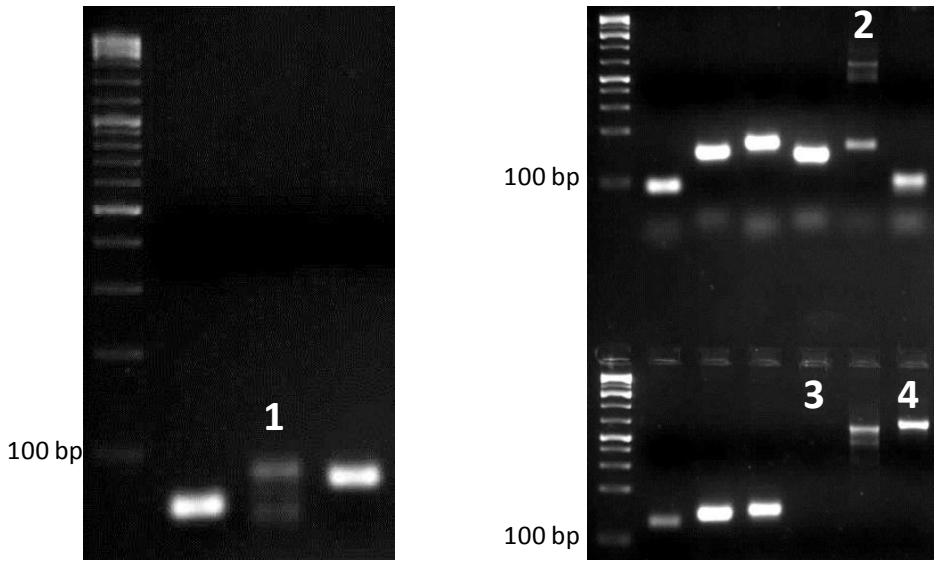


Figure 6.2. Examples of primer specificity confirmation by agarose gel electrophoresis. Selected qPCR products were tested; 1 – CCL19; 2 – IL19; 3 – CD40; 4 – NOS2.

Amplicons from a subset of 10 qPCR reactions that produced only a single species were excised from the agarose gel, cleaned-up and sequenced. Results for selected alignments are shown in Figure 6.3.

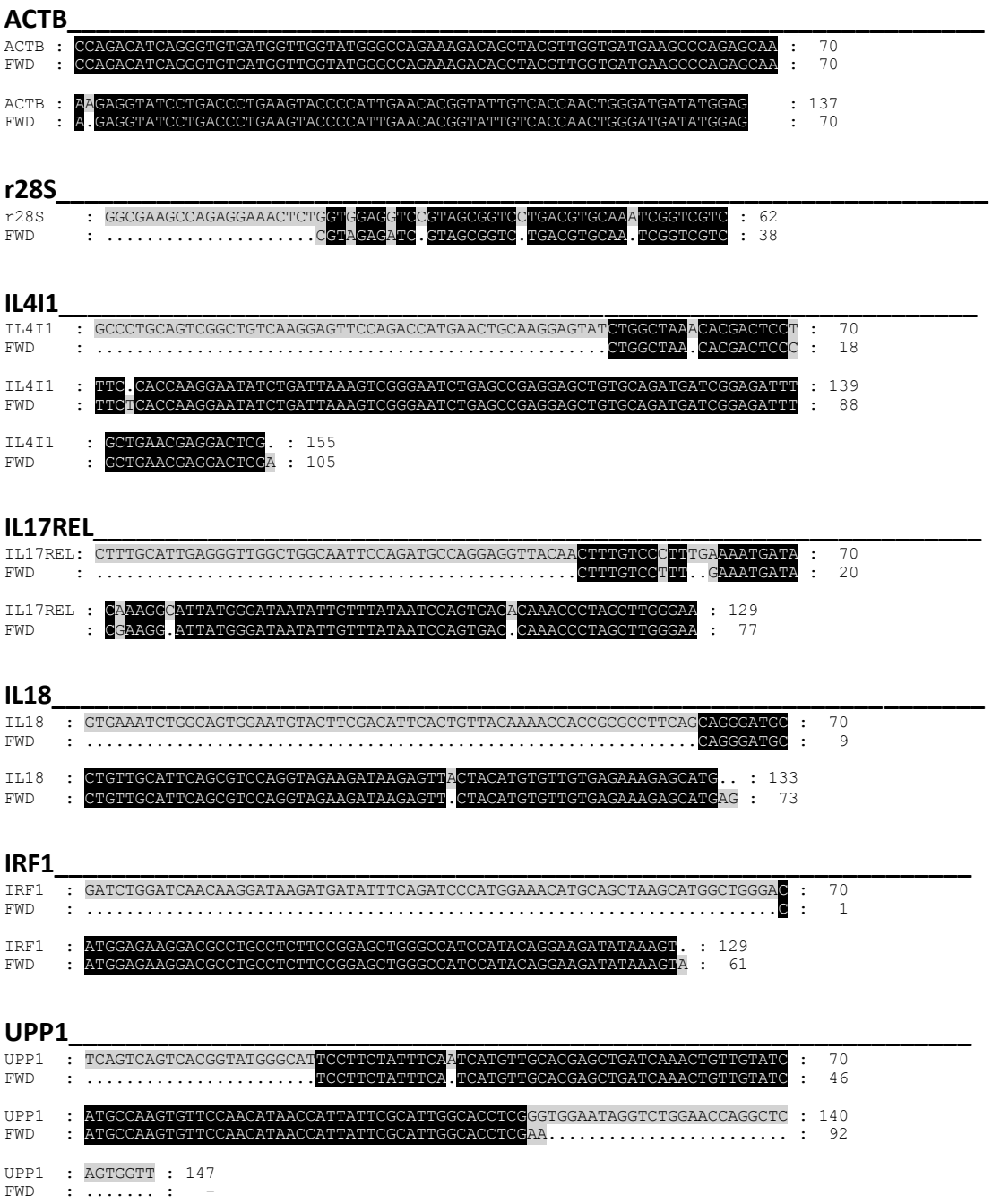


Figure 6.3. Sequence alignments of selected qPCR products.

6.3.2 **Optimisation of the 96.96 qPCR Dynamic Array via pilot studies**

For the first test run, cDNA samples were generated using SuperScript III reverse transcription kit where 2 µg of RNA was used. Complementary cDNA was diluted 1:5 prior to preamplification. A reaction volume of 5 µl was prepared

comprising 2.5 µl of PreAmp Mix and 2.5 µl 200 nM primer mix stock which included primers for r28S and ENSGALG00000015395 genes. The thermal reaction consisted of 16 cycles. Preamplified cDNA was diluted 1:4 in low EDTA-TE buffer without Exonuclease I treatment. Results are presented as a heat map in Figure 6.4.

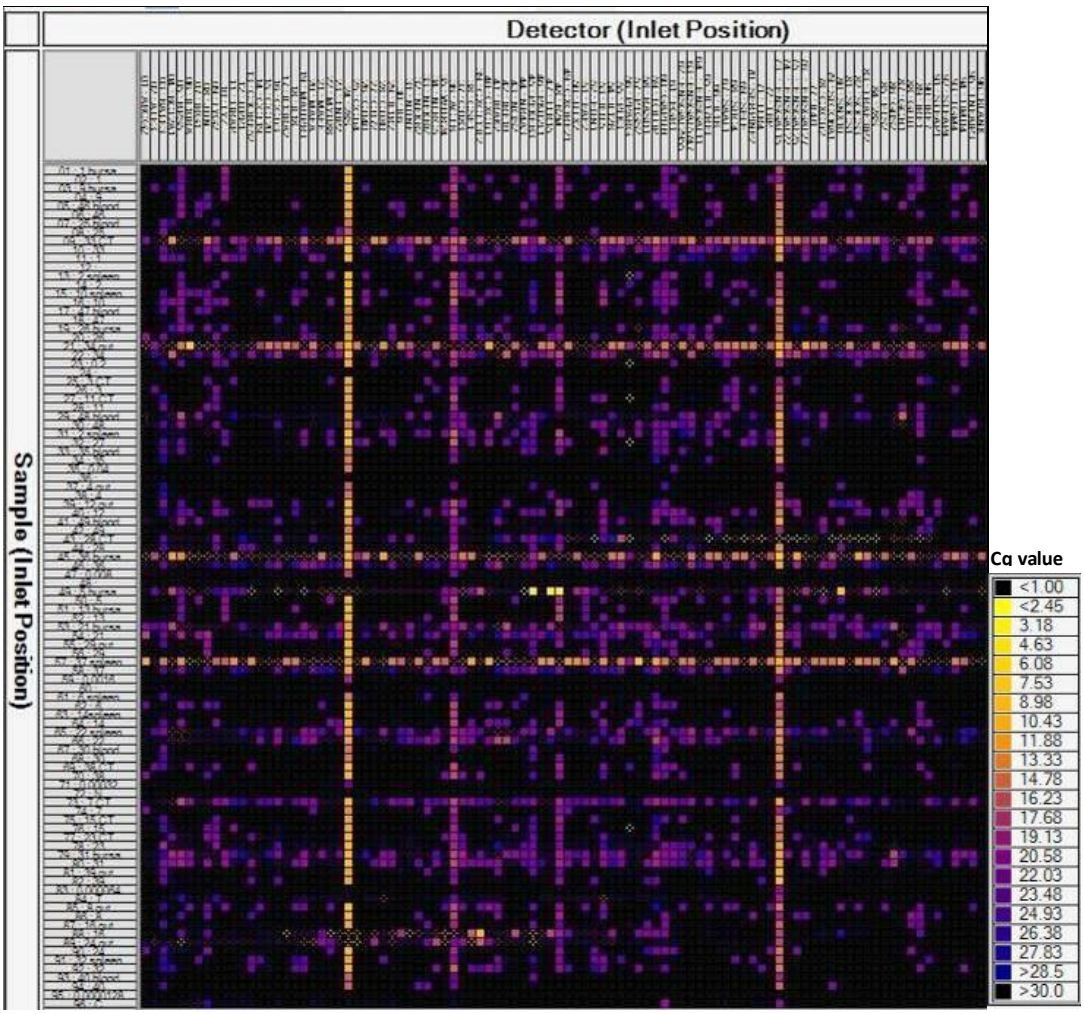


Figure 6.4. Heat map view of qPCR results from first optimisation of 96.96 Dynamic Array. Reactions were performed with 95 genes including 6 reference genes, in columns and 40 samples from Aviagen broilers in rows (technical duplicates). Each square represents one of the 9,216 chambers with contained qPCR reactions with colour-coded level of Cq values.

Examination of amplification curves for all genes indicated that many target transcripts were only detected very late in the qPCR cycling programme, (around 26-27 cycles, which is near the completion of qPCR reaction), as indicated by navy blue colour on the heat map. In case of genes that were amplified early in the qPCR reaction (e.g. r28S, ENSGALG00000015395) the melting curve examination showed specific amplification. For most of the genes with an amplification signal, melting

curve analysis showed non-specific products caused by residual primers. The first 96.96 Dynamic Array run using preamplified cDNA did not produce positive results in the case of most of the genes tested, as it was not properly optimised. Although the preamplification step was performed as described in Rødgaard *et al.* (2012), where PreAmp mix volume was 5 µl and DNA used was diluted 1:5, not many genes were expressed.

Several changes in sample preparation were applied to resolve the problems encountered during first run. In the next pilot study, the complementary DNA was reversely transcribed from not more than 100 ng/µl of total RNA. Newly made cDNA was not diluted before preamplification. The primer mix (200 nM) did not contain primers for r28S and ENSGALG00000015395 transcripts, as these genes proved to be highly abundant and could bias the efficiency of the preamplification reaction. The crucial step in multiplex PCR is the removal of unincorporated primers. In this study exonuclease I, from *Escherichia coli* (Lehman and Nussbaum, 1964) was used to digest primers that were not incorporated into newly made amplicons. The changes in sample preparation in this second pilot study did not result in useable qPCR data. Only r28S and ENSGALG00000015395 transcripts were amplified, despite absence of their primer pairs in the preamplification mix (data not shown). To test the hypothesis of preamplification inhibition, a third test run was performed where cDNA without preamplification was used. The cDNA from samples predicted to have high levels of the target transcripts was selected to make serial dilutions, specifically from the HD11 cell line stimulated with LPS and CD40 ligand, heterophils stimulated with *Salmonella* Enteritidis, or spleens from IBDV and MDV infected birds and splenocytes stimulated with LPS. The results of the third pilot trial are presented in Figure 6.5. Non-preamplified cDNA qPCR resulted in stronger fluorescence signals than preamplified samples in previous tests. Although this third pilot experiment confirmed that the RNA is reverse transcribed there was a need to confirm if different samples from infection studies would yield similar results with and without preamplification.

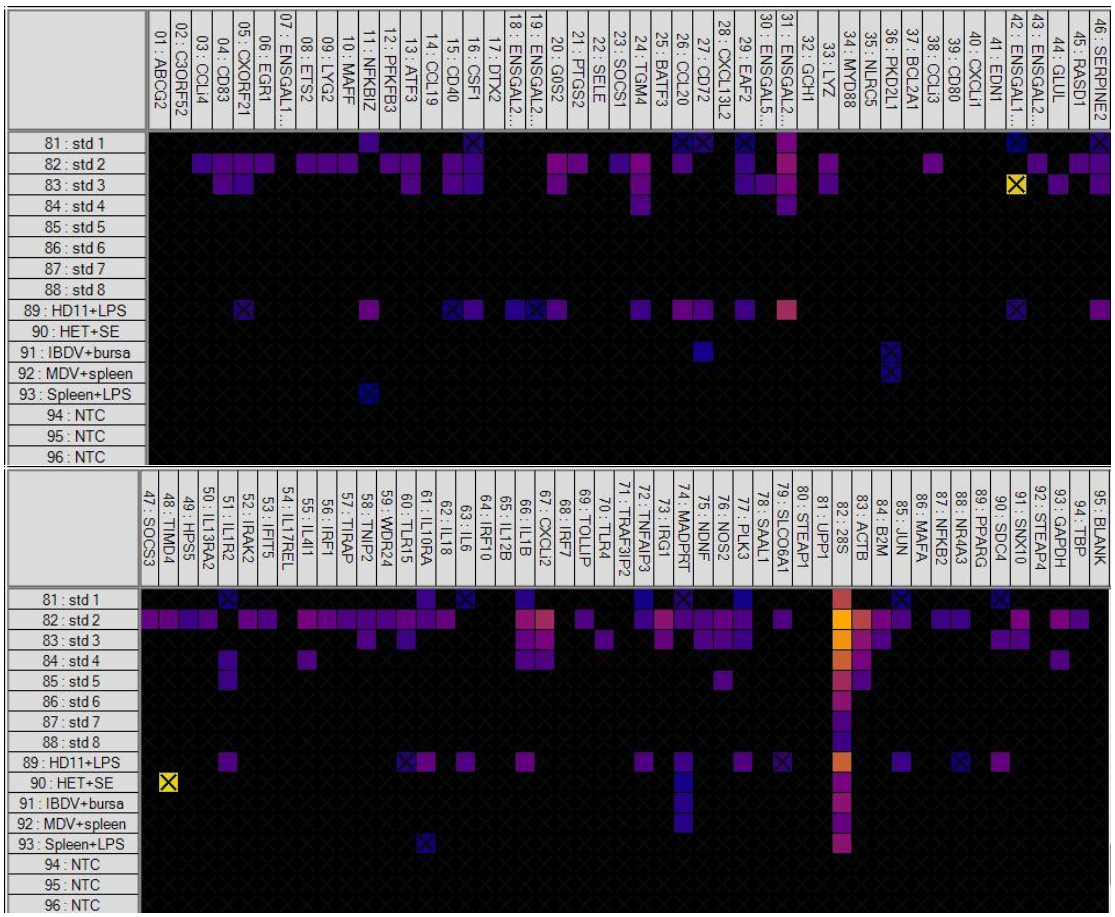


Figure 6.5.Heat map view of the third pilot study using serial dilutions of samples predicted to have high levels of the target cDNA. Samples were not preamplified prior the use in 96.96 Dynamic Array chip. Chambers containing higher concentrations of cDNA have detectable levels of fluorescence.

The fourth test run was performed using archived samples (cDNA and preamplified cDNA), specifically spleen and thymus from MDV infected birds; spleen and bursa from IBDV infected birds; spleen and caecal tonsils from *Campylobacter* colonisation studies; duodenum infected with *Clostridium perfringens* (CP4 virulent strain); spleen and Harderian gland from IBV infected line N birds; HD11 cell line stimulated with LPS and CD40 ligand; jejunum infected with *Eimeria maxima* and caeca infected with *Eimeria tenella*. RNA samples were reverse transcribed as previously described. The preamplification protocol was modified where 2.5 µl of PreAmp master mix was combined with 1.25 µl of cDNA or preamp cDNA (not diluted) and 1.25 µl primer mix (200 nM). Archived preamplified cDNA samples were not amplified in the qPCR. Neither archived samples results in positive qPCR, apart from r28S and ENSGALG00000015395 cDNA. The majority of the tested genes

were not amplified in selected samples from infection studies and most of the fluorescence signal recovered was very weak (data not shown).

The preamplification step is claimed to be crucial for use of 96.96 chip as the volumes of the reactions are very small (6 nl) and low expressed genes may not be detected. The qPCR performed on cDNA returned positive results but only in case of genes with high mRNA levels. Preamplified cDNA produced positive outcomes only for genes that were not included in preamplification primer mix. Therefore, there is a need to optimise the protocol for preamplification, as the recommended guidelines did not yield positive results.

6.3.3 **Preamplification optimisation**

The preamplification or specific target amplification is a multiplex PCR with a limited number of cycles where simultaneous gene expression of many targets occurs (Mengual *et al.,* 2008). As only primers for genes of interest are used in the PCR, no other target is amplified and the relationship between targets is preserved (Korenkova *et al.,* 2015). Although is it the essential part of the workflow in these type of applications it is the least studied step in qPCR. Preamplification should increase the yield of qPCR and highly expressed samples should have Cq ~ 5-15 cycles, where low expressed targets are characterised by Cq values around cycle 20. The cDNA for two samples – caecal tonsils and bursa from Aviagen broilers were prepared using 500 ng of total RNA and the SuperScript III kit. β-actin (ACTB) mRNA gene expression was examined in selected samples. Three preamplification mixes ranging from 2.5 µl (Mix 1), 5 µl (Mix 2) to 7.5 µl (Mix 3) of PreAmp master mix and different concentrations of cDNA were tested in conventional qPCR machine. Two cycle numbers for thermal cycling were tested and preamplified cDNA was diluted ten times after exonuclease I treatment. Results are shown in Figure 6.6. Non-preamplified cDNA was amplified earlier in reaction cycle compared to preamplified cDNA. Both caecal tonsils and bursa samples preamplified in three different reactions resulted in Cq values higher than 25 for 15-cycle PreAmp and Cq equal or

higher than 30 for 18-cycle PreAmp. The lowest Cq values was observed for 15-cycle mix 3 where the highest volume of PreAmp master mix was used.

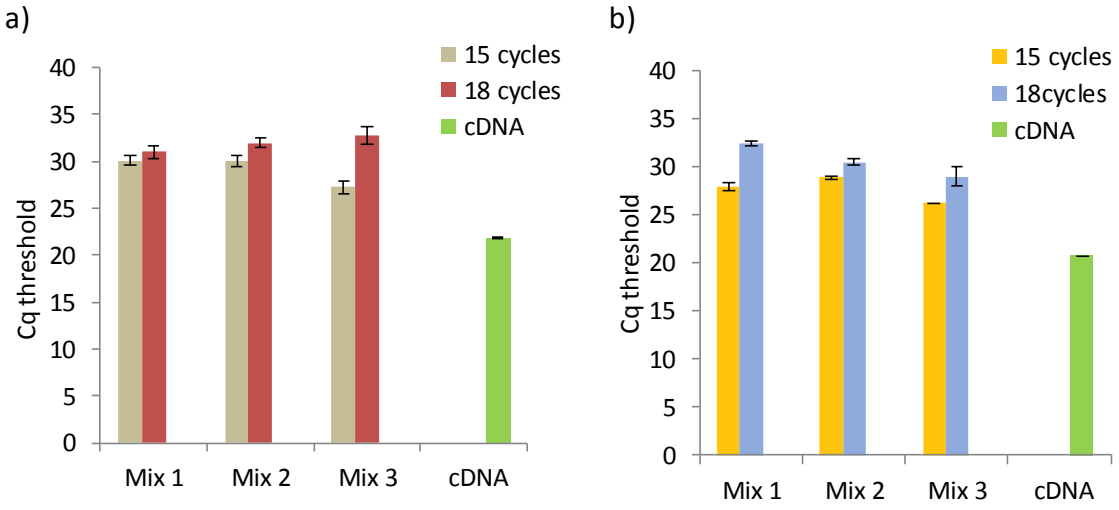


Figure 6.6. Preamplification test in 5 µl performed using a standard qPCR machine ; expression of ACTB in a) cDNA of caecal tonsils and b) cDNA of bursa. Three master mixes tested in two reactions using bursa and caecal tissues. Non-preamplified cDNA (1/5 dilution) used as a control in qPCR. The error bars represent SD from three technical replicates.

The next optimisation attempt involved increasing the volume of PreAmp Master mix to 20 µl and decreasing cycle numbers to 10. Samples were treated with exonuclease I. Different concentrations of RNA were used in reverse transcription reaction. Results are shown in Figure 6.7. Increasing the reaction volume to 20 µl resulted in positive preamplification despite the lower number of thermal cycling. Preamplified samples, reverse transcribed from 250 ng of total RNA and both neat and diluted 1/10 were amplified earlier than non-preamplified cDNA. Only bursa cDNA made from 125 ng of total RNA, preamplified and diluted 10 times resulted in a Cq value being higher than non-preamplified cDNA.

According to the manufacturer's description the PreAmp Master Mix was optimised on total RNA reverse transcribed using a High Capacity Reverse Transcription Kit. The next step was to use the same RT kit and test newly made preamplified cDNA and diluted 10 times in qPCR. The test was performed using cDNA made from 100 ng/µl RNA, as recommended by the manufacturer. Two RT reaction volumes were made, 10 µl and 20 µl, to test the same RNA concentration.

Preamplification mix consisted of 2.5 µl PreAmp Master Mix, 1.25 µl of primer mix (200 nM) and 1.25 µl of neat cDNA. Samples were treated with exonuclease I. Results are shown in Figure 6.8.

