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Comparative tick gene expression in Brahman and Holstein-Friesian cattle

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Abstract

This project funded a small trial to provide tick and animal samples to complement milestones within the larger tick vaccine project funded by the Beef CRC and the Qld Smart State Innovation Fund (2005-2010). The main aim of this study was to compare the gene expression of ticks raised on resistant cattle (Brahman) with ticks raised on susceptible cattle (Holstein-Friesian). Tick gene expression profiles from ticks collected from resistant cattle revealed over 200 genes not expressed by ticks raised on susceptible cattle. These genes are under further investigation as putative vaccine candidates. A secondary aim of this trial was to collect and study skin samples from naïve and infested Brahman and Holstein-Friesian cattle to add to gene expression data collected previously. In addition, skin biopsy samples taken from cattle before and after infestation were examined using immuno-histochemistry to determine the differences in the skin immune responses at the tick:host interface. Understanding the behaviour and physiology of ticks on resistant breeds, and the bovine skin responses leading to strong immunity of resistant breeds will contribute to the development of a new tick vaccine.

Executive Summary

This activity was undertaken to support a larger tick vaccine research project currently funded by the Beef CRC and the Qld Smart State Innovation Fund, which aims to identify new tick vaccine candidates. Tick vaccine research was previously highlighted as a high priority for industry largely due to the rising tick resistance to acaricides. The previous tick vaccine TickGARD is now commercially unavailable and the beef industry demands an alternative with higher efficacy following an annual boost. New acaricides to treat tick-infested cattle are not immediately forthcoming and the need to protect cattle and allow continued access to markets for Australian beef export industry is a high priority. A recent gap analysis (November 2007) within the tick vaccine research project identified the need for support of additional cattle trials at various stages of the research. This small trial involved the recruitment of 3 naïve Brahman cattle (resistant) and 3 naïve Holstein-Friesian for the collection of both ticks and host samples for further analysis.

The first objective was to study the differential gene expression of ticks collected from resistant and susceptible breeds. To date, most tick research is undertaken using susceptible breeds as this allows efficient tick propagation and collection for further laboratory study. Our research group identified that the study of ticks collected from resistant or immune breeds needed to be addressed to identify whether tick gene expression is different, and whether the genes expressed by ticks collected from resistant breeds could be exploited as putative vaccine candidates. This question was raised during the project's 'gap analysis' in November 2007 and it was decided that a commercial tick microarray would be used to identify these differences. Results identified over 200 transcripts produced by ticks attempting to attach to Brahman cattle previously not identified during studies undertaken using susceptible breeds as tick hosts. These transcripts have now been added to the list of candidates being examined by the group and will undergo *in silico* and *in vitro* screening to define a short-list for *in vivo* study in 09/10. This is the first known study where gene expression of ticks collected from resistant breeds has been compared with that of ticks collected from susceptible breeds.

A secondary objective was to collect skin samples from these cattle to complement a tick challenge trial undertaken using these breeds. Aside from understanding tick behaviour and gene expression, a large component of the research includes a detailed analysis of the host-skin interface to better understand how immunity develops. Skin samples taken from the naïve Brahman and Holstein-Friesian animals were submitted for bovine microarray analysis to demonstrate baseline differences between the breeds. Results demonstrated that naïve Brahmans appear to be innately 'ready' to respond. This was confirmed by immuno-histochemical studies of the types of immune cells in the skin. This study demonstrated innate differences in the cell types present in the Brahman and Holstein-Friesian skin prior to infestation. Additionally, after tick infestation the cell types present in the skin of Brahman and Holstein-Friesian cattle were dissimilar, indicating different patterns of cell recruitment in the two breeds.

The control of cattle ticks is vital to the continued success of the beef industry in terms of compliance with regulatory protocols for intrastate, interstate and international livestock movement and to enhance animal welfare through avoiding stress and debilitation. It costs $US2.5 billion to manage ticks and tick-borne diseases in tropical and sub-tropical regions of the world. Tick infested areas include the coastal regions of Queensland and northern New South Wales and across Northern Australia. The cattle industry in northern Australia incurs approximately $175 million in annual losses due to the impact of ticks. In addition, ticks are developing resistance to the wide range of acaricides used to treat cattle. Thus increasingly it will become less feasible to treat cattle as acaricides decrease in effectiveness. In Queensland, an effective vaccine would minimise the use of synthetic acaricides applied to treat cattle for ticks, thereby, decreasing chemical residues in milk, meat and

the environment. Managing tick infestations with an effective tick vaccine would reduce the use of chemical treatments, which in turn may mitigate non-tariff trade barriers associated with pesticide use. The economic benefits from reduced input costs and increased productivity due to a reduction in parasites, improved animal welfare and increased market access (due to decreased chemical residues) has been estimated at $98m (G. Griffith, DTRDI/DSD application 2005) with a potential international market value of a further $US100 million in vaccine exports per annum.

The data gathered from these cattle and the tick samples will assist to:

- Supplement the list of tick vaccine candidates identified to date
- Supplement the understanding of differential breed responses to ticks to identify the protective responses; this is required for a successful new tick vaccine
- Increase the groups' capability to deliver on Beef CRC/SSIF milestones and tasks associated with the identification of putative tick vaccine candidates.

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1 Background

1.1 Background – Study of tick gene expression and identification of tick vaccine candidates

Ticks are present in many parts of the world with *Rhipicephalus (Boophilus) microplus*, the cattle tick, being the most widely distributed species and the most important economically. Originally from Asia, this one-host tick species has colonised most of the world's tropical and sub-tropical countries (McCosker, 1979). These ticks transmit protozoan (*Babesia bovis* and *B. bigemina*) and bacterial (*Anaplasma marginale*) organisms that cause babesiosis and anaplasmosis ('tick fever'). The tick-disease complex is the most important affecting world-wide livestock production (deCastro 1977), leading to severe economic losses in milk and beef production and restriction in traffic of animals costing >$US2.5b annually.

Cattle are particularly vulnerable when they first encounter ticks but develop a degree of resistance after repeated exposure. *Bos indicus* cattle and crosses (tropical breeds which predominate in northern Australia) develop stronger resistance than do *Bos taurus* (British & European breeds). Chemical treatments (acaricides) are used to control ticks, however ticks have developed resistance to most current acaricides, and there is a market imperative to reduce chemical residues in both cattle and the environment. Control of ticks is required to minimise production losses and reduce annual industry losses of $175m due to the impact of ticks and tick-borne diseases and costs of treatment to ensure compliance with regulatory protocols for intrastate, interstate and international livestock movement (MLA Report, Playford – Strategic Bovine Services 2005).

Figure 1. Tick infested areas in Australia

The "Livestock Revolution" is a global food model that predicts the consumption of meat in developing countries will grow by 2.8% per year between early 1990s and 2020 (Delgado *et al*. 1990, 2002). Australia is the world's number one beef exporter supplying 23% of the world beef trade and is well placed to supply this predicted increased beef demand from the Asian markets. In 2003, domestic and export sales of beef exceeded $9.6 billion, with Queensland contributing to at least one third of this total ($3.2 billion) (CRC Stage 2 submission). Tick infested areas include the coastal regions and across northern Australia (Figure 1). Managing tick infestations with an effective tick vaccine would reduce the use of chemical treatments. The economic benefits from reduced input costs and increased productivity due to a reduction in parasites, improved animal welfare and increased market access (due to decreased chemical residues) has been estimated at $98m (Business case, Tick vaccine project submission – Smart State Innovation Fund, December 2005).

Queensland is the most developed tropical/sub-tropical region in the world and therefore well placed to lead the development of new tick vaccine. The current tick vaccine (Tick*GARD*$^{®PLUS}$) is based on a concealed tick gut antigen (Rand et al. 1989), is not effective against all tick stages (de la Fuente *et al*. 1998) and appears to have variable effects against ticks from different geographical locations (Garcia-Garcia *et al*. 2000). As immunity induced by Tick*GARD*$^{®PLUS}$ is not boosted through field exposure, protective titres are of short duration (Jonsson et al., 2000) and multiple booster shots are required following primary vaccination. Subsequently it has been poorly adopted by the beef industry and is now no longer manufactured commercially. Nonetheless, the concept of vaccination

against ticks is very popular with beef and dairy industry stakeholders. The industry has identified that a tick vaccine with 12-month duration of immunity and 90% efficacy is a top priority for Australia (MLA, Playford 2005).

