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Bovine Colostrum And Gene Expression In E. Coli.

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BOVINE COLOSTRUM AND GENE EXPRESSION OF *E.COLI*

by

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BIOGRAPHICAL SKETCH

Savanna Love Schepis was born in Walla Walla, Washington on August 21, 1982. She grew up in upstate New York attending junior and then high school. After graduating, she attended Guilford College and graduated in May 2006 with a Bachelor of Science degree with a double major in Health Science and Biology. After graduation, Savanna worked for one year at Plaza Veterinary Hospital as a veterinary assistant and waitressed as well. Savanna then came to North Carolina Agricultural and Technical State University and joined the graduate program in Animal Health Sciences in the fall of 2007. Savanna received the 2009 Graduate Student Award for academic excellence from the School of Agriculture and Environmental Science (SAES). She has presented her work at symposiums, which include: Ronald E. McNair Symposium, The NC A&T Annual Life & Physical Sciences Research Symposium, The N.C. Opt-Ed Alliance Day, and the Southern Section of the American Society of Animal Science conferences. Her current research involved the effect of exposure to colostrum on the expression of bacterial genes associated with bovine bacterial culture and growth, RNA analysis using microfluidics and spectrophotometry and gene expression studies using microarray analysis and RT-PCR. This research was supported by the United States Department of Agriculture Evans Allen Program in the School of Agriculture and Environmental Sciences at NC A&T State University.

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LIST OF SYMBOLS AND NOMENCLATURE

-CHS	Non-treated, heat shocked *E. coli* samples
-CNHS	Non-treated, non-heat shocked *E. coli* samples
+CHS	Colostrum-treated, heat shocked *E. coli* samples
+CNHS	Colostrum-treated, non-heat shocked *E. coli* samples
aceE	Down-regulated heat shock gene; pyruvate dehydrogenase, decarboxylase component
ANOVA	Analysis of variance
ATP	Adenosine-Tri-Phosphate
atpD	ATP synthase subunit B
bp	Base pairs
Buffer RLT	Guanidine isothiocyanate containing lysis buffer 25-50% concentration used for lysis
Buffer RPE	Washing buffer for removing chaotropic salts from the bacterial lysate (RNeasy Purification Ethanol)
Buffer RW1	Washing buffer to wash out any remaining protein after lysis (also contains Guanidine isothiocyanate 2.5-10% concentration)
^{o}C	Degrees Centigrade
cDNA	Complementary DNA derived from reverse transcription of RNA
CLN	Colostrinin^{TM}
cm	Centimeters
Cy	Cyanine
dCTP	Deoxycytidine Triphosphate

DEPC	Diethyl Pyrocarbonate (ethylene-diaminetetraacetic) acid
dnaK	Molecular chaperone DnaK
DNase	Deoxyribonuclease; an enzyme which degrades DNA
dNTP	Deoxynucleotide Triphosphate
E. coli	*Escherichia coli*
EDTA	Ethylene Diamine Tetra acetic acid
EtBr	Ethidium Bromide
fdoG	Down-regulated heat shock gene; formate dehydrogenase-O, major subunit
frdD	Fumarate reductase subunit D
groES	Co-chaperonin GroES
grpE	Hsp 24 nucleotide exchange factor
hfq	RNA-binding protein Hfq
hslU	ATP-dependent protease ATP-binding subunit
hslV	Up-regulated heat shock gene; ATP-dependent protease peptidase subunit
HSP	Heat Shock Protein
HSP's	Heat Shock Proteins
K-12	*Escherichia coli* K-12 strain
Kb	Kilobytes
LB	Luria-Bertani Broth
ldhA	Up-regulated heat shock gene; D-lactate dehydrogenase
LPS	Lipopolysaccharide

LSD	Least significant difference
µg	Micrograms
min	Minutes
µl	Microliters
ml	Milliliters
mM	Millimolar
MMLV-RT	Moloney Murine Leukemia Virus Reverse Transcriptase
mscL	Non-regulated heat shock gene; large conductance mechanosensitive channel
NC A&T	North Carolina Agricultural and Technical State University
nm	Nanometers
orf	Open reading frame
PCR	Polymerase Chain Reaction
PDGF	Platelet derived growth factor
RNA	Ribonucleic acid consisting of a string of covalently-bound nucleotides
RNase	Ribonuclease; an enzyme which degrades RNA
rplB	Non-regulated heat shock gene; 50 S ribosomal protein L2
rpm	Rotations per Minute
rRNA	Ribosomal RNA
RT	Reverse Transcription
S	Svedberg Units, Sedimentation Coefficient
SCC	Somatic Cell Count

SDS	Sodium Dodecyl Sulfate
sec	Seconds
σ32	Sigma 32
SSC	Solution consisting of trisodium citrate and sodium chloride used in different concentrations during washing steps of microarray
TCA	Tricarboxylic acid
TE	Tris-Cl, EDTA
TGF A	Transforming growth factor A
TGF B	Transforming growth factor B
TNF ά	Tumor Necrosis Factor ά.
tolB	Translocation protein TolB precursor
UV	Ultraviolet
V	Volts
WB1	Wash Buffer 1; Microarray washing buffer containing 2x SSC and 0.1% SDS
WB2	Wash Buffer 2; Microarray washing buffer containing 1x SSC
WB3	Wash Buffer 3; Microarray washing buffer containing 0.1x SSC

ABSTRACT

Schepis, Savanna Love. BOVINE COLOSTRUM AND GENE EXPRESSION IN *E. COLI*. **(Major Advisor: Mulumebet Worku)**, North Carolina Agricultural and Technical State University.

Escherichia coli cause mastitis upon entry into the mammary gland. Colostrum is the first milk produced by the lactating mother and provides immune nutrients for protection of the newborn. Bacterial pathogenesis may be modulated by exposure to colostral components. The objective of this study was to evaluate the effect of host immune factors in colostrum on *E. coli* K-12 growth, RNA transcription, and gene expression. Samples of *E. coli* K-12 were grown to mid-log in LB (Luria Bertani) broth and colostrum-treated LB broth. One of each culture was heat shocked to create one control and three different treatments. Growth was inhibited for the first 20 minutes in colostrum treated samples. RNA from control and treated samples was isolated using the RNeasy (Qiagen, CA) kits. The RNA was evaluated using a ND-1000 NanoDrop Spectrophotometer (NanoDrop Technologies Inc., DE) and Agilent 2100 bioanalyzer (Agilent Technologies, CA). The concentrations of samples were significantly different for each treatment ($p<0.0001$) and the purity was found to be not significantly affected ($p>0.05$). The effect of the treatments on transcription of six E. coli genes (fdoG, aceE, hslV, ldhA, rplB, and mscL) was evaluated using RT-PCR and agarose gel electrophoresis, and 93 genes were evaluated using *E. coli* K12 Starter V2 microarray chips (Ocimum Biosolutions, CA). Microarray data was analyzed using Imagene® 9.0 (Biodiscovery, CA). All six genes selected for PCR analysis were successfully amplified using specific primers. Colostrum, heat shock, and the combination of colostrum and

heat shock had an effect on bands present. Analysis of all data from microarray experiments showed that both treatment and dye had an effect, however in dye swap experiment there were no dye effects on gene expression. Evaluation of gene expression was only conducted on the dye swap experiment. Colostrum treatment resulted in an up-regulation of 54.1% of the genes on slide 1 and 38.0% of the genes on slide 2. In conclusion, major findings from this study indicate that colostrum and heat shock have an effect on *E. coli* growth, transcription, and gene expression.

CHAPTER 1

Introduction

Bovine mastitis presents a major challenge to the cattle industry worldwide, regardless of the implementation of several different control strategies. Mastitis leads to multibillion dollar economic losses by diminishing milk production and quality (Shpigel et al, 2008). Mastitis is as an inflammation of the mammary gland, primarily due to bacterial infection through teat canals. Acute mastitis infections induce severe inflammatory response that results in extensive mammary tissue damage and even death in some cases, and *E. coli* is the leading cause of acute mastitis in dairy animals (Shpigel et al, 2008). It is for this reason that *E. coli*-induced bovine mastitis has become an area of intense investigation (Long et al., 2001). The clinical signs of mastitis are due to inflammation caused by the endotoxins released during bacterial lysis. Antibacterial defense involves activation of neutrophils, which form the most important body defense mechanism. (Burvenich et al., 2003). However, the host defense does not always succeed in destroying the infection. Preventing bacterial growth in the teat canal, especially while in the presence of milk components, is an important place to prevent the disease.

E. coli have characteristic growth curves that can be influenced by environmental factors such as nutrients like colostrum. Further, environmental factors, such as colostrum, also impact gene expression. Colostrum is the first milk produced by the lactating mother and provides immune nutrients for protection of the newborn. *E. coli*

cause mastitis upon entry into the mammary gland where exposure to colostral components can occur and impact bacterial pathogenesis. Understanding the effect of environmental factors in bacterial growth and regulation of gene expression is essential to determining the factors affecting bacterial pathogenesis. Determining these factors will help for the development of interventions to combat infectious diseases.

Gene expression profiling is an effective approach available to evaluate how substrates affect bacterial cells. It surveys the genes expressed by the bacteria as it grows in the substrate and how it reacts to different stimuli. Gene expression profiling can be performed with microarray experiments. The objectives of this study were to evaluate the effect of host immune factors in colostrum on growth, RNA transcription, and gene expression in *E. coli* K-12.

CHAPTER 2

Literature Review

Mastitis

Mastitis is an inflammation of the mammary gland that is characterized by several physical and chemical alterations of the milk and corresponding pathological changes in the mammary tissue depending on the type of the disease. Mastitis can be caused by any foreign matter in or injury to the internal tissues of the mammary gland, but infectious microorganisms, Gram-positive and Gram-negative bacteria especially, are the predominant cause (Soback and Saran, 2005). It occurs in all mammals, but it is especially problematic in the cattle industry, where it can cause huge economic losses.

Annual losses due to mastitis, in 2003, were estimated at more than $2 billion for the dairy industry and another $400 million for the beef industry in the United States alone (Paape, 2003). Economic losses of mastitis can be due to milk production losses, decreases in milk quality, discarded milk, and costs of treatment (drugs, labor, veterinarians). Mastitis causes decreased milk production in dairy cows, which results in decreased income for the producer. Mastitis decreases the quality of milk by causing unstable and rancid taste, lowering the cheese yield, or decreased shelf life (Hogeveen and Osteras, 2005). Mastitis can even cause an extreme decrease in milk quality by causing watery milk that is clotted or has flakes in it, which then has to be discarded, further impacting income (Burvenich et al., 2003). Administering antibiotics to help combat the infections not only cost money, but takes treated cattle out of production for a

period of time, until the medicine is no longer in the cattle's system. Beef cattle cannot be slaughtered during this time period, and dairy cattle cannot be used for milk production. If milk from treated cattle ends up in the mass supply, the producer from where it originated can be heavily fined.

There are several different classifications of mastitis. The classification depends on the criteria such as the development of the disease (duration length or appearance of clinical signs), symptoms, source of pathogens, method of transmission, and pathogens themselves (Hamann, 2005). Mastitis can be classified as contagious or environmental classification depending on the suspected source of pathogen and method of transmission. The disease can be classified as acute or chronic depending on the duration length of infections and the appearance onset of clinical signs (Kirk, 1997). Mastitis infections may also be classified by symptoms, and depending on the degree of inflammation or the severity of the inflammatory response may be clinical or subclinical (Hamann, 2005).

Clinical mastitis has visible signs of the disease, either mild or severe. Mild signs include flakes or clots in the milk and a possible slight swelling of the infected quarter (Crist et al., 1994). Severe signs include abnormal secretions, a hot, swollen quarter or udder, fever, rapid pulse, loss of appetite, dehydration, depression, and even death (Crist et al., 1994; Long et al., 2001).

Subclinical mastitis is the most common form of mastitis (Crist et al., 1994). It is 15 to 40 times more common than clinical mastitis (Crist et al., 1994). There is no detectable change in the udder and no gross changes in the milk; however, the somatic cell count (SSC) of the milk will be elevated and a culturing of the milk will detect

bacteria in it. It also causes decreased production and decreased milk quality (Crist et al., 1994).

Subacute clinical mastitis is a condition in which abnormalities of the udder and secretion are readily observable. This form of mastitis can vary in severity, depending in part on the microorganism causing the infection. Changes in the milk, such as flakes, clots, and a watery appearance are the most obvious abnormalities (Nickerson et al,. 1995). Heat, swelling, and udder sensitivity are slight or absent (Nickerson et al., 1995).

Acute mastitis is characterized by sudden onset, redness, swelling, hardness, pain, grossly abnormal milk, and reduced milk yield (Hurley and Morin, 2003). Possible systemic symptoms can include fever, loss of appetite, reduced rumen function, rapid pulse, dehydration, weakness, and depression. The disease is termed peracute mastitis when the onset is very rapid and the signs are very severe (Hurley and Morin, 2003).

Chronic mastitis is an udder infection that is persistent. It may alternate between subclinical and clinical phases, or it may remain in a subclinical phase indefinitely (Jones, 1998). The clinical signs may persist for long periods of time or the signs may persist in a sub-clinical form for months or years with occasional clinical flare-ups (Jones, 1998).

Mastitis Causing Pathogens

Mastitis can be caused by several different pathogens, but only the major ones are of importance since they are the main cause of economic loss in the cattle industry. The major pathogens include *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae*, *Klebsiella spp.*, *Enterobacter spp*., and *Streptococcus uberis*, and environmental pathogens that include coliforms (Kudi et al., 2009). Other pathogens like

Mycoplasma are also known to cause outbreaks, but a majority of mastitis is caused by environmental pathogens (Kahn et al., 2005).

Environmental Mastitis

Due to the fact that a majority of mastitis is caused by environmental pathogens, environmental mastitis is the major mastitis problem (Fox, 2009). Environmental mastitis is sometimes referred to as environment-to-cow mastitis (Ingalls, 2003). The primary habitat of bacteria causing environmental mastitis is in the environment. Significant sources include organic bedding materials, manure covered alleyways, and wet or damp area exposure (Smith and Hogan, 1993). Environmental conditions that can increase exposure to the pathogens include: overcrowding, poor ventilation, inadequate manure removal from all areas of contact, poorly maintained stalls, access to farm ponds or muddy exercise lots, dirty maternity areas, and a general lack of farm cleanliness and sanitation (Hogan and Smith, 1987). Infection can occur during environmental contact of the teats at milking time or between milking, especially if there is damage or weakening of the sphincter of the teat canal that closes it off once milk is done passing through.

The major environmental pathogens that cause environmental mastitis include coliform bacteria, *Streptococcus* species, *Staphylococcus* species, and *Pseudomonas* species (Schukken et al., 2005). Coliforms are lactose-fermenting gram-negative rods of the family Enterobacteriaceae and include *Escherichia coli*, *Klebsiella* spp., *Enterobacter* spp., and *Citrobacter* spp. (Kahn et al., 2005).

Escherichia coli Mastitis

Escherichia coli (*E. coli)* is an important pathogen that causes mastitis in dairy cows (Dogan et al., 2005). *E. coli* is the head of the large bacterial family, Enterobacteriaceae, the enteric bacteria, which are faculatively anaerobic Gram-negative rods that live in the intestinal tracts of animals in health and disease. Over 700 antigenic types or serotypes of *E. coli* have been recognized based on O, H, and K antigens, although it is generally accepted that the type of strain does not play a major role in the severity of the mastitis (Burvenich et al., 2003). *E. coli* is massively excreted via feces into the environment by cattle. It will remain in the environment of cattle as long as it produces feces, which is why *E. coli* mastitis presents such a challenge (Burvenich et al, 2003). Between 5 and 24% of all mastitis cases are caused by *E. coli* (Dopfer et al. 1999; Bradley and Green, 2001).

E. coli mastitis infections cause inflammation of the mammary gland in dairy cows with local and sometimes severe systemic clinical symptoms. Clinical infections are diagnosed by red, swollen appearance of the gland and flakes or clots, which are protein aggregates, in the milk (Kerr and Wellnitz, 2003). Systemic symptoms are mostly a result of endotoxin release, or lipopolysaccharide (LPS) (Burvenich et al, 2003). This disease affects many high producing cows in dairy herds and may cause several cases of death per year in the most severe cases (Schroeder, 1997).

Prevention of *E. coli* mastitis has been based on maintaining clean environmental conditions – especially during the early and late stages of the non-lactating period, because of the impairment of mammary gland defense mechanisms at those times – and

ensuring that recognized control methods relating to the general hygiene of the herd are in place (Maunsell et al., 1998). Although, even utilizing these methods, it is still a costly problem today, making it the focus of much research.

Pathogenesis of E. coli

Bacteria have two means by which they can cause disease: invasiveness and toxigenesis. Invasiveness is the ability of the bacteria to invade tissues. It involves adherence and initial multiplication, production of extracellular substances – such as invasins – which facilitate invasion, and ability to bypass or overcome host defense mechanisms (Todar, 2008). Toxigenesis is the ability to produce toxins, which can be either exotoxins or endotoxins. Endotoxins refer to the LPS component of the outer membrane of Gram-negative bacteria, such as *E. coli*, which may be released from growing bacterial cells and cells that are lysed as a result of the host immune defense or antibiotics (Todar, 2008). The endotoxins can then be transported by blood and lymph causing cytotoxic effects at tissue sites other than that of the original point of infection (Todar, 2008).

Although attachment of *E. coli* to mammary epithelial cells seems not to be essential for the pathogenesis of clinical coliform mastitis, *E. coli* has several fimbrial and afimbrial adhesions that mediate adhesion to host epithelial cells through surface compounds like proteins, glycolipids, and carbohydrates (Burvenich et al., 2003; Le Bourgunec, 2005). Invasion of cells, especially non-phagocytic cells, provide bacteria with a survival advantage, allowing them to better resist detection and clearance by both the innate and adaptive immune system (Finlay and Cossart, 1997). *E. coli* strains that

manage to avoid rapid clearance from the mammary gland with milk flow can establish bacterial reservoirs, which can persist for several months with their ability to avoid host defenses and even gain protection from antibiotics (Dogan et al., 2005). The most common filamentous bacterial appendages of *E. coli* are the type 1 pili that promote bacterial adhesions to various types of eukaryotic cells (Dogan et al., 2005). These pili are composed of several subunits, an example being a mannose-binding lectin called FimH that is responsible for promoting bacterial adherence and colonization of mucosal surfaces (Hommais et al., 2003; Dogan et al., 2005). FimH has been shown as an important virulence factor for uropathogenic *E. coli* and adherent invasive *E. coli*; however, in mammary cell invasion blocking FimH only slightly decreases inhibition of adhesion, indicating that there are other mechanisms for invasion (Martinez et al., 2000; Boudeau et al., 2001; Dogan et al., 2005).

Invasion alone does not cause *E. coli* mastitis, toxigenesis plays a major role in the disease. The presence of *E. coli* in the udder is not the cause of the disease, the endotoxins produced by the *E. coli* are the cause (Hartman et al., 1976). The biological activity of endotoxin is associated with the lipopolysaccharide, or LPS (Todar, 2008). Toxicity is associated with the lipid component, termed Lipid A, and antigenicity is associated with the polysaccharide components, termed O antigens (Todar, 2008). LPS is responsible for many pathophysiological signs observed during Gram-negative bacterial infections in ruminants such as fever, changes in the number of circulating leukocytes, complement activation, activation of macrophages, increased vascular permeability,

changes in plasma levels of metabolites, minerals, acute phase reactants, and hormones (Burvenich et al., 2003).