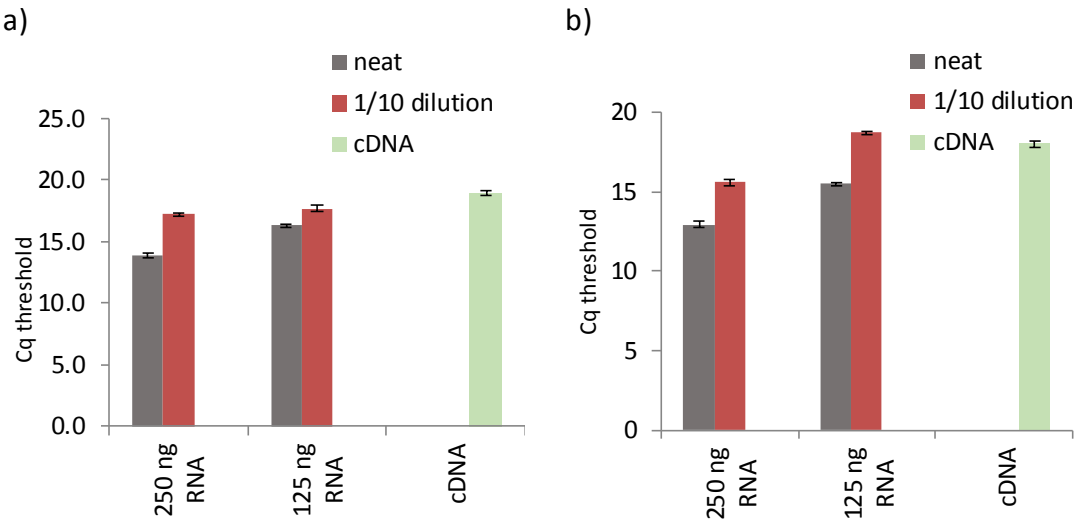


Figure 6.7. Preamplification test in 20 µl reaction volume and 10-cycle PCR ; expression of ACTB in a) caecal tonsils cDNA; b) bursa cDNA. Two concentrations, 250 ng and 125 ng, of RNA were used to make cDNA. Non-preamplified cDNA (1/5 dilution) was used as a control in the qPCR. The error bars represent SD from three technical replicates.

There was no difference observed in Cq values between different volumes of RT reactions. Both, 10 µl and 20 µl reactions resulted in similar Cq values and concentrations of cDNA (~2400 ng/µl). The mRNA expression of ACTB in both samples was at similar levels when cDNA and preamplified cDNA was used as template in qPCR. Preamplified cDNA had higher Cq values than non-preamplified cDNA but lower than previously reversely transcribed samples using SuperScript III kit, as shown in Figure 6.6 (Mix 1). Therefore, the High Capacity Reverse Transcription kit was used in further experiments to generate cDNA.

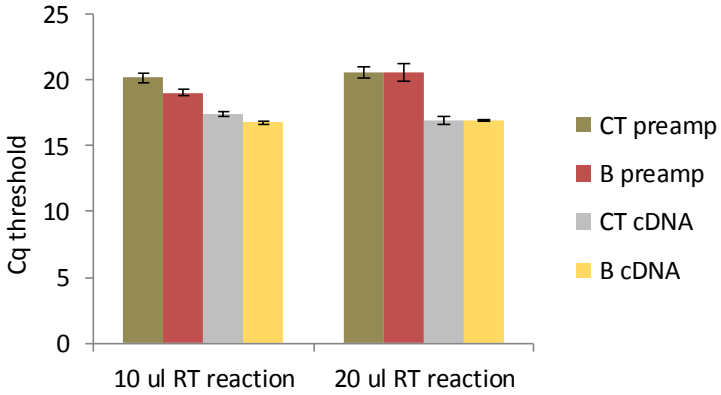


Figure 6.8. Preamplification test of cDNA made using a High Capacity Reverse Transcription Kit and 100 ng/µl total RNA in 10 ul and 20 ul reactions. Ceacal tonsils (CT) and bursa (B) tissue RNA was tested. Non-preamplified cDNA was diluted 1/5. The error bars represent SD from replicates (n=3).

The next step was to examine if standard RNA isolated from COS-7 cells transfected with plasmids containing IL-1β, IL-6, IL-8, IL-12β, IL-18 and CXCLi2 inserts will produce positive signals with and without preamplification when tested using 96.96 Dynamic Array. To make cDNA, High Capacity Reverse Transcription in 10 µl reaction, using 100 ng/µl was performed. Newly synthesised cDNA, in final concentration of 5 ng/µl of reaction, was used in 5 µl preamplification reaction. Preamplified cDNA was then serially diluted (1:2) and used as a template in 96.96 Dynamic Array along with non-preamplified cDNA in serial dilutions.

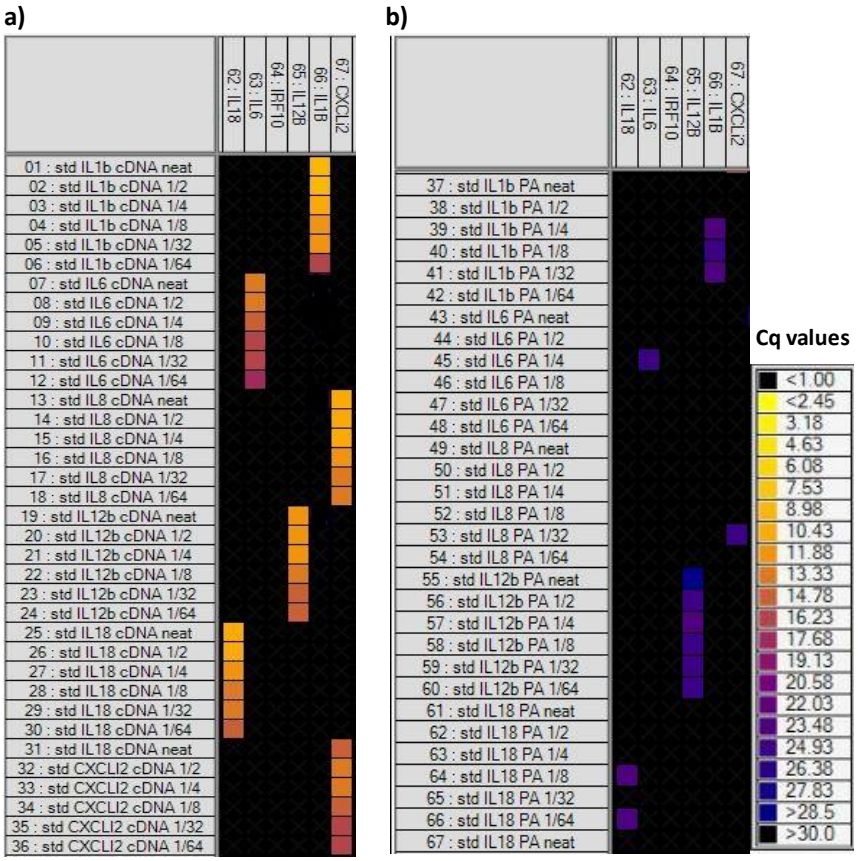


Figure 6.9. Heat map view of a) non-preamplified cDNA and b) preamplified cDNA corresponding to control transcripts. Samples were reverse transcribed from standard IL-1β, IL-6, IL-8, IL-12β, IL-18 and CXCLi2 RNA, serially diluted 1:2. Increase in dilution caused decrease in detected fluoresce of qPCR reaction in non-preamplified samples (a). Preamplified samples did not resulted in positive qPCR for most of the genes, apart from IL-12β (b).

As shown in Figure 6.9a, serial dilutions of cDNA without preamplification resulted in specific and efficient detection of the target cDNAs with decreasing fluorescence signal in qPCRs with each two fold serial dilution, which is characterised by change in colour that corresponded with higher cycle number. Amplification of preamplified cDNA in 96.96 Dynamic Array qPCR was unsuccessful, as shown in Figure 6.9b. For most of the samples tested the concentration of cDNA after preamplification was too low due to possible inhibition of reaction. It can be therefore concluded that use of higher concentrations of cDNA in higher than recommended volumes of PreAmp master mix would give positive results.

A very important aspect of PCR setup is a dilution of the original sample. Lowering the concentration of a template is common practice in single-template assays but in the case of mixed templates, dilutions may lead to loss of low or

moderately concentrated targets. Another test of the preamplification reaction was performed using final concentration of cDNA in the preamplification reaction 185 ng/µl in 20 µl volume. Ten samples from two chickens, each representing a different farm, were selected and pooled for cDNA synthesis using High Capacity Reverse Transcription Kit where 100 ng/µl of total RNA was used. As the RT reaction produces ~2500 – 3500 ng/µl of cDNA, samples were diluted 1:5 before the preamplification reaction. Results are shown in Figure 6.10. The increase in volume of the reaction and concentration of cDNA resulted in successful preamplification. In the preamplification optimisation, both template dilutions and concentration of PreAmp master mix were changed in order to eliminate inhibition. The greater reaction volume with appropriate cDNA concentration worked but the reason for inhibition of reaction in previous optimisation attempts is unknown. Having optimised the reaction, the next step involved testing the same settings in 96.96 Dynamic Array chip.

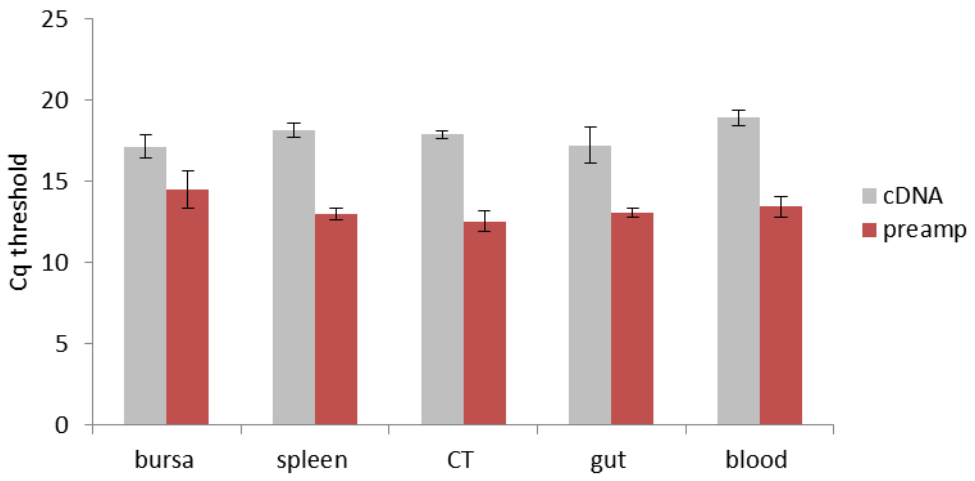


Figure 6.10. Preamplification test with the use of higher cDNA concentration and higher reaction volume. The mRNA expression of ACTB in ten samples from sibling test and pedigree farms was tested. Higher concentrations of cDNA (185 ng/µl) were used in 20 µl preamplification reactions. Non-preamplified cDNA used as a control. The error bars represent SD from four replicates.

6.3.4 Application of the optimised qPCR protocol to RNA from tissues from Aviagen broilers on pedigree and sibling test farms

Having optimised the preamplification protocol (Figure 6.10), samples from the remaining Aviagen broilers were prepared in the same manner to be used as a template in 96.96 Dynamic Array qPCR. Samples were treated with exonuclease I before qPCR in 96.96 Dynamic Array. Results are shown in Figure 6.11. The expression of 89 genes related to immune responses, as well as 6 reference genes was detected, as shown in the heat map based on colour coding of the expression level before pre-processing. The majority of the data fell in the of range about Cq = 5 – 23. Melting curves for all the primer pair were examined. Melting curves for 'no template' controls, for reference genes, for genes where primers did not span exon-intron boundaries are shown in Appendix 1, Figure 2-5. The same reaction settings were implemented in an additional 96.96 Dynamic Array to confirm whether all steps, from RNA extraction, reverse transcription to preamplification, results in reproducible data. RNA from eight tissues (four per farm) was isolated and reversely transcribed as previously described. Newly synthesised cDNA was preamplified in 20 µl reactions using 185 ng/µl of the template.

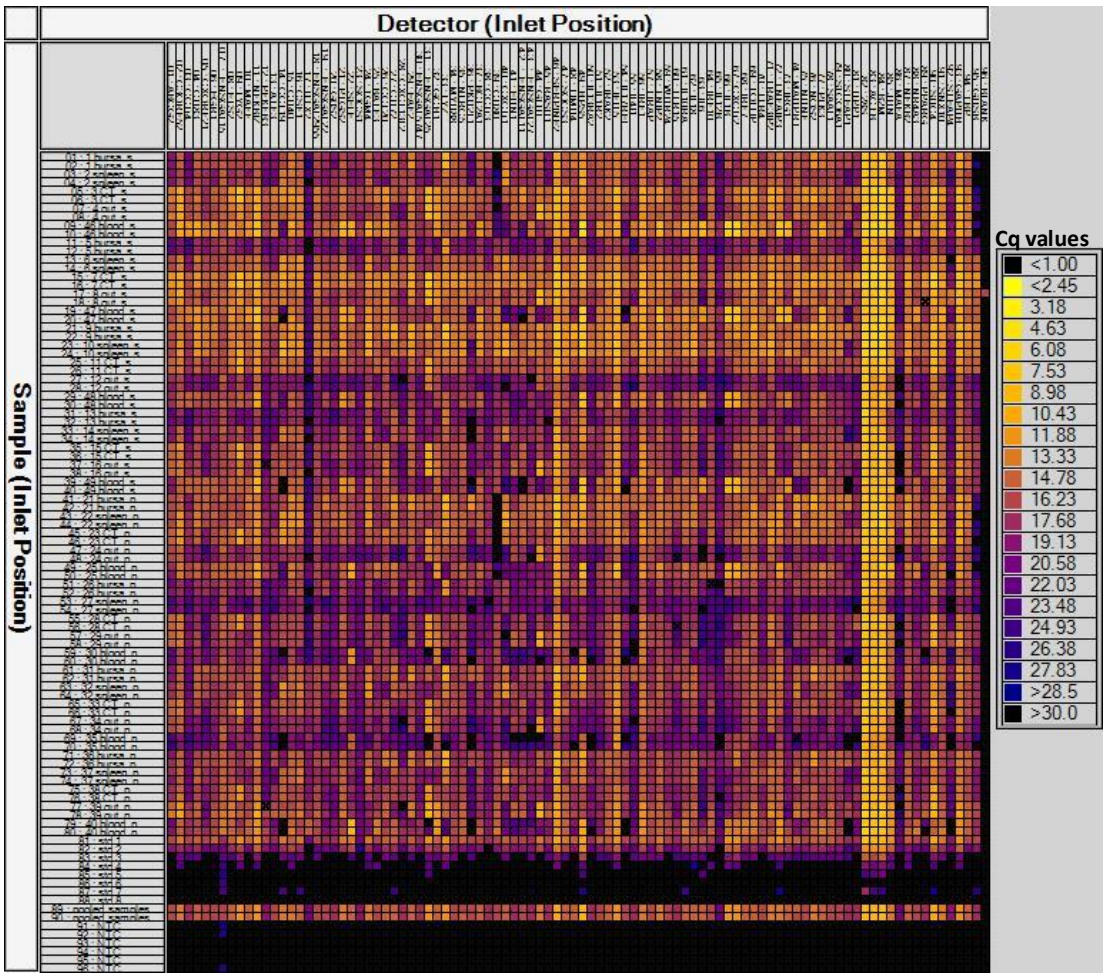


Figure 6.11. Heat map view of 96.96 Dynamic Array qPCR for immune-related genes in broilers on pedigree and sibling test farms. cDNA (185 ng/µl) preamplified in 20 µl was used as a template. Samples (blood, spleen, bursa, caecal tonsils and ileum) were collected from 8 chickens (4 per farm). 40 samples were used in a duplicate as the templates for amplification of 89 genes of interest and 6 reference genes. The bottom part of the heat map corresponds to serial dilutions for standard curve calculation, no template control (NTC) and positive control chambers.

PBL samples were not available for RNA extraction. Whole PBL samples were used in RNA extraction for the first run in Figure 6.11. Preamplified cDNA from eight tissues was tested in quadruplicates on the 96.96 Dynamic Array chip. Results of this experiment are shown in Figure 6.12. Most of the genes were amplified similarly to the previous run (Figure 6.11). Only the column designated for IL-12β did not show amplification as the primer pair for this gene was missed in preparation of assay mixes. All replicates of the same sample showed Cq values reaching the same level with minimal differences (~0.5 Cq) between individual repeats. Missing data were likely to be a result of the genes being expressed at very

low levels, which can be seen as lack of fluorescence detection in most of the replicates. The last column of the chip did not produce results as water, instead of primers, was added to the assay mix as a negative control. Overall the data from this repeated study show that the optimised protocol and qPCR platform is capable of reliably reporting transcript levels across repeated studies.

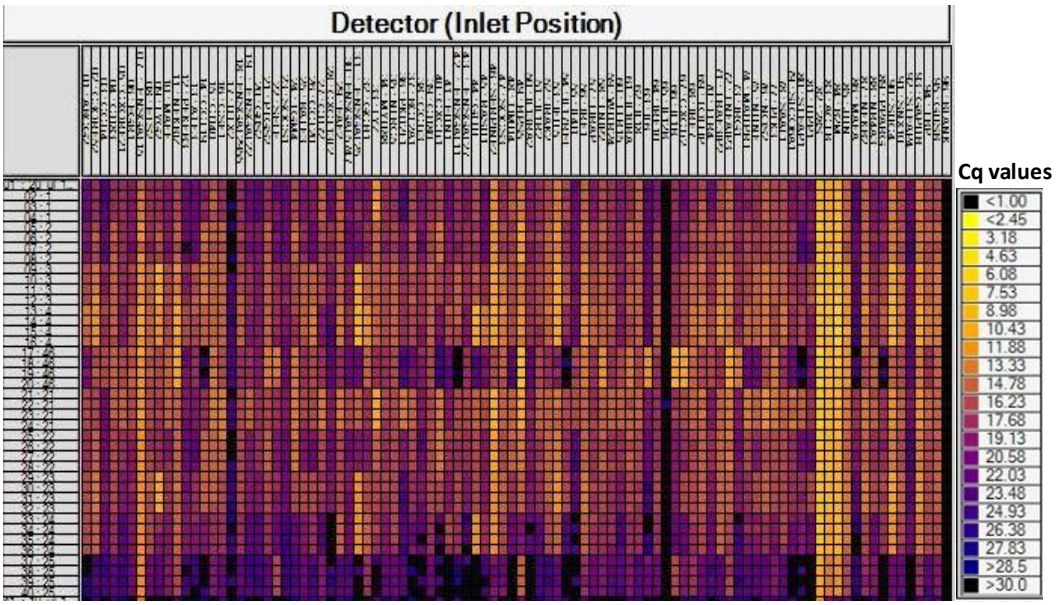


Figure 6.12. Heat map view of 89 genes mRNA expression detected by 96.96 Dynamic Array qPCR in 8 tissue samples tested in quadruplicates. The test established the reproducibility of the settings used. The maximum difference between quadruplicate Cq values did not exceed ~0.5 Cq confirming accuracy of measured mRNA gene expression within the chip.

6.3.5 **Data pre-processing**

Data that did not give rise to any Cq value was represented by 999 which was then translated to NAN (not a number) by GenEx software. Missing data were rare, as evident from the reliable repetition of values across Figure 6.11 and Figure 6.12, however rarely failed reactions were detected, possibly owing to the nano-scale volumes handled, bubbles in microfluidic channels or issues with the quantity or integrity of the samples or reagents loaded. Missing data due to PCR failure were restored based on replicate information. Out of 9,216 reactions, 84 were filled in with replicate mean value. The rest of the missing (54 reactions) data were filled in with values calculated by imputation.

6.3.6 The analysis of tissue gene expression from Aviagen broilers raised in two hygienic settings

The normalised data were analysed in GenEx software using principal component analysis and t-test statistical methods to explore the differences in gene expression between two housing environments (pedigree and sibling test farms) and within farms based on gene expression between organs.

Principal Components Analysis (PCA) is a useful, explanatory technique for simplifying complex and multivariable data sets (Basilevsky, 1994; Pearson, 1901). The whole data set from both farms was analysed using PCA statistical test in GenEx Enterprise to compare the global gene expression profiles of five chicken tissues. The preliminary PCA plot indicated broad differences between the PBL samples and the rest of the tissues (Figure 6.13).

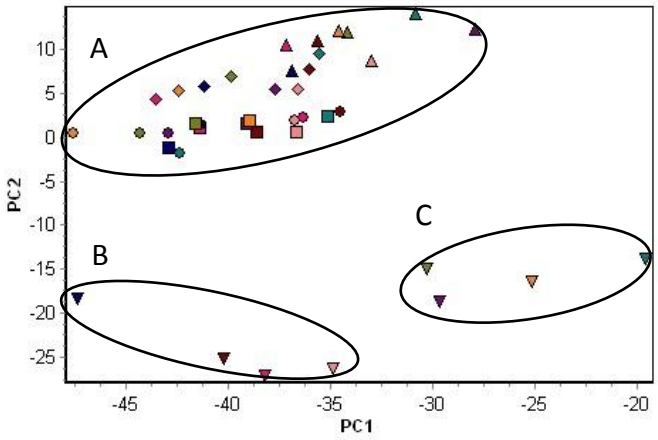


Figure 6.13. Principal component analysis indicating broad similarities and differences in transcription of immune-related genes in analysed tissues. Data points represent individual samples of sibling test and pedigree farms; A – bursa (circles), caecal tonsils (diamonds), spleen (squares), ileum (up-pointing triangles) from sibling test and pedigree farms; B – PBL (down-pointing triangles) collected from chickens raised on sibling test farm; C – PBL collected from chickens raised on pedigree farm.

The PCA results of the whole data showed the difference between PBL samples from two farms (zones B and C in Figure 6.13). The rest of the samples were clustered together and there were no clear sets consisting of tissues from a particular farm but there was visible grouping of samples depending on the tissue type. PCA was also performed on the same data set with PBL samples removed. This second analysis showed heterogeneity within the subset where bursa and spleen

samples grouped together and caecal tonsils group slightly overlapped with ileum group but there was no separation based on the farm type (Figure 6.14).

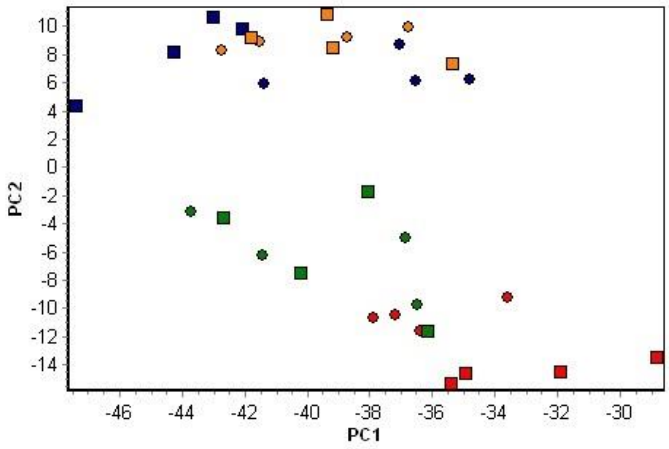


Figure 6.14. Principal component analysis of qPCR data for immune-related genes across tissues of birds reared on the pedigree and sibling test farms without PBL samples ; bursa (dark blue), spleen (orange), caecal tonsils (green), ileum (red). Circles represent samples from sibling test farm and squares represents samples from pedigree farm.

Differences in gene expression between and within the farms

The data from the 96.96 dynamic array was analysed in GenEx to explore the differences in expression of the selected immune-related genes between farms. When comparing the levels of gene expression between the farms where all tissues were taken under consideration, only 13 genes out of 92 tested proved to be significantly differentially expressed ($P \leq 0.05$). The expression levels of all genes tested at both farms are shown in Figure 6.15.