The Beef CRC/Qld Smart State Innovation Fund (SSIF) tick vaccine research program has combined the *in silico* analysis of available tick sequence resources with tick gene discovery in order to identify putative tick vaccine candidates for further laboratory screening. This is termed a genome-based or reverse vaccinology approach. Approximately 14,000 *Rhipicephalus (Boophilus) microplus* sequences were available to the project through the US Department of Agriculture (USDA) at the tick genome database (BmGI2, Wang et al 2007). The project also identified novel tick genes associated with tick attachment and feeding utilising ticks from Hereford (susceptible) cattle using subtractive hybridisation methods. Using bioinformatics and molecular biology, the team has identified approximately 200 vaccine candidates for further study (Beef CRC 07/08 progress report).

Adding value to this study is the in-depth analysis of the host immune response. The 'concealed' tick gut antigen in Tick*GARD*$^{®PLUS}$ produced a short-lived immunity because natural infestations do not stimulate a response to concealed antigens such as Bm86. An ideal vaccine would incorporate the 'concealed antigen' approach while inducing an anamnestic ("remembered") response following natural infestations, thus eliminating the need for repeat vaccination. Molecules released during tick attachment and feeding stimulate innate and acquired immune responses. The ability of the host to respond to these molecules results in different levels of resistance. The foundation for the host's control of ticks is that tick infestations induce immune responses in cattle that are manifested as decreased tick feeding and reduced tick reproductive efficiency. Much of the research on responses of cattle to infestations with *R. microplus* was undertaken in the 1970s and was therefore limited in scope. Research during the 1980s and early 1990s focused on the development of a tick vaccine based on fractionation of native antigens and the development of recombinant antigens.

Previously the group had utilised a technique called subtraction hybridisation to improve the understanding of the molecular pathways involved in tick pathogenesis. Skin is the tick-host interface and blood-feeding by ticks is a complex and a long highly ordered process. Attachment and feeding takes several days to complete and involves sawing through the epidermis by means of toothed chelicerae, inserting the mouthparts into the resulting wound site followed by the formation of a feeding pool (Nuttall & Labuda 2004). For ixodid species, the majority of the blood meal is not taken up until the last day of attachment (Kemp et al 1982). Such a profound physical and chemical assault on the host should provoke strong haemostatic, inflammatory and immune responses. However, despite the host's armoury of rejection mechanisms, the tick manages to remain attached and achieve engorgement. Successful protection of the host against ticks relies upon the tick ingesting host immune factors during feeding. It is viewed that the site of pathogen adaptation to the very specific environment created by the intimate and dramatic interplay between host and parasite - the tick feeding site.

To date, no study has endeavoured to isolate candidates from ticks that are attempting to attach or feed on the 'resistant' host such as Brahman cattle. Through a 'gaps analysis' of the project initiated by the group, we identified that one more gene discovery experiment necessary. By undertaking such a study we ensured that the candidate list is not biased towards ticks collected from susceptible breeds. In this instance we have applied the tick microarray based on the USDA tick sequence library and available through a commercial company (NimbleGen) – to isolate novel tick sequences associated with tick attachment to Brahman cattle.

2 Project Objectives

By May 2008:

2.1 Complete analysis of gene expression data from ticks at different life cycle stages collected from resistant and susceptible breeds

2.2 Supplement immunological studies by completing immuno-histochemical analysis at tick-bite sites from resistant and susceptible breeds and provide baseline host gene expression data from naïve cattle

3 Methodology

3.1 Trial animals

Six naive female cattle from two breeds (Holstein-Friesian and Brahman) were recruited for this trial. To induce an immune response in the cattle, each animal was infested weekly for 6 weeks with 50,000 larvae of *R. microplus* (Brahman cattle) or 10,000 larvae (Holstein-Friesian cattle). One week after the last infestation all the cattle were challenged with 20,000 of larvae of *R. microplus* and the number of ticks for each animal were counted on days 19, 20 and 21 after the challenge infestation.
Animal ethics approval was obtained from the UQ Animal Ethics and Experimentation Committee (UAEEC) – 20th December 2007 AEC Approval No. SVS/872/07/CRC and the animals were held at Pinjarra Hills Farm (UQ).

Planned trial schedule:
January 2008:

- Recruit 3 naïve Brahman and 3 naïve Holstein-Friesian cattle (pre-vaccinated with tick fever) to Pinjarra Hills Veterinary Farm
- Skin biopsies taken for microarray analysis
- Skin biopsies collected for cell culture (establish cell culture models for future in vitro study of tick vaccine candidates). (To date we have used cell cultures exposed to tick antigens; these are currently being analysed. This proposal was an additional opportunity to collect skin tissue from these cattle for further culture studies.)
- Infest Brahman cattle with 50,000 tick larvae and Holstein-Friesian cattle with 10,000 larvae per week for 6 weeks
- Collect attached larvae (24hrs)
- Collect unattached 'frustrated' larvae (24hrs)

February 2008:

- Continue infesting cattle with ticks
- Collect adult female ticks
- Send tick samples for microarray analysis (tick microarray gene expression analysis - NimbleGen USA)
- Continue to monitor and collect ticks for storage

March 2008:

- Compare expression profiles of ticks from Brahman and Holstein-Friesian cattle and confirm by testing tick material collected from animals
- Meet March CRC milestone – *Isolation of novel tick sequences 31.03.08*

March/April – treat and sell experimental cattle (refund net income to MLA).

A trial extension was obtained from the UAEEC because adequate larval tick samples were not able to be collected initially and to enable samples to be collected for immuno-histochemistry, the animals needed to achieve tick count stability reflective of their breeds and levels of resistance/susceptibility.

Ticks were collected according to the schedule described above and methods for tick extraction for analysis are described below in Section 3.2. For skin biopsy collection, cattle were restrained in a crush and administered 5 ml of lignocaine HCl (2%) epidurally between vertebrae Ca1 and Ca2 to induce analgesia around the tail base and escutcheon region. An 8 mm biopsy punch was used to take four full-thickness biopsies of the skin prior to the first artificial tick infestation. Two biopsies were placed directly into liquid nitrogen (for extraction of RNA – Section 3.3) and two were stored as described below for immuno-histochemical analysis Section 3.4).

3.2 Tick gene expression

The *R. microplus* sequence database (BmiGI2, Wang et al. 2007) was used to design a custom oligo array through a commercial company NimbleGen by collaborators at the US Department of Agriculture (USDA) and Washington State University (WSU). Tick samples collected from the animals above were immediately frozen in order to extract total RNA according to the specifications required by this company. Briefly, total RNA were obtained by TRIzol method (Invitrogen) follow by mRNA extraction by Poly (A) Purist^{TM}-MAG (Magnetic mRNA Purification Kit) AMbion®. The cDNA synthesis were realized by SuperScript^{TM} Double Stranded cDNA Synthesis Kit (Invitrogen) with RNase A (10 mg/ml) treatment as recommend NimbleGen for cDNA preparation (see Appendix 1).

A total of 10 samples were prepared for microarray gene expression analysis. cDNA and or RNA was prepared from tick collections from each breed (Brahman/B, Holstein-Friesian/H) of the following samples for microarray submission (1 and 2 denote biological replicates):

- Larvae (pre- infestation) – L1, L2 (control samples)
- 'Frustrated' larvae (larvae are placed into organza bags left on cattle for ~24hrs, to examine the genes expressed when ticks are attempting to attach) – BFL1, BFL2, HFL1, HFL2
- Attached adult female ticks (~16 days) – BAT1, BAT2, HAT1, HAT2.