Mastitis results once bacteria pass through the teat duct and multiply in milk-producing tissues (Schroeder, 1997). *E. coli* invades the udder through the teat canal where it grows and initiates a prompt inflammatory reaction. *E. coli* can breach the teat duct in several ways: between milkings bacteria may pass through the teat duct by multiplying inside the duct, by physical movement resulting from pressure placed on the teat end as the cow moves about, or by physical damage to the sphincter that controls the entrance to the mammary duct causing it to remain slightly open instead of completely sealing it off to the environment (Schroeder, 1997). Once bacteria have entered the gland, it can multiply sufficiently to trigger the inflammatory response with increasing numbers of neutrophils appearing. This neutrophil infiltration is one of the first steps of the inflammatory reaction because they have to prevent escape and multiplication of the pathogens (Burvenich et al., 2003). Vasodilatation occurs resulting in increased blood flow to the gland, which causes an increase in the vascular permeability. Inflammatory products such as prostaglandins, leukotrienes, proteases and toxic oxygen metabolites increase capillary permeability in the gland. Swelling then occurs due to filtration of fluid into the tissue. Phagocytosis is a complex process by which phagocytes – such as macrophages or neutrophils – move into the tissue, recognize the bacteria as foreign material, and then ingest and destroy the bacteria. Phagocytes – leukocytes (white blood cells) that engulf invading micro-organisms – destroy them, leave the blood vessels and enter the tissue by chemotaxis (Schroeder, 1997). Chemotaxis is movement of

phagocytes in response to a chemical stimulus, and is phagocytes into contact with the bacteria at the site of the infection and is critical for initiating the recognition (Todar, 2008). Tissue repair occurs once the bacteria have been destroyed; however, tissue scarring may occur and may be temporarily or permanently damaged (Hurley and Morin, 2003). The amount of damage usually is dependent on the duration of the infection, the longer the infection, the more damage to the surrounding tissue caused by the phagocytes. After elimination of the invading pathogens, neutrophil reaction subsides and promotes a prominent mononuclear (macrophage) reaction. Macrophages do not only replace neutrophils, but also actively participate in their removal. They also cause more damage to the surrounding tissue than neutrophils, so in cases of chronic infection, severe tissue damage can occur (Kumar et al., 2007).

Pathogenic *E. coli* have developed numerous ways to bypass or overcome the immune defenses of the host, which contributes to the virulence of the microbe and the pathology of the disease (Todar, 2008).

Heat Shock Proteins

Protein-damaging stresses - including heat shock, cold, altered pH and oxygen deprivation induce the expression of a subgroup of molecular chaperones, called heat shock proteins (HSP's), which consist of several protein families designated by their molecular weight (Antigenics, 2008; Makarow and Braakman, 2006). The environmental stresses make it more difficult for proteins to form their proper structures and cause some already structured proteins to unfold. The functions of proteins are determined by their three-dimensional structure. When excessive stress is applied to proteins, chains of

amino acids - which are folded into spirals, loops and sheets - begin to lose their shapes. When the interior of these proteins is exposed, proteins can adhere and form globs. This can make them dysfunctional (Brandt et al., 2008). HSP's are a class of proteins that protect cells from environmental stress damage by binding to partially denatured proteins, dissociating protein aggregates, and regulating the correct folding and intracellular translocation of newly synthesized polypeptides (Leppa and Sistonen, 1997). HSP expression can be transcriptionally regulated (Leppa and Sistonen, 1997). The function of HSP's and the presence of heat shock-inducible transcription are extremely well conserved throughout evolution; however, the transcriptional regulatory mechanisms are distinct in prokaryotes and eukaryotes (Arsène et al., 2000). The regulation of the expression of HSP's in *E. coli* – including DnaK, DnaJ, GrpE, GroEL, and GroES – is regulated by the product of the *rpoH* gene, namely, the stress-inducible sigma 32 (σ32) subunit of RNA polymerase (Arsène et al., 2000). Under non-stressful conditions, σ32 is maintained at low levels due to its rapid turnover and upon exposure to heat shock, the concentration of σ32 is greatly increased through enhanced synthesis and increased stability, which results in preferred transcription of σ32-dependent heat shock genes (Arsène et al., 2000). A negative feedback system controls the σ32-mediated transcription (Arsène et al., 2000). Three of the heat shock genes, dnaK, dnaJ, and grpE, are special because mutations in any one of these lead to constitutive levels of heat shock gene expression, implying that their products negatively auto regulate their own synthesis (Liberek and Georgopoulos, 1993). The accumulating DnaK-DnaJ-GrpE chaperone machinery binds to σ32 and inhibits its activity (Tomoyasu et al., 1998). Moreover,

binding of DnaK-DnaJ to the σ32 promotes its degradation by the ATP-dependent metalloprotease FtsH (Tatsuta et al., 1998). Therefore, availability of DnaK/DnaJ is a direct sensor of cellular stress and a regulator of heat shock transcription (Tomoyasu et al, 1998). Thomas and Baneyx (1998) have found that temperature increases up-regulate two groups of HSP's in *E. coli* – HSP's and holoenzymes – and found these genes to cooperate with the major chaperone systems in the management of thermal stress.

When the stresses occur that may lead to a heat shock response, the induction of HSP synthesis is vital for pathogen survival (Zugel and Kaufman, 1999). These increased pathogen HSP levels in cells lead to rapid degradation of HSP by the host processing machinery, which means that the pathogen-derived determinants may then be efficiently presented by host cells and promote recognition of the infected cells by the immune system (Zugel and Kaufman, 1999). Due to the high conservation of the determinants among various microbial pathogens, HSP are major antigens (Zugel and Kaufman, 1999). These strong cellular and humoral immune responses lead to inflammatory reactions, which mean that the HSP's can be utilized as targets for future therapeutic applications (Leppa and Sistonen, 1997).

Colostrum

Colostrum is the first natural food for the newborn calf, secreted during the first few days after calving, and is important for the health of calves. It is a thick yellowy substance rich in nutrients – such as fats, proteins, sugars, and vitamins and minerals – that are important for the survival of a newborn calf. Colostrum is formed during the last

2 weeks of the non-lactating period – termed colostrogenesis) and is secreted for 2 to 4 days after the lactation has started (Maunsell et al., 1998; Thapa, 2005).

There are ninety known components in colostrum, the most important being biologically active molecules that are essential for specific functions, such as growth factors, and immune factors (Thapa, 2005; Pakkanen and Aalto, 1997). Growth factors stimulate growth, help in regeneration, and accelerate the repair of aged original muscle, skin, collagen, bone, cartilage, and nerve tissue (Thapa, 2005).

The important growth factors in colostrum include platelet derived growth factor (PDGF), transforming growth factors α and β (TGF α and TGF β), vitamins and minerals, and amino acids (Thapa, 2005). PDGF assists in cell division in connective tissue, smooth muscle, and fibroblasts, and assists neuron survival and regeneration (Thapa, 2005). TGF's stimulate the proliferation of cells in connective tissue and assist in the formation of bone marrow and cartilage (Thapa, 2005). Vitamins and minerals are the most important nutrients essential for the normal metabolism, growth, and development, and they make colostrum serve as an antioxidant in the body (Thapa, 2005). Vitamins C, E, and A are in more than adequate amounts in colostrum (Thapa, 2005). The amino acids are the building blocks of proteins, and are required for growth and development of a newborn calf.

The important immune factors in colostrum are specific antibodies, immunoglobulins (Ig's), proline rich polypeptides (PRP's), cytokines, lactoferrin, lymphokines, oligopolysaccharides and glycol-conjugate sugars, and lysozymes (Thapa, 2005). The specific antibodies, which are the dam's antibodies, confer passive immunity

to several microorganisms – viruses, bacteria, yeast, and fungus – the dam has encountered, but the immunity is only temporary until the calf can build up its own resistance. Immunoglobins combat several different forms of infections, and the passive transfer of colostral bovine IgG at birth is imperative to the survival of the calf. Immunoglobins are involved in opsonization, where the F_{ab} end of the Ig molecule and complement – particularly C3b – bind to the antigen or foreign body, flagging it for phagocytosis (Hurley and Morin, 2003). The F_c portion binds to specific F_c receptors on the phagocytes and the phagocytes proceed to destroy the antigen (Hurley and Morin, 2003). Therefore, the antibody molecule acts as a linker between the antigen and the phagocyte. Failure of adequate transfer of colostral IgG is associated with increased disease morbidity and mortality in calves (Maunsell et al., 1998). PRP's have been shown to stimulate the thymus to regulate the immune system in the body and it stimulates the immune system (Thapa, 2005). Lactoferrin has been shown to inhibit the growth of several microbes, including *E. coli, Salmonella typhimiurium, Shigella dysenteria, Listeria monocytogenes, Streptococcus mutans, Bacillus stearothermophilus, and Bacillus subtilis* (Pakkanen and Aalto, 1997; Ellison et al., 1988). Lactoferrin is an iron-binding and transport protein that enhances iron absorption (Walzem, 1999). Although a study showed that lactoferrin working independently is extremely effective in inhibiting bacterial growth, it is also effective in binding and neutralizing endotoxins (Steijins and Hooijdonk, 2000). Cytokines are interleukins that regulate duration and intensity of immune responses and boost T cell activity (Thapa, 2005). Lymphokines, a subset of cytokines produced by lymphocytes, are peptides that involved in mediating

immune responses (Kumar et al., 2008). Oligopolysaccharides and glycoconjugate sugars attract and bind to pathogenic bacteria and prevent their entry in the mucosal lining (Rawal et al., 2008). Lysozymes are capable of destroying bacteria on contact, and work by attacking peptidoglycans found in the cell walls of bacteria (Rawal et al., 2008; Pellegrini et al., 1992).

Colostrinin™ (CLN™) is derived from the colostrum of mammals, and is characterized as a complex mixture of low-molecular weight polypeptides comprised of high proline – around twenty-two percent – and low percentages of glycine, alanine, arginine, and histidine (Szaniszlo et al., 2009). CLN™ is a proline-rich polypeptide complex derived from mammalian colostrum. The complex and the peptides within it are viewed as having potential utility in neurodegenerative illnesses such as Alzheimer's disease, Parkinson's disease, multiple sclerosis and amyotrophic lateral sclerosis (Szaniszlo et al., 2009). CLN™ was originally identified by scientists working in Poland, where early clinical studies, dating back to 1995, had indicated a significant potential benefit to Alzheimer's disease sufferers (ReGen Therapeutics Plc., 2010). The natural origin and nature of CLN™ means that it conforms to the general criteria for non-pharmaceutical health supplements (ReGen Therapeutics Plc., 2010).

The peptides found in CLN™ are not phosphorylated or glycosylated and fifty percent of the total mass is attributed to the hydrophobic amino acids (Szaniszlo et al., 2009). At first, the immune regulatory effect of CLN™ was demonstrated on the humoral immune response, but later, it was also shown to be an inducer of cytokines and chemokines (Szaniszlo et al., 2009). Cytokines and chemokines regulate and determine

the nature of immune responses, control immune cell trafficking, and control the cellular arrangement of immune organs (Kumar et al., 2008). It has also been shown to modulate maturation and differentiation of murine thymocytes (Szaniszlo et al., 2009).

Exposure of cells to CLN^{TM} can do many things. CLN^{TM} decreases intracellular oxidative stress levels, reduces 4-hydroxynonenal-mediated cellular damage, suppresses 4-hydroxynonenal-induced cellular signaling in cells, induces complex signaling pathways, and mediates cell differentiation in a manner comparable to hormones and neurotrophins (Szaniszlo et al., 2009). CLN^{TM} impacts on signaling pathways and modulates gene expression in cellular redox regulation, cell proliferation, and differentiation (Szaniszlo et al., 2009).

Gene Expression

Gene expression analysis of *E. coli* requires isolation of RNA (ribonucleic acid). RNA is a nucleic acid consisting of a string of covalently bound nucleotides. Each nucleotide is composed of a nitrogenous base, a ribose sugar, and a phosphate. It is bio-chemically distinguished from DNA by the presence of an additional hydroxyl group attached to each pentose ring (a ribose sugar instead of deoxyribose as in DNA), as well as by the use of the uracil nitrogen base instead of thymine. One of the main functions of RNA is copying genetic information from DNA (transcription) and translating it into proteins (translation) (Micklos et al., 2003). RNA quality and quantity is important for transcription.

RNA synthesis, in prokaryotes, due to a lack of a nuclear membrane, is carried out in the cytoplasm where the DNA is present, versus a eukaryote where it starts in the

nucleus and migrates to the cytoplasm (Birge, 2005). Polysomes, which are mRNA's with multiple ribosomes attached, are found in prokaryotes; however, in eukaryotes each mRNA has only one ribosome attached (Birge, 2005). Both eukaryotic and prokaryotic mRNA are predominantly single stranded but the molecule has a tendency to form internal hydrogen bonding to give a secondary structure that needs to be broken during any cDNA synthesis, or even some analytical techniques that measure purity and concentration (Birge, 2005). Prokaryotic ribosomes are 70 S - the small subunit being 30 S and the large 50 S –the subunits themselves composed of 23 S and 16 S and the 23 S, respectively - while eukaryotic ribosomes are 18 S and 28 S (Micklos et al., 2003).

In general, RNA is a more unstable molecule than DNA, and requires much more care when working with it (Qiagen, 2001). This instability is largely due to the presence of ribonucleases (RNases), that break down RNA molecules their endonucleatic and exonucleatic activity (Qiagen, 2001; Werner-Washburne and Davidson, 2002). RNases are very stable, do not require cofactors, are effective in very small quantities, and are difficult to inactivate (Qiagen, 2001). RNases play a role in maintaining a much shorter half-life for prokaryotic RNA than eukaryotic RNA (Carpoussis, 2001). A shorter half-life has partly to do with gene expression and with protection of the cell from viruses (Birge, 2005). The lack of a poly(A) tail and the extremely short bacterial RNA half-life represents hurdles for the application of microarray technology to prokaryotic research (Wei et al., 2000). RNase contamination can come from human skin and dust particles, which can carry bacteria and molds; which mean isolation and analysis of RNA requires specialized techniques.

Techniques Used to Analyze E. coli Genomes

For a long time *E. coli* has served as an important model organism in studying fundamental cellular and molecular processes, such as analyzing reactions of organisms to ecological and physiological changes, along with importance in biomedical research, and for this reason, it is perhaps the best characterized and studied bacterium (Reed et al., 2003). In 1997 the sequence of the genome of *E. coli* K-12 strain MG1655, was completed. *E. coli* has served as a model organism for basic studies of biochemistry, physiology, genetics, and biotechnology (Blattner et al., 1997). The genome of *E. coli* K-12 is similar to the *E. coli* strains that might infect cattle (Willenbrock et al., 2007).

By using a microarray representing the complete *E. coli* genome or parts of it, it is possible to analyze simultaneously in a single hybridization experiment, the expression of relevant *E. coli* genes. By analyzing regulation of gene expression under various conditions, unique relationships between gene functions and gene clustering are discovered (Fukiya et al., 2004). DNA microarray technology has changed the way scientists study gene expression. On a single microarray chip, a large set of genes is arrayed in a compact manner to investigate the expression levels of thousands of genes (Pollack et al., 1999). Microarrays, also termed biochips, consist of highly ordered matrices of DNA – or other material such as oligonucleotides probes– that are placed in a solid platform as dots (probes) and tagged with a fluorescent 'target'. Each spot usually represents a gene and the amount of gene expression is quantified by detection of the luminance intensity using lasers or other forms of light (Howbrook et al., 2003). The light emitted from the fluorescent probes is then detected and converted to an image file

which generates numeric data of the different intensities detected in each spot. The intensities calculated are translated into quantitative data for the transcription of each gene signifying if the gene is turned on or off and by how much (up or down regulation), or if it is simply untouched. The data produced is processed with statistical methods and further explored for biological information and manipulated with bioinformatics tools (Shena and Davis, 1999).

Total or messenger RNA is used as starting material for the microarray preparation. In order to prepare for hybridization, first strand complementary DNA (cDNA) is synthesized from good quality total RNA using oligo-d (T) primers or random primers for bacteria (Rhodius et al., 2002). During the reverse transcription fluorescence labeled dyes are incorporated into the first strand cDNA and the RNA template is degraded. The cDNA is hybridized onto a prefabricated array which can be a section or complete genome of an organism (Rhodius et al., 2002). Following hybridization, the cDNA is then assessed and analyzed from the signal intensities (Rhodius et al., 2002). The signals from the fluorescent tag of each of the cDNA's are evaluated separately, and then they are both used to calculate treated/control expression ratios (Rhodius et al., 2002). There are several different available gene expression microarrays today, with templates for human, rat mouse, model organisms (Arabidopsis, yeast, Zebrafish), some plants, some microbial genomes such as *E. coli* K-12) (Ocimum Biosolutions, 2010; Rhodius et al., 2002).

Tools for Microarray Analysis

Computers and specially designed software are necessary tools for microarray analysis. The complexity of the experiment, or volume of data put out during the experiment, determines the type of computer necessary to perform the analysis. An average computer may be used for basic experiments; however, pricier computers with more memory and faster processors are required for more complex or higher data volume experiments so the system isn't overloaded. The analysis is performed by specialized software. The analysis process can be divided into two steps – image processing and data analysis (Hegde et al., 2000). There are a number of image processing and data analysis software available to perform these tasks. Some combine image processing and data analysis in the same software, while others are separate. Jaguar™ 2.0 (Affymetrix Inc, CA) is an example of a combined image processing and data analysis software that is packaged with the Affymetrix® 428 array scanner (Affymetrix Inc, CA), however requires an older windows system to run it, making it difficult to perform some of the data analyses quickly and effectively. MAGIC (MicroArray Genome Imaging & Clustering) Tool (Davidson College, VA) is another example of combined image processing and data analysis software that is a free open source software available for download on the internet. However, free software isn't always compatible with the format of the data, which causes a need for commercial software. Imagene® 9.0 (Biodiscovery, CA) is an example of a commercial image processing and data analysis software that offers a free 15 day trial of their software, which allows the scientist to see if the software is a proper fit for their experiment before purchasing either a year or

lifetime license. The software is mostly an imaging processing software with small amounts of data analysis. If further analysis is needed, another piece of software made by the same company (Biodiscovery, CA), Nexus Expression^{TM}-Lite, is required. All of the software are good, each with their own advantages and disadvantages; however, it is the requirements of the experiment and the amount of funds available that determine exactly which software is obtained.