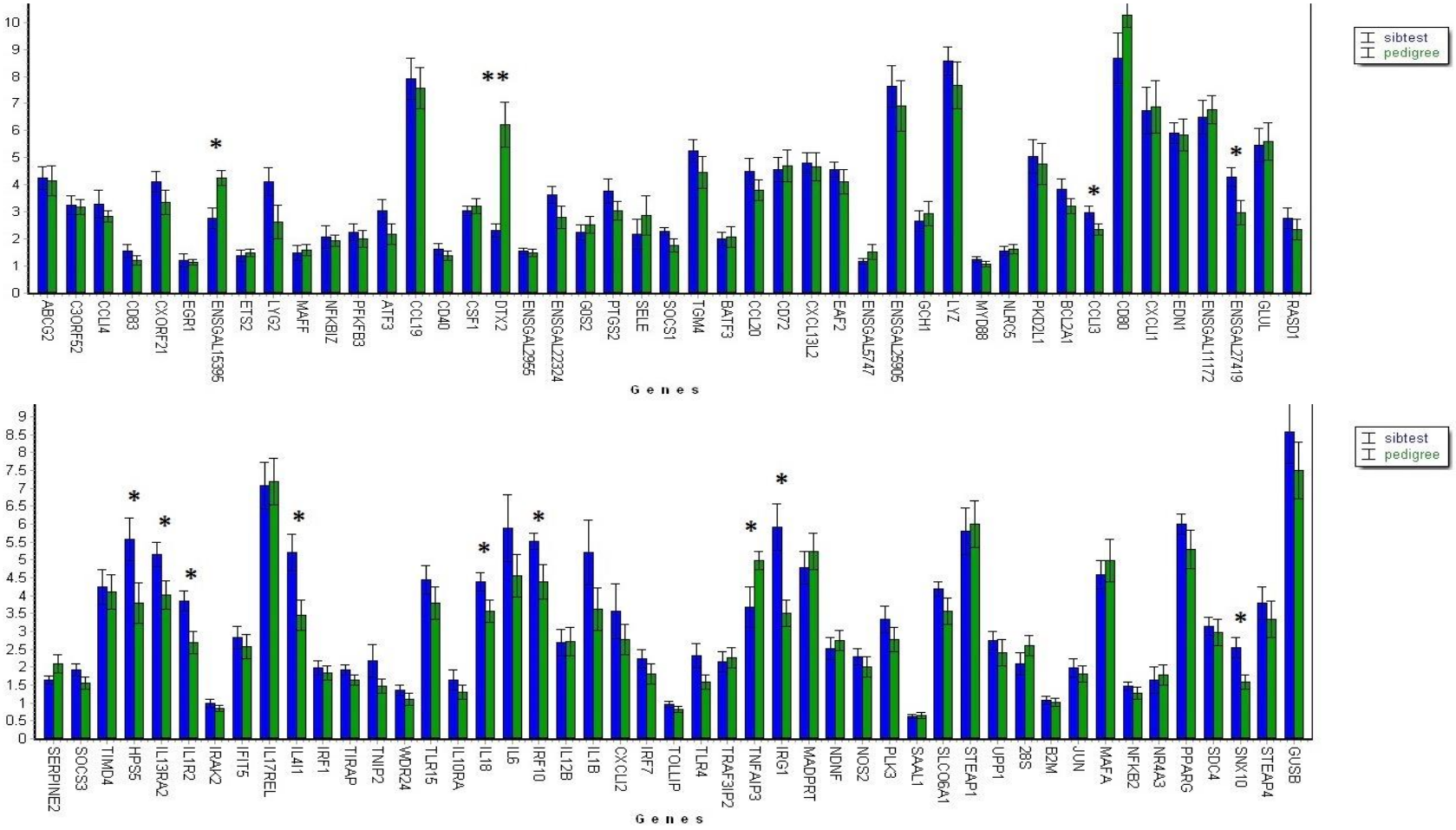


Figure 6.15. Relative expression (mean ± SEM) of genes expressed in tissues collected from pedigree (green) and sibling test (blue) farms. The significantly differentially expressed genes are indicated with * P ≤ 0.05 or ** P ≤ 0.0005; the data point represents all 20 tissue samples per farm type.

To explore the differences between farms, individual tissues were analysed and compared. As PCA results previously showed, the biggest differences between the two farms were observed in PBL samples. When PBL samples from pedigree (n = 4) and sibling test (n = 4) farms were compared, 51 genes were shown to be significantly differentially expressed (DE) with 10 genes highly significantly DE ($P \leq 0.0005$) and 41 significantly DE ($p \leq 0.05$) (Figure 6.16).

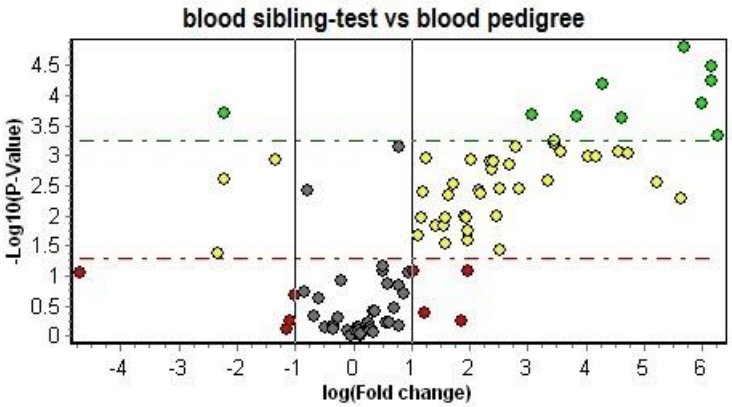


Figure 6.16. Volcano plot of genes expressed in chicken blood (PBL) collected from pedigree and sibling test farms. Scattered points represent genes; the x-axis is the log2 fold change for the ratio sibling test vs pedigree farm, whereas y-axis is the log10 p-value. Green colour represents genes that are highly significantly DE (p value ≤ 0.0005) and yellow colour represents gene that are significantly DE (p value ≤ 0.05).

The number of differentially expressed genes was substantially lower in other tissues tested. The same analysis which compared gene expression between the two farms resulted in 19 genes significantly DE with 2 highly significantly DE in the bursa (Figure 6.17a), 12 genes significantly DE with 1 highly significantly DE in the spleen (Figure 6.17b), 9 genes significantly DE in the caecal tonsils (Figure 6.17c) and 23 genes significantly DE with 1 highly significantly DE in the ileum (Figure 6.17d). Based on the number of genes that are differentially expressed at different levels of biosecurity in housing environment, PBL could potentially be used as the indicator of immune functions.

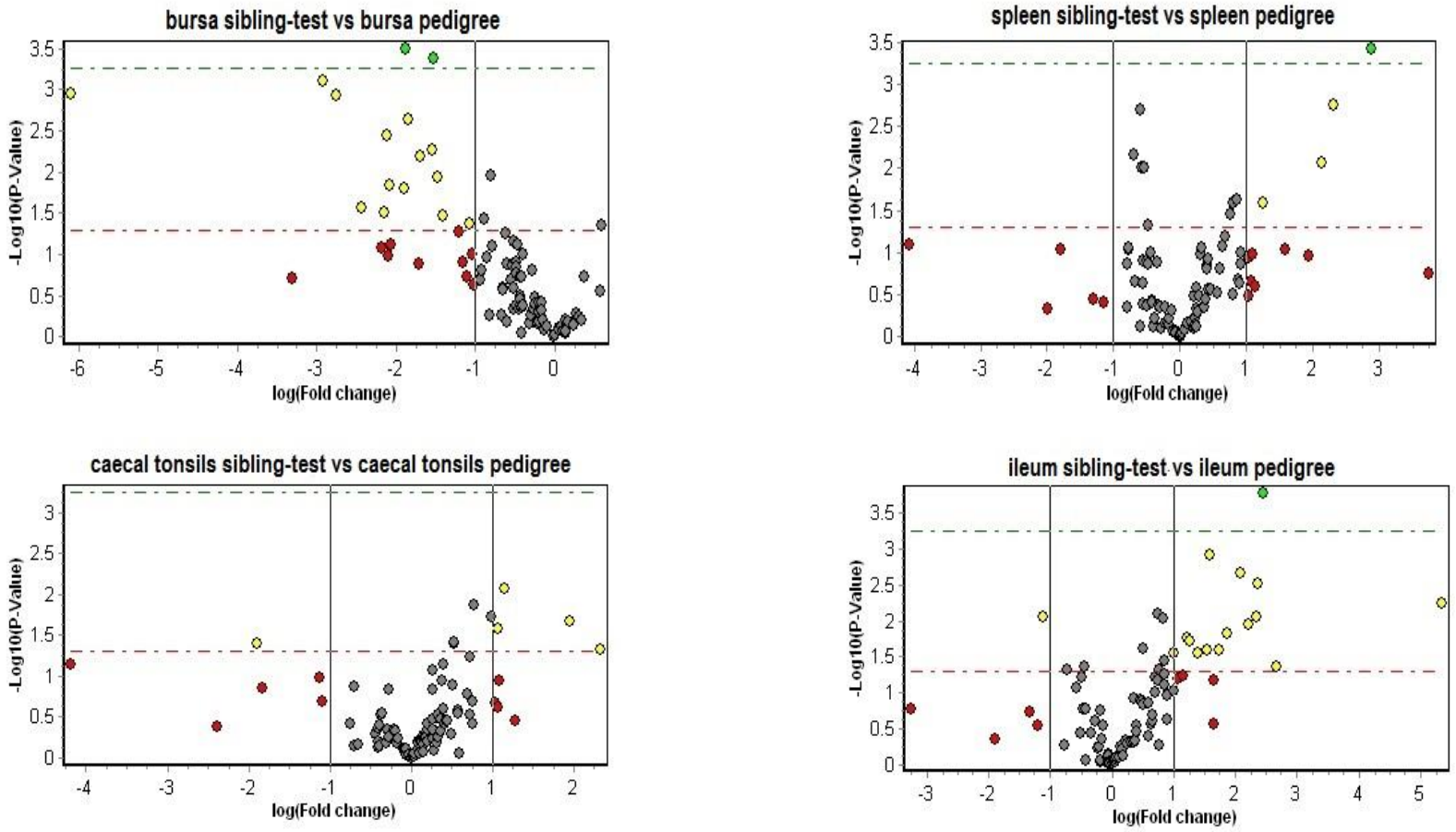


Figure 6.17. Volcano plot of immune-related genes expressed in tissues collected from chickens raised on pedigree and sibling test farms ; a) bursa, b) spleen, c) caecal tonsils, d) ileum. Scattered points represent genes; the x-axis is the log2 fold change for the ratio sibling test vs pedigree farm, whereas y-axis is the log10 p-value. Green colour represents genes that are highly significantly DE (p value ≤ 0.0005) and yellow colour represents gene that are significantly DE (p value ≤ 0.05).

The analysis of tissues from different farms showed that samples of bursa, spleen, caecal tonsils and ileum (Figure 6.18 a-d) were highly positively correlated between farms whereas PBL responses were characterised by a lack of correlation (Figure 6.18e).

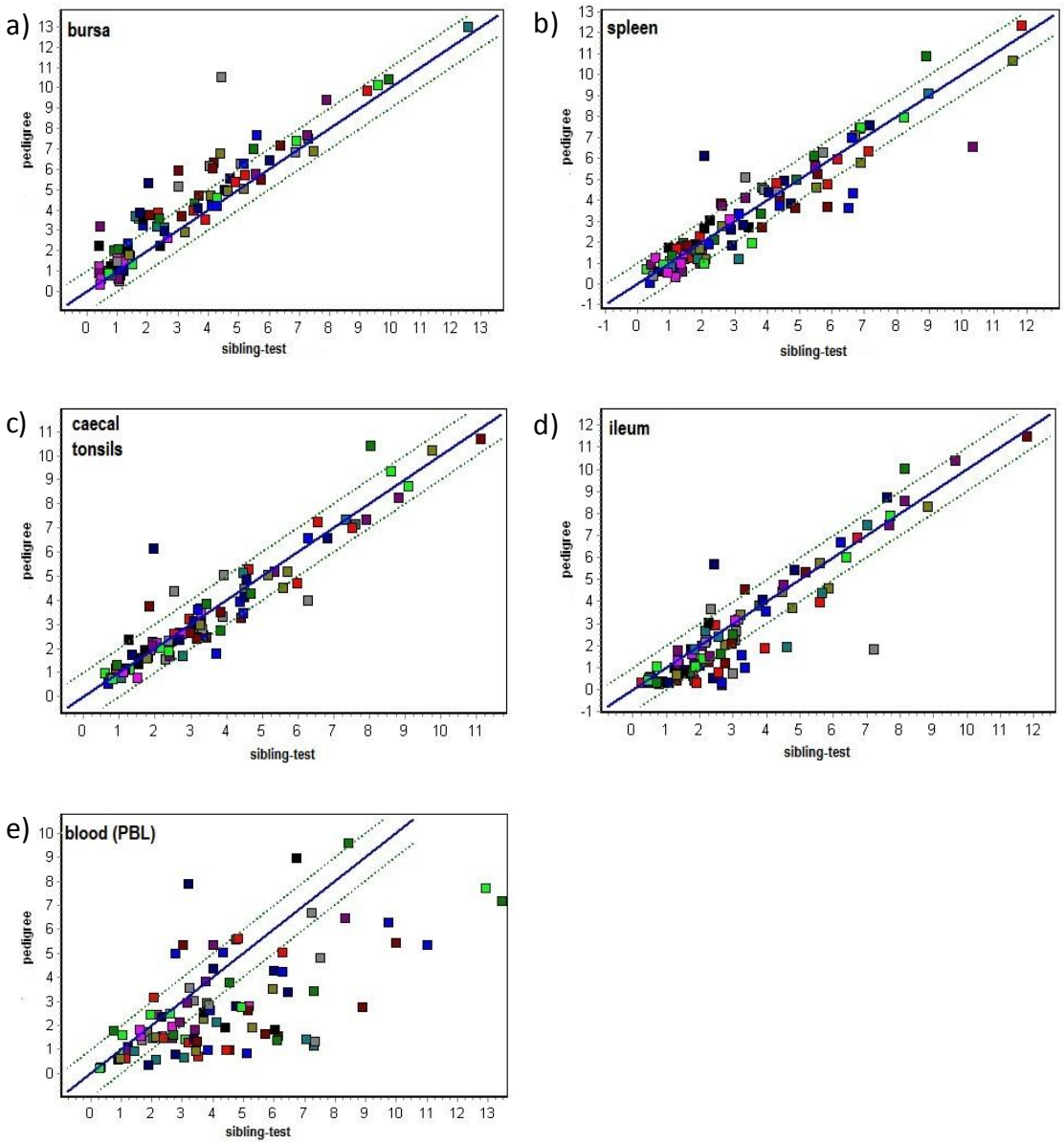


Figure 6.18. Correlation of the magnitude and direction of gene expression in tissues from chickens raised on pedigree (y axis) and sibling test (x axis) farms a) bursa, b) spleen, c) caecal tonsils, d) ileum and e) blood (PBL).

The variation between birds within an organ was analysed to determine whether the differences seen in an organ originates from particular bird being higher responder to pathogens present in the environment. Out of 92 genes expressed at different levels, 25 were significantly DE with five of them being highly significant (Figure 6.19). The significance of gene expression was due to the differential response of just one or two of birds regardless of farm type they were housed in. In many cases the level of expression of a particular gene varied significantly between the birds from the same farm, for example expression of DTX2 gene was very high in birds 7 and 8, both from pedigree farm whereas bird 5 and 6 showed a level of expression similar to their siblings raised on sibling test farm (birds 1-4). Another example is the CD80 gene, which was expressed on higher levels in birds 2, 3, 4, 6, 7 and 8 but not in birds 1 and 5 which were housed in different environments. The three reference genes that were not selected as normalisers (ribosomal r28S, B2M and GUSB), had levels of mRNA expression that were significantly different across birds, confirming that they were not a good control of gene expression in this sample set. For the bird vs bird analysis all tissues were combined therefore the differences seen between birds stem from differential responses detected in particular tissues.

Overall, the sample group (four birds per farm) is not enough to overcome variance within the group (environment). There was no clear variation in gene expression observed between birds. The gene expression resulted is small number of differentially expressed genes and the level of variation changed within and between farms.

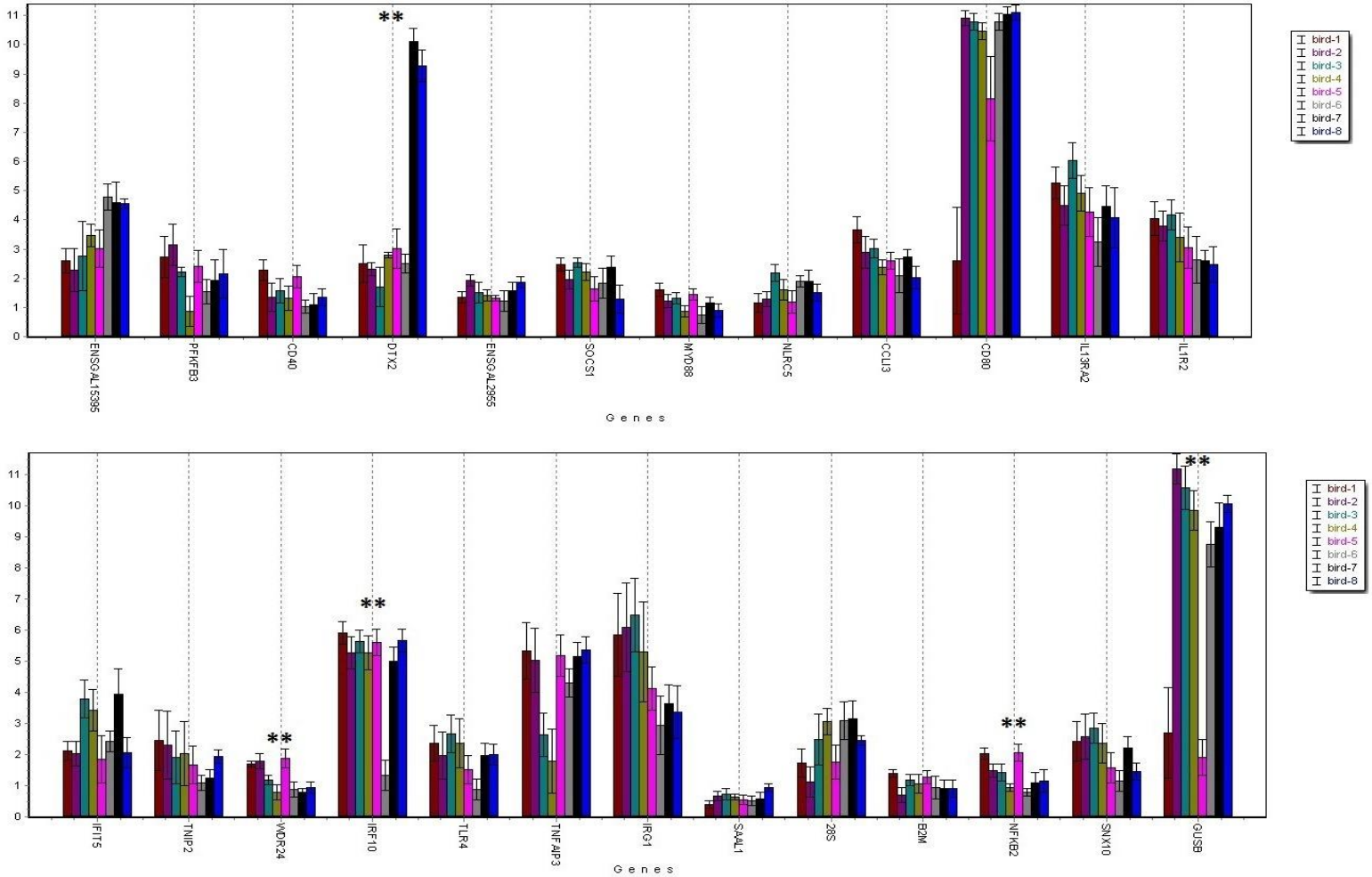


Figure 6.19. Relative expression (mean ± SEM) of significantly DE genes between birds from the sibling test farm (1-4) and pedigree farm (5-8) ; ** $p \leq 0.0005$.

PBL gene expression analysis

The PBL proved to have the highest number of differentially expressed genes and differed between farms (Figure 6.20). Among the 51 genes with expression significantly different between farms, 46 had higher mRNA levels in PBL samples collected from birds from the sibling test farm. The lower level of biosecurity at the sibling test farm may be associated with the observed upregulation of genes involved in immune responses: e.g. toll-like receptors (TLR4, TLR15), cytokines (IL-1β, IL-6,IL-12β, IL-18), and chemokines (CCLi4, CXCLi2, CCL20). Other genes that were expressed at significantly higher levels are also known to be regulated during infections.

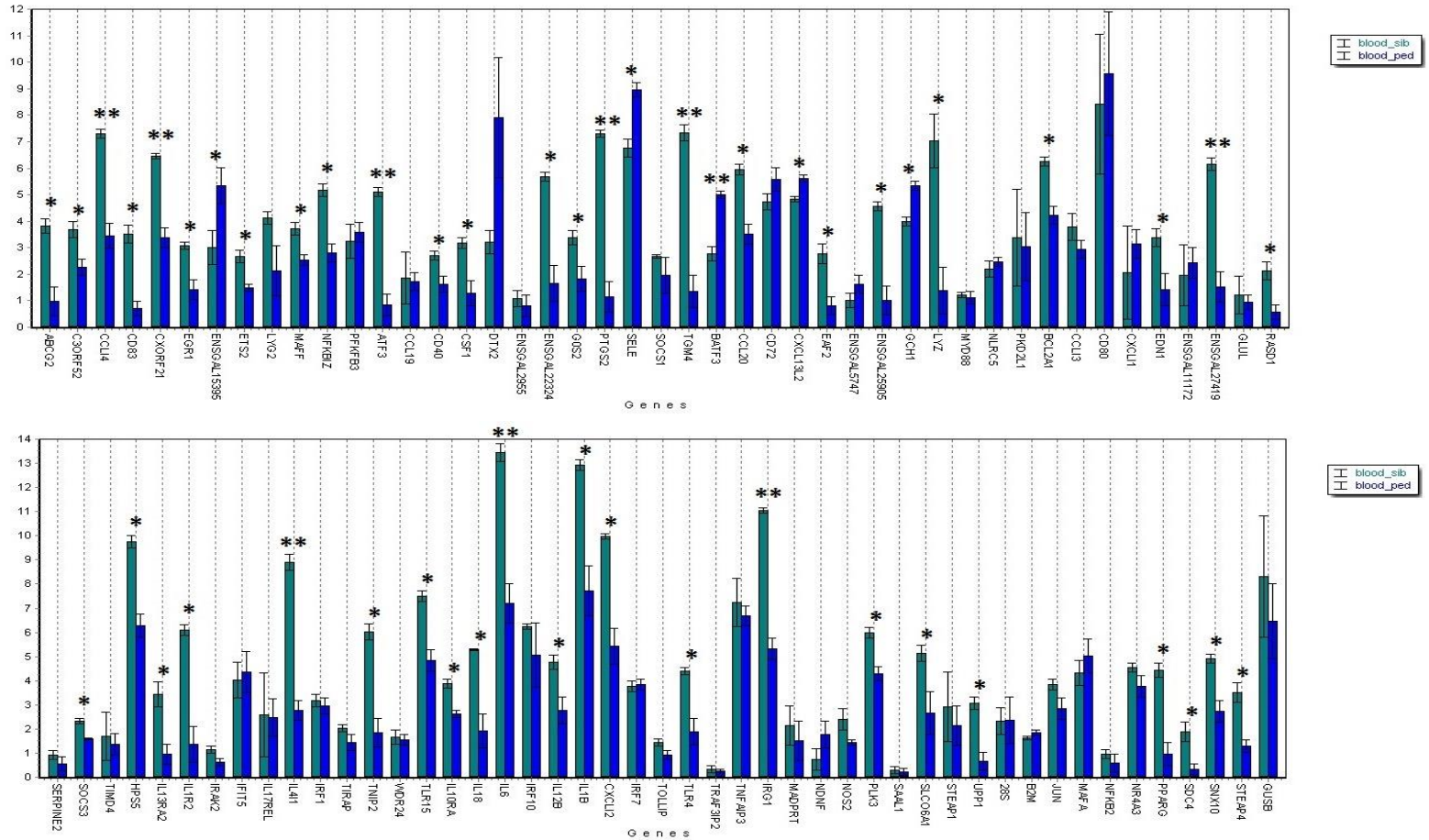


Figure 6.20. Relative expression (mean ± SEM) of genes expressed in PBL collected from pedigree (blue) and sibling test (green) farms. Genes expressed significantly different (* $p \leq 0.05$; ** - $p \leq 0.0005$)

6.4 Validation of differentially expressed genes by qPCR

To validate the 96.96 Dynamic Array results, three transcripts (DTX2, IRG1, SAAL1) were analysed by qPCR in a conventional 96 well format. These genes were selected because of their significantly different regulation (DTX2 downregulated and IRG1 upregulated) and no change in expression (SAAL1) when gene expression in all tissues from both farms was compared. Results showed that SAAL1 and IRG1 exhibited a similar transcriptional profile to that of 96.96 array. The down-regulation of DTX2 was not detected, as it was observed in data generated using the 96.96 Dynamic Array. Comparison of both 96.96 array and qPCR statistical test is shown in Figure 6.21.