The above cDNA or RNA samples are sent to NimbleGen's service division in Iceland via International Courier on dry ice. Following array/slide hybridisation, the result output is provided in files listing values for the relative signal intensities of each probe (representing each tick gene) on the array for each RNA/cDNA from each tick preparation, with probes spotted twice on each array (herein referred to as blocks). Thus a list is provided for each array or slide experiment representing each of the 10 samples submitted. Statistical methods are used to normalise the signal intensities relative to controls and relative between samples under comparison (Bolstad et al. 2003; Irizarry et al. 2003). The comparative gene expression is then analysed using a fitted mixed model $yijkr=mu+BSrk+Gi+GTij+Eijkr$ where $yijkr$ is the log2 RMA normalized signal intensities; i =gene, j=treatment, r=block, k=slide/array, the main fixed effect is $BSrk$ (block by slide interaction), Gi is the main random effect of gene; $GTij$ is the random interaction term of the gene by treatment; E is just the error term; and normal assumptions for the random effects – iid are assumed. The model was fitted using VCE4.0 (Groeneveld & Garcia-Cortes, 1998). Differentially expressed (DE) genes were considered as those which deviate 3 or more standard deviations from the mean. The comparison used here is the difference between the same tick samples from the different breeds e.g. frustrated larvae from Brahman compared to frustrated larvae from Holstein-Friesian using averages of array results from biological replicate samples prior to the comparison. The putative identity of the lists of

up and down regulated tick sequences (DE genes) are then subsequently analysed to determine function. Sequences correlating to the DE identified on the arrays were submitted to global predicted amino acid searches (tBLASTx, Altschul et al. 1997) as well as analysis through functional databases including COG/KOG and CDD to assign putative function.

The genes were characterised based on their EuKaryotic Orthologous Groups (KOG). KOG is a eukaryote-specific version of the Clusters of Orthologous Groups (COG) tool for identifying ortholog and paralog proteins (Tatusov et al. 2003). The KOG tool provides a way of describing the function of predicted genes using four functional groups:

1. Information storage and processing – which includes sub-categories to describe genes associated with RNA processing, transcription, translation, replication and chromatin structure
2. Cellular processes and signalling – which includes cell division, defense mechanisms, signal transduction, cell wall/membrane biogenesis, cell motility, cytoskeleton, extracellular structures, Intracellular trafficking, secretion, vesicular transport, post-translational modification, protein turnover and chaperones.
3. Metabolism – which includes energy production and conversion, carbohydrate/amino acid/nucleotide/coenzyme/lipid/inorganic ion transport and metabolism; and secondary metabolite biosynthesis, transport and catabolism
4. Poorly characterised – which are essentially 'unknown' but can include categories such as a general function prediction only, function unknown and completely novel sequences previously not identified (which we have described as 'unique')

Each of the above functional groups is subdivided into KOG classifications identified by letters of the alphabet. Within each classification, groups of orthologous or paralogous proteins ("KOGs") are assigned a KOG ID.

The poorly characterised group was further analysed using the conserved domain database (CDD) in order to further characterise the genes by their putative protein function associated with a domain hit (Marchler-Bauer *et al.* 2003).

3.3 Host microarray analysis

RNA Extraction from Skin Biopsies

Skin biopsies stored in liquid nitrogen (LN_2) were removed and each was wrapped individually in heavy-duty aluminium foil and returned briefly to LN_2. The biopsy was then pulverised with a hammer on a metal block (both of which were stored at -80°C) and the pulverised tissue was transferred to a 2 ml tube contained 1500 µl of chilled Trizol reagent (Invitrogen) and three small glass beads. The tissue was then homogenised in a tissue homogeniser for 2 min and the tubes returned to ice. RNA extraction was then performed according to the manufacturer's instructions for Trizol reagent (Invitrogen). DNase treatment and RNA cleanup was performed using a RNeasy-mini cleanup kit (Qiagen) and the RNA eluted from the column in a final volume of 50 µl. Duplicate samples (within animal) were combined to ensure sufficient RNA from each animal for gene expression analysis. RNA concentration was determined using a spectrophotometer and 15 µg total RNA was aliquoted for microarray analysis. All RNA was stored at -80°C until analysis.

Microarray Analysis

Whole genome expression analysis was carried out by the Australian Genome Research Facility (Melbourne) using the Affymetrix GeneChip Bovine Array (see Affymetrix Web site for additional information) containing 24,128 probe sets representing ~23,000 bovine transcripts and 19,000 UniGene clusters. Target preparation and microarray processing procedures were performed as described in the Affymetrix GeneChip Expression Analysis manual. Scanning was performed with an

Agilent Microarray Scanner (Agilent Technologies, Palo Alto, CA). Statistical analysis of differential expression was tested using a per gene linear model on the log2 expression intensities obtained using MAS, RMA and GCRMA summarization methods (Bolstad et al. 2003, Irizarry et al. 2003, R Development Core Team 2008; Gentleman et al.2004). The final list of differentially expressed genes is comprised of those which were detected as significantly differentially expressed for at least two out of the three summarization methods (MAS, RMA and GCRMA) and flagged as present in at least 50% of the samples.

3.4 Host immunohistochemistry

Experimental scheme and tissue preparation

Skin biopsies (8 mm diameter) were collected from all animals before infestation and after infestation at sites where tick larvae were feeding (as described in the trial schedule). Skin biopsies were collected 24 hr after the primary infestation (PI) as well as 24 hr after the infestations performed one week, and three weeks after the primary infestation. Skin biopsies were also collected 24 hr after the challenge infestation. The skin biopsies were embedded in Tissue-Tek O.C.T. compound (Sakura Finetechnical Co., Tokyo, Japan) that was frozen in isopentane cooled with liquid nitrogen. The embedded samples were stored -80 ºC until use.

Immunohistochemistry

The host immune cells infiltrating the areas around the mouthparts of the larvae were identified by double immunofluorescence staining. Cryosections, 6 μm thick, cut from the skin biopsies were mounted on Polysine™ glass slides (Menzel-Glaser, Germany), dried overnight at room temperature (RT) and fixed in cold ethanol (4 ºC) for 8 min. After background staining was blocked with Image-iT FX signal enhancer (Invitrogen, Carlsbad, California, USA) followed by 10% [v/v] goat sera in 1% [w/v] BSA/PBS, the cryosections were incubated overnight at 4ºC in a humidified chamber with monoclonal antibodies specific for different leukocyte receptors (Table 1) diluted in 1% [w/v] BSA/PBS. IgG1 and IgG2a mouse monoclonal antibodies (DakoCytomation, Carpinteria, California, USA) in similar concentrations to the receptor specific antibodies were used as negative controls. After washing with phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4 and 1.4 mM KH_2PO_4) (PBS) the cryosections were incubated with goat anti-mouse isotype-specific antibodies labelled with FITC or Texas Red (1/400 [v/v] in 1% [w/v] BSA/PBS, Invitrogen, Carlsbad, California, USA) for 40 min at RT. After washing with PBS the nuclei were stained with DAPI dilactate (Invitrogen, Carlsbad, California, USA) and the slides were mounted with mounting medium (KPL, Gaithersburg, Maryland, USA). The slides were examined and photographed using an epifluorescent microscope, Olympus BX 51 (Olympus, Tokyo, Japan), equipped with a digital camera (Model DP 70, Olympus, Tokyo, Japan). The positively labelled cells were manually counted with a computer image analysis software (NIS-Elements Advanced Research, Nikon, Japan) in 10-12 successive high power fields (40× objective) located in the superficial dermis around the mouth parts of the ticks. The results are expressed as positively labelled cells per field.

Table 1. Monoclonal antibodies used to characterize the leukocytes infiltrating the skin around the tick mouth parts

Monoclonal antibody designation	Source	Antigen specificity	Isotype	Cellular expression	Dilution
MM1A	VMRD	CD3	IgG1	T cells	1/800
CH138	VMRD		IgM	Granulocytes	1/400
MCA837G	Serotec	CD8	IgG2a	T cytotoxic cells	1/50
HM57	DakoCytomation	CD79ά	IgG1	B cells	1/100
IL-A29*	International Livestock Research Institute (ILRI)	WC1	IgG1	Gamma delta T cells	1/25
IL-A21*	ILRI	MHC II	IgG2a	Macrophages, dendritic cells, B cells, activated T cells	1/200
IL-A12*	ILRI	CD4	IgG2a	T helper cells	1/25
IL-A111*	ILRI	CD25	IgG1	Activated cells	1/25

* Monoclonal antibodies from tissue culture supernatant.

4 Results and Discussion

4.1 Animal trial

Three Brahman heifers aged 15 months and 3 Holstein-Friesian heifers aged 7 months were sourced from tick-free areas of New South Wales for the experiment. All cattle were vaccinated against the tick-fever causing organisms *Babasia bovis, B. bigemina* and *Anaplasma marginale* two months prior to the commencement of the trial. Animals were transported by road using a licensed livestock carrier to the University of Queensland's Pinjarra Hills Veterinary Farm for the commencement of the trial.