Validation of Results

Reverse transcription followed by the polymerase chain reaction (PCR) is another technique of choice to analyze mRNA expression derived from various sources, such as microarray (Pfaffl, 2001). It is often used alongside microarray expression analyses, as a type of verification of the microarray results. Current publication guidelines require that all microarray results are confirmed by an independent gene expression profiling method. Reverse transcriptase PCR offers a simple rapid approach to evaluate primers. To measure gene expression using PCR, the mRNA is reverse-transcribed into cDNA, then amplified to larger amounts using PCR. PCR is mainly used to duplicate a certain fragment sequence of DNA a million times precisely (Tsai and Sue, 2007). The amplification process of PCR can be divided into three consecutive temperature sectors; the denaturation sector, the annealing sector, and the extension sector (Tsai and Sue, 2007). The denaturation sector involves the disassemblement of the double-stranded DNA segment into two single strands at a high temperature of 94 ºC (Tsai and Sue, 2007). The annealing sector involves the single-stranded DNA to be attached to a specific primer, at the primer-designated temperature; usually 5-10 ºC lower than the primer

melting temperature if not specified, but a temperature gradient reaction can be performed if the information is not known (Tsai and Sue, 2007; Qiagen, 2010). The extension sector involves the association of cDNA singles with primers which are extended into double-stranded DNA's (Tsai and Sue, 2007). The combination of reverse-transcription and PCR is known as reverse transcriptase polymerase chain reaction, or RT-PCR, which is not to be confused with real time PCR (Tsai and Sue, 2007). RT-PCR involves exceptional sensitivity, along with a dynamic range, to achieve high accuracy, and requires PCR primers for all genes of interest (Tsai and Sue, 2007).

CHAPTER 3

Materials and Methods

Bacteria

A strain of *Escherichia coli* K12 was obtained from Carolina Biological (Burlington, NC). The *E. coli* was streaked onto LB agar plates to grow isolated colonies. Following an overnight incubation at 37 °C, the plates were wrapped in Parafilm (Pechiney Plastic Packaging Company, IL) to prevent drying and stored at -20 °C in the refrigerator until they were needed. For all experiments, single colonies of E. coli were used to inoculate LB for overnight suspensions.

Overnight Suspension

Overnight cultures were used for preparing mid-log cultures. In order to calculate mid-log phase for the bacteria, an overnight suspension was prepared using isolated *E. coli* colonies. A cell mass was scraped from a freshly streaked LB agar plate containing isolated colonies of *E. coli.*, then transferred into a sterile 10 ml culture tube containing 5 ml of sterilized LB Broth. The culture was incubated for 24 hours at 37 °C, with continuous agitation.

Colostrum Preparation

Colostrum Plus (Symbiotics, CA), a colostrum powder, was obtained from a commercial vendor**.** The colostrum powder (0.96 g or one dose) was added to 1 liter of LB broth and mixed thoroughly in a shaking water bath for 1 hour at room temperature. Freshly prepared solutions were used to treat *E. coli.*

Preparation of Cells at Mid-Log

Bacteria display a characteristic four-phase pattern of growth in liquid culture (Figure 3.1). The initial lag phase is a period of slow growth during which the bacteria adapt to the conditions in the fresh medium. This is followed by a log phase during which growth is exponential, doubling every replication cycle. The stationary phase occurs when the nutrients become limiting and the rate of multiplication equals the rate of death. The logarithmic decline phase occurs when cells die faster than they are replaced.

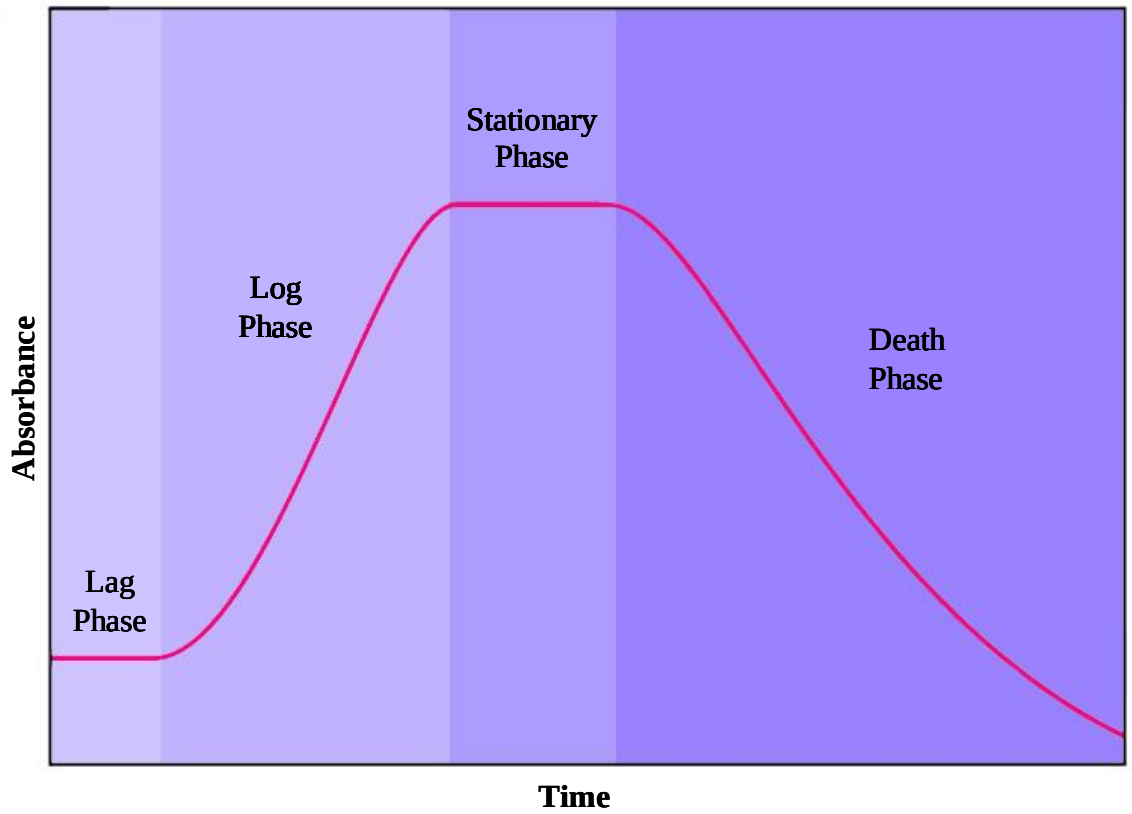

Figure 3.1. Typical growth curve

The mid-log population of cells - cells in the middle of the logarithmic phase of growth - was used for RNA isolation of the bacteria and were obtained from populations grown in untreated LB broth and colostrum-treated LB broth. Following incubation, 1 ml of the overnight suspension was transferred into one flask containing 100 ml of untreated LB broth and one flask containing 100 ml of colostrum-treated LB broth, and grown to mid-log. At inoculation, a 1-ml sample was sterilely withdrawn and the absorbance (optical density at 550 nm) was measured in a spectrophotometer (Sun Instruments Corp., CA). This procedure was repeated at approximately 20-minute intervals until the culture reached the stationary phase. Absorbance readings were recorded.

Serial Dilutions

To correlate the optical density of the culture with the actual number of viable *E. coli* cells, 500 ml of untreated LB with 5 ml of *E. coli* overnight culture and 500 ml of colostrum-treated LB with 5 ml of *E. coli* overnight culture was prepared. A 10-ml aliquot (time = 0) of each culture was immediately removed and placed on ice to arrest cell growth. The remaining of each culture was incubated at 37 °C with vigorous shaking in a shaking water bath. Additional aliquots were removed from each shaking culture every 20 minutes for a total of 120 minutes. Each aliquot was held on ice until ready for the optical density measurement at 550 nm. A 10^2 dilution of untreated cultures was prepared by mixing 10 µl of the aliquot with 990 µl of fresh untreated LB broth. A 10^2 dilution of colostrum-treated cultures was prepared by mixing 10 µl of the aliquot with 990 µl of fresh colostrum-treated LB broth. Three serial dilutions were prepared for samples from each time point. An untreated 10^4 dilution was prepared by mixing 10 µl

of the untreated 10^2 culture with 990 µl of untreated LB. An untreated 10^5 dilution was prepared by mixing 100 µl of the untreated 10^4 dilution with 900 µl of untreated LB and an untreated 10^6 dilution was prepared by mixing 100 µl of the untreated 10^5 dilution with 900 µl of untreated LB broth. A colostrum-treated 10^4 dilution was prepared by mixing 10 µl of the colostrum-treated 10^2 culture with 990 µl of colostrum-treated LB. A colostrum-treated 10^5 dilution was prepared by mixing 100 µl of the colostrum-treated 10^4 dilution with 900 µl of colostrum-treated LB and a colostrum-treated 10^6 dilution was prepared by mixing 100 µl of the colostrum-treated 10^5 dilution with 900 µl of colostrum-treated LB broth. A 100 µl sample of each dilution was spread onto an LB agar plate, for a total of three plates for each time point (aliquot). Each plate was labeled with the appropriate time point, dilution, and treatment, inverted and incubated (15-20 minutes, 37 °C). Following incubation, a dilution plate for each time point was selected containing between 30 and 300 bacterial colonies. The numbers of colonies were multiplied by the appropriate dilution factor to give the cell number per milliliter in the original aliquot. A 1 ml sample was sterilely withdrawn and the absorbance (optical density at 550 nm) was measured.

Growth Curve

The normal growth curve of *E. coli* can determine the generation time of the culture. Bacterial growth optical densities were collected for a total of 140 minutes. Following incubation, 1 ml of the overnight suspension was sterilely transferred into a flask containing 100 ml of untreated LB broth and a flask containing 100 ml of colostrum-treated LB broth, 1 ml of overnight suspension in each flask, and grown to

mid-log in a 37 ºC water bath. Immediately after inoculation, a 1-ml sample was sterilely withdrawn and the absorbance (optical density at 550 nm) was measured. This procedure was repeated at approximately 20-minute intervals until 140 minutes had been reached. *E. coli* culture reaches an OD550 of 0.3-0.5 in 1 hour and 30 minutes, under ideal conditions. To determine the correct amount of starting material, OD readings should be between 0.05 and 0.3 to ensure significance. It is essential to use the correct number of bacteria in order to obtain optimal RNA yield and purity within the RNeasy columns (Qiagen, CA).

Treatment of Bacteria

Using sterile technique, an overnight suspension of bacteria was inoculated into two flasks of 100 ml of LB and two flasks of 100 ml of colostrum-treated LB and allowed to grow for 80 minutes in an environmental shaker at 37 ^{o}C. One flask of LB culture and one flask of colostrum-treated LB culture were then taken and placed in a 40 ^{o}C stationary bath for 7 minutes for heat shock. The other flask of LB and colostrum-treated cultures remained in a stationary 37 ^{o}C water bath. Each culture was split into two 50 ml polypropylene conical tubes (Corning) for the RNeasy Maxi Protocol (Qiagen, CA). All samples were centrifuged (10 minutes, 5000 x g, 4 °C) to pellet the bacteria and the supernatant was decanted, and disposed according to Good Laboratory Practices. Each pellet was re-suspended with 10 ml of LB broth prepared in DEPC-treated water and combined to give a total of four bacteria samples for the RNeasy Maxi Protocol. The tubes were centrifuged (5000 x g, 10 minutes, 4 °C) to pellet the cells and the supernatant was then discarded.

RNA Isolation

RNA isolations were done using the RNeasy Maxi protocol for isolation of total RNA as recommended by the manufacturer with slight modifications (Appendix A for detailed protocol of RNeasy Maxi Protocol for Isolation of Total RNA, CA). Viable *E. coli* cells were used for the isolation of RNA and all reagents and glassware, if not supplied with the kits, were treated with DEPC to eliminate RNase contamination. Isolations were repeated, and combined, four times using the RNeasy Maxi Protocol for Isolation of Total RNA (Qiagen, CA). All bacterial cultures were harvested by centrifuging (5000 x g, 5 minutes, 4 °C). All subsequent steps for the RNeasy protocols were performed at room temperature and after harvesting the cells, all centrifugation steps were performed at 25 °C. *E. coli* cells (2.5×10^9 for untreated and 2.6×10^9 for colostrum-treated) were used as starting material for the RNeasy Maxi Protocol for RNA isolation. The purified RNA was stored at -70 °C until needed.

Purity and Concentration of RNA

The purity and concentration of RNA following the RNA isolation was determined using the ND-1000 NanoDrop Spectrophotometer (NanoDrop Technologies, Inc., DE). The OD 260 nm and 280 nm ratio provided an estimate of the purity of RNA with respect to contaminants that absorb in the UV. Concentration was determined at an OD 260 nm. Pure RNA has an A_{260}/ A_{280} ratio of 1.9-2.1. A 10X dilution of the RNA samples in DEPC-treated water was necessary in order for the sample concentrations to fall in the measuring range of the equipment. Eight repetitions of each RNA sample were measured so an average purity and concentration could be obtained for each treatment. A

statistical analysis (ANOVA) was performed using SAS version 9.2 (SAS Institute, Inc., NC) to determine if RNA purity or concentrations were significantly different due to treatment.

Characterization of RNA Quality Using the Agilent 2100 Bioanalyzer

The Agilent 2100 Bioanalyzer (Agilent Technologies, CA) and the RNA 6000 LabChip® kit provided a rapid alternative for the characterization of the RNA samples. The basis of the instrument is a microfluidic network that is driven by electrical potentials. It is a capillary electrophoresis instrument where a network of channels is connected to a number of power supplies. By programming these power supplies, fluids and ions are moved in specific directions. The network comprised a single separation channel with fixed-point laser induced fluorescence detection. This network is comprised in a single chip and all samples on the chip are analyzed sequentially. A disposable RNA chip was used to determine the concentration, the purity and the integrity of the RNA samples in approximately 25 minutes and provided two visual representations of each sample and an electropherogram.

A gel mix was prepared as recommended by the manufacturer by adding 550 µl of the RNA 600 gel matrix (red top) into a spin filter, centrifuged at full speed for 10 minutes. Following the centrifugation, 65 µl filtered gel aliquots were prepared. To prepare the gel-dye mix, the RNA 600 Nano-dye concentrate was vortexed and 1 µl was added to a 65 µl aliquot of the filtered gel and centrifuged for 10 minutes. The gel-dye was loaded into wells of a disposable RNA chip by placing the RNA chip on the chip priming station supplied with the kit. The syringe gasket on the chip priming station was

properly sealed to prevent large air bubbles from forming under the gel-dye. Five µl of RNA 6000 Nano Marker and 1 µl of RNA 6000 Ladder (Ambion Inc., TX) were pipetted into the appropriate wells as designated by the manufacturer. To minimize secondary structure, each RNA sample and the RNA 6000 Ladder (Ambion Inc., TX) was denatured for 2 minutes in a heating block at 70 °C before loaded. A 1 µl sample of the ladder was pipetted into the well marked with the ladder symbol and 1 µl of each RNA sample were pipetted into the appropriate wells. Colostrum-treated, non-heat shocked samples were loaded into wells 1-3. Colostrum-treated, heat shocked samples were loaded into wells 4-6. Untreated, non-heat shocked samples were loaded into wells 7-9. Untreated, heat shocked samples were loaded into wells 10-12. The chip was then placed on the vortexing station for 1 minute at 240 rpm and then checked for any air bubbles that might interfere with analysis. The run was started within 5 minutes of preparation. The bioanalyzer software was activated before insertion of the chip. The chip was inserted correctly into the Agilent bioanalyzer and the Prokaryote Total RNA Nano assay was selected. The assay was started and run for 20-30 minutes. After every run was completed, the bioanalyzer was cleaned using 350 µl of RNase-free water pipetted into an electrode cleaner. (Refer to Appendix D for detailed Agilent 2100 Bioanalyzer Protocol using the RNA 600 Nano LabChip kit and RNA concentration)

Reverse Transcriptase PCR Analysis

The effects of colostrum, heat shock, and the combination of colostrum and heat shock treatment of transcription of six *E. coli* genes (fdoG, aceE, hslV, ldhA, rplB, and mscL) was evaluated using RT-PCR. The primer sequences used for these six heat shock

genes are shown in Figure 3.1. These six primers were obtained from Eurofins MWG Operon (Alabama) and chosen based on data from the microarray gene list (Ocimum Biosolutions Inc., MD) that stated the genes whose expression are either down-regulated, up-regulated, or non-regulated during heat shock; two different genes for each expression effect were randomly chosen. The genes that are down-regulated during heat shock are fdoG and aceE. The genes that are up-regulated during heat shock are hslV and ldhA. The genes that are non-regulated during heat shock are rplB and mscL.

Table 3.1. Primer sequences used in PCR.

Gene	Direction	Regulation	Sequence 5' to 3'
fdoG	Forward	Down-regulated	GCCCAGGTCAGCAGAAGGCAGTT
fdoG	Reverse		CCMACCTTTTCCACATTCACAAG
aceE	Forward	Down-regulated	GCCTCAGAACGTTTCCCAAATGA
aceE	Reverse		CCCGCCAGACGCGGGTTAACTTT
mscL	Forward	Up-regulated	GCCAGCATTATTAAAGAATTTCG
mscL	Reverse		CCAGAGCGGTTATTCTGCTCTTT
rplB	Forward	Up-regulated	GCCGCAGTTGTTAAATGTAAACC
rplB	Reverse		CCTTTGCTACGGCGACGTACGAT
hslV	Forward	Non-regulated	GCCACAACTATAGTAAGCGTACG
hslV	Reverse		CCCGCTTTGTAGCTTAATTCTTC
ldhA	Forward	Non-regulated	GCCAAACTCGCCGTTTATAGCAC
ldhA	Reverse		CCAACCAGTTCGTTCGGGCAGGT

Approximately 2 µg of RNA was added to 2 µl of random primers (Invitrogen, CA), and 12 µl of nuclease-free water. The reagents were then mixed, spun briefly, and incubated at 75 ºC for 3 minutes to denature the RNA. After incubation the tubes were

placed on ice and 2 µl of 10X RT Buffer (Ambion Inc., TX), 4 µl of dNTP mix (Ambion Inc., TX), 1 µl RNase Inhibitor (Ambion Inc., TX), and 1 µl MMLV-RT (Ambion Inc., TX) were added to the reaction mix. The reagents were mixed, spun briefly, and incubated at 42 ºC for 1 hour to allow for cDNA synthesis. Following this incubation, the reaction was incubated again at 92 ºC for 10 minutes to inactivate the reverse transcriptase. After the inactivation of the reverse transcriptase 5 µl of the cDNA mix, 5 µl of 10X PCR buffer (Ambion Inc., TX), 2.5 µl of dNTP mix (Ambion Inc., TX), 50 µl nuclease-free water, 2.5 µl PCR primers (Ocimum Biosolutions Inc., MD), and 1 µl of thermostable DNA polymerase (Ambion Inc., TX) was added to a PCR tube and mixed gently. The PCR mixture was then placed in the thermocycler Primus® 96™ (MWG AG Biotech). The thermocycler conditions for the primers varied based on the melting points of each primer, which were provided by Eurofins MWG Operon (AL). More specifically, only the amplification temperatures differed between each gene, while the times remained the same. The denaturation and extension temperatures and times were the same for all genes. Tables 3.2 to 3.7 give the conditions for each primer.

Table 3.2. Specific PCR conditions for aceE gene.

Gene Description	Regulation	Sequence Length (bp)	PCR Step	Temperature (ºC)	Time
Pyruvate dehydrogenase, decarboxylase component	Down-regulated	2664	Denaturation	94.0	5 min
			Amplification	94.0	30 sec
				60.5	30 sec
				72.0	1 min
			Extension	94.0	5 min

Table 3.3. Specific PCR conditions for fdoG gene.