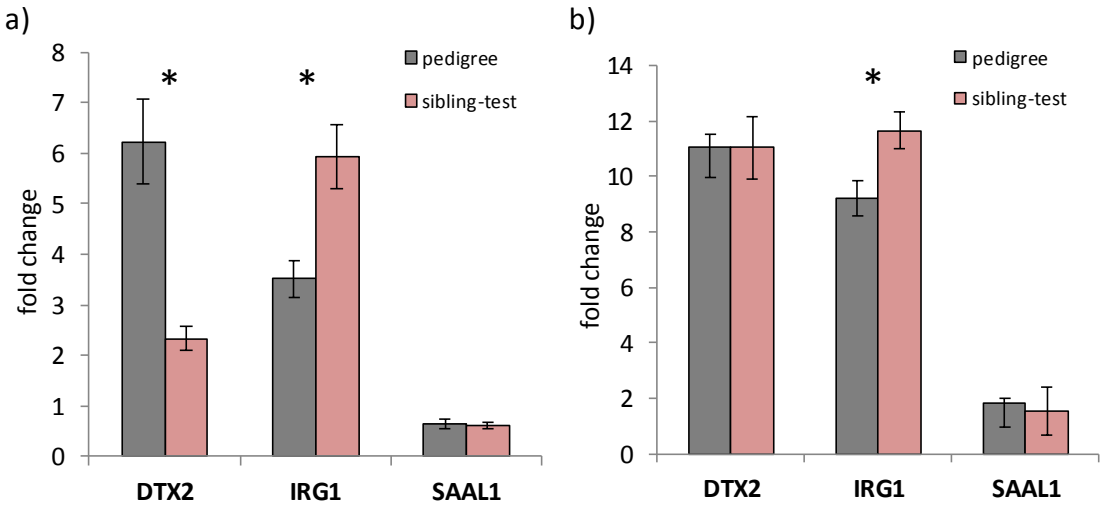


Figure 6.21. Validation of 96.96 Dynamic Array qPCR results for selected 3 transcripts : DTX2, SAAL1, IRG1; a) fold change in expression of three genes tested between sibling test and pedigree farm in 96.96 Dynamic Array, b) results of fold change calculation for three genes of interest, as measured in conventional 96 well format; each bar represent average of 40 biological replicates ± SEM * - level of significance $p \leq 0.05$.

6.5 Discussion

Poultry breeders have to overcome many challenges caused by diseases that lead to decreased yield and bird welfare. Chickens with improved innate resistance could be introduced in breeding programs to minimise the losses but there are no methods available that would select the chickens with superior immune responsiveness to pathogens. Therefore, the idea to develop a cost-effective and rapid technique to select birds characterised with higher level of immune performance seemed reasonable. The work presented in this chapter was performed in order to develop and validate a new diagnostic tool for immune gene expression in the chicken. The 96.96 Dynamic Array from Fluidigm was selected as the platform of choice.

As with any qPCR method, each step for the BioMark System had to be optimised. Without intensive quality control, several steps in qPCR workflow could influence the accuracy of the results. The use of a dye-based technique of DNA detection required extensive primer design and optimisation. Although the design of primers to span intron-exon boundaries is recommended (Wang and Seed, 2003) it does not guarantee that pseudogenes as intronless copies will not be amplified. In this study, contamination with DNA was avoided by applying the DNase treatment during the RNA extraction process. This is the most efficient strategy to remove or decrease the number of gDNA copies (Derveaux *et al.,* 2010) to the level where abundance of gDNA does not interfere significantly with quantification of mRNA (Bustin and Nolan, 2013). Melting curve analysis was performed at the end of the PCR cycles and confirmed the specificity of primer annealing with perfect melting curves having a single sharp peak (Taylor *et al.,* 2010). All of the primers used in this study were successfully validated. The stability of reference genes selected in Chapter 5 was also confirmed based on the Cq values generated during high-throughput qPCR.

The use of the Fluidigm platform requires a preamplification step. The samples need to be highly concentrated to ensure the template DNA for

amplification is present in the mix. This method of enrichment of samples has been used in many studies (Noutsias *et al.,* 2008; Sindelka *et al.*, 2010). The process of preamplification should be optimised before the main experiment using high throughput qPCR platforms (Rusnakova *et al.,* 2013). In experiments presented in this chapter, samples were tested with and without preamplification where different concentrations of cDNA were used with various volumes of TaqMan PreAmp master mix. Three cycle numbers of PCR reactions were tested to determine if the length of reaction changes the amount of amplification. As described in Korenkova *et al.* (2015), different concentrations of RNA, number of cycles and dilutions of cDNA were all very crucial for the preamplification reaction. These studies using various combinations showed that for the BioMark System preamplification works most efficiently in 15-18 reaction cycles and with higher concentrations of cDNA samples diluted either 20x or 40x. In experiments presented in this chapter, neither higher number of cycles of PCR reaction nor the different concentrations of cDNA used resulted in preamplified samples having lower Cq values that non-preamplified cDNA. Korenkova *et al.* (2015) observed that increase of number of cycles (21 and 24) caused exhaustion of primers and reagents by high abundant templates and lower level of success of reaction, therefore it is not recommended, especially if the targets have unknown level of abundance.

To validate the utility of 96.96 Dynamic Array chip as a diagnostic tool a number of samples from Aviagen broilers were used. In this study, the differences in immune responses between chickens raised on two farms with high and low levels of biosecurity were analysed. Detection of differences in immune performance between birds within and between the farms, were performed to establish which tissue delivers the most informative data.

The study consisted of two different farms, where four birds were random selected from the population and five tissues were sampled. PCA analysis of the qPCR results transformed the data into easily visualised two-dimensional plots. Although birds were not infected with any particular pathogen, they were

vaccinated, the hygiene levels differed between farms with presence of common pathogens such as *Campylobacter*, *Eimeria* species and *E. coli* was known at the sibling test farm. The difference in gene expression profiles were most obvious in blood samples when the complete data sets, including all tissues from both farms, was analysed in PCA. The lists of genes that were significantly differentially expressed in individual tissues were not common for all tissues. Most of the genes DE in blood were also DE in other tissues but there was no pattern characterised by the same profile of expression with any particular tissue. That is the reason why blood samples were separated from other tissues when visualised in the PCA. The analysis of the data set with exclusion of the blood samples revealed the similarities between the gene expression profiles in bursa and spleen and between caecal tonsils and ileum. Surprisingly a stronger concordance was observed between bursa and spleen. The caecal tonsils and ileum are closely related and are part of the gut-associated lymphoid tissue (GALT) system together with the bursa of Fabricius (Befus *et al.,* 1980). Nevertheless, gene expression profiles in caecal tonsils and ileum separated from bursa samples that were assembled in cluster with the spleen. All tissues were equally exposed to the same biotic stimuli, yet there in no indication that any of the tissues showed significant changes in the transcription of immune-related genes.

The birds used in this experiment were three-weeks of age and their immunological responses were coordinated by a nearly mature immune system. As the birds were sampled at only one timepoint it is difficult to speculate if the gene expression observed at this particular level was triggered by matured cells and organs involved and if the response would differ if the birds were sampled earlier and later in their life. The cytokine expression in the caecum was studied throughout the chicken's life span in the experiments done by Crhanova *et al.* (2011) which revealed that IL-8, IL-17 and partially IFN-γ expression changed with gut flora development. The relative expression of other genes tested IL-1β, IL-18 and iNOS was unstable up to day 25 after which the low levels of expression continued to be unchanged. The same study involved infections with *Salmonella*

Enteritidis and showed that the level of response to the pathogenic bacteria was dependent on the age of the birds infected. The stimulation of younger chickens caused higher levels of cytokine gene expression and although those levels continued to decrease they were higher at day 42 compared to the gene expression in birds infected later in their life. The patterns of immune response to gut microflora and pathogenic microorganisms may include overlapping pathways, therefore it is difficult to say whether the responses observed in sibling test farm birds were activated by only commensal microbes. The levels of the gene expression may not give the clear answer as the microflora of the environment is unknown but it could be speculated that the responses triggered at the sibling test farm reached higher levels compared to pedigree farm owing to presence of common pathogenic microorganisms.

It could be expected that PBL and spleen tissue gene expression would have more genes in common, as the spleen is very rich in blood. Spleen accumulates leukocytes and is the main site of antigen presentation and processing. However in the present study principal component analysis reliably found differences in the profile of expression of the immune-related genes expressed at these sites. A microarray study by Nie *et al.* (2010) on basal expression of genes in eight tissues, including bursa and small intestine, found that nearly half of the genes were expressed in all tissues and small percentage were tissue-specific.

It is difficult to establish if the blood is the best estimator of immune vigour compared to other tissues, based on the initial data. The PBL showed the highest number of the DE genes that included genes with proinflammatory functions. In the same samples, many significantly DE genes were involved in diminishing the inflammation by regulating the overexpression of the proinflammatory genes. The PBL consist of many cells programmed to confer microorganisms as the first line of innate defence, where cell populations in other tissues are also involved in later stages. It would be preferential to select the PBL for developing a diagnostic test for

immune robustness in chickens as the circulating cells detect inflammatory state in whole organism.

The test of mRNA gene expression in samples from both farms was not performed using whole transcriptome gene expression platform and only eighty-nine genes were included. Many of the immune-related genes, listed in Chapter 4, were selected based on upregulation during infection or stimulation with constituents of pathogens. Therefore, it is difficult to differentiate between immune responsiveness of healthy chickens and chickens responding to infection using the selected genes, as the phenotype of immune robustness at mRNA level are not known. It could be assumed that the selected panel of genes was not appropriate to detect differences in immune responses in other tissues, apart from blood leukocytes. It would be feasible to test the same hypotheses in additional timepoints and examine the outcomes from different immune/colonisation stages. The low numbers of significantly expressed genes between individual birds from both farms could suggest that the level of variation did not depend on the level of biosecurity. This could be explained by the individual ability of bird to detect the invading microbes but also by the unequal level of immune responses within the bird, between organs. Another obstacle in interpretation of the results is the differences in the vaccination schemes used at the two farms. The differences in the gene expression in PBL may plausibly be due to different vaccines and times of administration. Therefore, the farm settings are not adequately controlled for measuring the innate immune robustness of healthy birds.

The results showed that there are differences in immune responses between farms but the number of birds used in this experiment was too low to select the birds characterised by improved immunity. The PBL isolated from the blood showed to be promising indicator of immune gene expression differences between farms but the variation between birds, based on only the blood samples, was impossible to estimate. Nevertheless, the Fluidigm chip with the proposed set of genes could be a convenient diagnostic tool for measuring the differential responses in the

future experiments. It has been optimised and verified in the field experiment but the experimental setup needs to be adjusted to avoid future problems with the data interpretation.

Chapter 7 General discussion

7.1 Overview of this study

The main aim of this project was to design and optimise a diagnostic tool for assessing innate immune responses in commercial chickens. All objectives stated in Chapter 1 were met and the tool has been optimised and validated. The 96.96 Dynamic Array IFC within the BioMark system (Fluidigm Corporation) was used in preliminary experiments involving 89 innate immune-related genes. Lymphoid tissues and blood were isolated from an Aviagen broiler line housed on their pedigree and sibling test farms that differ in levels of hygiene. The test validated the usability of the chip and evaluated immune performance based on expression of selected genes. Crucial steps in the workflow were also optimised. The optimisation of preamplification reaction was the first major challenge, as the recommended and/or published protocols did not produce useable data. Reference gene normalisation was also performed before the final high-throughput qPCR analysis and the data from the Fluidigm chip confirmed the stability of transcription of the selected reference genes across animals and tissues. The importance of proper selection and validation of reference genes has been underlined several times in Chapter 5 and supported with numerous published examples.

Supplementary to the studies presented, a first author manuscript has been accepted for publication in the Journal of Veterinary Immunology and Immunopathology to select reference genes for qPCR analysis of chicken gene expression, this time using lymphoid tissue isolated from the RI-J line. This study confirmed that TBP is the most stable gene in bursal cells, thymocytes and splenocytes, consistent with findings presented in Chapters 5 and 6. The selection of genes used in Chapter 6 was performed based on published studies and the RNA-seq analysis of chicken effector cells stimulated with LPS. The cloning and sequencing of selected candidate genes confirmed their presence in the chicken genome and made the design of assays more accurate. The design and validation of a new diagnostic tool has been performed successfully and according to the MIQE guidelines for qPCR analysis.

7.2 Challenges and limitations of the study

The project started by evaluating effector cell phenotypes and their mRNA gene expression as screening methods for innate immune function to be used in breeding programmes. The techniques presented in this work involved weeklong cell cultures. These methods were confirmed to be time-consuming and together with the requirement for greater number of birds to perform heterophil experiments, they were not considered as viable future diagnostic tools. Although previous studies on commercial and inbred lines showed that resistance to one pathogen could be associated with resistance to other pathogens, the birds used in the experiments presented in this thesis were not challenged with multiple pathogens simultaneously as occurs in commercial settings. The mRNA levels of only a few cytokines and chemokines is unlikely to be sufficient to select birds with higher immune responsiveness. Therefore, the decision was made to select genes that are involved in innate immune responses to a plethora of infections and implement them in new high-throughput qPCR diagnostic tool.

The selection of genes to be used in the qPCR platform was based on published experiments with widely different design. Most of the studies used to generate the list utilised microarray platforms to distinguish the genes significantly differentially expressed in infected cells or tissues. The comparison of the data from different platforms is difficult as they are based on diverse protocols, from manufacturing, hybridisation and final analysis (Brazma *et al*., 2001). Therefore, direction, magnitude and significance of gene expression in a particular infection study may differ when analysed using different platforms.

The current study design did not result in detection of birds characterised with overall greater responses in any of the farms tested. The intensity of immune responses differed between farms as shown in Figure 6.15, which is not surprising as the level of hygiene varied greatly. The analysis of all datasets showed that the greatest difference between farms is visible in blood samples (Figure 6.13). The analysis of immune responses based on lymphoid tissues did not show any of the

organs to be more responsive to the environmental challenges. Therefore, it suggests that the blood would yield the most information about the immune state of each bird sampled. That hypothesis needs to be tested in future experiments with larger numbers of birds and more defined challenge at a given timepoint.

The genes used to characterise immune function in this study were selected based on their activation upon infection or stimulation of effector cells with pathogen-associated molecular patterns. This makes the analysis of results problematic. The elevated expression of particular genes may not be a result of a high state of innate immune readiness in healthy birds, but rather could be a reflection of disease. Moreover, it is not possible to fully interpret the meaning of responses detected when the nature of pathogen challenge, time of measurement relative to time of infection and the relative exposure (pathogen load) is not precisely controlled.

A further challenge in evaluating immune responses is interpretation of gene expression as a being indicative of robustness, resistance or tolerance. The definition of robustness in farm animals was explained by Knap (2005) as "the ability to combine high production potential with resilience to stressors, allowing for unproblematic expression of high production potential in wide variety of environmental conditions". Disease resistance could be defined as the ability to reduce the pathogen occurrence by inhibiting infection and pathogen growth (Best *et al*., 2008). Tolerance to pathogen may be described as the ability to limit the damage by counteracting the detrimental impact (Rohr *et al*., 2010). The tolerant animal would be able to maintain productivity despite increasing load of pathogens. For breeders, both, maintenance of productivity and ability to reduce pathogen burden, are equally important. Previous studies on lines selected for higher heterophils mRNA gene expression showed that the parental and F1 progeny with higher resistance resulted in fewer *Campylobacter jejuni* colonies from cloacal swabs (Li *et al*., 2008c). This could suggest that selection based on mRNA gene expression levels may lead to generation of a line that is robust, as it not only

maintains the expected phenotype but also reduces the prevalence of pathogens. The use of gene expression data simultaneously with information about other traits, for example weight gain and feed conversion ratio, could help to refine the phenotype of robustness and ensure it does not come at the expense of other production-relevant traits.

7.3 Future applications

In addition to previously used pro-inflammatory cytokines and chemokines to evaluate innate immune responses of inbred and commercial lines (Wigley *et al*., 2006; Swaggerty *et al*., 2008) the panel of immune-related genes selected for this project includes various genes involved in downstream signalling after pathogen/agonist recognition. The additional carefully selected genes in the panel could result in more precise selection of chickens based on their immune responses, once the immune robustness phenotype is better understood. Collection of samples at different timepoints would be practical to examine different maturity stages of immune system and changes in responses. This approach could clarify if the expression of the selected panel of genes is dependent on the age of the birds. In the future, when the chip is used as a diagnostic tool, only one appropriate time-point may be necessary to predict the robustness. Apart from additional time-points, vaccination schedules would have to be standardised. Birds used in the preliminary chip test, housed on the pedigree farm or sibling test farm received different vaccines at hatch and on different days throughout their lives, which had the potential to change the nature and magnitude of immune responses and explain some of the differences detected.

The chip could also be used as a tool to study immune readiness at the point of hatch or even during late stages of embryo development. Mortality in the first week post-hatch is an important aspect of chicken breeding and is used as an indicator of the occurrence of welfare problems. The transitional period from embryonic stage to the post-hatch chick and further to the broiler farm is a major challenge to the digestive, immune and thermoregulatory systems. The

performance of the newly hatched chickens in the first days is indicative of the performance during rearing weeks at the farm (Yassin *et al*., 2009). Therefore, the gene expression screening of newly-hatched chicks, yet to have significant contact with pathogens or acquired microbiota could help to predict how the flock will perform in later stages.

The value of the multiplex PCR platform for immune-related genes could also be tested in a wider range of environmental settings, for example on test farms using birds of the same genotype but reared in cold and hot environments, at different stocking densities, intensive indoor vs. outdoor free-range systems and so on. This will be important to improving the performance of poultry to meet fast increasing demand owing to growth of the human population and rising affluence and urbanisation in developing countries. Expansion of breeding under hot temperatures is also very important because of global climate change. Heat stress is known to result in decreased productivity and increased mortality (Turnpenny *et al*., 2001). It has been shown that diverse lines demonstrate different, heritable responses to chronic heat exposure (Lu *et al*., 2007). Several heat shock proteins and genes responsible for glucose transportation have been shown to be involved in responses to heat stress (Garriga *et al*., 2006; Lei *et al*., 2009; Yan *et al*., 2009). These, and many other expression quantitative trait loci (eQTL) associated with heritable resistance to other production-relevant phenotypes, could be added to the qPCR panel to test differences in transcript expression between farms as a guide to selection.

Apart from commercial lines tests, the optimised diagnostic tool could be applied in studies of inbred lines when challenged with pathogens. That could help to select genes that carry the information about resistance or susceptibility to disease, for example in the inbred lines housed by the National Avian Research Facility that differ in resistance to *Eimeria, Salmonella, Campylobacter* and other agents. Results could narrow down the list of candidate genes associated with differential resistance. The limited number of genes tested, compared to whole

transcriptome analysis, would lower the costs of experiments even further. However, it would not allow for the discovery of novel genes or transcript variants associated with resistance, as happens in RNA-seq differential expression studies. Projects involving a vaccine challenge or infection could also benefit from applying this platform by adding to knowledge of the nature, magnitude and timing of responses to vaccination and pathogen challenge. Gene expression studies offer very exciting avenues for solving the problems of existing and emerging diseases. Blood transcriptome analysis as a diagnostic tool has been successfully used to profile human patients with sepsis, where candidate transcriptional signatures of differential diagnosis were identified (Pankla *et al*., 2009). Microarrays and other gene expression platforms that measure host responses to infections could serve as a disease progression monitoring systems. It has become obvious that genomic and transcriptomics approaches cannot elucidate all the problems but play a part in selection programs. The complexity of biology and host interaction with the environment make the selection for immune robustness difficult. In-depth information of fields such as epigenetics, alternative splicing, miRNA regulation would add to knowledge of candidate biomarkers at multiple levels. The Chicken genome is still in draft version and lacks much genetic and functional annotation, therefore it would be advantageous to annotate the genome at similar levels to that of human (Cheng *et al*., 2013).

7.4 Future challenges of poultry breeding

Breeding for disease resistance in farmed animals has made remarkable progress. Despite the advances, difficulties still exist (Jie *et al*., 2011). The costs of breeding for disease resistance are still high as challenging chicken flocks with pathogens to select resistant individuals leads to death of susceptible birds and the number of birds used need to be very high. Identification of the disease resistance phenotype can also be problematic, as the disease may not affect each bird to the same level. Therefore, susceptible animals may not have been identified as they are not exposed to the pathogen, resistant birds may have sub-clinical infections and play a role of pathogen reservoir/carrier. In addition, the interaction between

pathogens makes the breeding of multi-resistant lines difficult and secondary infections can cause higher mortality than the primary infection (Jie *et al*., 2011).

Knowledge of genes involved in disease resistance can be implemented in creating transgenic chickens, e.g. inactivating viral receptors or introducing or upregulating resistance-associated genes. Recently, genetically modified chickens able to suppress avian influenza A virus transmission have been generated by constitutive expression of a decoy RNA resembling a viral vRNA segement to interfere with virus replication (Lyall *et al*., 2011). The creation of birds resistant to AI could lead to reduced costs of production caused by AI outbreaks and lower risk of transmission of the virus to human population. The chickens resistant to AI phenotypes do not show significant differences in hatching weights. Future transgenic chicken lines overexpressing particular immune-related genes, or indeed birds given immuno-modulatory substances to boost immune function, could be screened using the qPCR panel with Fluidigm chip to understand the basis of any beneficial phenotypes. For decades it has proved challenge to introduce disease resistance by selection without affecting other production traits, therefore transgenesis may aid the fight against the diseases (Whitelaw and Sang, 2005).