An Animal Ethics modification was obtained during the trial to allow:

1) The shaving of cattle to affix bags in order to improve the collection of attached larvae
2) Sufficient rest from infestation in order for the resistant breeds to stabilise tick numbers (to ensure collection of appropriate skin sections for immuno-histochemistry).

Table 2. Actual sampling schedule

Date	Day of trial	Treatment/samples taken
24/01/08	Day 0	Skin biopsies for bovine microarray and immuno-histochemistry Infest Brahman cattle with 50,000 tick larvae and Holstein-Friesian cattle with 10,000. One Brahman (BO2) arrived with a large leg wound – treated locally and with intramuscular antibiotic.
25/02/08	Day 1	Collect attached larvae. Skin biopsies
31/01	Day 7	Infest with ticks
01/02/08	Day 8	Skin biopsies
7/02/08	Day 14	Infest with ticks
8/02/08	Day 15	Collect ticks
14/02/08	Day 21	Infest with ticks
15/02/08	Day 22	Skin biopsies
20/02/08	Day 27	Modification application submitted to UQ AEEC
21/02/08	Day 28	Infest with ticks
28/02/08	Day 35	Infest with ticks
3/03/08	Day 39	Infest with ticks. One Holstein-Friesian and one Brahman were infested under controlled environment. Ticks were localized on a small shaved area of the animal's back using a glued fabric bag (to collect attached larvae).
4/03/08	Day 40	Skin biopsies
11/03/08	Day 47	Brahman B02 was euthanased.
18/03/08	Day 54	Collect ticks
24/04/08	Day 60	Holstein-Friesian (Ear Tag: 181 QHWW1109XBC00283) died. Remaining 2 Holstein-Friesians treated with acaricide (Amitraz).
25/04/08	Day 61	Holstein-Friesian (Ear Tag 183 NLIS Tag: QHWW1109XBC00282) died.
7/05/08	Day 104	Blood collection from Brahman, tick infestation (Brahmans)
8/05/08	Day 105	Final skin biopsy immuno-histochemistry (Brahman only). Acaricide treatment (amitraz)
18/06/08		Animals sold (2 Brahmans, 1 Holstein-Friesian)

One Brahman sustained an injury to the front right leg during transport to Pinjarra Hills. The wound did not respond to treatment and the animal was euthanased about 5 weeks into the trial. An incident report was submitted to the UAEEC. The tick infestations had a strong effect on the Holstein-Friesian cattle as expected. An extremely high level of infestation was seen as a result of higher than expected re-infestation from the paddocks in which the cattle were kept. This resulted in the unexpected sudden death of one of the Holstein-Friesian heifers and the detection of severe anaemia in another. The remaining Holstein-Friesian heifers were treated with acaricide and veterinary intervention was sought for the anaemic heifer, including blood transfusion, but it also died. An incident report was submitted to the UQ AEEC. All the required tick and host skin biopsy samples were obtained as planned for this trial. Table 3 summarises the tick counts from the trial at 6 weeks.

Table 3. Numbers of ticks per side following infestation of cattle with 20,000 larvae 6 weeks after primary infestation

Breed	Animal number	Count – 26/3/08	Count – 27/3/08	Count – 28/3/08	Total	Average
Holstein-Friesian	H180	177	82	40	299	
	H181	500	250	95	845	570
	H183	315	180	70	565	
Brahman	B01	125	50	25	200	135
	B03	25	20	25	70	

4.2 Tick gene expression

RNA for microarray gene expression analysis was prepared from ticks collected from the various stages. Tables 4 and 5 summarise the samples shipped to NimbleGen in April and June 2008.

Table 4. Samples for first shipment to NimbleGen in April 2008

Samples	Animal	Description	cDNA	total μg RNA	λ260/ 280	λ260/ 230
H-FL1	183	Holstein frustrated larvae	1.8 μg	-	1.87	2.91
B-FL1	BO1	Brahman frustrated larvae	2.8 μg	-	1.87	2.58
H-AT1	181-183	Holstein – adult female 1	-	25 μg	2.09	1.7
B-AT1	BO1-BO3	Brahman – adult female 1	-	25 μg	2.08	1.6
L1	N/A	Control larvae 1	-	23 μg	2.0	2.0
B-AT2	BO3	Brahman – adult female 2	-	36μg	2.08	1.7
H-AT2	181	Holstein – adult female 2	-	27μg	2.08	2.13
L2		Control larvae 2	-	24 μg	1.99	1.9

Of the above samples shipped, the cDNA (H-FL1, B-FL1) met NimbleGen quality control standards, however, all RNA preparations except H-AT1 had degraded during prolonged shipment to the company (service division in Iceland). It was thus decided that we would prepare cDNA for replacements and subsequent samples rather than RNA which is highly labile. This is the first shipment of samples to NimbleGen for tick microarray analysis from Australia, thus there was no precedent to follow. The second shipment was sent early June and results have not yet been received from NimbleGen (Table 5). Tables 6, 7 and 8 refer to results and analyses from the H-FL1 and B-FL1 samples sent in first shipment.

Table 5. Samples for the 2nd shipment to NimbleGen – June 2008 (asterisk denotes replacement sample from 1st shipment)

Samples	Volume	Concentration	Total cDNA
B-AT1*	12μl	160ng/μl	2.0 μg
B-AT2*	12μl	234ng/ml	2.8 μg
B-FL2	15μl	232 ng/μl	3.5 μg
H-FL2	17μl	124 ng/μl	2.0 μg
H-AT2	15μl	104 ng/μl	1.56 μg
H-AT1*	13μl	203 ng/ml	2.6 μg
L1*	10μl	244 ng/ml	2.44 μg

A total of 222 genes were up-regulated in frustrated larvae associated with Brahman attachment compared with Holstein-Friesian ticks (Table 6). Many of the functional categories confirmed association in replication, development and metabolism, with 126 transcripts as either proteins with unknown function or unique tick sequences. These 'unknowns' (poorly characterised) were submitted to CDD analysis which identified transcripts as putatively associated with replication and protein turnover Table 7 – indicating the heightened readiness for 'growth' and perhaps attempts to manipulate the host. A small list of down-regulated genes includes those associated with cuticle and cell wall development (Table 8) which is logical given that the larvae were not attached and not increasing their cuticle or size during this pre-attachment phase. It is important to note that the tetraspanin and tick histamine binding proteins up-regulated by ticks attempting to attach to the Brahman cattle have already been identified as vaccine candidates resulting from our *in silico* selection to date. Thus this data does support our previous selection (Task 9.12 *Select 50 candidates*, 30th June 2008).

Table 6. Summary of 222 genes up-regulated in larvae pre-attaching to Brahman cattle

Predicted functional categories	No.	Examples within these categories
INFORMATION STORAGE & PROCESSING		
Translation, ribosomal structure	5	
Transcription	2	
Replication, recombination and repair	17	
Chromatin structure	2	
CELLULAR PROCESSES & SIGNALLING		
Cell division	1	
Defense mechanisms	4	Tumour necrosis factor receptor
Signal transduction	2	
Cell motility	1	
Cell wall, membranes	10	
Cytoskeleton	1	
Protein turnover, chaperones	8	
METABOLISM		
Energy production and conversion	10	
Inorganic ion transport and metabolism	5	B-cell receptor protein
Lipid transport and metabolism	3	tick histamine binding proteins
Nucleotide transport and metabolism	2	
Amino acid transport and metabolism	10	tetraspanin, heme binding proteins
Coenzyme transport and metabolism	5	
Carbohydrate transport and metabolism	6	
Secondary metabolites biosynthesis, transport and catabolism	2	
POORLY CHARACTERISED		
Protein function unknown	17	
Unknown	99	
Unique	10	
Total	222	