Gene Description	Regulation	Sequence Length (bp)	PCR Step	Temperature (ºC)	Time
Formate dehydrogenase-O, major subunit	Down-regulated	3051	Denaturation	94.0	5 min
			Amplification	94.0	30 sec
				60.0	30 sec
				72.0	1 min
			Extension	94.0	5 min

Table 3.4. Specific PCR conditions for hslV gene.

Gene Description	Regulation	Sequence Length (bp)	PCR Step	Temperature (ºC)	Time
ATP-dependent protease peptidase subunit	Up-regulated	531	Denaturation	94.0	5 min
			Amplification	94.0	30 sec
				56.9	30 sec
				72.0	1 min
			Extension	94.0	5 min

Table 3.5. Specific PCR conditions for ldhA gene.

Gene Description	Regulation	Sequence Length (bp)	PCR Step	Temperature (ºC)	Time
D-lactate dehydrogenase	Up-regulated	990	Denaturation	94.0	5 min
			Amplification	94.0	30 sec
				61.4	30 sec
				72.0	1 min
			Extension	94.0	5 min

Table 3.6. Specific PCR conditions for mscL gene.

Gene Description	Regulation	Sequence Length (bp)	PCR Step	Temperature (ºC)	Time
Large conductance mechano-sensitive channel	Non-regulated	400	Denaturation	94.0	5 min
			Amplification	94.0	30 sec
				55.1	30 sec
				72.0	1 min
			Extension	94.0	5 min

Table 3.7. Specific PCR conditions for rplB gene.

Gene Description	Regulation	Sequence Length (bp)	PCR Step	Temperature (ºC)	Time
50S ribosomal protein L2	Non-regulated	822	Denaturation	94.0	5 min
			Amplification	94.0	30 sec
				58.7	30 sec
				72.0	1 min
			Extension	94.0	5 min

Agarose Gel Electrophoresis

Amplified samples were separated on a 2% agarose gel in 0.5X TBE buffer. Eight µl of the PCR product was added to the gel along with 3 µl of loading dye (Ambion Inc., TX). A DNA ladder was added to the first lane of the gel (Ambion Inc., TX). Gels underwent electrophoresis at 120 volts for about an hour. After electrophoresis gels were flooded with ethidium bromide in a gel box for 5 minutes and then washed with distilled water for 30 minutes. The gels were observed and photographed with a gel documentation system. (BIO-RAD GEL DOC 2000, CA).

Microarray Analysis

Three different microarray experiments were conducted. The first compared the control (non-treated, no heat shock) to the colostrum-treated sample without heat shock. The second compared the control to the non-treated sample with heat shock. The third compared the colostrum-treated with heat shock to the colostrum-treated sample without heat shock.

RNA Preparation

Isolated RNA was prepared as above. Further purification of the isolated RNA was needed before labeled-cDNA synthesis could be performed; therefore a DNase digest was performed as recommended by the microarray manufacturer (Ocimum Biosolutions Inc., MD). The recommended starting material for the DNase digest is 100 µg of total RNA. Approximately 100 µg of RNA was dissolved in 70 µl of nuclease-free water. A reaction mix was prepared with the 70 µl of Total RNA, 18 µl 25 mM $MgCl_2$, 10 µl of 200 mM Tris-HCl (pH 8.0), and 2 µl RNase-free DNase I 10 U/µl (Roche, Basel France) and incubated for 30 minutes at 30 ºC. After the incubation, 1 volume of phenol/chloroform/isoamylaclohol 25:24:1 (Fisher Scientific, PA) was added to the digest, vortexed until the phases were completely mixed, and centrifuged for 1 minute at 14,000 rpm. The upper layer was transferred to a new tube and 1 volume of chloroform (Fisher Scientific, PA) was added, the new layers vortexed until the phases were completely mixed, and centrifuged for 1 minute at 14,000 rpm. The upper layer was transferred into a new tube and 1/10 volume of 3 M NaCl and 2 volumes of 100% ethanol were added, mixed, and then stored overnight at -20 ºC. Tube was then centrifuged at

14,000 rpm for 10 minutes at 4 ºC and the supernatant discarded. The pellet was covered with 500 µl of 70% ethanol, centrifuged at 14,000 rpm for 7 minutes at 4 ºC, the supernatant discarded, and the pellet dried. The RNA concentration was adjusted to 10 µg/µl by adding 10 µl of nuclease-free water. The purified RNA quantity and quality was then checked using ND-1000 NanoDrop Spectrophotometer (NanoDrop Technologies, Inc., DE).

Labeled-cDNA Synthesis

The recommended starting material for cDNA synthesis was at least 50 µg of total RNA (Ocimum Biosolutions Inc., MD). Three different microarray experiments were prepared. The first compared the control (non-treated, no heat shock) to a colostrum treated sample without heat shock. To label the cDNA, Cy3-labeled dCTPs were used for the colostrum-treated sample without heat shock, and Cy5-labeled dCTPs were used for the control. The second compared the control to a non-treated sample with heat shock. To label the cDNA, Cy3-labeled dCTPs were used for the control, and Cy5-labeled dCTPs were used for the colostrum-treated sample with heat shock. The third compared a colostrum-treated sample with heat shock to a colostrum-treated sample without heat shock. To label the cDNA, Cy3-labeled dCTPs were used for a colostrum-treated without heat shock, and Cy5-labeled dCTPs were used for a colostrum-treated sample with heat shock. Fluorescent dyes are destroyed by light; therefore, to protect the fluorescently labeled cDNA, the procedure was performed in dim lighting. The cDNA synthesis reactions were performed with dye swaps to ensure consistency. The incorporation of Cy5 tends to be less efficient than that of Cy3 because of greater steric

hindrance, and Cy5 is susceptible to rapid degradation by atmospheric ozone at levels present in most laboratories. Three cDNA synthesis reactions were performed using 50 μg of- total RNA and cDNA synthesis was performed twice using more than 100 μg of total RNA. The reaction mixture was incubated (65 ^{o}C, 10 minutes, room temperature,10 minutes) and the on ice (2 minutes) followed by the addition of reaction buffer (5X RT reaction buffer, Superscript II kit, Invitrogen, CA), dNTP master mix, Cy3-dCTP (PerkinElmer, PA), Cy5-dCTP (PerkinElmer, PA), 0.1 M DTT (Invitrogen, CA) and Superscript II (Invitrogen, CA). The reaction was incubated (42 ^{o}C, 2 hours). Following incubation, the reaction was terminated by the addition of NaOH to degrade the RNA template. The reaction was neutralized by adding HCl and unincorporated dNTPs were removed using a PCR Purification Kit (Qiagen, CA). The cDNA was eluted with nuclease-free water provided with the PCR Purification Kit (Qiagen, CA) as recommended by the PCR Purification protocol (Qiagen, CA). Equal amounts of Cy3 and Cy5 labeled cDNA were combined. The purified cDNA was then dried using a vacufuge (Eppendorf, Germany).

Microarray Chips

In the present study, *E. coli* K12 Starter V2 array chips (Ocimum Biosolutions Inc., MD) on glass slides were used for expression profiling of *E. coli*. The chip's content contained 93 *E. coli* genes, 1 *Arabidopsis* control, 1 internal control and 2 replicates of genes for a total of 96 spots. The spotted genes on the arrays were separated into grids. The *E. coli* Starter V2 arrays consist of 2 identical grids with a total of 192

spots as shown in Figure 3.2. The internal control and *Arabidopsis* control oligonucleotides were used as controls for successful hybridization.

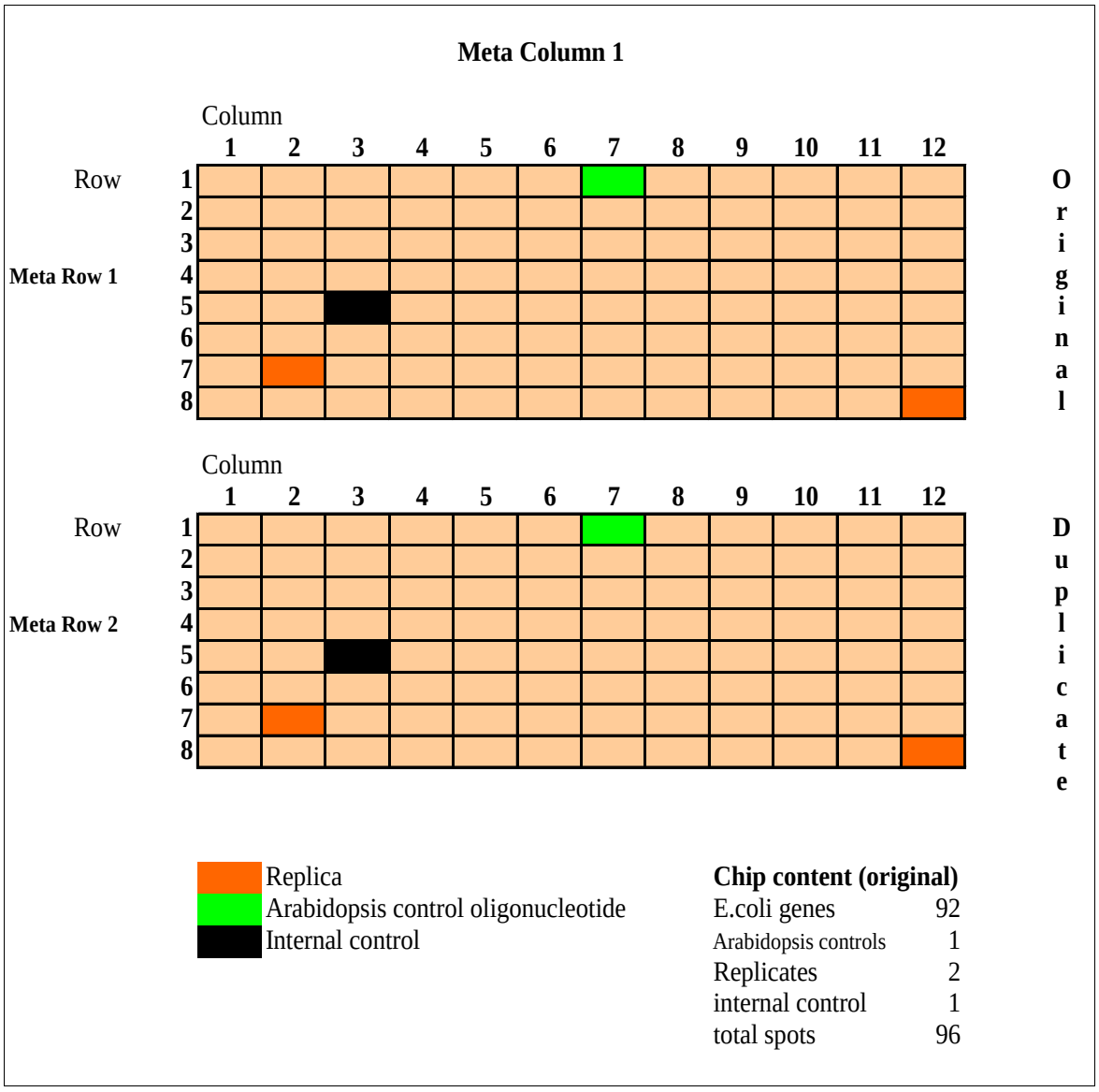

Figure 3.2. Chip Content and Orientation of *E. coli* K12 Starter V2 microarray slide (Source: Ocimum Biosolutions Inc., MD).

Six different reactions – three different comparisons, including dye swaps – were performed utilizing six microarray slides. Slides 1 and 2 compared untreated, non-heat shocked samples to colostrum-treated, non-heat shocked samples. Slides 3 and 5 compared untreated, non-heat shocked samples to untreated, heat shocked samples. Slides 4 and 6 compared colostrum-treated, non-heat shocked samples to colostrum-treated, heat shocked samples.

Hybridization

Hybridization of labeled cDNA to the array slides was performed as recommended by the kit and array manufacturer (Ocimum Biosolutions Inc., MD). Briefly, to locate the arrays, the microarray Gene Frames® (Ocimum Biosolutions Inc., MD) were used with a salt-based hybridization buffer. The salt-based hybridization buffer was preheated (42 ^{o}C, 10 minutes) and 120 µl of hybridization buffer was added to concentrated cDNA. The labeled cDNA/hybridization mixture was then heated (3 minutes, 95 ^{o}C) and incubated on ice (maximum of 3 minutes). The mixture was centrifuged briefly. The array finder was used to locate the spotted area on the Ocimum-array. To prepare the Gene Frames® (Ocimum Biosolutions Inc., MD), the thick polyester sheet was removed ensuring the frame remained bound to the thin polyester sheet. With the slide on a flat surface, the blue frame was laid over the microarray. The thin polyester sheet was removed and 120 µl of cDNA/hybridization mixture was applied to the Gene Frames® (Ocimum Biosolutions Inc., MD). The polyester cover slip was placed over the Gene Frames® (Ocimum Biosolutions Inc., MD) taking care to spread the mixture out evenly without trapping air. The gas-tight sealing system of the microarray

Gene Frames® (Ocimum Biosolutions Inc., MD) prevented reagent loss due to evaporation, thus improving hybridization.

The slides were placed into a wet hybridization chamber, which was placed into a damp gel box and incubated in the dark with intermediate shaking for 24 hours. After the hybridization, the Gene Frame® (Ocimum Biosolutions Inc., MD) was carefully removed.

Washing

The hybridized arrays were placed into a clean slide staining jar filled with 200 ml of pre-warmed washing buffer 1 and washed for 5 minutes with gentle agitation. The hybridized arrays were then washed in washing buffer 2 and washing buffer 3 for 5 minutes each as performed with the washing buffer 1. The slides were placed into 50-ml conical tubes and dried by centrifugation (1800 rpm, 2 minutes). (See Appendix E for a complete Ocimum *E. coli* K12 V2 Array Protocol from Ocimum Biosolutions, Gaithersburg, MD).

Scanning Procedure

Following the washing steps of the microarray slides, laser detection of Cy-3 and Cy-5 fluorescence after hybridization was immediately performed using the epifluorescence confocal microscope Affymetrix® 428™ Array Scanner (Affymetrix, CA). The Affymetrix® 428™ Array Scanner uses lasers to excite fluorphores at 532 nm and 635 nm. The Jaguar™ version 2.0 software controls the 428 scanner and computes intensity results. During scanning, two photomultiplier tubes with different filters collect and convert the fluorescent emissions into electrical current. The Affymetrix® 428™ Array Scanner converts this current into a numeric value through an analog to digital

converter. These digital intensity values were collected from pixels (10 x1 10 microns) on the array surface and are saved to an image data *.tiff files using Jaguar software.

The Cy3 dye has peak absorption at 550 nm and a peak emission at 570 nm. The Cy5 dye has peak absorption at 649 nm and a peak emission of 670 nm. The laser was used to scan the Cy3 in conjunction with the 570 nm filter and Cy5 in conjunction with a 665 nm filter. One scan at 10 microns was performed for each sample for labeled Cy3 and Cy5 dyes.

Data Acquisition

Microarray data was acquired using Jaguar™ version 2.0 software, which controlled the Affymetrix® 428™ array scanner and provided a complete system for image acquisition.

Microarray Image Analysis

The images of each microarray slide obtained were analyzed using Imagene® 9.0. Imagene® 9.0 is purchasable software developed by Biodiscovery Inc. (El Segundo, CA). The software was used during a 15-day free demo of the software.

Data Processing

When the microarray experiments were performed, the raw data was recorded in the form of one picture file that contained two separate images. These files were in the format of a TIFF file with the suffix is *.tiff. These were gray scale images with the data from each color dye captured in a combined file; therefore a single file has both Cy3 and Cy5 data. The specially designed software takes the *.tiff file and generates a color associated with the different intensities of gray. If red and green dyes are co-localized,

the software will depict a yellow color. The *.tiff file was loaded into the Imagene 9.0 software; which showed the separate Cy3 and Cy5 images in grayscale, along with a red-green-yellow color image that contained both Cy3 and Cy5 spots. The spot coordinates then had to be matched with the gene names. Since known *E. coli* gene locations were used, a text file was created that was interpreted by Imagene® 9.0 software to identify each spot. This was a critical step for subsequent data analysis. The gene list was loaded into the Imagene® 9.0 software and matched with the spots on the microarray (See Appendix F for the *Escherichia coli* Gene List).

Quantifying Spots

The *.tiff files were then converted into numerical values. Spots were located using Imagene® 9.0 software's method of gridding. Grids were placed around each located spot to ensure uniformity of spot intensities. Position and size of the identified spots were modified to fit the observed spots if the program missed any due to background noise or weak spots. Once the software knew where to locate the florescent dye data, it quantified the amount of red and green present in the *.tiff file. These numbers were used to calculate ratios of expression. Calculation of the expression ratios of the corresponding spots allowed pairwise comparison of the relative transcript levels for each *E. coli* gene under the treatments.

Comparing Genes

The data was displayed in a few ways. First, the ratios were log transformed by the Imagene® 9.0 software to make an induction and repression on the same scale. The highest ten and lowest ten expressed genes were identified for each slide. Then, the

highest five expressed genes for the dye swap (slide 1 and slide 2) were compared to observe if the dye treatment had any effects on the expression levels of those genes. NCBI database was used to identify gene function.

Statistical Analysis

Analysis of variance (ANOVA) was performed using the statistical analysis software SAS version 9.2 (SAS Institute, Inc., NC) to analyze the effect of treatment (-CNHS, -CHS, +CNHS, and +CHS), dye (Cy3 and Cy5), and the interaction of both treatment and dye on the level of gene expression. Because the size of data is large, it can be assumed the data is approximately normal and the variances are equal; therefore, a parametric statistical test like ANOVA is suitable. The ANOVA test was conducted with the gene expression as a dependent variable and the treatments and dyes as independent variables.

CHAPTER 4

Results

Growth Curve

The growth patterns of untreated and colostrum-treated *E. coli* samples are shown in Figure 4.1. Slight differences in the growth curve were observed. The growth curve of the colostrum-treated samples was slightly steeper following a short lag phase in the growth. The OD readings of untreated samples were the highest, but the final OD readings of the samples only differed by 0.11. Colostrum did not change the time of mid-log growth, even though initial slow growth may have occurred. Both cultures reached an OD_{550} of 0.3-0.5 in approximately 1 hour and 50 minutes. This pattern was observed in 3 experiments.