7.5 Conclusions

The research described here could potentially aid the selection for improved immune robustness. The technical optimisation and validation of a new tool to simultaneously quantify expression of tens of relevant immune-related genes will prime research in many areas of avian biology, especially to define baseline immune gene expression for selection, the basis of differential resistance, and host responses to infection, vaccination or immuno-modulatory substances. The chip with the selected panel of genes now needs to be tested at different time-points in experiments where genotypically identical chickens are placed in different environments in which more variables are defined and controlled. Results from mRNA gene expression studies, together with information about other phenotypes could help to distinguish birds that are more robust.

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Appendix 1

Table 1. Primers for sequencing cDNA inserts in pGEM-T.

Primer name	Plasmid name	Sequence (5'-3')
T7 (forward)	pGEM T-Easy	TAATACGACTCACTATAGG
Sp6 (reverse)	pGEM T-Easy	ATTTAGGTGACACTATAG

Table 2. Probes and primers used in RT-qPCR

RNA target	Probe/primer sequence (5'-3')	Accession number
r28S	Probe: (FAM)-AGGACCGCTACGGACCTCCACCA-(TAMRA) F: GGCGAAGCCAGAGGAAACT R: GACGACCGATTTGCACGTC	FM165415
IL-1β	Probe: (FAM)-CCACACTGCAGCTGGAGGAAGCC-(TAMRA) F: GCTCTACATGTCGTGTGTGATGAG R: TGTCGATGTCCCGCATGA	AJ245728
IL-6	Probe: (FAM)-AGGAGAAATGCCTGACGAAGCTCTCCA-(TAMRA) F: GCTCGCCGGCTTCGA R: GGTAGGTCTGAAAGGCGAACAG	AJ309540
IL-18	Probe: (FAM)-CCGCGCCTTCAGCAGGGATG-(TAMRA) F: AGGTGAAATCTGGCAGTGGAAT R: ACCTGGACGCTGAATGCAA	A3416937
CXCLi1	Probe: (FAM)-TCGCTGAACGTGCTTGAGCCATACCTT-(TAMRA) F: TGGCTCTTCTCCTGATCTCAATG R: GCACTGGCATCGGAGTTCA	Y14971
CXCLi2	Probe: (FAM)-TCTTTACCAGCGTCCTACCTTGCGACA-(TAMRA) F: GCCCTCCTCCTGGTTTCAG R: TGGCACCGCAGCTCATT	AJ009800
TGF-β4	Probe: (FAM)-ACCCAAAGGTTATATGGCCAACTTCTGCAT-(TAMRA) F: AGGATCTGCAGTGGAAGTGGAT R: CCCCGGGTTGGTTGGT	M31160

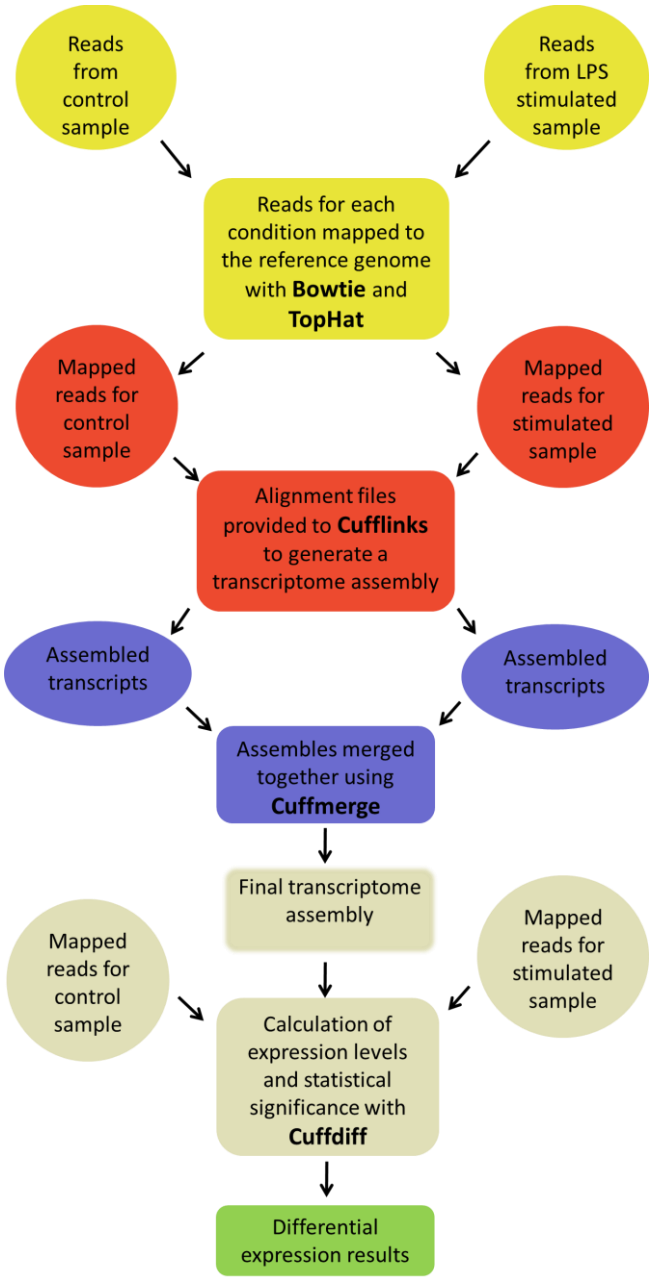


Figure 1. RNA-seq differential expression pipeline (Trapnell *et al.,* 2012)

Table 3. Primers designed to clone genes of interest

Gene	Forward	Tm [°C]	Reverse	Tm [°C]	length (bp)
ABCG2	ATGGGAACTGCTCAAAACAACAG	56.1	TTATGTGAACTTCCTCATAAACCGGAG	56.6	2106
ATF3	GAGGAACTTAGATTCGCCATC	53	TTAACCTTGTAATGTTCCTTCTTTGATC	53.8	419
BATF3	GAGCCACGAAGAAGACGATAAGAAGG	58.8	TCATCTGGGCAGGCAGC	58.2	292
BCL2A1	ATGGAAACTGCTGAGTTCTATTACG	54.9	TCAGTGGTACTCTCTGAACAAG	53.9	524
C3ORF52	ATGAGCTGGCTCCGTGC	58.4	CATGTTGATGTGAGAGAAACTGGGTC	57.6	700
CD40	ATGGGGCGGCTCGGGC	65	TCACAGCTGCTCCTGCTCGGC	64.5	831
CD72	ATGGCCCAGAGCGTGCTCTAC	61.8	TCACCCTTCGGCCAGCAGA	61.6	972
CD80	ATGAAGATGGGGTGCCTGAAGAG	58	TCATAGAGATGACATTTCACATGTCAATTTACAGC	58	950
CD83	ATGGCTTCAGCAGCCTAC	53	TTAGATAGAACTTGAAGTAAGTCCAC	52	648
CXORF21	ATGCTGTCAGAAGGTTACCTTTACAG	56.7	CTATGCATTAGTGTTGCTATACTGAGAAATATG	57	870
DTX2	ATGGCAGCAGCTCAGGGAGCAG	64.2	CTGTTCCAGGCCACCTTCAGGAGCTC	64.7	1475
EAF2	ATGAACGGGATGGCCCCG	60.8	TCAGTCATCACTATCGCTTCCAGATTCAC	59.6	795
EDN1	ATGGATTGCAGCCGCCTG	58.9	CTAGAATGTTTTCAGGCTTTTCCAGATGC	58.2	618
EGR1	ATGGCTGCGGCCAAG	56.5	TTAGCAAATCTCAATTGTCCTTGGAGA	56.5	1532
ENSGALG00000002955	ATGAAGATGTTCAGGTGGAGGTG	56.8	CAAGTCTATCCTTTCCCTGGAGATC	56.4	1167
ENSGALG00000005747	ATGACTCGCTGCCAGGTG	57.8	TCATTCATTCTGGCAGCTTTTATAGTCC	57	1056

Gene	Forward	Tm [°C]	Reverse	Tm [°C]	length (bp)
ENSGALG00000011172	ATGCACCAGAGCAGCATCAATGG	60.2	GGTCCAGGCACTCCGC	59.3	1315
ENSGALG00000015395	ATGCTTCTGCTTCAGGCCTGC	60.6	TCAGTGTTCCTCTGGACTTGTAGAGACAG	60.5	1056
ENSGALG00000022324	ATGAGGATGCCGCTTCC	55.1	TTAAACATTCACTGTGCTTTCTTGTGG	56.4	1008
ENSGALG00000025905	ATGAAGATGGAGACAGGGGAT	55.3	TTAAATTTGTGATATCATTCTCAGTGCAGG	55.9	933
ENSGALG00000027419	ATGATGGTTACTTGCCTGCTCCTCTC	60.3	TCACGTGTTGGGCACTGCAG	60.7	918
ETS2	ATGAGTGAATTTGCGATCAGAAACATG	56.3	TCAGTCCTCGGTGTCAGG	56.2	1440
G0S2	ATGGAAACCATGCACGAGCTG	58.3	TTAGGATGCATGCTGCCTGG	57.8	299
GCH1	TACGCACGGCGGGAGGG	63.5	TCAGCTCCTGATGAGCGTCAAGAACTCTTC	62.6	711
GLUL	ATGGCCACCTCGGC	55.5	TTAGTTCTTGTACTCAAAAGGCTCGTC	56.8	1122
HPS5	ATGAGGCTCTGTATCTGCTTCGTG	58.6	TCAGTATTTGTTAGGAAGTCCTGCGG	58.4	384
IFIT5	ATGAGTACCATTTCCAAGAATTCC	53	TCAGCTTGAGAGGGAAAGTCG	52	1440
IL10RA	ATGGCCCTCTGCGCTGC	61.9	CTTCTGGCACAGGAACAGCTGCT	61.8	900
IL17REL	ATGATAAGTGTTCATGTTCTGATTCTTC	53.7	GTGGAACTGAATAATCTACATCTGTC	53.3	1266
IL1R2	ATTTCAAGACACTTTCCCTCGCTCTTC	58.9	CCTGCCAGGATGACACAGG	58.1	1149
IL4I1	ATGGCTGCGATGGTTCTCTTCC	59.7	TCAGAGCTCTCCCTTCTCCACG	60.1	1572
IRAK2	GTGTGGTCTTAGCAGAGATA	51	TTATCTACATTTACCTTGGGATG	50	803
IRF1	ATGCCCGTCTCAAGGATGCG	65	TTACAAGCTGCAGGAGATGGC	60	942

Gene	Forward	Tm [°C]	Reverse	Tm [°C]	length (bp)
IRF10	ATGGCGGAGCCGGGGTC	63	GCCAGCAGTCTGAGTGATTGACATCCTCA	63	620
JUN (AP1)	ATGGAGCCTACTTTCTACGAGGATG	58	TCAAAACGTTTGCAACTGTTGTGTTAG	58	932
LYG2	GAACATAGCAAATGTTGAAACAACTGG	55.5	TCAGTATCCATTTCTCTGAAAGAACTTG	55	541
LYZ	ATGGGTCTTTGCTAATCTTGGTGCTTTGC	62	TCACAGCCGGCAGCCTCTG	63	444
MADPRT	ATGGAGCACGCCATTCTGG	58.1	CACGGAAGACATTGTGGCATCG	58.2	481
MAFA	ATGGCCTCGGAGTTGGCC	60.7	TCACATGAAGAAGTCAGCGGCAGTAG	60.4	861
MAFF	ATGGCTGCGGATGGGCTG	61.4	CTAGGAGTAGGCGGCCTGGTTG	61.5	450
MYD88	ATGGCTACGGTACCCGTGGGTG	64	TCACCAAGTGCTGGATGCTATTGCTCGC	64	1131
NDNF	ATGCAGAAGCTGCCTACC	55.1	CTAACAGAACTTCCTCGTTTTCACC	55.7	1651
NFKB2	ATGGTGGAGCAGAAGGAGCC	59.6	GCTTGTCGCTGACGTCCC	59.1	2646
NFKBIZ	ATGGGGGGAGGAAAGCAGCACA	58	TCAAAACAGCGACGCTCTCTGC	65	1192
NLRC5	CTGGCAGAACTAGATCTCTCCAGGAATCAG	60.8	TCACTCAATCTTCCGGAGGTGCTC	60.5	1263
NOS2	ATGCTGTGCCCATGGCAG	59	TTATATTCTTTTGACTTCATGTGGGAACACAG	57	3411
NR4A3	ATGCCCTGTGTGCAAGCGCAG	63.6	TCAGAAGGGCAAGGTGTCCAGAAACAGC	64	1829
PFKFB3	GCCAATTCCCCCACTGTGATAG	57.9	CGGCAGCCATAAGCCACTG	59.2	1189
PKD2L1	ATGGAGGGGAAGTGCTGCTTCTAC	60.7	GCTGCAGCTCATCCTTCTGGC	61	1558
PLA2G5	ATGAATGCTCTCCTTGCATTGGC	58.3	TCACCTGCACTTGCACCTG	58.2	417

Gene	Forward	Tm [°C]	Reverse	Tm [°C]	length (bp)
PLK3	ATGACGGACCTCTCCAGCAACAAAAC	61.1	CTAGGCATCAGCCCACTCCTGG	61.6	1734
PPARG	ATGGTTGACACAGAAATGC	51	CGCCATTAATATAAGTCTTTATAGATTTC	51	1433
PTGS2	ATGACAACAGGATTTGATCGGTATG	55.2	CCTGGATACAGTTCCATAGCATCTAT	55.8	1379
RASD1	ATGAAACTGGCAGCGATGATCAAGAAG	59.4	CTAGCTGATCACACAGCGGTCC	59.7	836
SAAL1	ATGGATCGCAACCCCTCGC	60.6	TTAGGTAGGGACCTTCAGGCTCGG	61.7	1407
SDC4	ATGCCGCTGCCCCGC	63.6	CCAAGGTCGTAGCTGCCCTCGTC	63.6	545
SELE	ATGGTGAATTGTTGGACATACC	53.2	TTAGACATTCTGGCATTCAGTAGT	53.8	1562
SERPINE2	ATGAACTGGCACTTCTCGC	55.5	TCAAGGTTTGTTTATTTGTCCCATAAAC	54.6	1329
SLCO6A1	ATGAAGGGCAACGACGGTAT	56.6	TTACGCATGTATAGCAGATACCATTTCTG	56.7	2037
SNX10	ATGACACCAAAACACGAA	49.5	TCACGATTCTTCAGAAGC	49.4	606
SOCS1	ATGGTAGCGCACAGCAAGGTGTCAGC	61	TTAGATCTGAAACGGGAAGGATT	59	624
SOCS3	ATGGTCACCCACAGCAAGTTCC	63	TTAGAGGGGGGCATCGTACTG	61	626
STEAP1	ATGGAGAAGAGAGAAGGTGATAGTC	55.3	GAACTCTCTCCAGGTCAAAGAG	54.5	759
STEAP4	ATGAATAAAAATTCTTCCAACATAATGGC	53.4	TTACACAGCAGACTTGTTGATAAC	53.3	1418
TGM4	ATGAGCCAAGACAGCGACCTGAAAG	61.1	CTAGTGGGTGACCGTGATGGCC	61.9	2129
TIRAP	ATGGCCGGATGGTTTAGGCGG	62	CTACTGCATTCCACTCGTGGAGCTCC	62.5	666
TNFAIP3	ATGGCTGGCCAACACATCCT	59.7	CTAGCCGTAGATCTGGGGGAACTG	60.4	2421

Gene	Forward	Tm [°C]	Reverse	Tm [°C]	length (bp)
TNIP2	ATGCACCTGGCCGG	55.8	TCATACAGTGAGGACATCTAAGTTCAC	55.9	1506
TOLLIP	ATGGCGACCACCGTCAGTAC	59	CTATGATTCTTCAGTCATCTGAAGCAAGGAG	58	824
TRAF3IP2	ATGGCTTCTGTGTCAGGCACTTTTGTG	61.5	TCATAACGGCACAACCTGGAGTGTTG	61	1598
UPP1	ATGGCTCCTGGTGTCT	52.8	TTATACTTTCCCAAGACTTTTCTTAATG	51.9	942
WDR24	ATGGATGAGAACCTGCTGGCC	59.8	TCAGGTGTACTCGCAGAGGTGG	60.6	2118

Table 4. Gene symbols and transcripts accession numbers for the final 89 genes.

	Gene symbol	Database number
1	ABCG2	ENSGALT00000009304
2	ATF3	XM_004935334.1(X1) XM_419429.4 (X2)
3	BATF3	XM_004935335.1; XM_419428.4
4	BCL2A1	ENSGALG00000006511
5	C3ORF52	XM_004938298.1 (x4); XM_416636.4 (x3); XM_004938299.1 (x5)
6	CCL19	XM_424980.4
7	CCL20	NM_204438.2
8	CCL4 (CCLi3)	NM_204720
9	CCL5 (CCLi4; ah294)	NM_001045832.1
10	CD40	ENSGALT00000039105 NM_204665.2
11	CD72	ENSGALT00000003752
12	CD80	NM_001079739
13	CD83	XM_418929.3
14	CSF1	NM_001193295.1
15	CXCL13L2	XM_420474.4 (X3); XM_004941025.1 (X5); XM_004941024.1 (X4); ENSGALT00000016832
16	CXCLi1	ENSGALT00000019072; NM_205018
17	CXORF21	ENSGALG00000016286
18	DTX2	XM_415763.4
19	EAF2	NM_001006525.1
20	EDN1	XM_418943.4
21	EGR1	ENSGALG00000007669
22	ENSGALG00000002955	ENSGALT00000004669
23	ENSGALG00000005747	ENSGALT00000009229
24	ENSGALG00000011172	ENSGALT00000018214
25	ENSGALG00000015395	ENSGALT00000030421
26	ENSGALG00000022324	ENSGALT00000035899
27	ENSGALG00000025905	ENSGALT00000042716
28	ENSGALG00000027419	ENSGALT00000046220
29	ETS2	NM_205312.1
30	G0S2	ENSGALG00000023933 NM_001190924.3
31	GCH1	NM_205223.1
32	GLUL	NM_205493.1
33	HPS5	XM_004941433.1 (X4); XM_421011.4 (X3); ENSGALT00000010127
34	IFIT5	XM_421662.3
35	IL10RA	NM_001039597.1
36	IL12B	NM_213571.1
37	IL13RA2	ENSGALT00000032399
38	IL17REL	XM_001232492.3 (X1); XM_004937501.1 (X2)
39	IL18	ENSGALG00000007874
40	IL1B	ENSGALG00000000534

	Gene symbol	Database number
41	IL1R2	XM_416914.4
42	IL4I1	ENSGALG00000000081
43	IL6	ENSGALG00000010915
44	IL8 (IL8L2; CXCLi2)	ENSGALG00000026098
45	IRAK2	NM_001030605.1
46	IRF1	NM_205415.1
47	IRF10	NM_204558.1
48	IRF7	ENSGALG00000014297
49	IRG1	ENSGALG00000016919
50	JUN (AP1)	NM_001031289.1
51	LYG2	ENSGALT00000027062; XM_416896.4
52	LYZ	NM_205281.1
53	MADPRT	NM_001033646.1
54	MAFA	NM_205025.1
55	MAFF	ENSGALT00000020063
56	MYD88	NM_001030962.1
57	NDNF	ENSGALT00000019508; XM_004941227.1; XM_004941228.1; XM_004941225.1; XM_420627.4; XM_004941226.1
58	NFKB2	ENSGALT00000009068
59	NFKBIZ	NM_001006254
60	NLRC5	XM_003641889
61	NOS2	NM_204961.1
62	NR4A3	XM_419081.4
63	PFKFB3	XM_004937473.1 (x11); XM_416472.4 (x7); XM_004937470.1 (x8); XM_004937472.1 (x10); XM_004937471.1 (x9)
64	PKD2L1	ENSGALT00000009261
65	PLK3	ENSGALG00000010129
66	PPARG	NM_001001460.1
67	PTGS2	NM_001167718.1; ENSGALT00000008125; ENSGALT00000044290
68	RASD1	ENSGALG00000004860; NM_001044636.1
69	SAAL1	ENSGALT00000045705
70	SDC4	NM_001007869.1
71	SELE	NCBI XM_422246.4
72	SERPINE2	NM_001083920.1
73	SLCO6A1	ENSGALT00000044226
74	SNX10	NM_001030986.1
75	SOCS1	NM_001137648.1
76	SOCS3	NM_204600.1
77	STEAP1	XM_418642.3 (X4); XM_004939274.1 (X6); XM_004939273.1 (X5
78	STEAP4	ENSGALG00000008997; XM_001235256.3
79	TGM4	ENSGALG00000011888
80	TIMD4	NM_001006149.1
81	TIRAP	NM_001024829.1

	Gene symbol	Database number
82	TLR15	NM_001037835.1
83	TLR4	NM_001030693.1
84	TNFAIP3	XM_003640919.2
85	TNIP2	NM_001031166.1
86	TOLLIP	NM_001006471.1
87	TRAF3IP2	XM_419782.2
88	UPP1	ENSGALT00000031003
89	WDR24	ENSGALT00000003862; NM_001030628.1

Table 5. Primers, alignment site and amplicon length for qPCR detection of transcripts of genes of interest using the 96.96 Dynamic Array in BioMark System (Fluidigm).