Table 7. Summary of the domain analysis of 126 genes

POORLY CHARACTERISED – domain analysis	126
INFORMATION STORAGE & PROCESSING	
RNA processing and modification	7
Translation, ribosomal structure	10
Replication, recombination and repair	29
Transcription	5
Chromatin structure	1
DNA binding domain	3
CELLULAR PROCESSING & SIGNALLING	
Cell division	2
Cell wall, membranes	3
Extracellular structures	2
Intracellular trafficking, secretion and vesicular transport	8
Defense mechanisms	5
Protein turnover, chaperones	13
Signal transduction	4
METABOLISM	
Energy production and conversion	4
Carbohydrate transport and metabolism	4
Amino acid transport and metabolism	7
Lipid transport and metabolism	7
Coenzyme transport and metabolism	1
Nucleotide transport and metabolism	2
Secondary metabolites, transport and catabolism	1
POORLY CHARACTERISED	
No known functional domains	15

Table 8. List of genes down-regulated in larvae attempting to attach to Brahman cattle

COG category and description	Domain (CDD)
[LKJ]_COG0513_Superfamily_II_DNA_and_RNA_helicases	A RNA processing and modification
[R]_COG2252_Permeases	L Replication
Un-named_protein_product	W extracellular structure
Unknown	S unknown
Unknown	M cell wall/membrane
[G]_COG0469_Pyruvate_kinase	M Insect cuticle protein
[O]_COG0071_Molecular_chaperone_(small_heat_shock_protein)	O protein turnover
[GEPR]_COG0477_Permeases_of_the_major_facilitator_superfamily	U Intracellular trafficking
Un-named_protein_product	C Energy conversion

The data collated above do not yet include comparative results including the control larval samples (L1, L2) or the biological replicates for these samples (B-FL2, H-FL2). We are currently awaiting the gene expression results from these and the adult female tick samples (B-AT1, B-AT2, H-AT1, H-AT2). The complete dataset will assist to eliminate 'housekeeping genes' from the analysis and provide a list of candidates which will either augment the current list of vaccine candidates (Beef CRC) or complement what has already been identified in our research to date – as described above for Task 9.12.

The data here complement the gene discovery phase of the Beef CRC project and by accessing ticks from Brahman infested cattle (MLA funded trial), the Beef CRC/SSIF funded components of gene discovery will benefit. The data collected from these experiments will contribute towards meeting the overall Beef CRC/SSIF milestones of defining vaccine candidates for *in vivo* study in 09/10 – Year 5 of the Beef CRC.

4.3 Host microarray analysis

Genes expressed differentially in skin of Holstein-Friesian and Brahman cattle
Ten transcripts were found to be differentially expressed in the skin between tick-naïve Holstein-Friesian and Brahman cattle. These are outlined in the table below.

Table 9. List of differentially expressed transcripts (confirmed using 3 statistical methods – MAS, RMA, GCRMA) identified in Brahman and Holstein-Friesian naïve skin samples

Gene Name	P -value MAS	P -value RMA	P – value GCRMA	Mean Fold Change	Bovine Reference Sequence (NCBI)	Higher Expression In:
Paraoxonase 3	1.22E-08	2.80E-10	4.90E-09	-5.07	NM_001075479	Holstein
Arachidonate 12-lipoxygenase pseudogene 2	1.29E-07	3.45E-11	2.12E-09	4.87	none	Brahman
Zyg-ll homolog B	3.14E-07	1.55E-07	5.11E-08	-3.07	XM_612919	Holstein
Membrane component, chromosome 11, surface marker 1	1.49E-06	4.73E-08	4.52E-08	3.32	none	Brahman
Ribosomal protein S27a	4.26E-06	1.32E-04	9.67E-07	1.85	NM_174778	Brahman
Transcribed sequence	9.71E-06	4.14E-06	9.65E-07	-1.32	none	Holstein
Hydroxysteroid (17-beta) dehydrogenase 13	5.64E-06	1.35E-05	9.95E-06	-5.46	NM_001046616	Holstein
Cytochrome P450, family 4, subfamily A, polypeptide 11	7.54E-05	1.17E-09	1.19E-06	3.35	none	Brahman
Forkhead box P1	4.83E-04	5.44E-07	5.18E-07	-2.89	XM_001249521	Holstein
Tumor necrosis factor (ligand) superfamily, member 10	1.00E-02	1.77E-06	9.06E-08	3.02	XM_583785	Brahman

The aim of this component of the study was to determine innate breed differences in gene expression in the skin of Holstein-Friesian and Brahman cattle. This information was needed to aid the interpretation of gene expression results from a pilot trial conducted on six Brahman and six Holstein-Friesian heifers in 2006. As the animals in the pilot trial originated from tick-infested areas of Queensland, pre-infestation samples were not required however this subsequently limited the interpretation of gene expression results. It was uncertain as to whether the differences in gene expression in skin samples taken at tick attachment sites (tick attached to the skin sample) resulted from tick infestation or whether they were merely innate breed differences. The data outlined above demonstrates that there are some minor innate differences in gene expression in the skin of naïve Holstein-Friesian and Brahman cattle, thus confirming that most of the differences in gene expression observed previously between the breeds are likely to be due to an effect of tick infestation. Combined results of this naïve analysis and those samples from the pilot study have

shown that over five-hundred genes are differentially expressed in each breed at tick attachment sites compared with tick-naïve skin samples. The final analysis of this data is associated with Beef CRC Task 7.1 *Complete analysis of Trial 1 samples (microarray and proteomics)* due 30/09/2008, which is on-track. The contribution from MLA towards the funding of these animals and funding these array analyses provided the resources to enhance our previous full microarray analysis (Brahman vs Holstein-Friesian responses to ticks) to meet this Beef CRC task.

4.4 Host immuno-histochemistry

Cattle were challenged with 20,000 larval ticks 6 weeks after primary infestation and tick counts were performed on days 19, 20 and 21 post-infestation. Cumulative tick counts indicated that there was a substantial difference between the two breeds (570 and 135 ticks per side for Holstein-Friesian cattle and Brahman cattle, respectively (see Table 3).

Preliminary results of the immuno-histochemistry indicate that naive Holstein-Friesian cattle have fewer CD3 cells (T cells), CD8 cells (T suppressor cells) and WC1 cells (gamma-delta T cells) than naive Brahman cattle (see Table 10). Infestation of cattle with cattle ticks resulted in an increase in CD3 cells and CD8 cells the skin of both breeds. However, numbers of WC1 cells only increased in Holstein-Friesian cattle following tick infestation. A change in the numbers of specific cells following tick infestation implies that this is response is induced by feeding ticks. Therefore the increase in WC1 cell numbers in the skin of the tick-susceptible Holstein-Friesian cattle seems to be a non-protective immune response. Figure 2 demonstrates the differential staining of WC1 markers in sections from both breeds both pre- and post- tick infestation.

Table 10. Average counts comparing CD3, CD8 and WC1 antibody staining of skin sections from Holstein-Friesian and Brahman artificially infested cattle compared to pre-infestation

Monoclonal antibody designation	Breed	Cells per field	
		Pre-infestation	6 weeks post-infestation
CD3	Holstein-Friesian	22.7	83.8
	Brahman	72.3	112.0
CD8	Holstein-Friesian	15.4	54.9
	Brahman	38.4	74.8
WC1 (see also Figure 2)	Holstein-Friesian	11.4	40.8
	Brahman	68.7	68.0