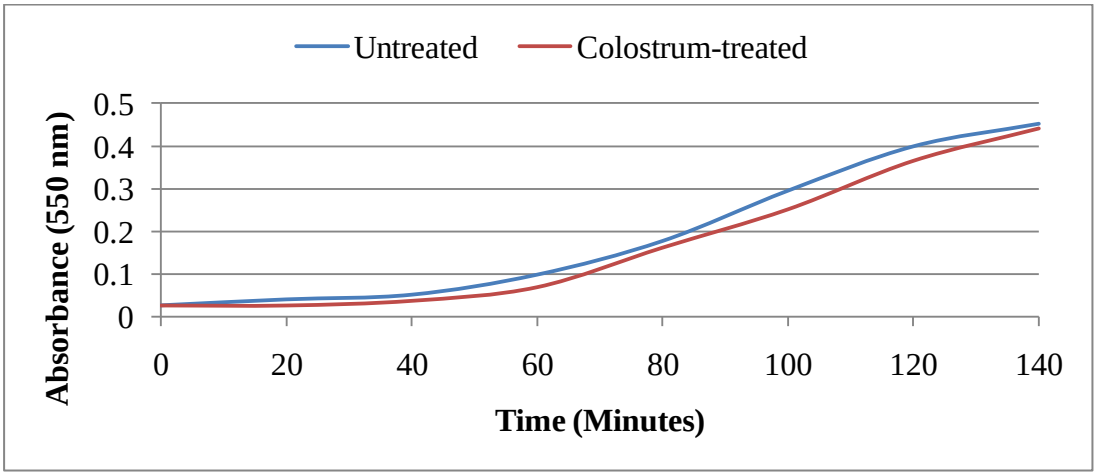

Figure 4.1. Representative *E. coli* K-12 growth curves. Bacteria were incubated in the presence of colostrum. Absorbance readings were taken every twenty minutes on a spectrophotometer. The untreated samples are represented by the blue line, while the colostrum-treated samples are represented by the red line.

Determination of Total Bacterial Counts Using Serial Dilutions and Plating

The total bacterial counts obtained from serial dilutions of aliquots for each time point for untreated and colostrum-treated samples, the number of colonies after incubation, the number of cells per milliliter, and the OD_{550} are presented in Tables 4.1 and 4.2. In untreated samples an OD reading of 0.178 at the 80-minute time point, and with a dilution of 10^6 yielded approximately 250 x 10^6 cells per milliliter yielded enough bacteria for RNA isolation using the RNeasy Maxi Kit.

Table 4.1. Numbers of bacteria harvested from untreated mid-log culture growth of *E. coli.* Serial dilutions of samples from each time point were conducted. The total number of colonies after plating and overnight incubation was used to calculate the number of cells per milliliter to correlate numbers to OD_{550} readings.

Time Aliquots (minutes)	Serial Dilutions	# of Colonies After Incubation	Cell Number per Milliliter	OD_{550}
60	10^2	Lawn	N/A	0.099
	10^4	136	136 x 10^4	
	10^5	101	101 x 10^5	
	10^6	5	5 x 10^6	
80	10^2	Lawn	N/A	0.178
	10^4	948	948 x 10^4	
	10^5	262	262 x 10^5	
	10^6	250	250 x 10^6	
100	10^2	Lawn	N/A	0.296
	10^4	1000	1000 x 10^4	
	10^5	77	77 x 10^5	
	10^6	6	6 x 10^6	
120	10^2	Lawn	N/A	0.400
	10^4	Lawn	N/A	
	10^5	230	230 x 10^5	
	10^6	112	112 x 10^6	

In colostrum-treated samples, the 80-minute time point, at a serial dilution of 10^6 resulted in approximately 6 x 10^6 cells per milliliter of the original aliquot. This dilution was chosen because of the total number of available cells per milliliter for RNA isolation. A total of 2.6 x 10^6 cells per milliliter of the original culture and 100 ml of culture were used as starting material for the RNA isolation with approximately 2.6 x 10^9 *E. coli* cells total from the untreated samples and 6 x 10^8 *E. coli* cells total for the colostrum-treated samples. The starting number of bacteria for RNA isolation was lower for colostrum-treated samples than untreated samples.

Table 4.2. Numbers of bacteria harvested from colostrum-treated mid-log cultures of *E. coli.* Serial dilutions of samples from each time point were conducted. The total number of colonies after plating and overnight incubation was used to calculate the number of cells per milliliter to correlate numbers to OD_{550} readings.

Time Aliquots (minutes)	Serial Dilutions	# of Colonies After Incubation	Cell Number per Milliliter	OD_{550}
60	10^2	Lawn	N/A	0.069
	10^4	103	103 x 10^4	
	10^5	76	76 x 10^5	
	10^6	1	1 x 10^6	
80	10^2	Lawn	N/A	0.162
	10^4	420	420 x 10^4	
	10^5	72	72 x 10^5	
	10^6	6	6 x 10^6	
100	10^2	Lawn	N/A	0.252
	10^4	960	960 x 10^4	
	10^5	137	137 x 10^5	
	10^6	31	31 x 10^6	
120	10^2	Lawn	N/A	0.366
	10^4	1325	1325 x 10^4	
	10^5	267	267 x 10^5	
	10^6	35	35 x 10^6	

RNA Characterization using the Agilent 2100 Bioanalyzer

The Agilent 2100 bioanalyzer software identified the 16S and the 23S ribosomal RNA peaks, which dominate the electropherogram and gels. Several chips were run on the bioanalyzer, only one of them yielding decent results, although they came from the same samples. The data was taken from the chip that yielded the most consistent results. An example of a gel for a faulty run is shown in Figure 4.2. Degradation of colostrum-treated samples can be seen in the gel as additional molecular weight bands compared to controls and the molecular weight marker. Lanes 7 - 11 have the 16 S and 23 S bands positioned higher than they are supposed to be in the gel, and lanes 7, 9, and 10 are flagged for errors. Lane 12 is positioned too high and is missing the 16 S band. An example of a gel for a good run on the bioanalyzer is shown in Figure 4.3. The 16 S (lower) and 23 S (upper) bands are clearly defined in all the lanes. An example of an electropherogram for a faulty run on the bioanalyzer is shown in Figure 4.4(a). An example of an electropherogram for a good run is shown in Figure 4.4(b). The marker, 16 S and 23 S peaks are labeled on each electropherogram. When the two are compared, the distortion in of both the 16 S and 23 S peaks can be clearly seen in the electropherogram from the faulty run. The baseline of electropherogram is also clearly distorted. Table 4.3 represents the best run of the total RNA concentrations for all treatment samples obtained from the bioanalyzer. Untreated, non-heat shocked samples had an average total RNA concentration of 167.7 ng/µl. Untreated, heat shocked samples had an average total RNA concentration of 294.3 ng/µl. Colostrum-treated, non-heat shock samples had an average total RNA concentration of 189.7 ng/µl. Colostrum-

treated, heat shock samples had an average total RNA concentration of 286.3 ng/µl. Several chips were run, yielding inconsistent purities and concentrations. Due to these inconsistencies, the ND-1000 NanoDrop was used for any further RNA analysis.

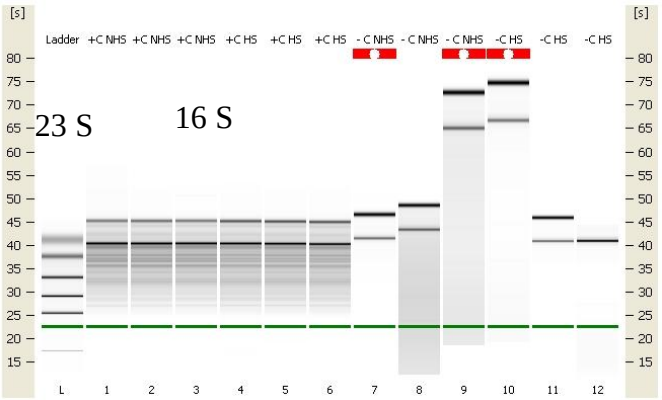

Figure 4.2. Agilent bioanalyzer RNA pseudo gel. This gel represents faulty run on the bioanalyzer. The first six lanes are contaminated by extra bands. The 16 S and 23 S bands are out of position in lanes 7- 11. Lane 12 is missing the 23 S band and the 16 S band is higher than it is supposed to be.

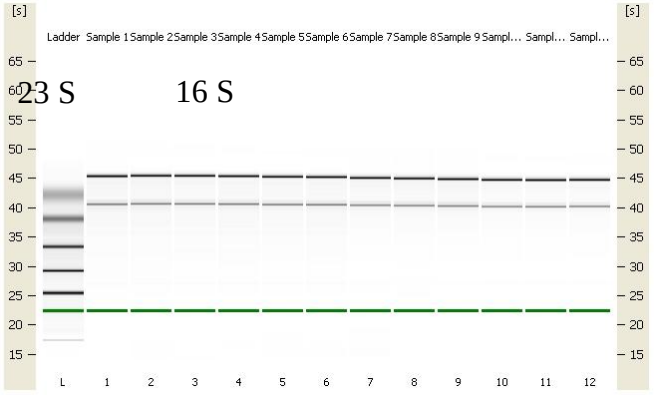

Figure 4.3. Agilent bioanalyzer RNA pseudo gel. This gel represents a good run on the bioanalyzer. The 16 S (lower) and 23 S (upper) bands are clearly defined in all the lanes. +CNHS samples in lanes 1-3. +CHS samples in lanes 4-6. –CNHS samples in lanes 7-9. –CHS samples in lanes 10-12.

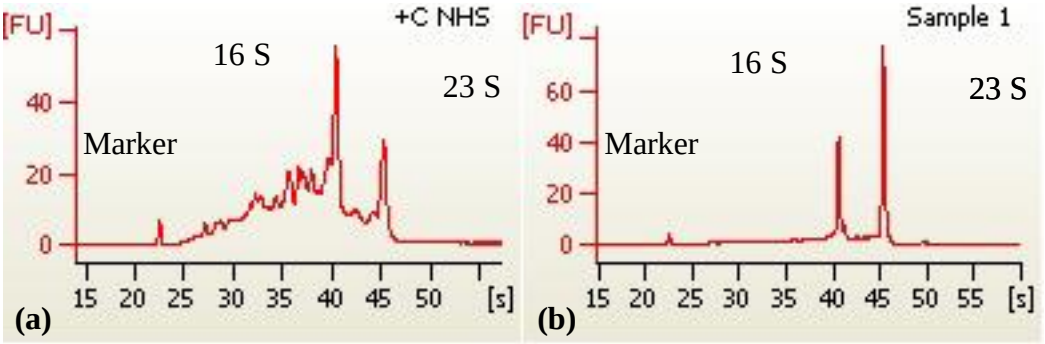

Figure 4.4. (a) Electropherogram from Agilent bioanalyzer (b) Electropherogram from Agilent bioanalyzer. (a) An example of an electropherogram from a faulty run on the bioanalyzer. There is a distortion in of both the 16 S and 23 S peaks and the baseline. (b) An example of an electropherogram from a good run on the bioanalyzer.

Table 4.3. Concentration of isolated total RNA evaluated using the Agilent 2100 Bioanalyzer. Total RNA concentrations for all treatments from 1 chip run on the Agilent 2100 Bioanalyzer.

Treatment	Concentration (ng/µl)
-CNHS	171
-CNHS	181
-CNHS	151
-CHS	278
-CHS	263
-CHS	342
+CNHS	186
+CNHS	194
+CNHS	189
+CHS	333
+CHS	274
+CHS	252

Concentration and Purity Using the NanoDrop Spectrophotometer

The concentrations and purities (280/260) for the four treatments are shown in Table 4.4. The concentrations of samples were found to be significantly different for

each treatment ($p < 0.0001$). The purity of isolated RNA was not significantly affected by treatment ($p>0.05$). The RNA concentrations varied for each of the four samples, as shown in table 4.4. RNA concentrations were highest in the colostrum-treated, non-heat-shocked samples (+CNHS), followed by the colostrum-treated, heat shocked samples (+CHS), then the non-treated, heat shocked samples (-CHS), and last, the non-treated, non-heat shocked samples (-CNHS). The non-treated, non-heat shocked samples (-CNHS) had an average concentration of 0.580 µg/µl and an average purity of 1.99. The non-treated, heat shocked samples (-CHS) had an average concentration of 0.662 µg/µl and an average purity of 1.94. The colostrum-treated, non-heat shocked samples (+CNHS) had an average concentration of 0.895 µg/µl and an average purity of 1.99. The colostrum-treated, heat shocked samples (+CHS) had an average concentration of 0.775 µg/µl and an average purity of 1.96.

Table 4.4. ND-1000 NanoDrop RNA concentrations and purities. Average RNA concentrations and purities for all treatments measured on the ND-1000 NanoDrop Spectrophotometer. RNA concentrations were significant due to treatment at $p < 0.0001$.

Treatment	Average Purity † (280/260)	Average RNA Concentration ‡ (µg/µl)
-CNHS	1.99	0.580
-CHS	1.94	0.662
+CNHS	1.99	0.895
+CHS	1.96	0.7755

† Means are not significantly different ($p > 0.05$)
‡ Means are significantly different ($p < 0.0001$)
N= 8

Polymerase Chain Reaction Analysis of Select Genes

A preliminary assay to evaluate individual gene expression using reverse transcriptase PCR (RT-PCR) with specific primers was conducted (Table 4.5). *E. coli* heat shock genes were successfully amplified (Figures 4.5-4.10). Table 4.5 summarizes the observed response to treatment.

Table 4.5. Observed response to treatment for PCR analysis. Gene ID, known response to heat shock, and response to each treatment. Band present denoted by +, double band denoted by +/+, and no band present denoted by -.

Gene	Known Response to Heat Shock	Response to Treatment			
		-CHS	-CNHS	+CHS	+CNHS
aceE	Up-regulation	+	-	+	+
fdoG	Up-regulation	+	+	+	+
hslV	Down-regulation	+	+/+	+/+	+/+
ldhA	Down-regulation	+	+/+	+	+/+
mscL	No effect	+	+	+	+
rplB	No effect	+	+/+	+	+/+

The hslV gene showed a loss of one of two bands only in response to the heat shock treatment without the addition of colostrum. The ldhA and rplB genes showed a loss of one of two bands in response to heat shock treatment. The mscL gene showed no change in the bands present, but colostrum treated sample bands were darker. Further optimization of the gel electrophoresis protocol is recommended. The DNA marker for several of the gels did not show up well, or in some cases at all, for unknown reasons. Large quantities of DNA remained in the wells and did not migrate through the gel. Purification of amplified DNA using Qiagen PCR Purification kits, dilution of amplified

DNA, or a reduction of the percentage of agarose in gel did not solve the problem without causing a loss of visible bands. Since a reducing the percentage of agarose in the gel didn't help, 2.0% gels were still utilized.

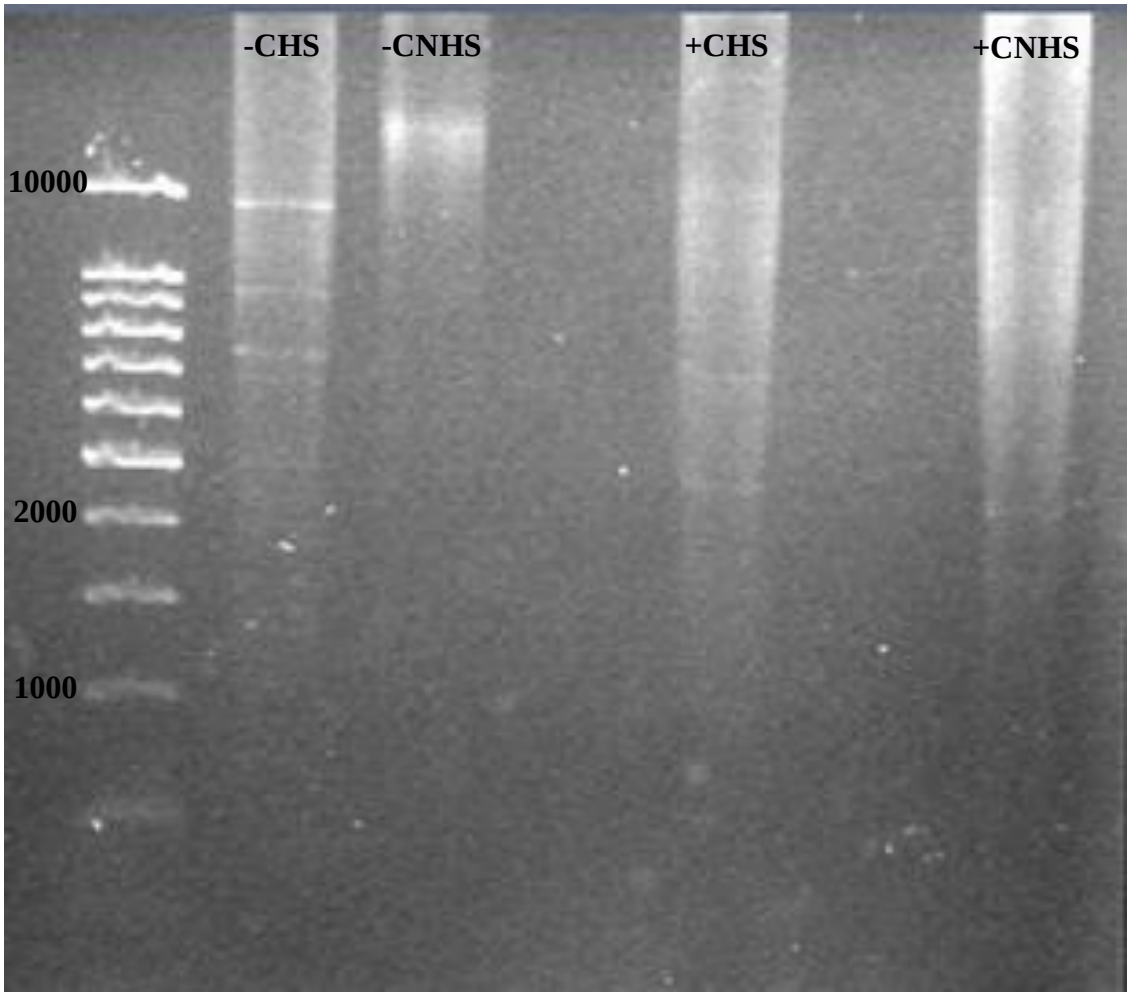

Figure 4.5. PCR analysis of *E. coli* heat shock gene aceE. This figure represents the best of 3 gel electrophoresis trials on 2.0% agarose gel. The arrows indicate where aceE bands should appear according to molecular weight. Lane 1 is the DNA marker, lane 2 is the -CHS treatment, lane 3 is the control (-CNHS), lane 6 is the +CHS treatment, and lane 8 is the +CNHS treatment.