Gene	Forward	Reverse	exon-intron	amplicon length (bp)
ABCG2	GGAGTAAGGTGCTCTGGTGAAGA	TCTCCCACATTGCCATGTTAGT	fwd 13-14 ex	73
ATF3	CATGAAAACGGAGTTTTCTCCTG	GTCTCCAGCTTTTCTGATTCTTTCTG	fwd 1-2 ex	141
BATF3	GGACAAACTTCACGAGGAATATGAAT	CCTTGGTATGGTCACAAAGTTCAT	fwd 1-2 ex	169
BCL2A1	ACCATATTTACTTTTGGAGGTCTTCTCA	ACCGTTTTCCCAGCCACC	fwd 1-2 ex	159
C3ORF52	GCAACGAGCAAGGAGAGAGATC	AGAGCTAGACTGATGAGAATGACCAG	fwd 1-2 ex	128
CCL19	TGAGATGTGTCCTGCATGTGTATG	GCCATGGGATGGGCTTCT	not ex-in	80
CCL20	AGCTGTCTGGTGAAGTCTGTGATATT	AGGATTTACGCAGGCTTTCAGT	rev 1-2 ex	77
CCL4 (CCLi3)	CTCTGCCCCAGTGGGACC	GACTTCGCGCTCCTTCTTTGT	fwd 1-2 ex	151
CCL5 (CCLi4; ah294)	CTCCGTTTGGGGCTGATACA	TGCTGCCTGTGGGCATTT	rev 2-3 ex	113
CD40	AAAACTGAGCCATGCCACTTCT	CCGGCTTGACTCACAGATCAC	fwd 1-2 ex	102
CD72	GCATGTCTGTCTGAACGGAGACT	AAGATGCACTTGCCATGGTAGA	fwd 4-5 ex	66
CD80	CCCTCTTTGTTACCGCTGACTT	CACACGTTCGTCGTTGAGGA	fwd 1-2 ex	152
CD83	ACCTGAGTGGCATCATCACATTAA	CACGTACAGGTAAAGAAGATGAGCAG	rev 3-4 ex	142
CSF1	TCGTCTGCAGCATCCATGAG	GTGCCGCTCGGTGATGAT	not ex-in	65
CXCL13L2	CCAACGGCAACCTGAACTG	CATATTTCCGCAAGGGAATGA	not ex-in	66
CXCLI1	AAGAGATCATCCTCACCCTGAAGA	GCTTCTTTTTTGCTGACATCCG	fwd 2-3 ex	115
CXORF21	CGCAGATATCACGAGAGAGGAA	TGCAAGAAGCGGCTGATG	one exon gene	70
DTX2	AATGGAGTCAGTGCTCTAAACCTCC	CCTCATCCACAGGAGTACCCTTC	fwd 2-3 ex	138
EAF2	AGGAGACCTTGAAGTTGGCA	GTGGAGTCGAGCCCTCAATA	not ex-in	71

Gene	Forward	Reverse	exon-intron	amplicon length (bp)
EDN1	CGTGTATTTCTGCCACCTGGAT	AGGGCCTCCAAGACCATAGG	not ex-in	76
EGR1	CCTTGCGGCAGACACTTTTC	CTACCATTTGGGGCTGGCT	fwd 1-2 ex	141
ENSGALG00000002955	GGAAACGCTCATCTGGGCTAT	TTTTTTCCGTTCCACTATCTGGAT	fwd 6-7 ex	81
ENSGALG00000005747	TGGATCCGGAGCCTCAAC	GGTCTGCGCAGCTCCAAA	one exon gene	65
ENSGALG00000011172	GGGAGACCTGCACGAAGCT	GCCAATGTTGGTGTAGATGTTACC	rev 1-2 ex	79
ENSGALG00000015395	GGAGACAACTGCAGAAACACAGAG	GAAGATCCTGGTGTCTGCCTCT	fwd 4-5 ex	148
ENSGALG00000022324	TCTCCCCACCGATGCTAGTG	GGCTAAACCAGAGAGAATGAGGAT	one exon gene	77
ENSGALG00000025905	TGACACTGCTTGTTCATCATTTCTT	GCCTCACAGTCTCCTGATTTAATTG	rev 4-5 ex	75
ENSGALG00000027419	GCATCAGGCTCTGTGCAGG	GTTGTCTTCAGCCCCATCCAT	fwd 1-2 ex	144
ETS2	GGAATGCTCAAGCGGCAA	TTCACAGTTGTTGGACTCGTAAGAAG	fwd 1-2 ex	150
G0S2	GAAGGGAATATAGAGGAGGAGAAGAGA	CTCCCGCTTCCGAGGAA	one exon gene	69
GCH1	GACTACAAGTCCAGGAACGCCT	TACCCCACGCATTACCATACAC	not ex-in	122
GLUL	GCCAGTCTGCAGACACAAATCT	GCCAGCCAAACGGATGAC	fwd 2-3 ex	132
HPS5	TCTGTATCTGCTTCGTGTTGCTCT	GCGCCTCCAGCTGCAT	fwd 1-2 ex	100
IFIT5	ATGAGTACCATTTCCAAGAATTCCTT	CAATCTGATCCTCTATTGATTCTTCCA	fwd 1-2 ex	127
IL10RA	CACAAAATCTATGGCACCAACAG	GTGCCCGTGGATCTTCATG	fwd 1-2 ex	66
IL12B	TGGGCAAATGATACGGTCAA	CAGAGTAGTTCTTTGCCTCACATTTT	not ex-in	83
IL13RA2	CTGCAAGGGAAACTGGAATCC	CGTGTGCTCCAGACCCTCATA	fwd 2-3 ex	150
IL17REL	CTTTGCATTGAGGGTTGGCT	TTCCCAAGCTAGGGTTTGTGTC	fwd 7-8 ex	129
IL18	GTGAAATCTGGCAGTGGAATGTACT	CATGCTCTTTCTCACAACACATGTAG	fwd 2-3 ex	133
IL1B	CAGCAGCCTCAGCGAAGAG	CTGTGGTGTGCTCAGAATCCA	fwd 1-2 ex	86
IL1R2	CCAAGAATCTGGGCAAAAGG	CTGGTACAGATATACACTCCTGAGTCTTC	rev 6-7 ex	69

Gene	Forward	Reverse	exon-intron	amplicon length (bp)
IL4I1	GCCCTGCAGTCGGCTGT	CGAGTCCTCGTTCAGCAAATCT	fwd 3-4 ex	155
IL6	GCTTCGACGAGGAGAAATGC	TGAATTCCAGGTAGGTCTGAAAGG	fwd 2-3 ex	72
IL8 (IL8L2; CXCLi2)	CTGTCGCAAGGTAGGACGCT	GCTATGATTTCAACATTCTTGCAGTG	fwd 1-2 ex	149
IRAK2	TGGAAGAGGTTGCCATTGATT	TGTTTCCATAATTTCCAGTACCTCTGT	rev 2-3 ex	95
IRF1	GATCTGGATCAACAAGGATAAGATGATAT	ACTTTATATCTTCCTGTATGGATGGCC	fwd 1-2 ex	129
IRF10	GGGATGCAGAGAAGGATGAGAA	GCTCTCCTCGGCCACATG	fwd 2-3 ex	83
IRF7	GCCTGAAGAAGTGCAAGGTCTT	TGCAAAACACCCTGAAGTCG	not ex-in	123
IRG1	AGTGGCTGTGCACTCAATGG	CACATTGAAAGCTAAGAGCAGGTC	fwd 2-3 ex	151
JUN (AP1)	CGAGCCCCCGGTGTATG	TTGTAGTTGGGTGCAGAGTTGAG	one exon gene	69
LYG2	GTTTAATGCAGGTTGACAAACGG	TGCTGTTCCTTACTCCATGTTGG	fwd 2-3 ex	142
LYZ	TACAGCCTGGGAAACTGGGT	TTCCTGGAGCCTGGGGTC	fwd 1-2 ex	155
MADPRT	ATAAAGGAGGTGGCGATGGA	GTGGATGCAGCCCTGGTACT	one exon gene	66
MAFA	GCAGAACAGGAGGACCTTGAAG	TCTGCTGGACCCGCTTGTAG	one exon gene	68
MAFF	AGCAGAAGATGGAGCTGGAATG	CTGCAGGGCCTCGTACTTG	fwd 1-2 ex	150
MYD88	GGCAGCGTGGAGGAGGA	TCCATGCCCATACGGATCAT	fwd 1-2 ex	150
NDNF	CACCCTGTGATGCTCCCCT	GTTCTCCTGAACCTTCTCCACTG	rev 2-3 ex	81
NFKB2	TGAGGTGCGGTTCTATGAGGAT	GGACGGTCAATTTTGGGCTT	not ex-in	132
NFKBIZ	GGAGCTCATCCGTCTCTTCTTG	GTTGCCATTGTAAGCCTTTGC	rev 9-10 ex	74
NLRC5	AGAGCCCTGGGTATGTAAGTTGAG	TTGCATATCATGATCGCAGTGA	rev 6-7 ex	90
NOS2	CAGCGGAAGGAGACAAACAGAG	AACTCTTCCAGGACCTCCAGG	not ex-in	109
NR4A3	CCAGCTTCAGCACCTTCATG	GAGGCGGATTGCATTTGG	not ex-in	82
PFKFB3	AACAAAAGTTTTCAATGTAGGGGAGT	CATCCCTCAGGGCAGCC	fwd 1-2 ex	134

Gene	Forward	Reverse	exon-intron	amplicon length (bp)
PKD2L1	GTTCTGAGGTTAGTGGTTGAGTTTCC	CGTATAAGCTTGACTGTCCGGATT	fwd 5-6 ex	80
PLK3	AAGGCCAAGAAGGGCTCTCT	CGGCAGATGGACGTCTTCA	fwd 7-8 ex	80
PPARG	TTTCAAGCATTTCTTCACCACACT	TGATTGCACTTTGGCAATCCT	fwd 1-2 ex	111
PTGS2	CTGTACAACACCGGAATTCTTCAC	GGAAATGTTGTTGATGATGTTCCA	fwd 1-2 ex	118
RASD1	TCACAGGTGACGTTTTCATCCT	GACCAGCGGCACCTCG	fwd 1-2 ex	143
SAAL1	GCAGCCAAACAAGTCCGC	GGAGCCTGCACAATAGCCTG	fwd 7-8 ex; rev 8-9	110
SDC4	AAACCTGTCCAACAAGATCTCCAT	CTGCAATGAGAGCTGTAAGAACTTCT	rev 4-5 ex	86
SELE	GCCGGGTTGTGAATTTGTTGA	CATTGAGCTGTGCATGACGA	fwd 1-2 ex	117
SERPINE2	ACACAACACCGCTCTCTGCTATC	ACCTGCACTCTTTTGGCTACCAT	not ex-in	91
SLCO6A1	TGGAACAGGCATGCTACATAACTT	ATCTCTGCCACAAACAGGGTAGTAT	fwd 8-9 ex	91
SNX10	GGTACGAGACCCCAGAACACA	ACAAATTCTCGGAAGCGTCG	not ex-in	132
SOCS1	GCACGCACTTCCGAACCTT	AAGCCGCAGGCATCCA	one exon gene	82
SOCS3	ACCCCAAACGCACCTACTACA	TGCCCGTTGACAGTCTTACG	one exon gene	121
STEAP1	GTCGCCAATCCACCAGAAGTA	CACCTGGGGAACAAATCCTG	not ex-in	113
STEAP4	GGATGCAAGTCGGCAGGT	CAGCCAAGAGAGATCCTTGATCTAA	fwd 1-2 ex	116
TGM4	GTCAAGGTACTTTCCAGTGTGGC	GCGTTCACTTCCGCGTACA	fwd 8-9 ex	103
TIMD4	CCTTCATAGTGCACACCATGTCA	TGCACTTGGAGTTTGGGCA	fwd 1-2 ex	147
TIRAP	CTGAAGGACATTGACAGGAAGGAT	TCTTCCAGGTAGCGCAGCA	rev 2-3 ex	122
TLR15	TTCAGACAAAGAAGAACAGAGGAAAA	TCGGTGCTCCACACAAGTCA	one exon gene	79
TLR4	GTCCGTGCCTGGAGGTCAT	CAATTTCAGACTGTTGAAACTGAGGT	fwd 1-2 ex	125
TNFAIP3	CAGATCCCACAGAGCCTCCTT	GGCGGCTCATTGCAGTTG	fwd 1-2 ex	157
TNIP2	GTAGCTCATGTGGAAGACTTAAATGC	GTGCTGAAGGTATGCTGTGCTG	fwd 3-4 ex	136

Gene	Forward	Reverse	exon-intron	amplicon length (bp)
TOLLIP	TGATGAGCGAGCCTTTTCAAT	CCTTCTTTGTCATCACCCTGC	fwd 3-4 ex	132
TRAF3IP2	ATTTGCCGGAAGAGTTGCG	GCAGGAAGTTCACAAATTTCATGAC	not ex-in	84
UPP1	CATGTACAAAGTGGGACCCGT	CGAGGTGCCAATGCGAATA	not ex-in	139
WDR24	CTCTGGCCAGTCGGAGAGTG	GCCGTTGTGGGCTGTGA	fwd 1-2 ex	148

Table 6. Primers designed for seven reference genes used in BioMark System (Fluidigm).

Gene symbol	Sequence	exon-intron	Amplicon length [bp]	Accession number
ACTB	F: CCAGACATCAGGGTGTGATGG R: CTCCATATCATCCCAGTTGGTGA	FWD 1-2 exon	137	AJ719605
B2M	F: TACTCCGACATGTCCTTCAACG R: TCAGAACTCGGGATCCCACTT	REV 2-3 exon	150	AB162661
GAPDH	F: GAAGGCTGGGGCTCATCTG R: CAGTTGGTGGTGCACGATG	FWD 3-4 exon	150	AF047874
GUSB	F: GGCAGACTGGTCCTGTTGTTG R: GGGTCCTGAGTGATGTCATTGA	FWD 1-2 exon	64	AJ720880
TBP	F: AGCTCTGGGATAGTGCCACAG R: ATAATAACAGCAGCAAAACGCTTG	REV 3-4 exon	134	AF221563
TUBAT	F: CAGCTCTCAGTGGCTGAAATCA R: CCTTGTTGCGGGTCACACTT	Do not span ex-in boundaries	77	M16030
r28S	F: GGCGAAGCCAGAGGAAACT R: GACGACCGATTTGCACGTC	Ribosomal RNA	62	FM165415

Table 7. Slope and reaction efficiencies for reference genes tested in tissue samples from birds reared on the pedigree farm.

Mean quantification cycle (Cq) values

ACTB	Bursa	Spleen	CT	Ileum	Blood		Dilution factor	Cq	Log10 (dilution factor)	Slope	Efficiency
Bird 1	19.9	16.2	18.3	19.6	23.6		1	19.5	0	-3.92	1.80
Bird 2	18.1	16.4	18.9	19.4	22.4		0.1	23.4	-1		
Bird 3	17.4	16.6	18.1	18.8	21.7		0.01	26.5	-2		
Bird 4	19.8	18.8	18.4	20.1	22.0		0.001	30.2	-3		
Bird 5	19.3	17.0	18.4	19.9	22.1		0.0001	35.8	-4		
B2M	**Bursa**	**Spleen**	**CT**	**Ileum**	**Blood**		Dilution factor	Cq	Log10 (dilution factor)	Slope	Efficiency
Bird 1	22.4	20.0	18.9	21.0	26.0		1	21.3	0	-3.58	1.90
Bird 2	20.8	19.4	21.0	21.4	24.8		0.1	24.9	-1		
Bird 3	19.4	19.5	20.4	20.3	25.0		0.01	28.7	-2		
Bird 4	20.7	21.5	20.6	22.2	24.8		0.001	32.3	-3		
Bird 5	21.4	20.3	21.0	21.8	25.5		0.0001	35.5	-4		
GAPDH	**Bursa**	**Spleen**	**CT**	**Ileum**	**Blood**		Dilution factor	Cq	Log10 (dilution factor)	Slope	Efficiency
Bird 1	24.0	21.0	22.6	22.4	28.1		1	23.5	0	-3.60	1.89
Bird 2	22.7	21.5	22.7	22.4	27.5		0.1	27.4	-1		
Bird 3	22.5	21.8	22.4	21.8	26.9		0.01	30.8	-2		
Bird 4	23.7	23.8	22.7	23.5	26.9		0.001	34.4	-3		
Bird 5	23.8	21.9	23.1	23.0	27.4						
GUSB	**Bursa**	**Spleen**	**CT**	**Ileum**	**Blood**		Dilution factor	Cq	Log10 (dilution factor)	Slope	Efficiency
Bird 1	23.0	26.2	27.7	28.3	31.9		1	27.2	0	-3.36	1.98
Bird 2	23.1	27.3	27.7	28.0	34.0		0.1	30.6	-1		
Bird 3	24.0	27.1	27.0	28.2	32.7		0.01	34.0	-2		
Bird 4	25.5	28.4	27.5	28.8	32.6		0.001		-3		
Bird 5	22.9	27.7	28.1	28.5	35.5						

TBP	Bursa	Spleen	CT	Ileum	Blood
Bird 1	28.7	25.7	28.0	28.9	33.3
Bird 2	27.3	26.3	28.2	29.0	32.6
Bird 3	27.1	26.7	27.9	28.1	31.5
Bird 4	28.4	28.7	28.0	29.5	31.7
Bird 5	28.4	26.7	28.5	29.2	32.1
TUBAT	Bursa	Spleen	CT	Ileum	Blood
Bird 1	24.7	28.6	33.0	34.2	36.5
Bird 2	23.9	31.5	33.9	34.2	38.1
Bird 3	25.2	31.3	33.7	33.8	35.3
Bird 4	27.0	32.6	31.9	35.2	36.0
Bird 5	23.7	31.7	33.6	34.2	38.2
r28S	Bursa	Spleen	CT	Ileum	Blood
Bird 1	11.9	9.8	10.8	10.5	16.6
Bird 2	10.8	9.8	10.9	10.9	15.5
Bird 3	10.7	10.0	10.8	10.4	14.6
Bird 4	11.7	11.2	11.0	12.2	15.3
Bird 5	11.8	10.3	11.1	10.7	14.5

Dilution factor	Cq	Log10 (dilution factor)	Slope	Efficiency
1	28.9	0	-3.83	1.82
0.1	32.8	-1		
Dilution factor	Cq	Log10 (dilution factor)	Slope	Efficiency
1	28.5	0	-3.23	2.04
0.1	31.8	-1		
0.01	35.0	-2		
Dilution factor	Cq	Log10 (dilution factor)	Slope	Efficiency
1	11.7	0	-3.67	1.87
0.1	15.5	-1		
0.01	18.9	-2		
0.001	22.9	-3		

Table 8. Slope and efficiencies for reference genes tested in tissue samples from birds reared on the sibling test farm.

Mean quantification cycle (Cq) values

ACTB	Bursa	Spleen	CT	Ileum	Blood
Bird 1	18.4	17.7	18.3	20.4	17.6
Bird 2	17.7	18.2	18.5	18.0	16.2
Bird 3	18.7	18.7	18.0	18.6	19.9
Bird 4	18.5	16.9	16.6	19.5	16.8
Bird 5	19.7	19.3	20.1	21.2	18.7

Dilution factor	Cq	Log10 (dilution factor)	Slope	Efficiency
1	19.4	0	-3.29	2.01
0.1	22.5	-1		
0.01	26.2	-2		
0.001	29.9	-3		
0.0001	32.6	-4		
0.00001	35.6	-5		

B2M	Bursa	Spleen	CT	Ileum	Blood
Bird 1	18.9	18.9	19.2	21.3	19.3
Bird 2	19.7	20.7	20.0	19.1	18.7
Bird 3	19.4	19.6	18.5	19.9	21.6
Bird 4	19.4	19.6	18.0	19.3	19.3
Bird 5	22.1	22.9	23.8	25.3	22.9

Dilution factor	Cq	Log10 (dilution factor)	Slope	Efficiency
1	20.9	0	-3.96	1.79
0.1	24.5	-1		
0.01	28.3	-2		
0.001	32.9	-3		

GAPDH	Bursa	Spleen	CT	Ileum	Blood
Bird 1	22.5	23.2	22.3	22.9	21.9
Bird 2	22.7	22.7	21.7	35.4	22.0
Bird 3	22.6	21.8	22.8	24.3	23.9
Bird 4	21.9	21.9	22.3	23.2	21.1
Bird 5	24.7	24.7	25.3	25.7	24.6

Dilution factor	Cq	Log10 (dilution factor)	Slope	Efficiency
1	23.6	0	-3.07	2.11
0.1	27.1	-1		
0.01	35.9	-2		
0.001	33.4	-3		
0.0001	35.9	-4		

GUSB	Bursa	Spleen	CT	Ileum	Blood
Bird 1	27.9	27.3	26.9	27.2	26.4
Bird 2	26.4	26.2	27.0	27.7	26.7

Dilution factor	Cq	Log10 (dilution factor)	Slope	Efficiency
1	26.9	0	-3.26	2.03
0.1	30.5	-1		

Bird 3	27.1	26.8	28.2	28.2	28.7		0.01	33.6	-2		
Bird 4	27.4	27.6	26.6	26.5	27.0		0.001	36.7	-3		
Bird 5	30.1	30.3	30.8	31.4	29.2						
TBP	Bursa	Spleen	CT	**Ileum**	Blood		Dilution factor	Cq	Log10 (dilution factor)	Slope	Efficiency
Bird 1	27.1	27.9	27.8	29.8	26.6		1	29.1	0	-3.24	2.03
Bird 2	27.3	28.4	28.1	26.9	26.7		0.1	32.1	-1		
Bird 3	27.7	27.5	26.9	28.3	29.3		0.01	35.5	-2		
Bird 4	27.5	27.0	26.9	29.3	27.4						
Bird 5	30.3	30.1	31.3	32.6	31.3						
TUBAT	Bursa	Spleen	CT	**Ileum**	Blood		Dilution factor	Cq	Log10 (dilution factor)	Slope	Efficiency
Bird 1	32.5	32.5	30.1	27.3	27.7		1	28.4	0	-3.68	1.87
Bird 2	26.9	27.0	27.7	30.2	31.2		0.1	31.8	-1		
Bird 3	30.2	26.9	33.5	33.3	33.9		0.01	35.7	-2		
Bird 4	33.2	30.1	32.8	29.6	30.0						
Bird 5	35.5	36.4	35.4	36.6	34.4						
r28S	Bursa	Spleen	CT	**Ileum**	Blood		Dilution factor	Cq	Log10 (dilution factor)	Slope	Efficiency
Bird 1	10.7	10.7	10.9	13.4	11.0		1	11.7	0	-3.67	1.87
Bird 2	10.8	12.7	11.2	10.6	10.4		0.1	15.5	-1		
Bird 3	10.8	11.0	10.5	10.8	12.8		0.01	18.9	-2		
Bird 4	10.6	10.1	10.0	13.4	10.7		0.001	22.8	-3		
Bird 5	10.8	11.2	13.4	13.8	10.3						

Table 9. Transformation of Cq values into quantities by ΔCt method - samples from pedigree farm.