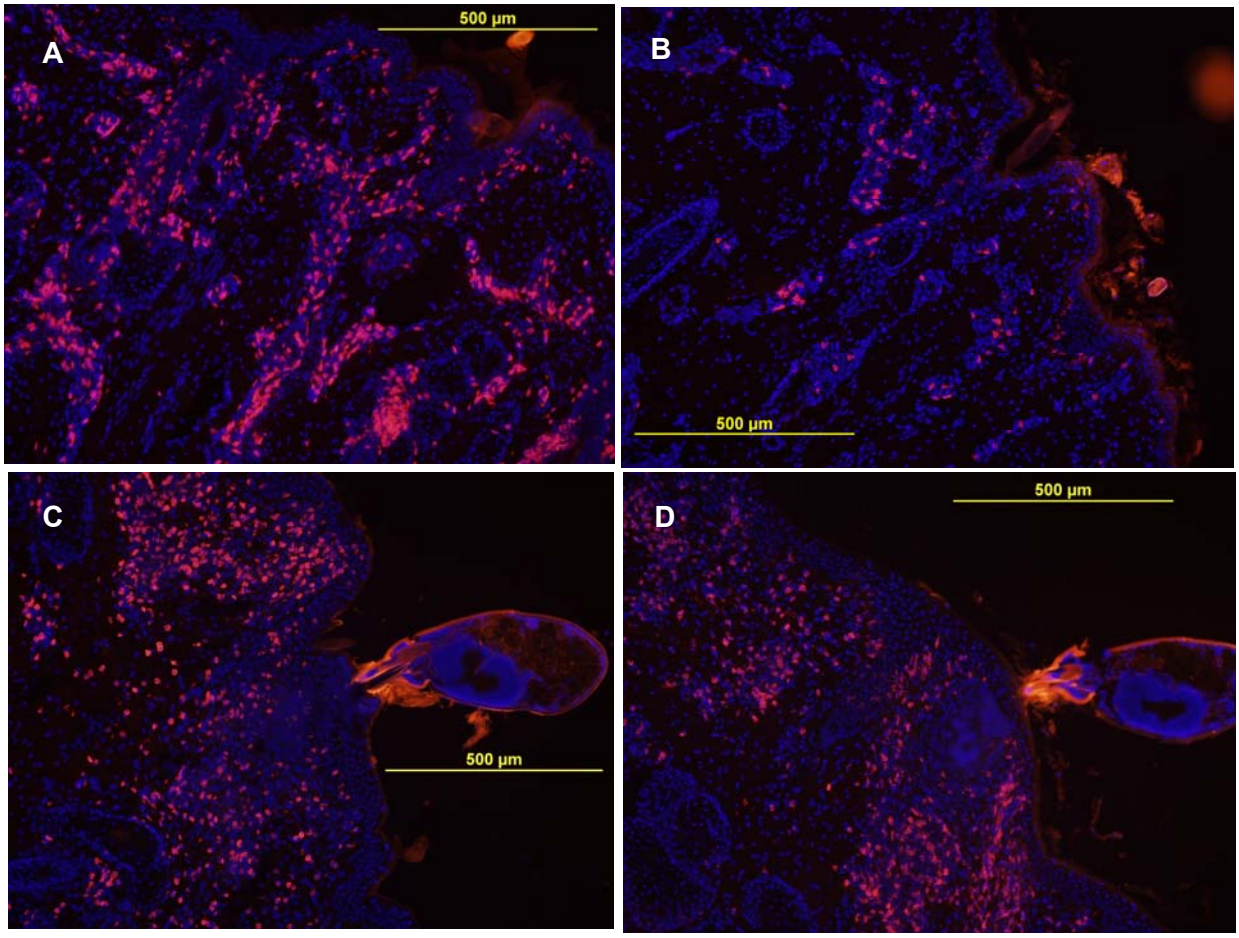


Figure 2. WC1 stained skin sections from A. naïve Brahman B. naïve Holstein-Friesian C. 6 week infested Brahman D. 6 week infested Holstein-Friesian

The final analysis of this data is associated with Beef CRC Task 7.1 *Complete analysis of Trial 1 samples (microarray and proteomics)* due 30/09/2008, which is on-track. The contribution from MLA towards the funding of these animals has provided the samples needed to complete the immuno-histochemical analysis of skin which in turn augments the skin gene expression data by providing the cellular evidence.

5 Success in Achieving Objectives

5.1 Complete analysis of gene expression data from ticks at different life cycle stages collected from resistant and susceptible breeds.

Tick samples from the trial animals were successfully collected and submitted to the commercial company for gene expression analysis. Preliminary data demonstrated an increase in gene expression (222 genes) by larvae attempting to attach to Brahman cattle in comparison to larvae attempting to attach to Holstein-Friesian cattle. Candidates previously identified using bioinformatics were found to be up-regulated in larvae attempting to attach to Brahman cattle. The complete dataset will assist to verify selected vaccine candidates which will be screened using *in vitro* screening tools in Year 4 of the Beef CRC/SSIF research (08/09). The main aim of this trial (MLA funded) was to collect ticks off Brahman cattle for gene expression studies (SSIF funded). *Task 9.23 Finalise list of tick antigens for in vivo screening, 30th September 2009.*

5.2 Supplement immunological studies by completing immuno-histochemical analysis at tick-bite sites from resistant and susceptible breeds and provide baseline host gene expression data from naïve cattle

The necessary samples from the cattle in order to complete this research were obtained successfully. Innate breed differences (gene expression – funded here by MLA) and immuno-histochemistry analysis pre- and post- infestation of Brahman and Holstein-Friesian cattle will supplement the final analysis of Trial 1 (Beef CRC funded) to meet *Task 7.1 Complete analysis of Trial 1 samples, 30th September 2008*.

6 Impact on Meat and Livestock Industry – now & in five years time

The economic costs of cattle tick include:

- Direct effects of the tick on cattle: loss of condition, anaemia and deaths, susceptibility to drought, damage to hides, slow growth rate;
- Risk of market being compromised by unacceptable chemical residues in meat or milk;
- Cost of controlling ticks, increased stock handling, costs of acaricides, cost of government regulations; and
- Costs of tick-borne diseases: deaths, slow growth, vaccine costs, treatment costs, handling costs.

The cost to Australia was $87m in 1973, $134 million in 1995 and $175m in 2005. The earlier estimates did not take into account government costs associated with control strategies and the costs of dipping yards (Playford 2005). On average, acaricides accounted for 11% of the costs, additional labour for 35% and production losses and animal deaths for 32%. The increasing resistance of ticks to acaricides is threatening the future viable use of acaricides. New strategies to manage tick burdens are urgently required.

Queensland supplies over 30% of Australia's cattle exports. Improved management of ticks will increase the value of these exports and by decreasing the costs involved with tick management will increase profits ($98m) for the State's Meat and livestock industries. A new tick vaccine is also potentially 'exportable' with an estimated conservative $US100-185m market predicted (G. Griffiths, Tick Vaccine project business case – SSIF submission).

Assuming an ultimate adoption rate of a new vaccine of 75% for beef and 50% for dairy, and the following annual increases in the adoption, the potential short term benefit for the beef and dairy industries has been calculated at a total of $32.5m:

- Beef industry: First 5 years – nil; 5% in year 6, 15% in year 7, 30% in year 8, 50% in year 9 and 75% adoption by year 10, thus up to $23m in present value terms
- Dairy industry: First 5 years – nil, 5% in year 6, 15% in year 7, 25% in year 8, 37% in year 9 and 50% adoption by year 10, thus up to $9.5m in present value terms.

7 Conclusions and Recommendation

Expression data from ticks collected from Brahman cattle has assisted the tick vaccine research team to scrutinise the current list of vaccine candidates identified during Beef CRC/SSIF research program i.e. if a gene previously identified using bioinformatics is identified in ticks collected from Brahmans the gene is added to the candidate list for further investigation; the process has also identified new candidates which will be added to the list for further investigation. Year 4 (08/09) of this research program aims to scrutinise candidates for *in vivo* screening in Year 5 (09/10).

Host gene expression data will assist to finalise analyses previously undertaken using pre-exposed Brahman and Holstein-Friesian cattle (Trial 1, Beef CRC). This study also allowed the collection of skin sections for immuno-histochemical analysis further verifying previous gene expression results. Understanding skin host immune responses will assist in the delivery of a successful vaccine.

8 Bibliography

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25, 3389-3402.

Bolstad, B.M., Irizarry, R. A., Astrand, M., and Speed, T. P. (2003) A comparison of normalization methods for high density oligonucleotide array data based on bias and variance. Bioinformatics 19; 185-193.

de la Fuente, J., Rodriguez, M., Redondo, M., Montero, C., Garcia-Garcia, J., Mendez, L., Serrano, E., Valdes, M., Enriquez, A. and Canales, M. (1998) Field studies and cost-effectiveness analysis of vaccination with Gavac(TM) against the cattle tick Boophilus microplus. Vaccine 16, 366-373.

deCastro, J.J. (1977) Sustainable tick and tickborne disease control in livestock improvement in developing countries. Vet Parasitol 71, 77-97.

Delgado, C., Rosegrand, M., Steinfeld, H., Ehui, S. and Courbois, C. (1999) Food and Agriculture Organization of the United Nations and International Livestock Research Institute. Livestock to 2020- the next food revolution. Food, Agriculture and the Environment Discussion Paper 28, International Food Policy Research Institute.