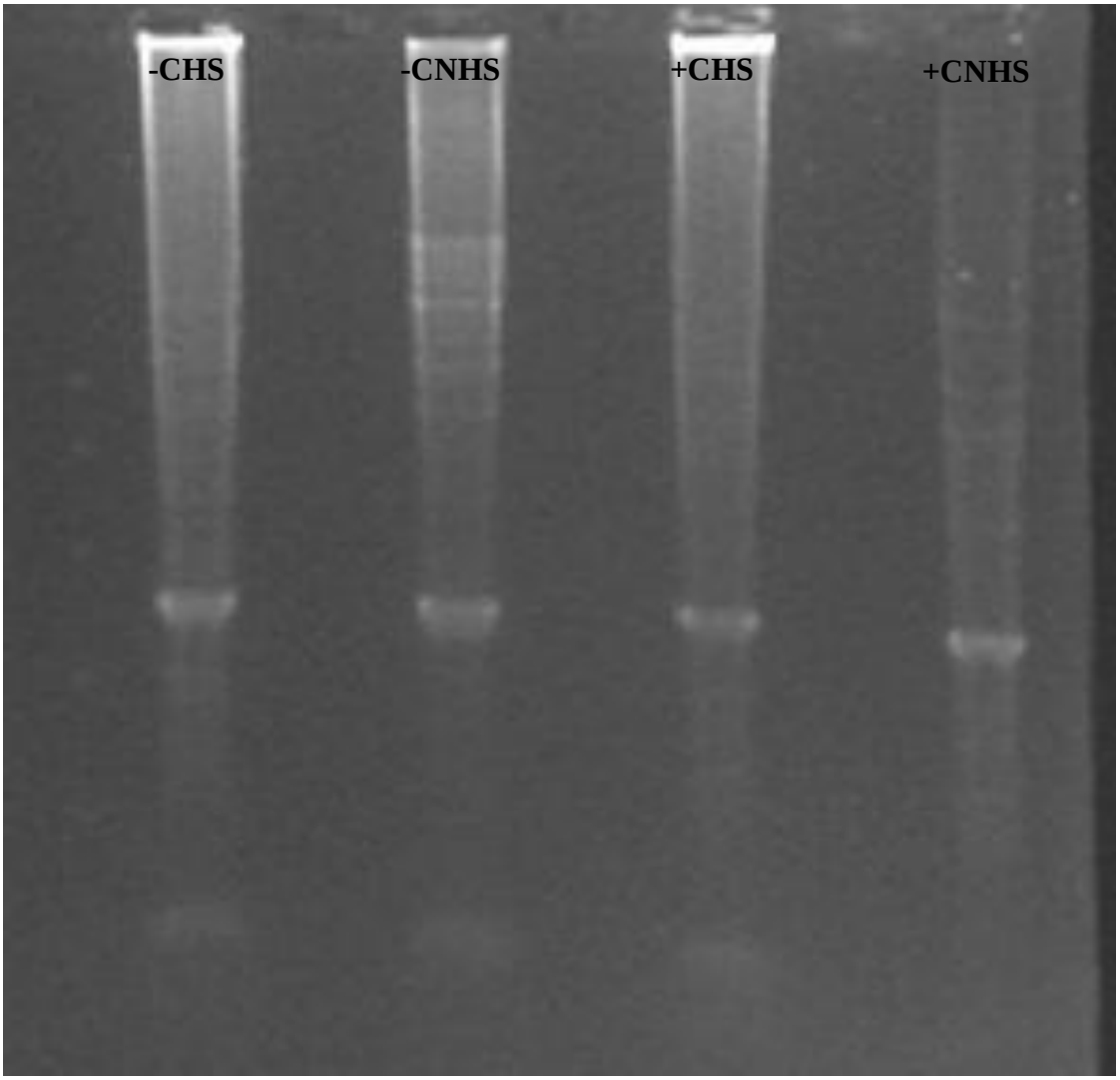

Figure 4.6. PCR analysis of *E. coli* heat shock gene fdoG. This figure represents the better of 2 gel electrophoresis trials on 2.0% agarose gel. The DNA marker did not show up well on these gels. The arrows indicate where fdoG bands appear. Lane 1 is the DNA marker, lane 2 is the -CHS treatment, lane 4 is the control (-CNHS), lane 6 is the +CHS treatment, and lane 8 is the +CNHS treatment.

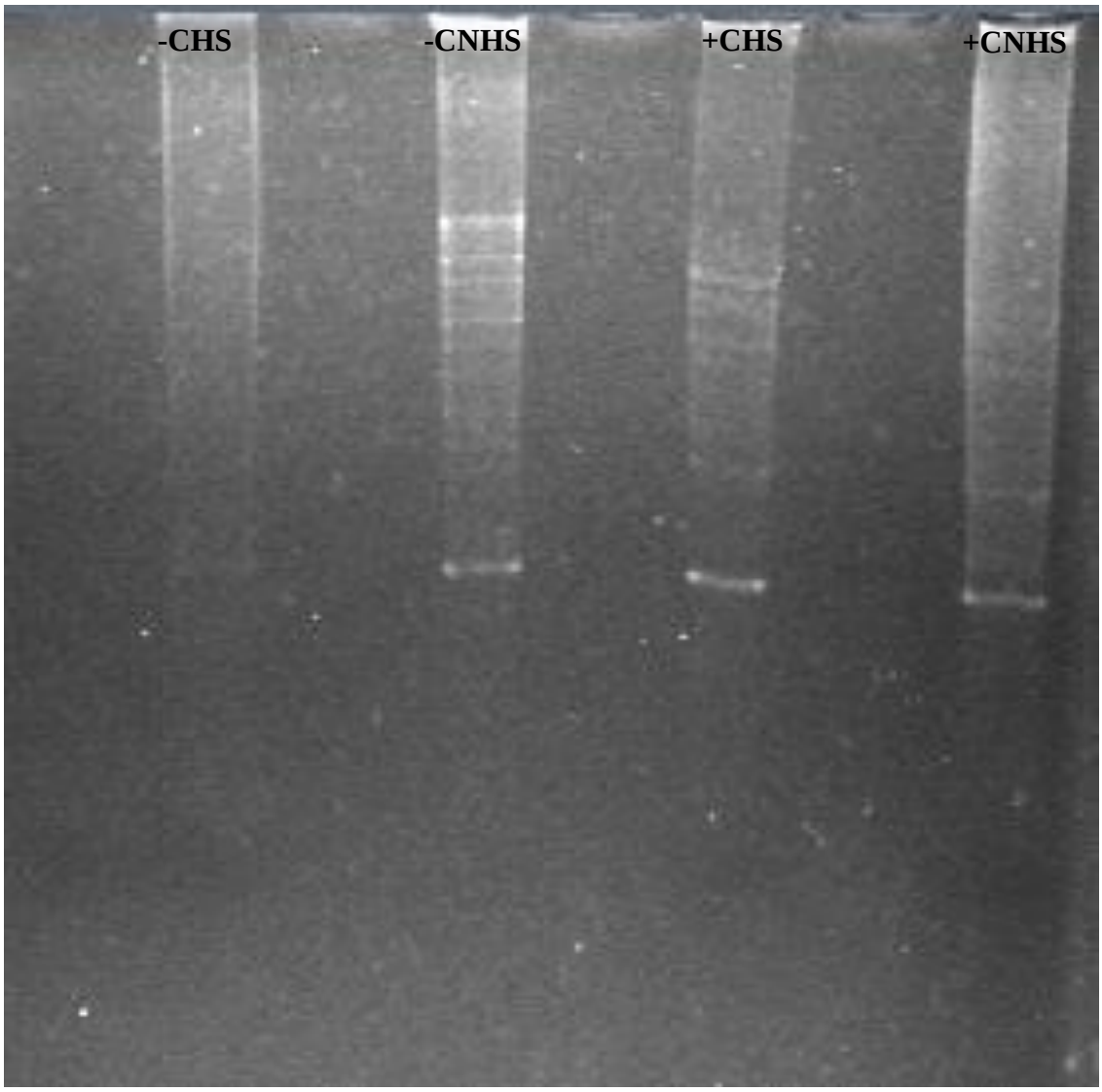

Figure 4.7. PCR analysis of *E. coli* heat shock gene hslV. This figure represents the best of 3 gel electrophoresis trials on 2.0% agarose gel. The DNA marker did not show up on these gels. The arrows indicate where hslV bands appear. Lane 1 is the DNA marker, lane 2 is the -CHS treatment, lane 4 is the control (-CNHS), lane 6 is the +CHS treatment, and lane 8 is the +CNHS treatment.

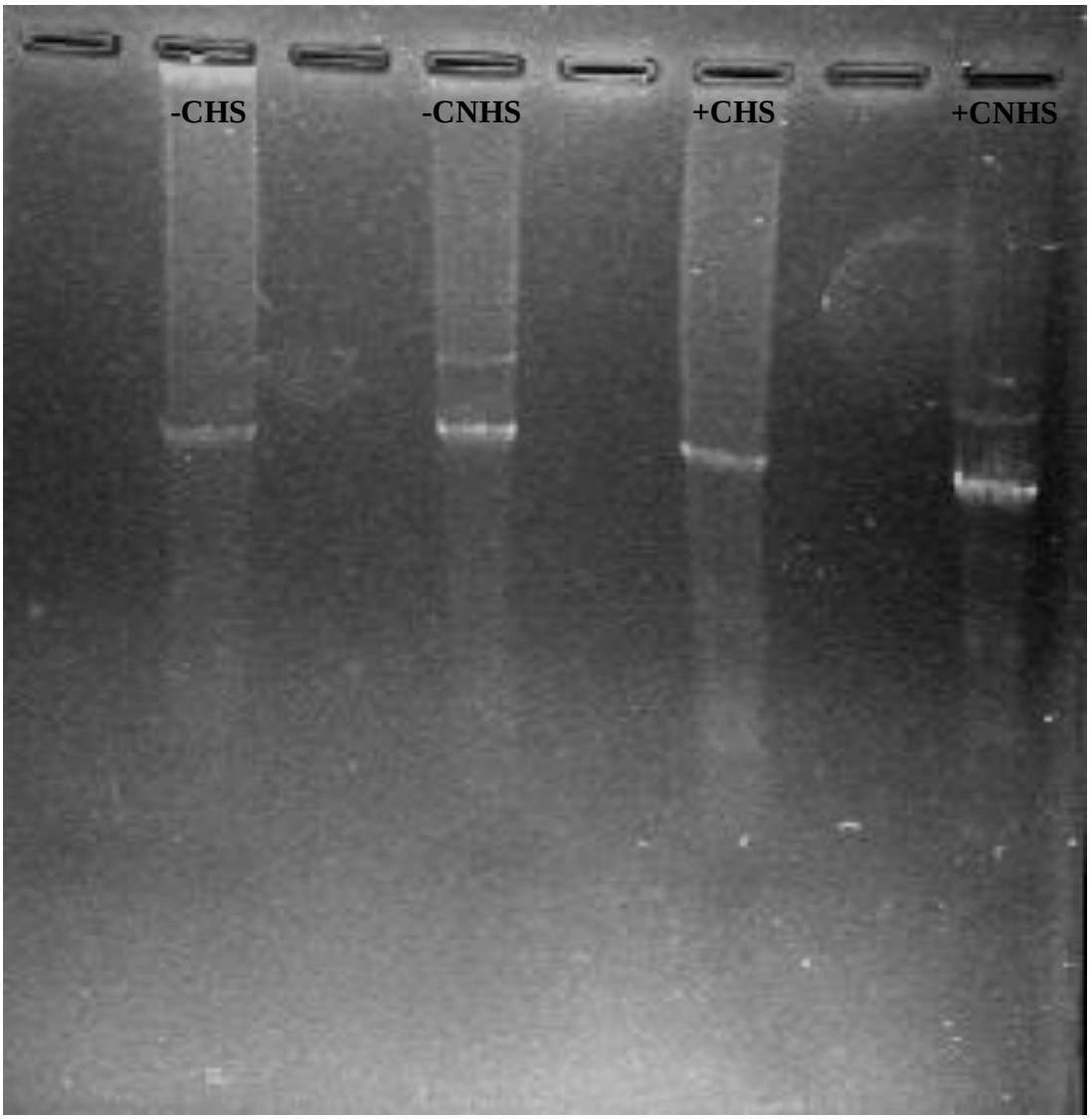

Figure 4.8. PCR analysis of *E. coli* heat shock gene ldhA. This figure represents the best of 3 gel electrophoresis trials on 2.0% agarose gel. The DNA marker did not show up on these gels. The arrows indicate where ldhA bands appear. Lane 1 is the DNA marker, lane 2 is the -CHS treatment, lane 4 is the control (-CNHS), lane 6 is the +CHS treatment, and lane 8 is the +CNHS treatment. The band is fainter for -CHS treatment and darker for the +CNHS treatment.

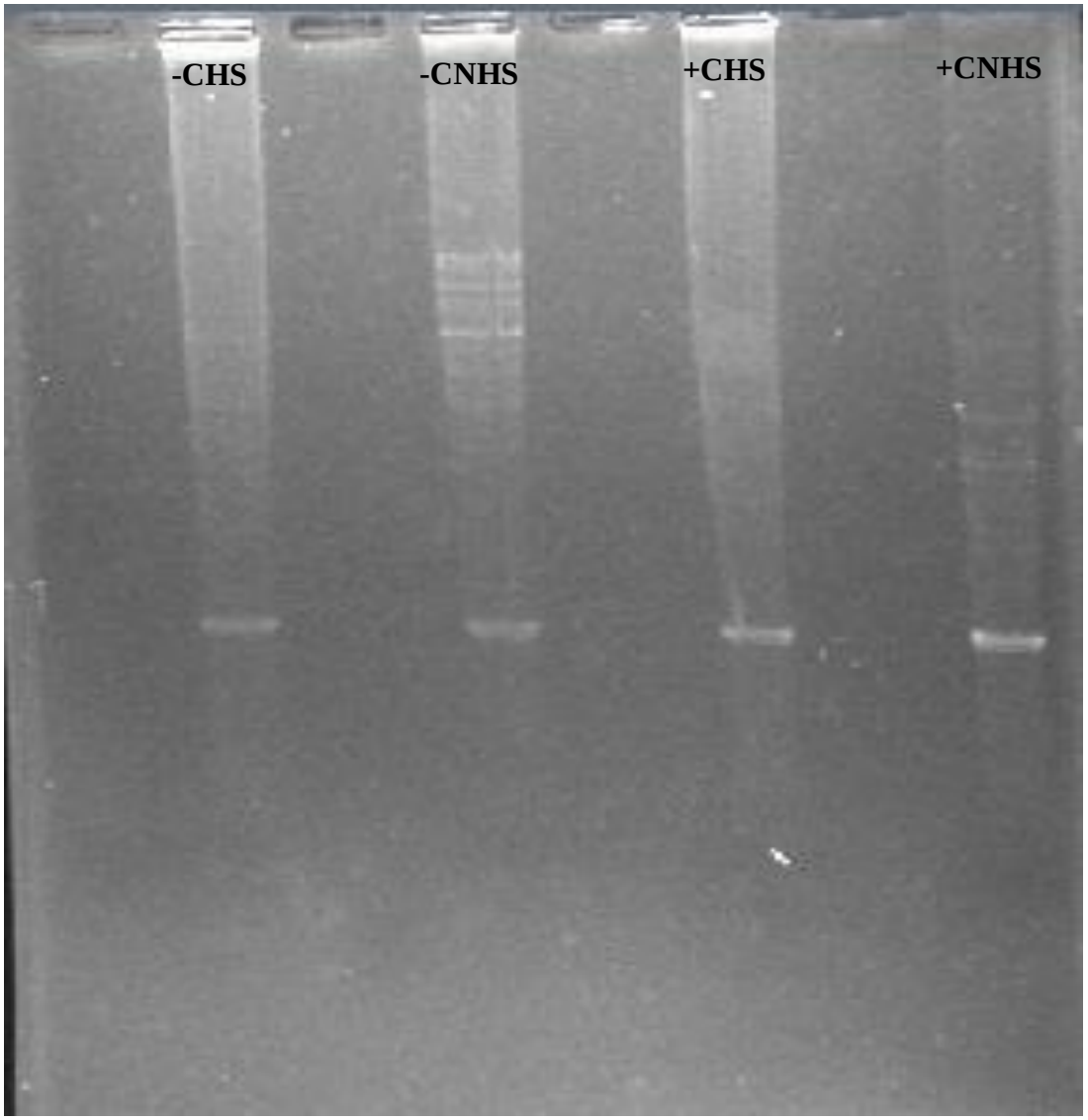

Figure 4.9. PCR analysis of *E. coli* heat shock gene mscL. This figure represents the better of 2 gel electrophoresis trials on 2.0% agarose gel. The DNA marker did not show up on these gels. The arrows indicate where mscL bands appear. Lane 1 is the DNA marker, lane 2 is the -CHS treatment, lane 4 is the control (-CNHS), lane 6 is the +CHS treatment, and lane 8 is the +CNHS treatment.

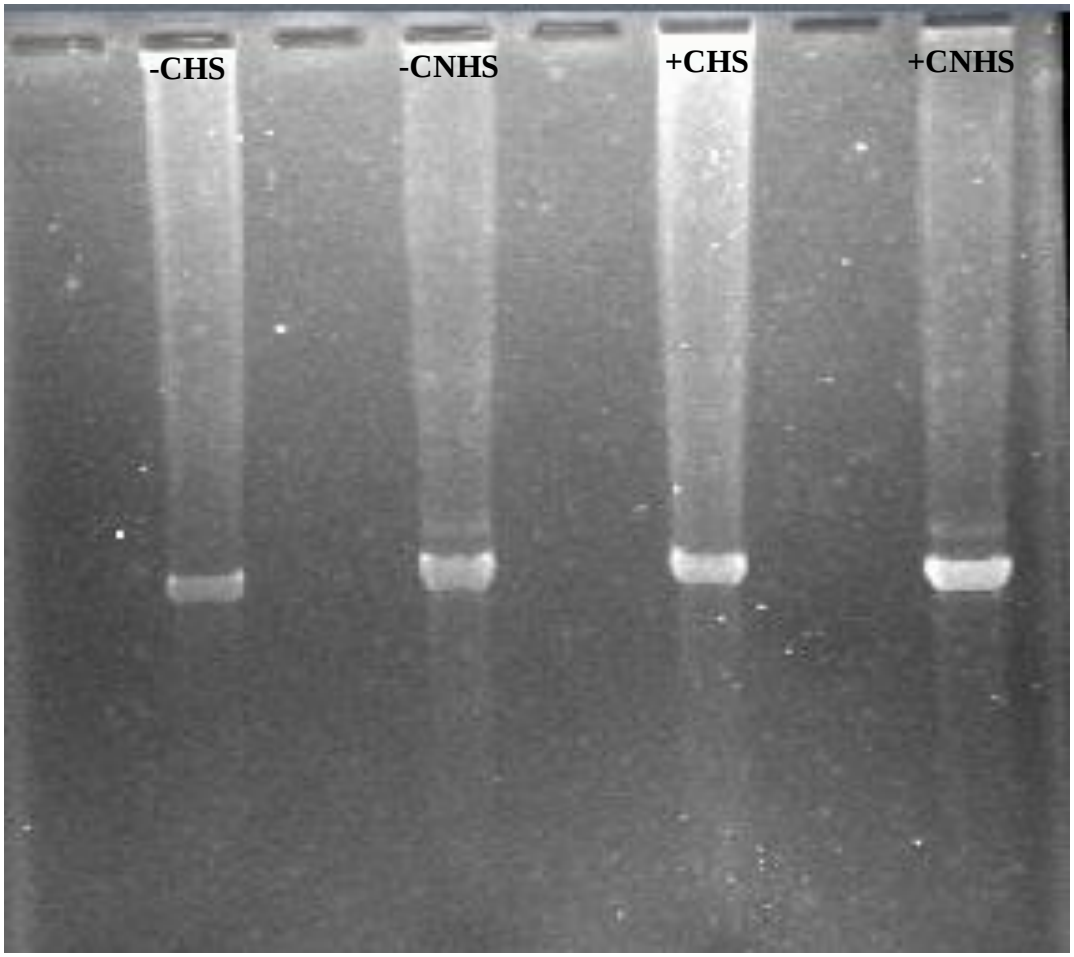

Figure 4.10. PCR analysis of *E. coli* heat shock gene rplB. This figure represents the better of 2 gel electrophoresis trials on 2.0% agarose gel. The DNA marker did not show up on these gels. The arrows indicate where rplB bands appear. Lane 1 is the DNA marker, lane 2 is the -CHS treatment, lane 4 is the control (-CNHS), lane 6 is the +CHS treatment, and lane 8 is the +CNHS treatment.