ACTB		Bursa	Spleen	CT	Ileum	Blood		Bursa	Spleen	CT	Ileum	Blood
ΔCq	Bird 1	-2.52	0	-0.16	-0.86	-1.84	E-ΔCq	0.23	1	0.91	0.60	0.34
	Bird 2	-0.69	-0.23	-0.81	-0.62	-0.63		0.67	0.87	0.62	0.69	0.69
	Bird 3	0	-0.39	0	0	0		1	0.79	1	1	1
	Bird 4	-2.34	-2.62	-0.30	-1.32	-0.31		0.25	0.22	0.84	0.46	0.84
	Bird 5	-1.90	-0.88	-0.33	-1.17	-0.37		0.33	0.60	0.83	0.50	0.80
B2M		Bursa	Spleen	CT	Ileum	Blood		Bursa	Spleen	CT	Ileum	Blood
ΔCq	Bird 1	-2.92	-0.64	0	-0.62	-1.15	E-ΔCq	0.15	0.66	1	0.67	0.48
	Bird 2	-1.39	0	-2.11	-1.02	0		0.41	1	0.26	0.52	1
	Bird 3	0	-0.15	-1.48	0	-0.17		1	0.91	0.39	1	0.89
	Bird 4	-1.23	-2.12	-1.66	-1.82	-0.01		0.45	0.25	0.34	0.31	0.99
	Bird 5	-1.99	-0.91	-2.03	-1.46	-0.67		0.28	0.56	0.27	0.39	0.65
GAPDH		Bursa	Spleen	CT	Ileum	Blood		Bursa	Spleen	CT	Ileum	Blood
ΔCq	Bird 1	-1.46	0	-0.22	-0.63	-1.21	E-ΔCq	0.39	1	0.87	0.67	0.46
	Bird 2	-0.17	-0.58	-0.32	-0.63	-0.59		0.90	0.69	0.82	0.67	0.69
	Bird 3	0	-0.81	0	0	0		1	0.59	1	1	1
	Bird 4	-1.19	-2.81	-0.30	-1.69	-0.04		0.47	0.17	0.83	0.34	0.97
	Bird 5	-1.30	-0.91	-0.76	-1.17	-0.51		0.44	0.56	0.62	0.47	0.72
GUSB		Bursa	Spleen	CT	Ileum	Blood		Bursa	Spleen	CT	Ileum	Blood
ΔCq	Bird 1	-0.13	0	-0.71	-0.27	0	E-ΔCq	0.92	1	0.62	0.83	1
	Bird 2	-0.18	-1.15	-0.69	0	-2.03		0.88	0.46	0.62	1	0.25
	Bird 3	-1.06	-0.90	0	-0.19	-0.76		0.48	0.54	1	0.88	0.59
	Bird 4	-2.63	-2.27	-0.48	-0.80	-0.63		0.16	0.21	0.72	0.58	0.65
	Bird 5	0	-1.48	-1.14	-0.45	-3.54		1	0.36	0.46	0.74	0.09
TBP		Bursa	Spleen	CT	Ileum	Blood		Bursa	Spleen	CT	Ileum	Blood

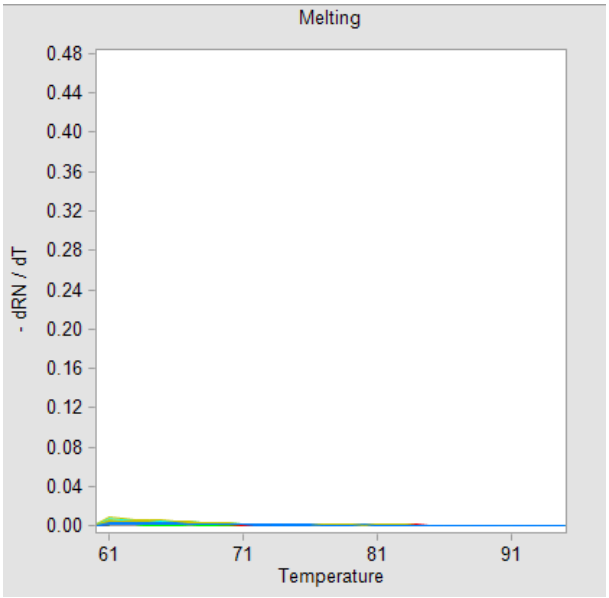
ΔCq	Bird 1	-1.66	0	-0.13	-0.79	-1.76		E-ΔCq	0.37	1	0.92	0.62	0.35
	Bird 2	-0.17	-0.65	-0.33	-0.93	-1.04			0.90	0.68	0.82	0.57	0.54
	Bird 3	0	-0.95	0	0	0			1	0.56	1	1	1
	Bird 4	-1.28	-3.03	-0.10	-1.45	-0.20			0.46	0.16	0.94	0.42	0.88
	Bird 5	-1.31	-1.02	-0.64	-1.13	-0.60			0.45	0.54	0.68	0.51	0.70
TUBAT		Bursa	Spleen	CT	Ileum	Blood			Bursa	Spleen	CT	Ileum	Blood
ΔCq	Bird 1	-1.08	0	-1.13	-0.41	-1.23		E-ΔCq	0.46	1	0.45	0.75	0.42
	Bird 2	-0.26	-2.88	-1.95	-0.39	-2.89			0.83	0.13	0.25	0.76	0.13
	Bird 3	-1.56	-2.65	-1.76	0	0			0.33	0.15	0.29	1	1
	Bird 4	-3.35	-3.92	0	-1.41	-0.74			0.09	0.06	1	0.37	0.59
	Bird 5	0	-3.02	-1.68	-0.33	-2.98			1	0.12	0.30	0.79	0.12
r28S		Bursa	Spleen	CT	Ileum	Blood			Bursa	Spleen	CT	Ileum	Blood
ΔCq	Bird 1	-1.20	-0.01	-0.05	-0.10	-2.08		E-ΔCq	0.47	0.995	0.97	0.94	0.27
	Bird 2	-0.13	0	-0.17	-0.53	-0.99			0.92	1	0.90	0.72	0.54
	Bird 3	0	-0.19	0	0	-0.07			1	0.89	1	1	0.96
	Bird 4	-1.00	-1.40	-0.22	-1.77	-0.76			0.54	0.41	0.87	0.33	0.62
	Bird 5	-1.08	-0.58	-0.34	-0.24	0			0.51	0.70	0.81	0.86	1

Table 10. Transformation of Cq values into quantities by ΔCt method - samples from sibling test farm.

ACTB		Bursa	Spleen	CT	Ileum	Blood		Bursa	Spleen	CT	Ileum	Blood
ΔCq	Bird 1	-0.63	-0.87	-1.69	-2.40	-1.39	E-ΔCq	0.65	0.545	0.31	0.19	0.38
	Bird 2	0	-1.27	-1.93	0	0		1	0.41	0.26	1	1
	Bird 3	-0.83	-1.87	-1.43	-0.56	-3.72		0.56	0.27	0.37	0.68	0.07
	Bird 4	-0.73	0	0	-1.51	-0.60		0.60	1	1	0.35	0.66
	Bird 5	-1.94	-2.38	-3.50	-3.12	-2.49		0.26	0.19	0.09	0.11	0.18
B2M		Bursa	Spleen	CT	Ileum	Blood		Bursa	Spleen	CT	Ileum	Blood
ΔCq	Bird 1	0	0	-1.19	-2.21	-0.61	E-ΔCq	1	1	0.50	0.28	0.70
	Bird 2	-0.82	-1.79	-1.94	0	0		0.62	0.35	0.32	1	1
	Bird 3	-0.43	-0.69	-0.44	-0.87	-2.88		0.78	0.67	0.78	0.60	0.19
	Bird 4	-0.44	-0.76	0	-0.29	-0.60		0.77	0.64	1	0.85	0.70
	Bird 5	-3.16	-4.01	-5.80	-6.30	-4.23		0.16	0.10	0.03	0.03	0.09
GAPDH		Bursa	Spleen	CT	Ileum	Blood		Bursa	Spleen	CT	Ileum	Blood
ΔCq	Bird 1	-0.62	-1.44	-0.58	0	-0.88	E-ΔCq	0.69	0.42	0.70	1	0.59
	Bird 2	-0.75	-0.91	0	-1.11	-0.90		0.63	0.58	1	0.51	0.58
	Bird 3	-0.72	0	-1.09	-1.46	-2.84		0.65	1	0.52	0.41	0.18
	Bird 4	0	-0.15	-0.58	-0.31	0		1	0.91	0.70	0.83	1
	Bird 5	-2.77	-2.90	-3.59	-2.80	-3.56		0.19	0.17	0.11	0.18	0.12
GUSB		Bursa	Spleen	CT	Ileum	Blood		Bursa	Spleen	CT	Ileum	Blood
ΔCq	Bird 1	-1.51	-1.09	-0.29	-0.74	0	E-ΔCq	0.34	0.46	0.81	0.59	1
	Bird 2	0	0	-0.39	-1.24	-0.28		1	1	0.76	0.42	0.82
	Bird 3	-0.68	-0.61	-1.66	-1.72	-2.23		0.62	0.65	0.31	0.30	0.21
	Bird 4	-0.97	-1.46	0	0	-0.57		0.50	0.36	1	1	0.67
	Bird 5	-3.69	-4.08	-4.25	-4.88	-2.75		0.07	0.06	0.05	0.03	0.14
TBP		Bursa	Spleen	CT	Ileum	Blood		Bursa	Spleen	CT	Ileum	Blood
ΔCq	Bird 1	0	-0.80	-0.93	-2.83	0	E-ΔCq	1	0.57	0.52	0.13	1
	Bird 2	-0.22	-1.38	-1.18	0	-0.19		0.86	0.37	0.43	1	0.87

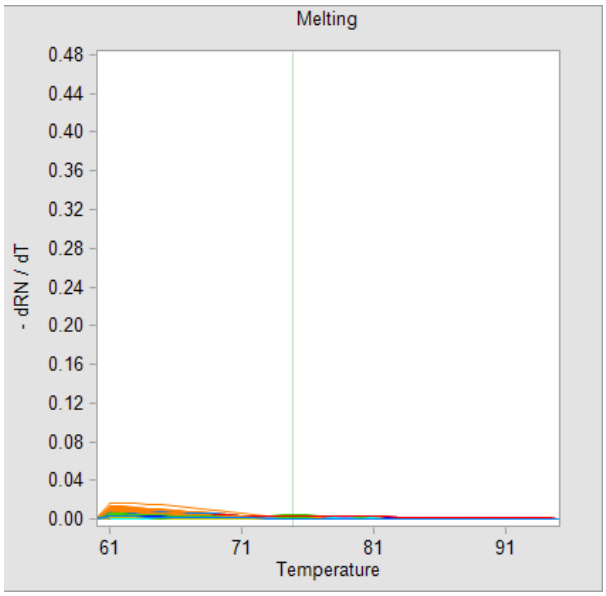
	Bird 3	-0.65	-0.50	-0.06	-1.31	-2.72		0.63	0.70	0.96	0.39	0.14
	Bird 4	-0.40	0	0	-2.38	-0.84		0.76	1	1	0.18	0.55
	Bird 5	-3.24	-3.09	-4.40	-5.63	-4.76		0.10	0.11	0.04	0.02	0.03
TUBAT		Bursa	Spleen	CT	Ileum	Blood		Bursa	Spleen	CT	Ileum	Blood
ΔCq	Bird 1	-5.61	-5.56	-2.38	0	0	E-ΔCq	0.03	0.03	0.23	1	1
	Bird 2	0	-0.07	0	-2.89	-3.58		1	0.96	1	0.16	0.11
	Bird 3	-3.24	0	-5.81	-5.95	-6.19		0.13	1	0.03	0.02	0.02
	Bird 4	-6.26	-3.23	-5.08	-2.31	-2.29		0.02	0.13	0.04	0.24	0.24
	Bird 5	-8.63	-9.44	-7.68	-9.24	-6.77		0.00	0.00	0.01	0.00	0.01
r28S		Bursa	Spleen	CT	Ileum	Blood		Bursa	Spleen	CT	Ileum	Blood
ΔCq	Bird 1	-0.07	-0.59	-0.87	-2.81	-0.67	E-ΔCq	0.96	0.69	0.58	0.17	0.66
	Bird 2	-0.14	-2.57	-1.14	0	-0.09		0.92	0.20	0.49	1	0.94
	Bird 3	-0.20	-0.87	-0.52	-2.39	-2.43		0.88	0.58	0.72	0.22	0.22
	Bird 4	0	0	0	-2.75	-0.36		1	1	1	0.18	0.80
	Bird 5	-0.13	-1.10	-3.35	-3.14	0		0.92	0.50	0.12	0.14	1

a)



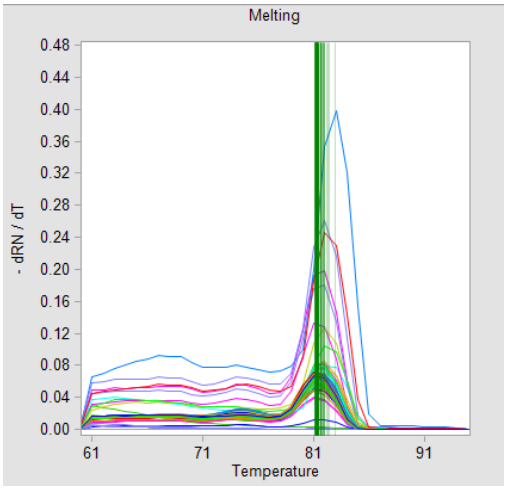
Reference genes NTC

b)

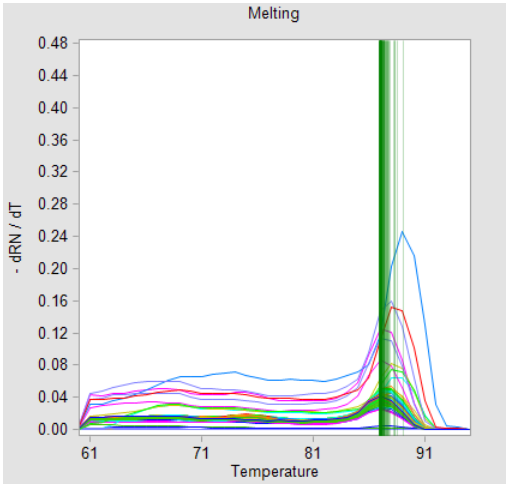


All test genes NTC

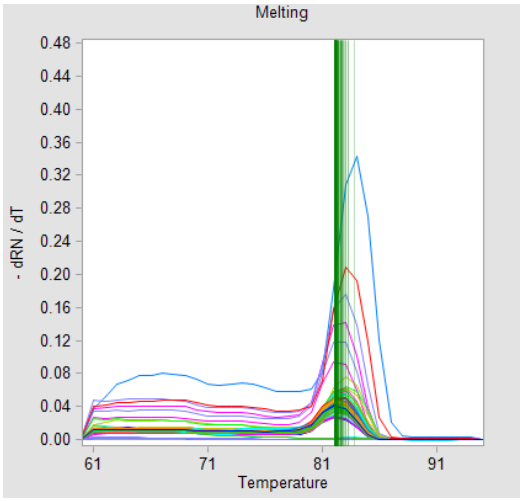
Figure 2. Melting curves for No Template Control (NTC) samples from qPCR analysis using 96.96 Dynamic Array platform; a) for reference genes, b) all genes tested.



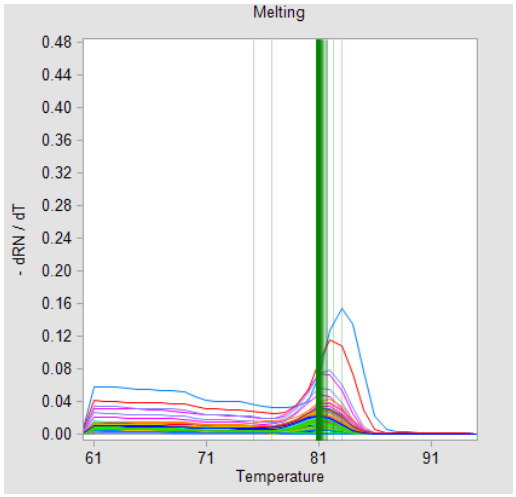
ACTB



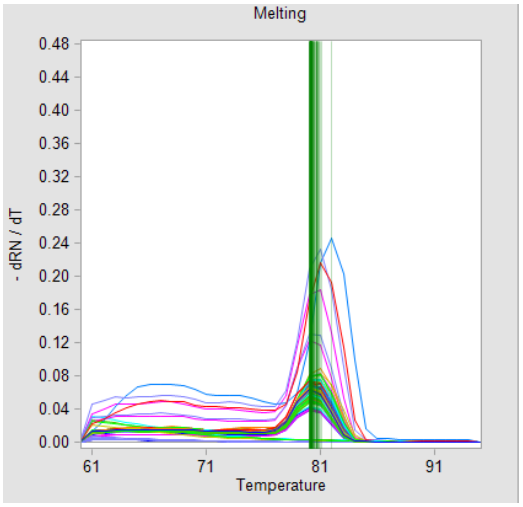
B2M



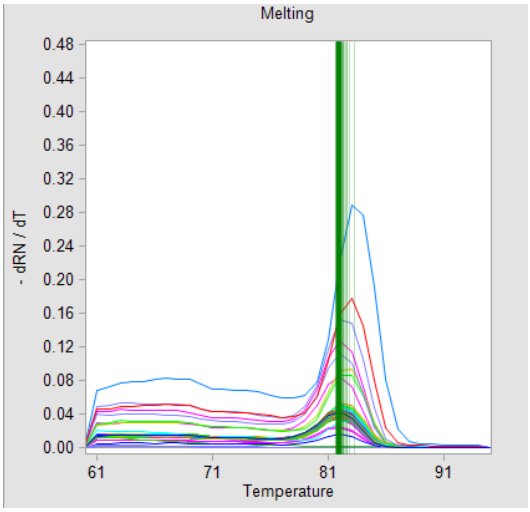
GAPDH



GUSB

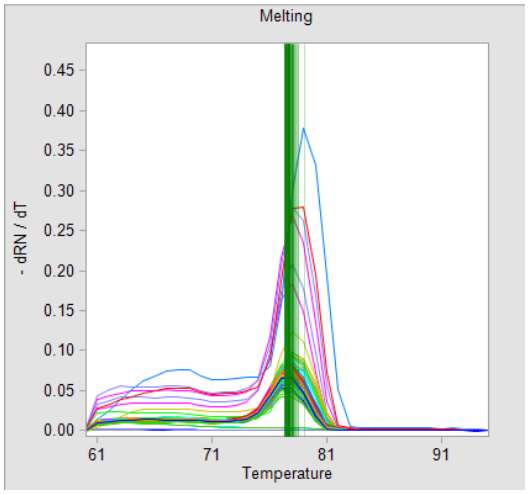


TBP

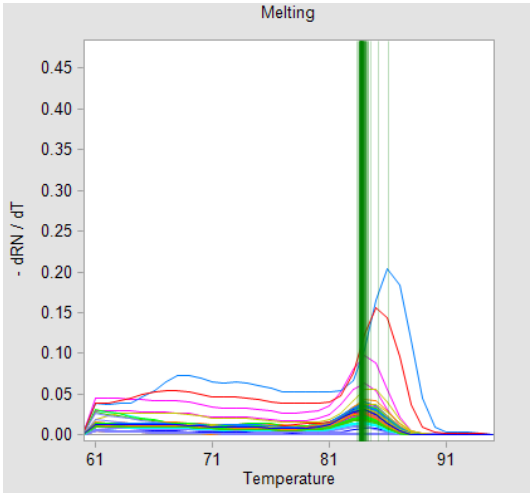


r28S

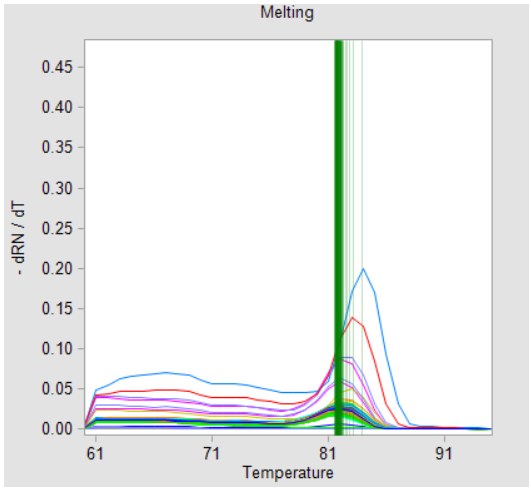
Figure 3. Melting curves for all reference genes for all conditions from analysis using 96.96 Dynamic Array.



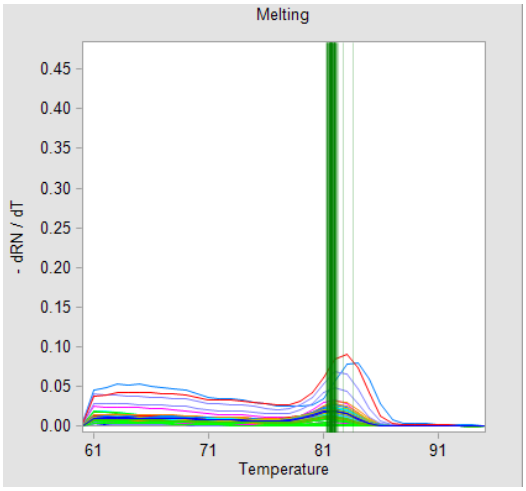
C3ORF52



CCL19



CSF1



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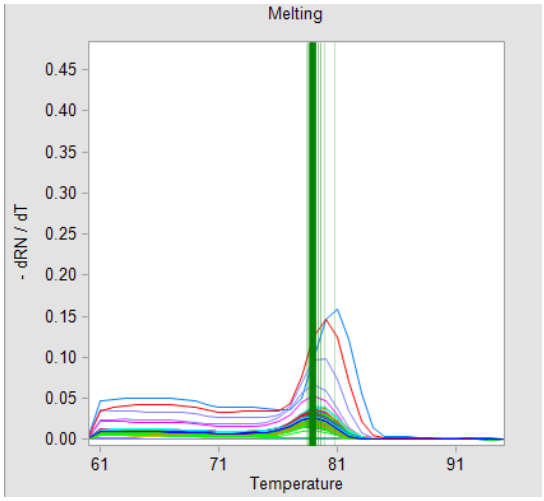

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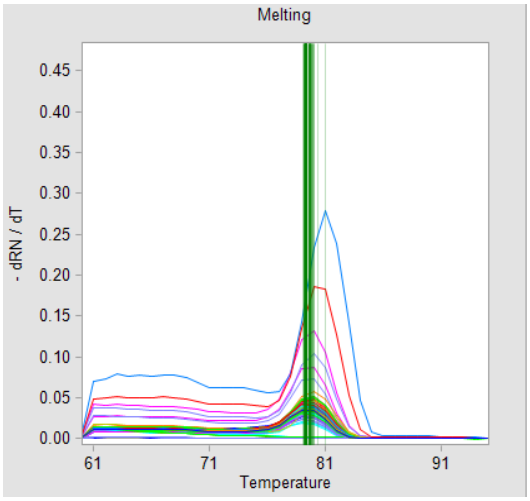
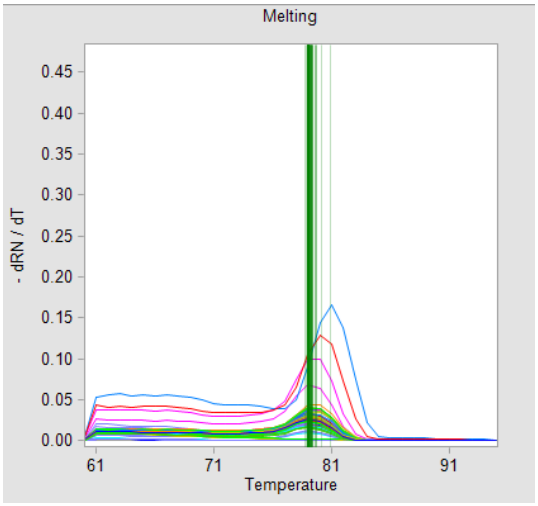


EAF2

Figure 4. Melting curves for genes where primers do not span exon/exon boundaries and with introns < 500 bp.