Delgado, C.L., Rosegrant, M.W. and Meijer, S. (2002) Livestock to 2020 - the revolution continues. Proceedings World Brahman Congress.

Garcia-Garcia, J.C., Montero, C., Redondo, M., Vargas, M., Canales, M., Boue, O., Rodriguez, M., Joglar, M., Machado, H. and Gonzalez, I.L. (2000) Control of ticks resistant to immunization with Bm86 in cattle vaccinated with the recombinant antigen Bm95 isolated from the cattle tick, Boophilus microplus. Vaccine 18, 2275-2287.

Gentleman, R.C., Carey V. J., Bates, D. M., Bolstad, B., Dettling, M., Dudoit, S., Ellis, B., Gautier, L., Ge, Y., Gentry, J., Hornik, K., Hothorn, T., Huber, W., Lacus, S., Irizarry, R., Leisch, F., Li,

C., Maechler, M., Rossini, A. J., Sawitzki, G., Smith, C., Smyth, G., Tierney, L., Yang, J. Y., Zhang, J. (2004) Bioconductor: open software development for computational biology and bioinformatics. Genome Biol. 5:R80.

Groeneveld, E., and Garcia-Cortes, L.A. (1998) VCE4.0, a (co)variance components package for frequentists and Bayesians. Proceedings 6th World Cong. Genet. Appl. Livest. Prod., Armidale, NSW, Australia; pp. 455-458.

Irizarry, R.A., Hobbs, B., Collin, F., Beazer-Barclay, Y.D., Antonellis, K. J., Scherf, U., Speed, T. P. (2003) Exploration, normalization and summaries of high density oligonucleotide array probe level data. Biostatistics 4; 249-264.

Jonsson N.N., Matschoss, A.L., Pepper, P., Green, P.E., Albrecht, M.S., Hungerford, J. and Ansell, J. (2000) Evaluation of TickGARD(PLUS), a novel vaccine against *Boophilus microplus*, in lactating Holstein-Friesian cows. Vet Parasitol 88(3-4), 275-285.

Kemp, D.H., Stone, B.F. and Binnington, K.C. (1982) Tick attachment and feeding: role of the mouthparts, feeding apparatus, salivary gland secretions and host response. In: F.D. Obenchain and R. Galun (Eds), Physiology of Ticks, Pergamon Press, Oxford, pp. 119-168.

Marchler-Bauer, A., Anderson, J.B., Derbyshire, M.K., DeWeese-Scott, C., Gonzales, N.R., Gwadz, M., Hao, L., He, S., Hurwitz, D.I., Jackson, J.D., Ke, Z., Krylov, D., Lanczycki, C., Liebert, C.A., Liu, C., Lu, F., Marchler, G.H., Mullokandov, M., Song, J.S., Thanki, N., Yamashita, R.A., Yin, J.J., Zhang, D. and Bryant, S.H. (2003) CDD: a conserved domain database for interactive domain family analysis. Nucleic Acids Res 35 (Database Issue), D237-40.

McCosker, P.J. (1979) Global aspects of the management and control of ticks of veterinary importance. Recent Advances in Acarology 2, 45-53.

Murrell, A., Campbell, N.J.H. and Barker, S.C. (2001) A total evidence phylogeny of ticks provides insight into the evolution of life cycles and biogeography. Mol Phylogen Evol 21, 244-258.

Nuttall, P.A. and Labuda, D. (2004) Tick-host interactions: saliva-activated transmission. Parasitology 129, S177-S189.

Playford, M. (Strategic Bovine Services). (2005) Review of research needs for cattle tick control, Phases I and II. June 2005. Meat & Livestock Australia Ltd., Locked Bag 991, North Sydney NSW 2059.

R Development Core Team (2008) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org.

Rand, K.N., Moore, T., Sriskantha, A., Spring, K., Tellam, R., Willadsen, P. and Cobon, G.S. (1989) Cloning and expression of a protective antigen from the cattle tick *Boophilus microplus.* PNAS 86(24), 9657-9661.

Tatusov, R.L., Fedorova, N.D., Jackson, J.D., Jacobs, A.R., Kiryutin, B., Koonin, E.V., Krylov, D.M., Mazumder, R., Mekhedov, S.L., Nikolskaya, A.N., Rao, B.S., , Smirnov, S., Sverdlov, A.V., Vasudevan, S., Wolf, Y.I., Yin, J.J. and Natale, D.A. (2003) The COG database: an updated version includes eukaryotes. BMC Bioinformatics 4, 41.

Wang, M., Guerrero, F.D., Pertea, G. and Nene, V.M. (2007) Global comparative analysis of ESTs from the southern cattle tick, *Rhipicephalus (Boophilus) microplus*. BMC Genomics 8, 368-382.

9 Appendices

9.1 Appendix 1 – NimbleGen cDNA and RNA preparation protocols

Protocols for

Gene Expression By Synthesis of Double Stranded cDNA

Outline

This protocol describes the process for creating cDNA from Total RNA or Poly A$^+$ RNA using Invitrogen's SuperScript™ Double-Stranded cDNA Synthesis Kit. NimbleGen recommends using the Qiagen RNAeasy kit for total RNA purification. For reduced 3' bias we recommend using Promega's ImProm-II first strand synthesis kit, combined with SuperScript™ Double-Stranded cDNA Synthesis Kit for second strand cDNA synthesis. This in only recommended when using non-standard expression designs, such as exon-based arrays or chromosome tiling arrays.

First Strand cDNA Synthesis Primer Options

First strand cDNA can be synthesized using either random hexamers or an oligo-dT primer. For standard gene expression experiments, NimbleGen requires the use of **random hexamers** when starting with **total RNA** from **prokaryotic** organisms, and recommends using an **oligo-dT** primer when starting with **total RNA** from **eukaryotic** organisms. When starting with **mRNA** from **eukaryotic** organisms, **random hexamers** can be used to reduce the 3'-bias that might be introduced during first strand extension

Protocol Information & Safety

- Wear gloves and take precautions to avoid sample contamination or degradation.
- RNA samples are sensitive to RNA degradation and we recommend using RNase Zap to clear all surfaces of RNases.
- RNA should be kept on ice at all times.
- Phenol:chloroform:isoamyl alcohol is dangerous and should be handled with caution. Wear the correct personal protective equipment when handling this chemical. See MSDS for more information.
- Keep RNase A in designated work area and use designated piptettes and tips to prevent RNase A contamination throughout the lab. Change gloves after handling RNases and clean work area with RNase Zap immediately after use.

Required Apparatus & Labware*

* See last page for a reagent supplier list

-Thermocycler
-Microcentrifuge with 14,000 x g capability
-Agilent 2100 Bioanalyzer or gel apparatus
-Vortexer Spectrophotometer (A NanoDrop Spectrophotometer is recommended)
-Speed vac Phase Lock tubes (optional)

Step 1. Spectrophotometric QC of RNA

1. Blank the spectrophotometer.
2. Measure each RNA sample.
3. Verify that all samples meet the following requirements:
   - $A_{260}/A_{280} \geq 1.7$
   - $A_{260}/A_{230} \geq 1.5$

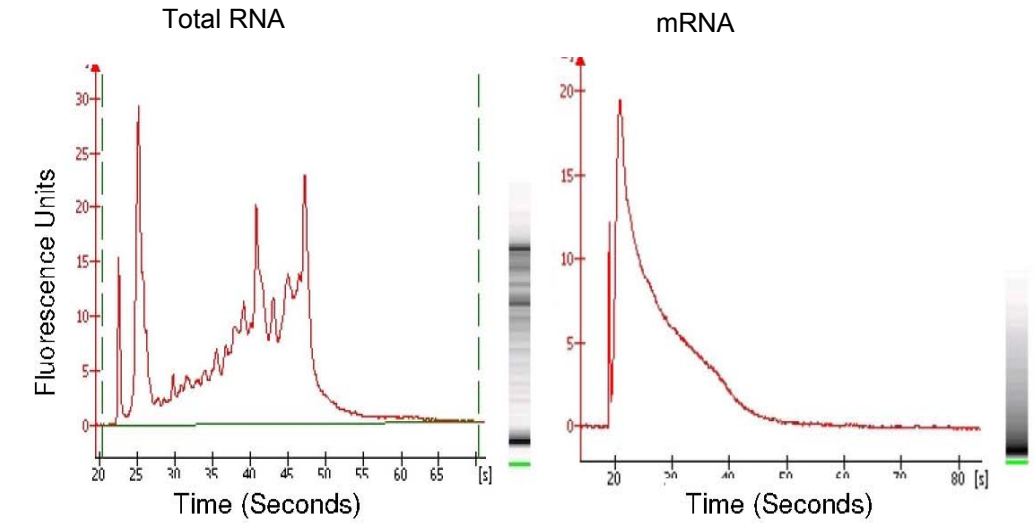
Example Bioanalyzer traces of degraded Total RNA and mRNA samples