Global E. coli Gene Expression Profiling Using Microarray Analysis

Successful hybridization was achieved on four microarray slides as indicated by spot intensities captured using the Jaguar analysis software and the *.tiff images. All slides were very faint. Slide 1 had a partial hybridization, only one of two grids containing spots, shown in Figure 4.11 (a). Slides 2 and 3 had complete hybridization,

both containing spots in both grids, shown in Figures 4.11 (b) and 4.11 (c). Slide 4 had complete hybridization; however, residual wash buffer contaminated the image blocking some of the spots, shown in Figure 4.11 (d). The best image was slide 3. The Cy3 images for all successfully hybridized slides are shown in Figures 4.12 (a), (b), (c), and (d). On all successfully hybridized slides, the Cy5 dye resulted in a low intensity and blurred *.tiff image for analysis. The Cy5 images for all successfully hybridized slides are shown in Figures 4.13 (a), (b), (c), and (d). The grid was aligned based on provided gene lists and array design (Ocimum Biosolutions, CA). The gridding allows for analysis programs to locate the spots and perform further analysis. The gridded images appeared as shown in Figure 4.14.

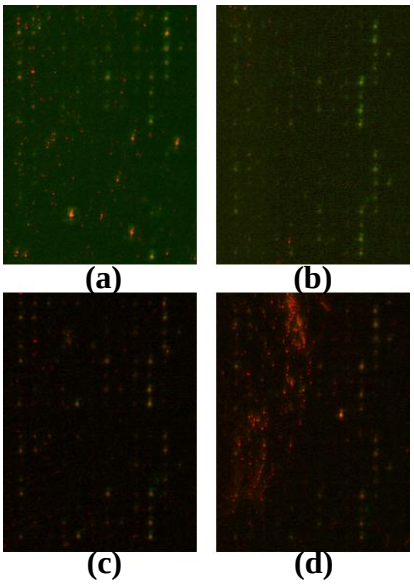

Figure 4.11. *.tiff file images for slides 1-4. Images of signal generated when Jaguar merges Cy5 and Cy3 image data together. (a) Slide 1 *.tiff image, +CNHS vs. -CNHS (b) Slide 2 *.tiff image, dye swap of 1 (c) Slide 3 *.tiff image, -CNHS vs. -CHS (d) Slide 4 *.tiff image, +CNHS vs. +CHS

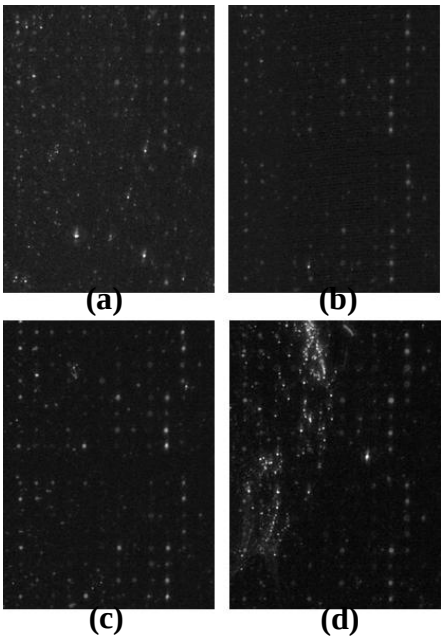

Figure 4.12. Cy3 images for slides 1-4. Images of signal generated when the microarray scanner was used to detect the Cy3 signals. (a) Slide 1 *.tiff image, +CNHS (b) Slide 2 *.tiff image, -CNHS (c) Slide 3 *.tiff image, -CHS (d) Slide 4 *.tiff image, +CHS

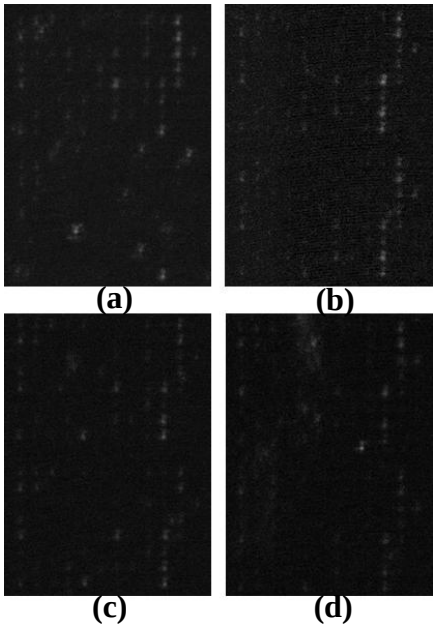

Figure 4.13. Cy5 images for slides 1-4. Images of signal generated when the microarray scanner was used to detect the Cy5 signals. (a) Slide 1 *.tiff image, -CNHS (b) Slide 2 *.tiff image, +CNHS (c) Slide 3 *.tiff image, -CNHS (d) Slide 4 *.tiff image, +CNHS

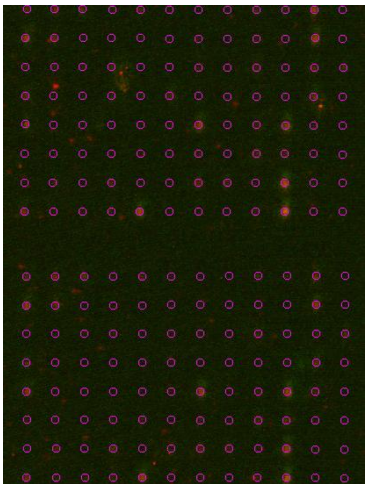

Figure 4.14. Example of a gridded microarray. Grid was aligned by Imagene® 9.0 based on provided gene lists and array design (Ocimum Biosolutions, CA). The grid is comprised of two metagrids, each with 96 spots, the second a duplicate of the first.

The gene expression values from the *.tiff files associated with each gridded spot was used to calculate ratios from both Cy3 and Cy5 associated samples. The data was normalized to place all the ratios on the same scale of magnitude and to eliminate any fractions. Ratios above 0 indicated up-regulation of the *E. coli* genes and below 0 indicated down-regulation or no regulation.

An analysis of variance was conducted to assess the effects of treatment and dye. Statistical analysis (ANOVA and LSD) of the dye swap gene expression data showed that dye did not affect gene expression, and that there was no interaction effect of the dye and treatment. The analysis also showed that colostrum treatment without heat shock did have a significant effect on gene expression ($p<0.1$). Further analysis that included data from experiments where no dye swap was conducted showed that both treatment and dye

had an effect. Evaluation of gene expression was only conducted on data from the dye swap experiment.

Slide 1 studied the effect of colostrum on *E. coli* gene expression. Analysis of slide 1 raw data with Imagene® 9.0 showed 52 out of the 96 genes were up regulated at different levels of expression. The five highest expressed genes were cydA, yi81_2, nagE, nagD, and dnaJ_2. The five lowest expressed genes were ycjX, chaA, rpsG, sucB, and hslV. A scatter plot analysis of the normalized Cy3 and Cy5 values of the 96 genes is shown in Figure 4.15. The five highest and lowest expressed *E. coli* genes for slide 1 are shown in Table 4.6.

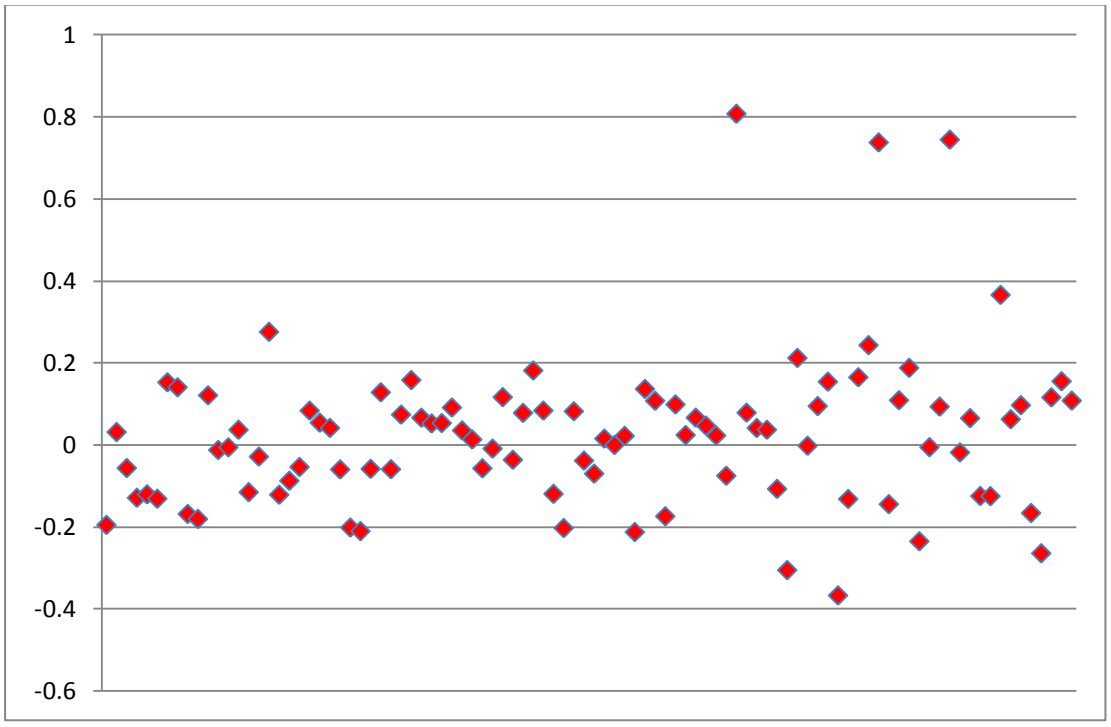

Figure 4.15. Slide 1 scatter plot. Effect of colostrum on *E. coli* gene expression. Genes above zero on the y-axis are the up-regulated genes. Genes below the zero on the y-axis are the down-regulated.

Table 4.6. Highest and lowest expressed *E. coli* genes for slide 1. The five highest and lowest expressed genes of slide 1 analyzed using Imagene® 9.0.

	Symbol	Gene	Description
Highest Expression	cydA	B0733	Polypeptide subunit I of Cytochrome D terminal oxidase
	nagE	B0679	PTS system, N-acetylglucosamine-specific enzyme IIABC
	nagD	B0675	N-acetylglucosamine metabolism
	dnaJ	B0015	Heat shock protein, chaperone with DnaK
	Yi81_1	B0016	IS186 hypothetical protein
Lowest Expression	ycjX	B1321	Putative EC2.1 enzymes
	chaA	B1216	Sodium-calcium/proton antiporter
	rpsG	B3341	30S ribosomal subunit protein S7, initiates assembly
	sucB	B0727	2-oxoglutarate dehydrogenase (dihydrolipoyltranssuccinase E2 component)
	hslV	B3931	Heat shock protein hslVU. ATPase subunit, homologous to chaperones

Slide 2, a dye swap of slide 1, also studied the effect of colostrum on *E. coli* gene expression. Analysis of slide 2 raw data with Imagene® 9.0 showed that 73 out of the 192 genes were up-regulated at different levels of expression. The five highest expressed genes were htpX, mgsA, nuoF, atpD, and yobF. The five lowest expressed genes were hscA, yeaN, hfq, cyoB, and rplB. A scatter plot analysis of the normalized Cy3 and Cy5 values of the 192 genes is shown in Figure 4.16. The five highest and lowest expressed *E. coli* genes for slide 2 are shown in Table 4.7.

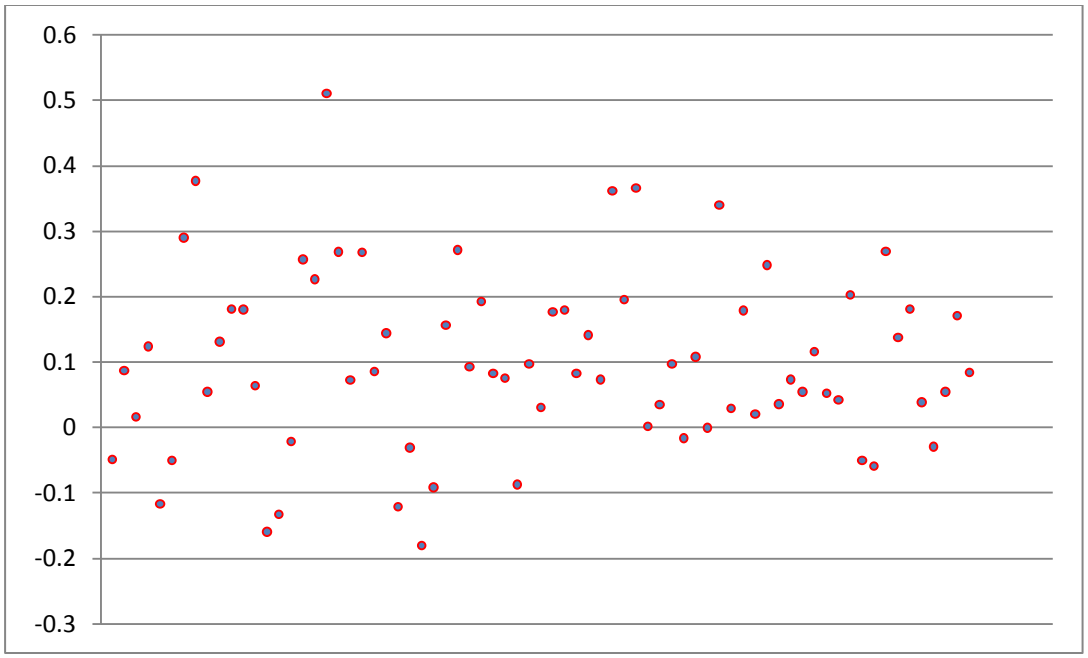

Figure 4.16. Slide 2 scatter plot. Effect of colostrum on *E. coli* gene expression. Genes above zero on the y-axis are the up-regulated genes. Genes below the zero on the y-axis are the down-regulated.

Table 4.7. Highest and lowest expressed *E. coli* genes for slide 2. The five highest and lowest expressed genes of slide 2 analyzed using Imagene® 9.0.

	Symbol	Gene	Description
Highest Expression	mgsA	B0963	Methylglyoxal synthase
	htpX	B1829	Heat shock protein, integral membrane protein
	nuoF	B2284	NADH dehydrogenase I chain F
	atpD		F1 sector, beta subunit of membrane-bound ATP synthase
	yobF	B1824	Open reading frame, hypothetical protein
Lowest Expression	yeaN	B1791	Putative amino acid/amine transport protein
	hscA	B2526	Heat shock protein, chaperone, member of Hsp70 protein family
	hfq	B4172	Host factor I for Bacteriophage Q beta replication, a growth-related protein
	cyoB	B0431	Cytochrome O ubiquinol oxidase subunit I
	rplB	B3317	50S ribosomal subunit protein L2

CHAPTER 5

Discussion and Conclusion

Escherichia coli cause mastitis by entering the mammary gland through the teat and causing inflammation of the mammary gland. *E. coli* is affected by exposure to immune components found in colostrum. These immune factors in include antibodies, Ig's, PRP's, cytokines, lactoferrin, lymphokines, oligopolysaccharides and glycolconjugate sugars, and lysozymes (Thapa, 2005). Colostrinin™ (CLN) – a PRP complex derived from mammalian colostrum – impacts signaling pathways and modulates gene expression in cellular redox regulation, cell proliferation, and differentiation (Szaniszlo et al., 2009). The objectives of this study were to evaluate the effect of host immune factors in colostrum on growth, RNA transcription, and gene expression in *E. coli* K-12.

In the present study, exposure of *E. coli* K-12 to colostrum resulted in different effects on growth. The short lag phase of the colostrum-treated *E. coli* in the growth curve comparison implies that colostrum has an inhibitory effect, even if only temporary, on *E. coli* cells. It is possible, since only a single dose of colostrum was used in the culture medium (LB broth), that continuous application of the colostrum may have continued to inhibit bacterial growth. The colostrum used in this experiment (Colostrum Plus®) is 100% pure bovine colostrum and is guaranteed to contain potent levels of key ingredients for the most immune support: 1.5% lactoferrin, 25% immunoglobulins, and 3% PRP's. Each of these immune factors is important because they are capable of causing

inhibition of bacterial growth. Lactoferrin has been shown to inhibit the growth of *E. coli*, along with several other microbes (Pakkanen and Aalto, 1997; Ellison et al., 1988). Immunoglobins are involved in opsonization, where the F_{ab} end of the Ig molecule and complement – particularly C3b – bind to the antigen or foreign body, flagging it for phagocytosis (Hurley and Morin, 2003). A study performed in 1976 showed that lactoferrin alone had a slightly inhibitory effect on the growth of *E. coli*, but the addition of specific antibody caused bacteriostasis (Griffiths and Humphreys, 1976). Lactoferrin helps deprive the bacteria of iron needed to reproduce. Studies have not yet been performed to observe the direct inhibitory effect of PRP's on bacteria, or specifically *E. coli.*

E. coli RNA quantity was impacted by exposure to colostrum and heat shock, when compared to controls. Analyses were performed with both the Agilent 2100 Bioanalyzer (Agilent Technologies, CA) and the ND-1000 NanoDrop (NanoDrop Technologies, Inc., DE). The isolation of total RNA was confirmed using an electropherogram generated on the Agilent 2100 Bioanalyzer. Initial analysis with the bioanalyzer showed degradation of RNA similar to those seen in lanes 1-6 of the pseudo gel shown in Figure 4.2. This showed that colostrum degraded the RNA and prompted the additional wash in LB broth made with DEPC-treated water just before RNA isolation. Degradation of the isolated RNA was no longer observed with the bioanalyzer after the adaptation of the additional wash. A powerful feature of the Agilent 2100 bioanalyzer is its ability to provide information about RNA integrity. As each RNA sample is analyzed, the software generates both a gel-like image and an electropherogram.

When analyzing total RNA, the areas under the 16S and 23S ribosomal RNA peaks are used to calculate the ratio of these two major ribosomal RNA species and these data are displayed along with quantitation data on individual electropherograms. Significant changes in the ratios of the 16S and 23S ribosomal RNA peaks are indicative of degraded RNA. Due to inconsistent concentrations on the same chip, expired Bioanalyzer reagents, and the need for exact concentrations, the ND-1000 NanoDrop was used for further RNA analysis. The NanoDrop assesses RNA concentration and purity using UV spectroscopy. Analysis with the NanoDrop showed that treatment had a significant effect on RNA concentration ($p<0.0001$) and not RNA purity, with the RNA isolated from the colostrum-treated samples without heat shock having the highest concentration, followed by the colostrum-treated samples with heat shock. The initial number of bacteria used for isolation was greater for untreated samples compared to colostrum-treated samples, so colostrum treatment may impact gene expression by increasing RNA transcription from DNA. The ND-1000 NanoDrop was shown to be a better way of measuring the concentration and purity of the RNA for this study. However, the Agilent 2100 Bioanalyzer was able to show degradation of the RNA due to the presence of colostrum, that the ND-1000 NanoDrop would not have been able to observe. The ND-1000 NanoDrop lacks the electropherogram images of the Agilent 2100 Bioanalyzer and the ability to distinguish RNA from DNA, but also requires smaller amounts of sample and requires no other reagents to run the analysis. The cost of running the analysis is also much cheaper and less time consuming.