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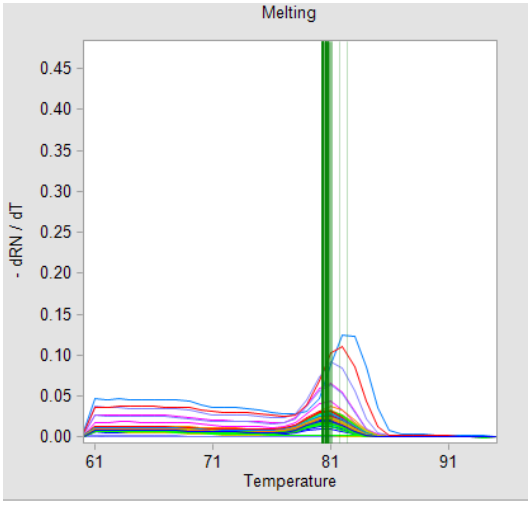

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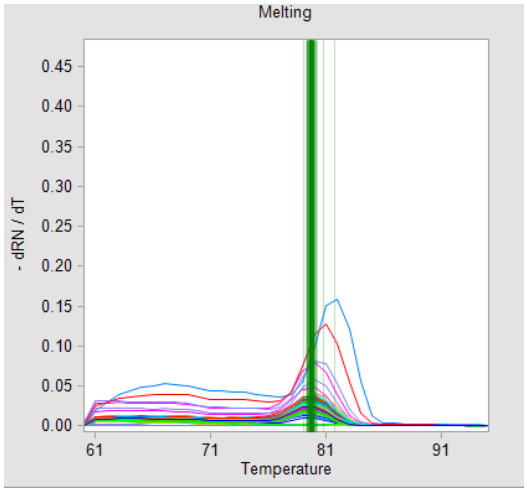

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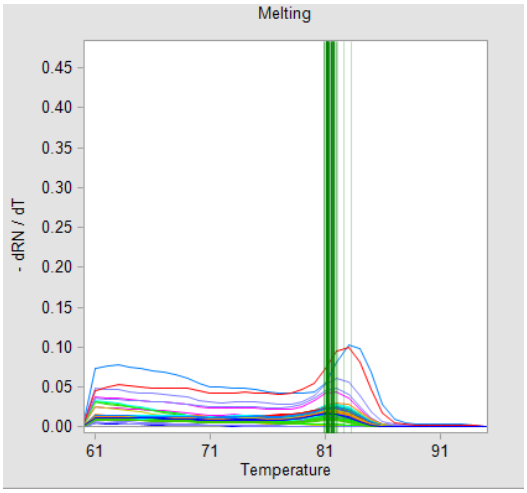
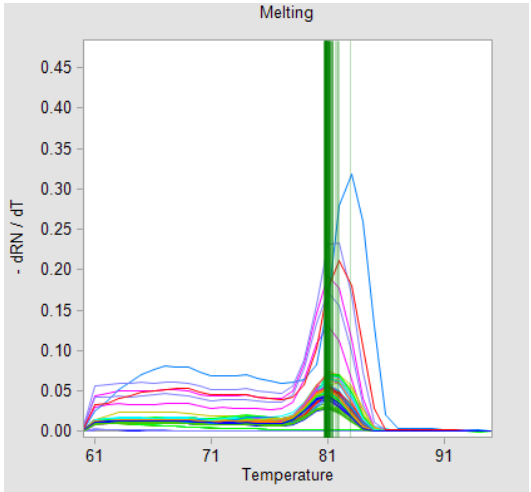
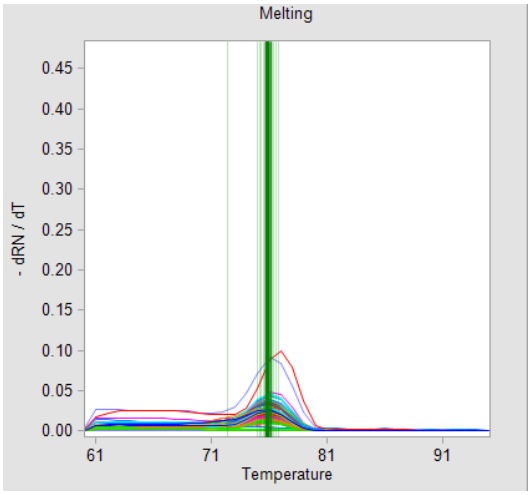


G0S2

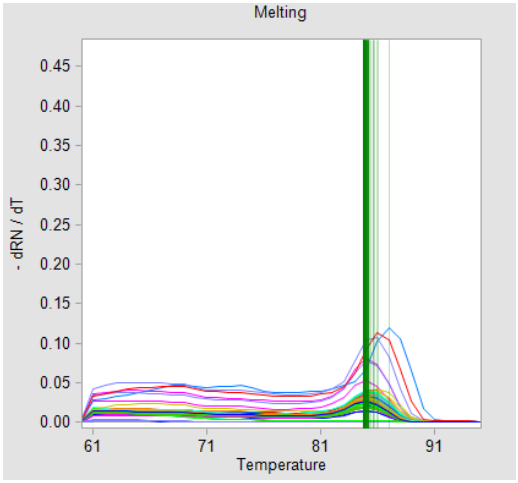


GCH1

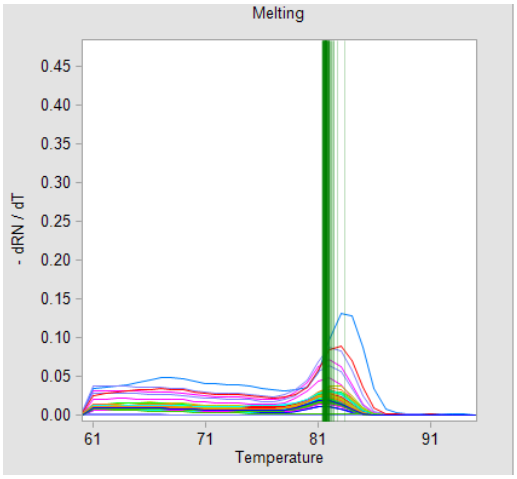


IL12B

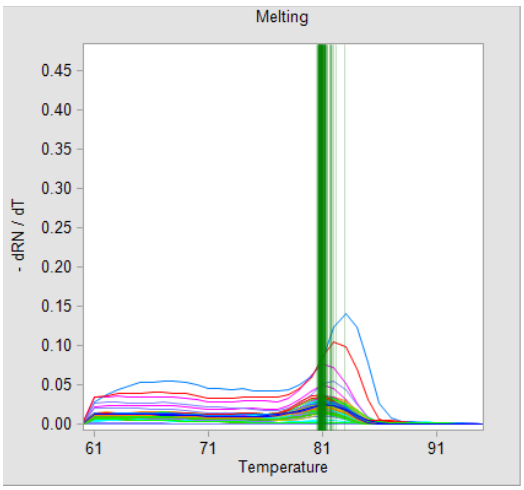
Figure 4 continued



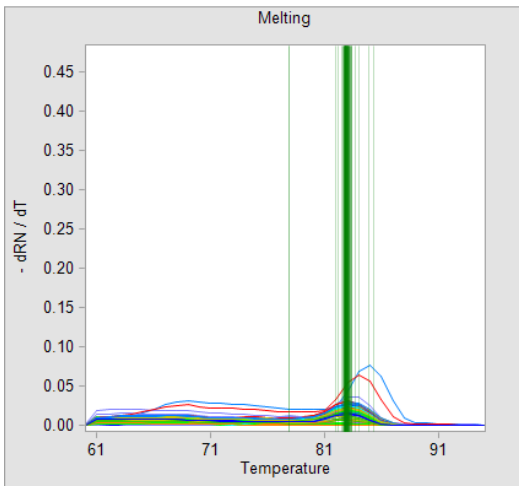
IRF7



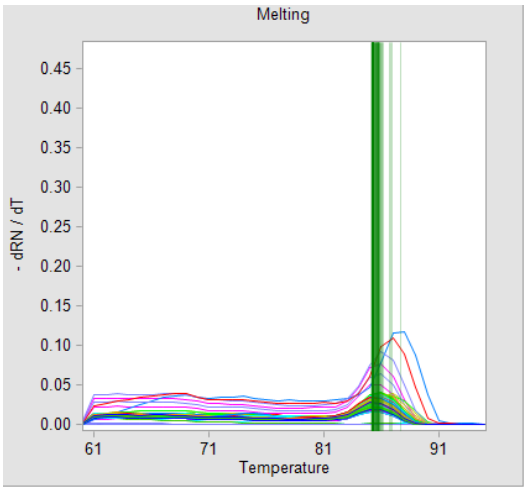
JUN



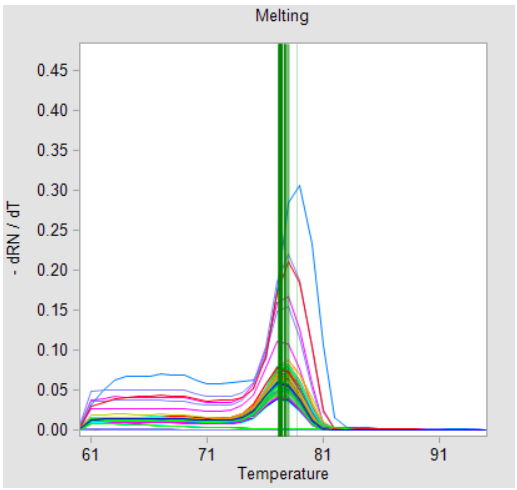
MADPRT



MAFA

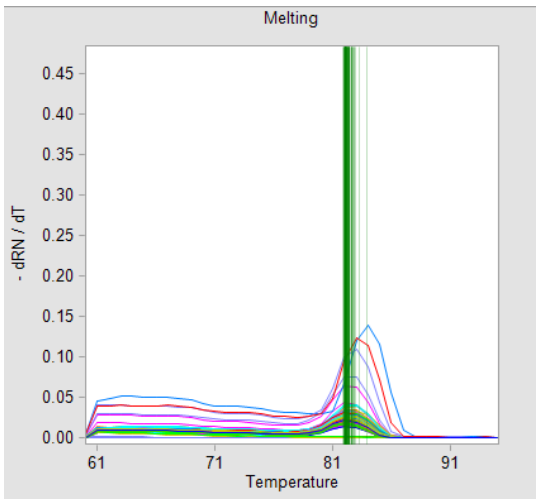


NFKB2

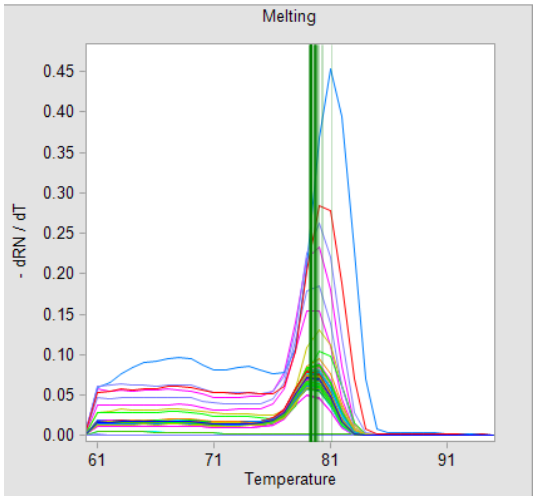


NOS2

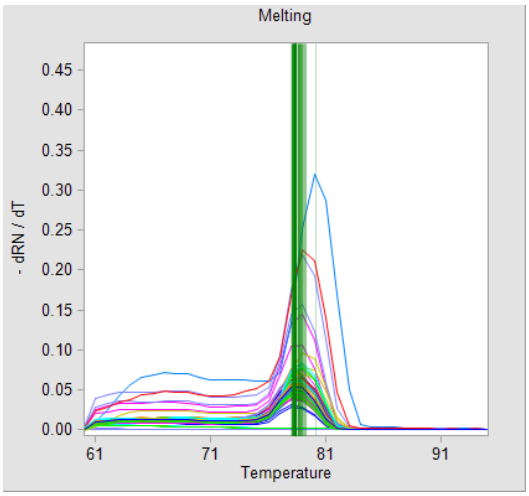
Figure 4 continued



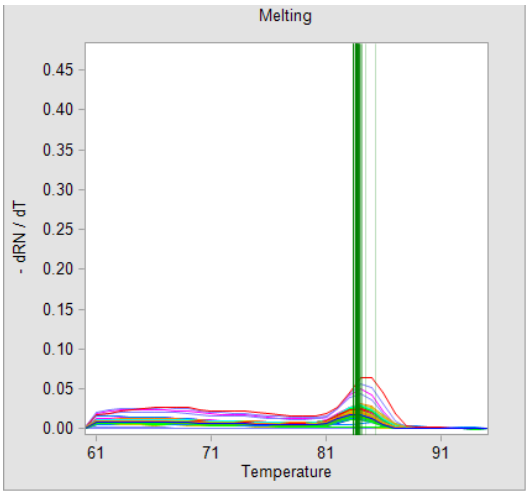
NR4A3



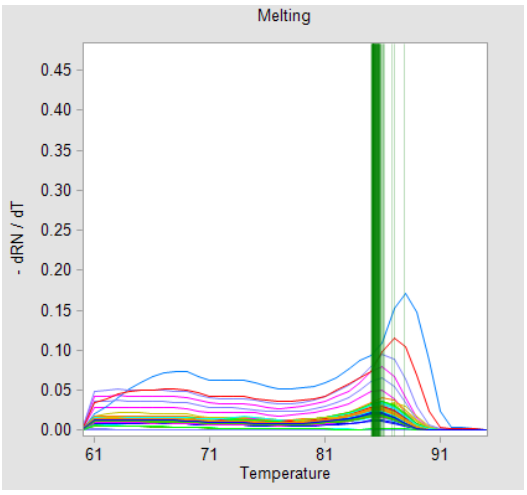
SERPINE2



SNX10



SOCS1



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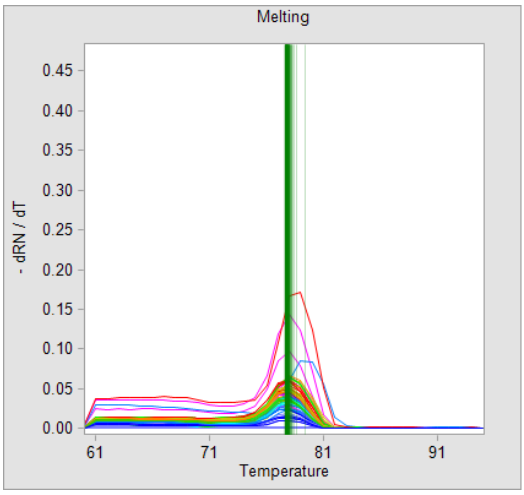
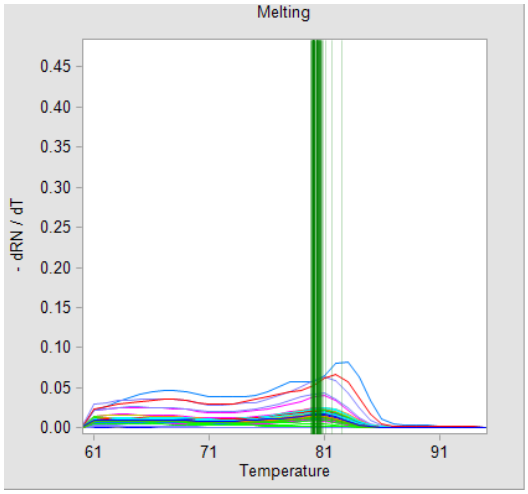


STEAP1

Figure 4 continued



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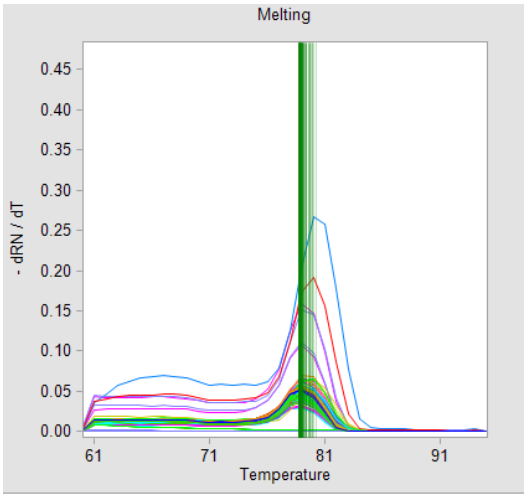

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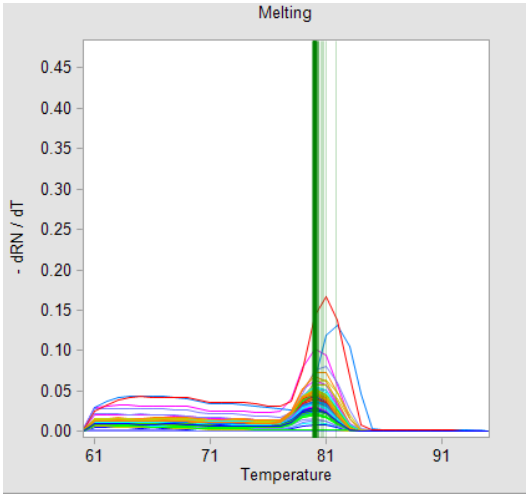
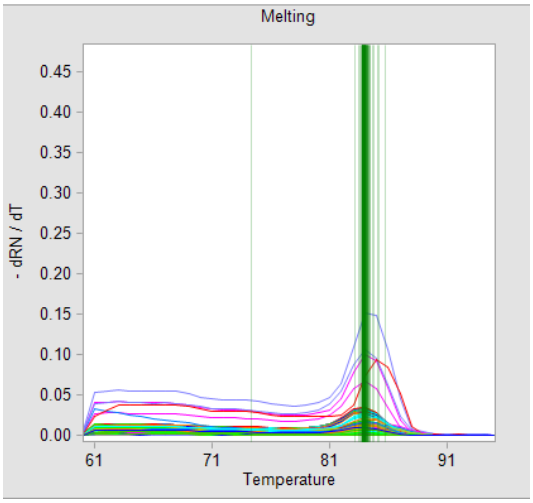
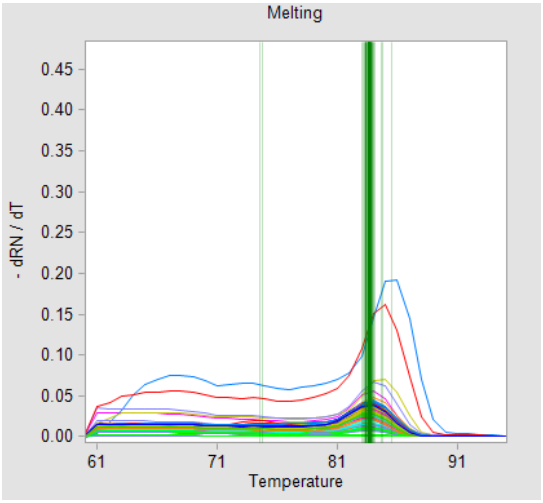


UPP1

Figure 4 continued



DTX2



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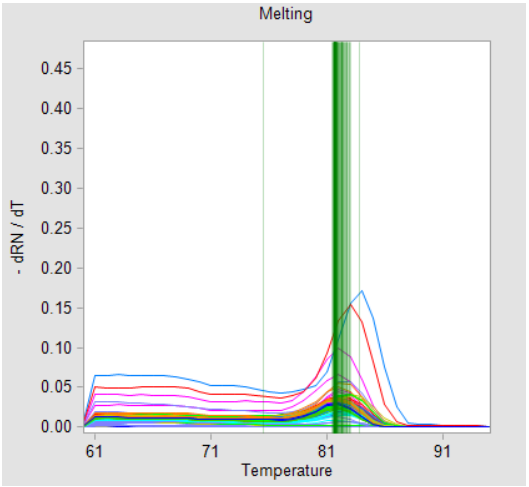

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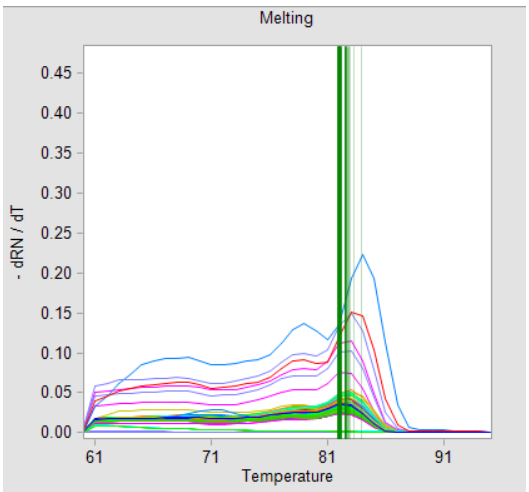
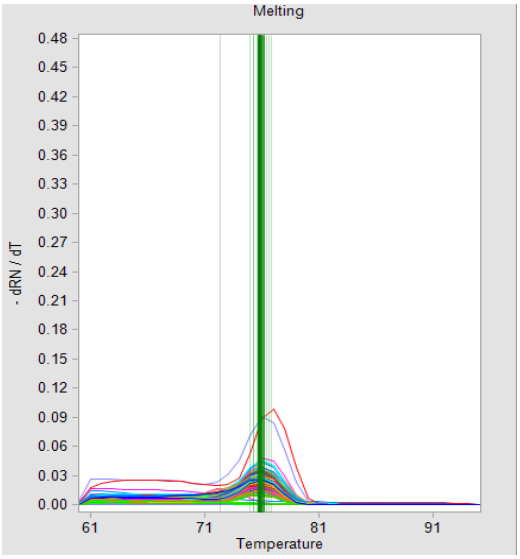


IRF1

Figure 5. Unusual melting curves with additional peaks at lower temperatures from qPCR analysis using 96.96 Dynamic Array.



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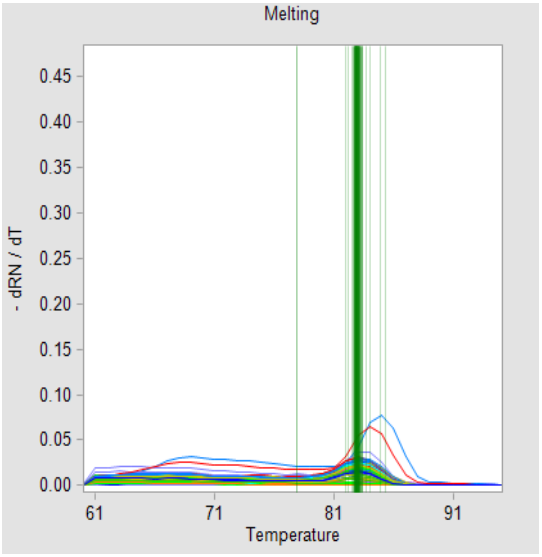
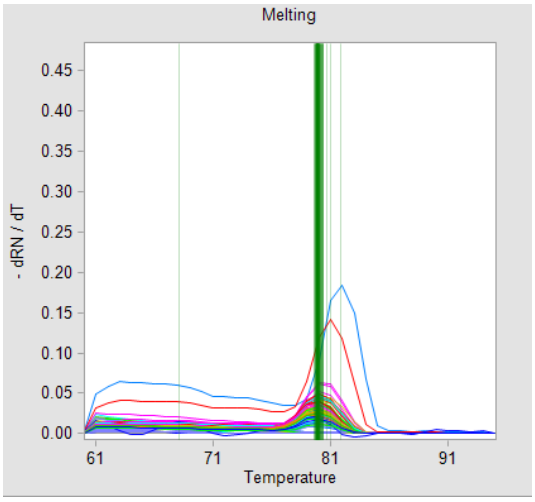
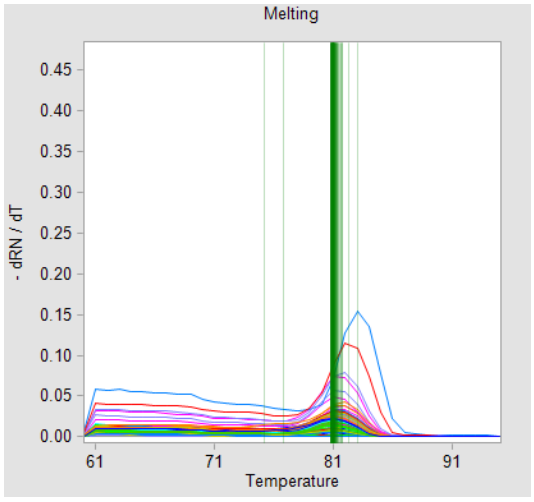


MAFA



STEAP4



GUSB

Figure 5 continued