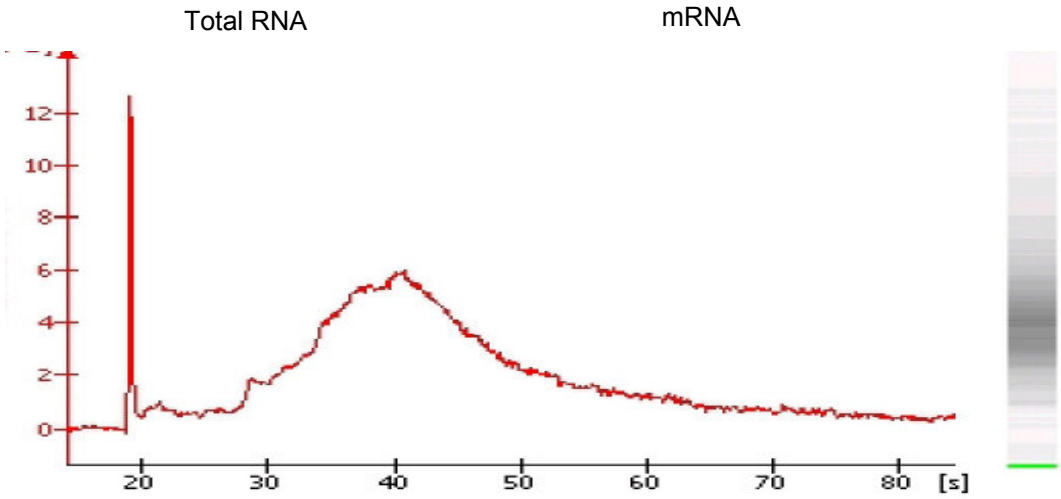
Step 2. Bioanalyzer/Gel QC of RNA

- Transfer 25-500 ng Total RNA or 25-250ng Poly A^+ RNA to a sterile microcentrifuge tube. Store the remainder of your sample on ice or at –80 ^{o}C
- Analyze the samples using the Agilent Bioanalyzer and RNA 600 Nano Assay Reagent Kit
- Review the bioanalyzer traces in comparison to the traces listed below, and visualize for degradation

Degraded samples appear as significantly lower intensity traces with the main peak area shifted to the left and typically exhibit much more noise in the trace. Samples exhibiting degradation should not be carried through labeling and hybridization, because there is an unacceptable risk of poor results.

NOTE: An agarose gel may be used instead of the Bioanalyzer to acess the quality of the RNA. See gel images next to Bioanalyzer electropherogram images on next page for a comparison:





Time (Seconds) Time (Seconds) Examples of good Total RNA and mRNA sample trace

Step 3. cDNA Synthesis

The Invitrogen Superscript™ Double Stranded cDNA Synthesis Kit should be used to synthesize double stranded cDNA. The kit **technical manual** should be followed exactly, with the **following exceptions.**

After second strand cDNA synthesis has been stopped with the addition of 0.5M EDTA and **before** the phenol:chloroform:isoamyl alcohol step, please:

Important!

Use caution when working with RNase A. Change gloves after use and use RNaseZap to clean work surface.

1 Add 1µl of 10mg/ml RNase A Solution to the tubes.

1 Mix tubes by gentle flicking or mix with a pipette. Quick spin.

2 Incubate tubes at 37 °C for 10 minutes.

3 Proceed with SuperScript™ Double-Stranded cDNA Synthesis Kit Technical Manual instructions. NimbleGen recommends using Phase-Lock Tubes for the phenol:CIAA clean-up.

Rehydrate samples to a concentration of 250ng/µl using water or TE.

Step 4. Spectrophotometric QC of cDNA

Before submitting samples to NimbleGen, a quality assessment is necessary to verify that the samples are of sufficient molecular weight and purity.

1. Blank the spectrophotometer.
2. Measure each cDNA sample.
3. Verify that all samples meet the following requirements:
   - Concentration = 250ng/µl
   - $A_{260}/A_{280} \geq 1.7$
   - $A_{260}/A_{230} \geq 1.5$

Step 5. Bioanalyzer/Gel QC of cDNA

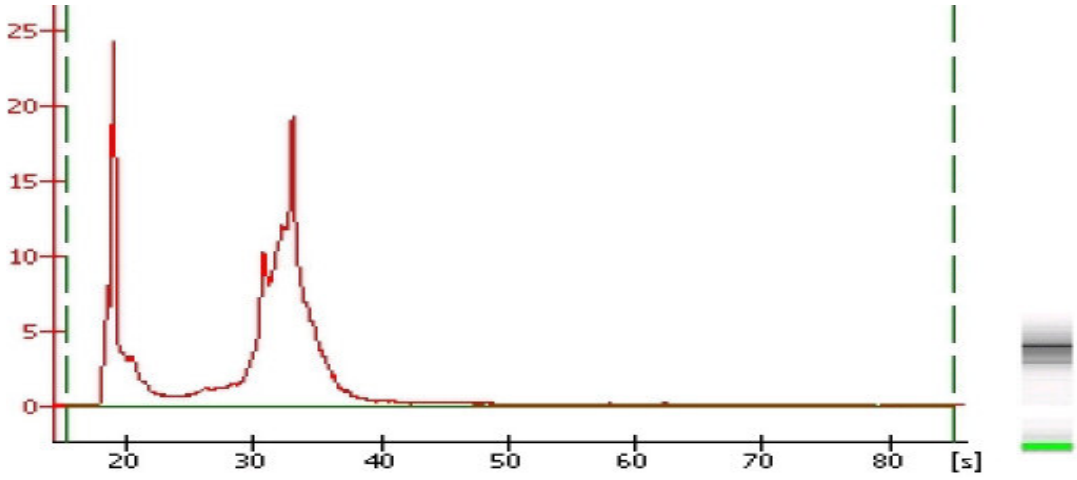
1 Transfer 100-250ng cDNA to a sterile microcentrifuge tube. Store the remainder of your sample on ice or at –20 °C.

2 Analyze the samples using the Agilent Bioanalyzer.

3 Compare the bioanalyzer traces to the traces listed below, and verify that all samples meet the following requirement for acceptance:

- Median size ≥ 400bp when compared to a DNA ladder

NOTE: An agarose gel may be used instead of the Bioanalyzer to acess the quality of the cDNA. See gel images next to Bioanalyzer electropherogram images on next page for a comparison:



Time (Seconds) Time (Seconds) Examples of good cDNA sample traces.

Sample Submission Requirements

§ Minimum 4µg of sample submitted § Samples are at a concentration of 250ng/µl § Minimum volume of 12µl § Samples have an $A_{260}/A_{280} \geq 1.7$ § Samples have an $A_{260}/A_{230} \geq 1.5$ § cDNA has a median size of ≥ 400bp § Sample Tube size 2.0mL or smaller

Component and Supplier List

Component	Vendor	Package Size	Item Number
SuperScript™ Double-Stranded cDNA synthesis kit	Invirtogen	10 reactions	11917-010
SuperScript™ Double-Stranded cDNA synthesis kit	Invirtogen	50 reactions	11917-020
SuperScript™ Double-Stranded cDNA synthesis kit	Invitrogen	100 reactions	11917-030
RNase A Solution	Novagen	1mL	70856-3
0.5M EDTA	Sigma Aldrich	100ml	E-7889
Phenol:chloroform:isoamyl alcohol	Ambion	100ml	9730
7.5M Ammonium Acetate	Sigma Aldrich	1 L	A-2706
Absolute Ethanol	Sigma Aldrich	500ml	E702-3
Phase Lock Tubes (light 1.5ml)	Fisher Scientific	200 tubes	E0032 007 961
Oligo dT Primer			