All six genes selected for PCR analysis were successfully amplified using specific primers. The gene aceE was up-regulated in response to colostrum exposure and heat shock. This gene, decarboxylase component of pyruvate dehydrogenase, is involved in the TCA cycle. This component of pyruvate dehydrogenase catalyzes the initial decarboxylation and oxidation of pyruvate and generates a reduced and acetylated form of the dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase (Stephens et al, 1983). The gene hslV had a loss of one of two bands in response to heat shock without colostrum treatment. This gene, proteasome-related peptidase subunit of heat shock protein hslVU, is involved in the degradation of misfolded proteins. The gene has been shown to be up-regulated in heat shock experiments. The mRNA production of this gene is increased upon heat shock because it causes protein misfolding (Lien et al, 2009). The genes ldhA and rplB had a loss of one of two bands in response to heat shock. The gene ldhA, fermentative D-lactate dehydrogenase, is involved in stress response. This gene enables both production and utilization of lactate, a fermentation end product and a carbon source for the growth of bacteria (Toyoda et al, 2009). The gene rplB, 50S ribosomal protein L2, is involved in binding Zn(II) and is known to be the most important constituent of the peptidyl transferase center (Mikulik et al, 2001). The genes fdoG and mscL experienced no effect due to treatments. The results of the heat shock treatment did not agree with the predicted results from Ocimum Biosolutions; however, the predicted values came from an experiment where the samples underwent a 50 ºC heat shock treatment. The heat shock treatment in study was only 3 degrees higher than the incubation temperature for growing the *E. coli* to mid-log (40 ºC). This heat shock

treatment was chosen to best simulate the change in udder temperature due to mastitic infection. The change in udder temperature depends on the severity of the infection. A 1.0 to 3.0 ºC rise in udder temperature is expected with a mastitis infection (Scott et al, 2003). To have the most possible impact on expression, the highest of the change in temperature was chosen for this study. This change in temperature may not have been enough to effect the expression of expected *E. coli* genes. The normal growth range of *E. coli* is from 21 ºC to 37 ºC, and the maximum growth at which balanced growth can occur is approximately 49 ºC (Luders et al., 2009). The growth rate of several strains of *E. coli*, including K-12, is influenced in the high temperature range (40-45 ºC) by the availability of exogenous methionine; growth being limited in the absence of methionine (Luders et al., 2009). This explains why heat shock treatment seemed to have some effect, but not the predicted effects obtained from the Ocimum Biosolutions microarray study. Colostrum had a definite effect on *E. coli* gene expression. Further analysis with real-time PCR would be beneficial in determining different levels of gene expression for each treatment.

In the present study, the Ocimum Biosolutions *E. coli* K12 V2 Array starter kit subset was used to survey a limited number of *E. coli* genes associated with heat shock and established a technique for evaluating the immune components in colostrum. Heat shock is another form of stress, usually created by the body of the organism (systemic fever or local inflammatory response) in order to defend against an invading organism. HSP's protect cells from environmental stress damage by binding to partially denatured proteins, dissociating protein aggregates, and regulating the correct folding and

intracellular translocation of newly synthesized polypeptides (Leppa and Sistonen, 1997). In *E. coli*, the regulation of the expression of HSP's in – including DnaK, DnaJ, GrpE, GroEL, and GroES – is regulated by the product of the *rpoH* gene, the stress-inducible sigma 32 (σ32) subunit of RNA polymerase (Arsène et al., 2000). Mutations in any one of the three heat shock genes, dnaK, dnaJ, and grpE lead to constitutive levels of heat shock gene expression, implying that their products negatively auto regulate their own synthesis (Liberek and Georgopoulos, 1993).

The microarray study was successful; however some problems were encountered in the study that limited some of the microarray analysis. Suggested troubleshooting can be found in Appendix G. Imagene 9.0 was used to effectively perform an analysis on the microarray data for the four slides. The effect of colostrum on *E. coli* gene expression was analyzed on slide 1. Colostrum treatment resulted in an up-regulation of 65.6% of the genes hybridized on the array slide. The five highest expressed genes were cydA, yi81_2, nagE, nagD, and dnaJ_2. The five lowest expressed genes were ycjX, chaA, rpsG, sucB, and hslV. The effect of colostrum on *E. coli* gene expression was also analyzed on slide 2; however, this slide was a dye swap of slide 1. Colostrum treatment resulted in an up-regulation of 38.0% of the genes hybridized on the array slide. This slide was used to determine if dye had an effect on gene expression, which according to SAS analysis had no significant effect. The five highest expressed genes were htpX, mgsA, nuoF, atpD, and yobF. The five lowest expressed genes were hscA, yeaN, hfq, cyoB, and rplB. Slide 1 and 2, did not share the same highest expressed genes, but this

could be due to the poor hybridization, rather than an effect of the dye. There wasn't a huge difference in normalized spot intensities from gene to gene.

In conclusion, major findings from this study indicate that bovine colostrum has an effect on *E. coli* growth, transcription, and gene expression. Bovine colostrum impacts *E. coli* gene expression at the level of transcription. This study provides an approach to evaluate the effects of colostrum components on gene expression in *E. coli.* This approach may help define the role of immune components in colostrum in combating infection with *E. coli* in the mammary gland. Further studies on the impact of colostrum on bacteria growth may help dairy farmers and animal health practitioners identify the best time to sample animals for the presence of infection during the peripartuient period.

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APPENDIX A

RNA ISOLATION PROTOCOLS

MATERIALS AND METHODS
RNeasy Maxi Protocol for Isolation of Total RNA from Bacteria
Source: Qiagen, Catalog # 75162

1. Harvest bacteria by centrifuging at 4000 rpm for 10 minutes at 4°C decant supernatant, carefully remove all remaining media by aspiration. After centrifuging, heat the centrifuge to 20-25°C since the same centrifuge will be used.
2. Loosen the bacterial pellets by flicking the bottom of the tube. Resuspend the bacteria thoroughly in the appropriate volume of lysozyme – containing TE Buffer. Incubate at room temperature for 2-5 minutes for Gram Negative bacteria. (Maxi kit – $5x10^9$-$2.5x10^{10}$- add 2 ml of lysozyme TE Buffer)
3. Add appropriate volume of Buffer RLT (7.5 mls) mix thoroughly by vortexing. *Ensure that B-ME is added to Buffer RLT before use. (80u in 8mls of Buffer RLT) Prepare 2
4. Centrifuge the bacterial lysate for 10 minutes at 4,000 rpm. Carefully transfer supernatant to a new 50ml tube (not supplied) by pepetting. Use only this supernatant (lysate) in subsequent steps.
5. Add the appropriate volume of ethanol (96-100%) to the lysate. (5.5mls of ethanol to each tube) Mix thoroughly by shaking. DO NOT centrifuge. If a precipitate forms, it will not affect the RNeasy procedure.
6. Apply the sample to an RNeasy Maxi column placed into a 50ml centrifuge tube (supplied). Maximum loading volume is 15mls. Close tube gently and centrifuge for 5 minutes at 4,000 rpms. Discard the flow through and reuse the collection tube in step 7. If the maximum amount of starting material is used, it may be necessary to increase the centrifugation time to 10 minutes in order to allow the lysate to completely pass through the column.
7. Add 15mls of Buffer RWI to the RNeasy column. Close the centrifuge tube gently, and centrifuge for 5 minutes at 4,000 rpms to wash the column. Discard the flow through. Reuse the collection tube in step 8.
8. Add 10ml of Buffer RPE to the RNeasy column. Close the centrifuge tube gently and centrifuge for 2 minutes at 4,000 rpm to wash the column. Discard the flow through reuse the collection tube in step 9.
9. Add another 10ml Buffer RPE to the RNeasy column. Close the centrifuge tube gently and centrifuge for 5 minutes at 4,000 rpms to dry the RNeasy silica-gel membrane. It is important to dry the RNeasy membrane since residual ethanol may interfere with downstream reaction.
10. To elute, transfer the RNeasy column to a new 50ml collection tube (supplied). Pipet 0.8ml of RNease – free water directly onto the RNeasy silica-gel membrane. Close the tube gently. Let stand for 1 minute, then centrifuge for 3 minutes at 4,000 rpms.
11. To obtain a higher RNA concentration, this second elution step should be performed using the first eluted from step 10. The yield will be 15-30% less than the yield obtained from using a second volume of RNase-free water, but the final concentration will be higher.

APPENDIX B

QUANTITATION OF RNA

Quantitation of RNA

Dilution = 10 ul of RNA sample + 490 ul DEPC-treated water

Measure absorbance of diluted sample in a 1 ml Rnase-free cuvette

Extinction coefficient of RNA = 40

Concentration of RNA sample = 40 x A_{260} x dilution factor

Total RNA yield = RNA concentration x volume of sample in ml

APPENDIX C

AGILENT 2100 BIOANALYZER PROTOCOL

MATERIALS AND METHODS

Agilent 2100 Bioanalyzer Protocol
RNA 6000 Nano Lab Chip Kit

Source: Agilent Technologies, Catalog # 5065-4476

Materials Needed: RNA 6000 Nano Chip Kit
RNA gel matrix
RNA dye concentrate
RNA chips
RNA 6000 Nano Markers
Spin Filters
RNA 6000 Ladder

1. Take out RNA Nano 6000 reagents (stored at 4°C) and placed them in the dark at room temperature for 30 minutes before starting.
2. Dispense enough RNA 6000 ladder (stored at -70°C) for use in one day (~1µl per chip). Dispense enough RNA sample for use in one day. (1µl per lane) Sample may evaporate when heated, a 2µl aliquot is a good idea.

Decontaminating the Electrodes:
The electrodes on the bioanalyzer before and after each chip run. Each box of chips contain two electrode cleaner chips – these are labeled RNase Zap and RNase – free H_2O. Slowly pipet 350µl RNase – free H_2O into the appropriate chip. Place the RNase Zap electrode cleaner chip into the bioanalyzer. Close the lid, incubate for 1 minute. Open the lid and remove the chip. Placethe RNase- free H_2O electrode chip cleaner chip into the bioanalyzer, close the lid, incubate for 10 seconds, open the lid and remove the chip. Allow the bioanalyzer to dry with the lid open for at least 10 seconds before use. ** Remove the RNase Zap and the RNase – free H_2O out of the electrode cleaner at the end of the day.**

Preparing the Gel:
1. Prepare 500µl of RNA 6000 Nano gel matrix (red) into the top receptable of a spin filter.
2. Place the spin filler in a micro-centrifuge and spin for 10 minutes at 4000 rpms.
3. Discard the filter according to good laboratory practices. Aliquot 65µl filtered gel into 0.5ml

RNase –free H_2O microfuge tubes that are included in the kit. Store the aliquots at 4°C, use within one month.

Preparing the Gel-Dye Mix:
1. Protect the dye concentrate from light.
2. Vortex RNA 6000 Nano dye concentrate (blue) for 10 seconds and spin down.
3. Add 1µl of dye to a 65µl aliquot of filtered gel.

4. Cap the tube, vortex thoroughly and visually inspect proper mixing of gel and dye. Store the dye concentrate at 4°C in the dark.
5. Spin tube for 10 minutes at room temperature at 14,000 rpms. Use prepared gel-dy mix within 1 day.

Loading the Gel-Dye Mix:

* Make sure that the chip priming station base plate is in the C position before loading the gel-dye mix. Make sure that the adjustable clip is set to the upper position.

1. Take a new RNA Nano chip out of its sealed log.
2. Place the chip on the Chip Priming Station
3. Pipette 9.0µl of the gel-dye mix at the bottom of well, marked G and dispense the gel-dye mix. When pipetting the gel-dye mix, make sure not the draw particles that may sit at the bottom of the gel-dye vial. Insert the tip of the pipette to the bottom of the chip well when dispensing. This prevents a large air bubble forming under the gel-dye.
4. Set the timer to 30 seconds. Make sure that the plunger is set to 1.0ml, then close the Chip Priming Station. The lock of the latch will click when the priming station is correctly closed.
5. Press the plunger until it is held by the syringe clip.
6. Wait for exactly 30 seconds and then release the plunger with the clip release mechanism.
7. Wait for 5 seconds, then slowly pull back the plunger to the 1ml position.
8. Open the chip priming station.
9. Pipette 9.0µl of the gel-dye mix in each of the wells marked G

Loading the RNA 6000 Nano Marker

1. Pipette 5µl of the RNA 6000 Nano Marker (green) into the well marked with the ladder and each of the 12 sample wells.

* Add 6µl of RNA 6000 Nano Marker (green) to each unused sample well!

Loading the ladder and samples

1. Aliquot the amount of RNA 6000 ladder that you use within a day into a RNase- free microcentrifuge tube and heat denature it for 2 minutes at 70°C before use.
2. Pipette 1µl of the ladder into the well marked with the ladder symbol.
3. To minimize secondary structure, heat denature ((70°C) for 2 minutes) the samples before loading the samples on the chip.
4. Pipette 1µl of each sample into each of the 12 ample wells.
5. Place the chip in the adapter of the vortex set – point (2400 rpms). Carefully remove extra liquid with a tissue.
6. Make sure that the run is started within 5 minutes.

Inserting the chip into the Aglient 2100 Bioanalyzer

1. Open the lid of the Afilent 2100 Bioanalyzer. Check that the electrode cartridge is inserted properly and the chip selector is in the 1 position.

2. Place the chip into the receptacle. The chip fits only one way.
3. Carefully close the lid. The electrodes in the cartridge fit into the wells of the chip.
4. The 2100 expert software screen shows that you have inserted a chip and closed the lid by displaying the chip icon at the top of left instrument panel.

Starting the Chip Run

1. In the instrument context, select the appropriate assay from menu.
   Assays > Electrophoresis > RNA > Prokaryote Total RNA Nano.
2. Accept the current File Prefix. Data is automatically saved to a file with a name using the prefix you entered.
3. Click the start button in the upper right of the window to start the chip run. The incoming row signals are displayed in the instrument context.
4. To enter sample information like sample names, select Data File link that is highlighted in blue or go to the Data and Assay context and select the chip summary tab. Complete the sample name table and press "Apply".
5. To review the raw signal trace, return to Instrument context.
6. When chip run is finished, the End of Run message appears.

Cleaning up after the RNA Nano Chip Run

1. Slowly fill one well with 350µl RNase-free water
2. Open lid, place electrode cleaner in bioanalyzer close lid, for 10 seconds.
3. Open lid, remove cleaner.
4. Wait 10 seconds to allow water to evaporate.

Checking RNA Nano Results

To review results of a chip run, click the Data & Assay context.

RNA Nano Ladder Results

Select the Gel or Electropherogram tab in the Data & Assay context. Select the ladder well. Major peaks of a successful ladder run: 6 RNA peaks, 1 marker peak

RNA Nano sample well Results

Major features for a successful total RNA run: 2 ribosomal peaks (w/successful sample preparation), 1 marker peak

RNA Concentration

RNA was concentrated by centrifugation (2 hours, at room temperature) in a speed vacuum (Savant).

APPENDIX D

MWG-BIOTECH *E. COLI* K 12 STARTER KIT V2

MATERIALS AND METHODS
MWG-Biotech *E. coli* Starter Kit V2
Source: MWG Biotech Ag., Catalog # 2110-0000

Required reagents

Purified tatal RNA, RNase-free water, reverse transcriptase (Superscript ll, Invitrgen*), dNTP-mastermix, Cy3/Cy5-dCTP (PerkinElmer Life Sciences), random hexamers, 1 M HCl, 1 M NaOH, TE-buffer (pH 7.5), all reagents should be filter sterilized.
*Alternatively Superscript lll can be used.

Preparation of the dNTP – master mix for reverse transcription: - Kit

Mix the following reagents:

Reagent	Volume
100mM dATP (final conc. 5mM)	5µl
100mM dGTP (final conc. 5mM)	5µl
100mM dTTP (final conc. 5mM)	5µl
100mM dCTP (final conc. 2mM)	2µl
RNase-free water	83µl

Required materials and equipment

Non-powdered gloves, thermocycler (MWG-Biotech Primus, Order No.: 4000-000010) or heating block for two different temperatures, DNase- and RNase-free tubes, microliter pipettes with RNase-free pipette tips, QlAquick PCR Purification Kit (Qiagen).

Procedure

The cDNA reaction is designed for a total volume of 20 – 40µl using random hexamers. Perform the cDNA reaction for each RNA sample in a separate tube. Use for one sample Cy3-labeled dCTPs, for the other sample Cy5-labeled dCTP in the reaction mix. In general, the amount of RNA depends on the copy number of genes to be interrogated. We recommend to start with an amount of at least 50µg total RNA. For the MWG E.coli O157 Array we recommend to start with 100µg of total RNA.

1. Preapare the following reaction mix:

Reagent	Volume (50µg RNA)	Volume (100 µg RNA)
Total RNA in RNase-free water	6.0µl	16.5µl
Random hexamers (3µg/µl)	3.0µl	3.0µl

2. Incubate reaction mix at 65°C for 10min. (Heat Block)
3. Incubate at room temperature for 10 min.
4. Chill on ice for 2 min.

5. Add the following reagents to the reaction mix. *Perform in dim light.

Reagent	Volume (50µg RNA)	Volume (100 µg RNA)
5x RT reaction buffer (Superscript ll Kit, Invitrogen)	4.0µl	8.0µl
dNTP – master mix	2.0µl	4.0µl
Cy3-dCTP or Cy5-dCTP (1mM)	2.0µl	4.0µl
0.1 M DTT (Superscript ll Kit, Invitrogen)	2.0µl	4.0µl
Superscript ll (200U, Invitrogen)	1.0µl	1.5µl

Mix gently and spin briefly.

1. Incubates at 42°C for 2 hours. (environmental shaker)
2. Terminate the reaction and degrade the RNA by adding 5µl 1M NaOH (10µl for 100µg of RNA)
3. Incubate at 65°C for 10 min. (Use Heat Block, Turn up after use.)
4. Neutralize by adding 5µl 1M HCl (10 µl for 100µg of RNA) and 200µl TE-buffer (pH 7.5).
5. Remove un-incorporated dNTPs, fluorescent dyes and random hexamers by using the PCR Purification Kit (Qiagen) according to the manufacture's instructions. Elute cDNA with nuclease-free water. (Hold back on Volume)
6. Take a sample from the cDNA to determine the concentration spectrophotometrically. (Do not discard the sample!).
7. Combine equal amounts of each cDNA (Cy3- and Cy5-labled cDNA) in one tube.
8. Evaporate the labeled and purified cDNA almost to dryness.
9. Depending on which hybridization protocol you will use re-dissolve the labeled cDNA in an appropriate amount of salt-based or formamide-based hybridization buffer (see **Hydridization Process**).
10. The labeled cDNA can be stored on ice for up to 2 hours, for longer storage (up to two days) freeze cDNA at -20°C. Always protect the labeled cDNA from light.

Hybridization Process

Required reagents

Labled cDNA in salt-based or formamide-based hybridization buffer

Required materials and equipment

Heating block at 95°C or boiling water bath, hybridization chamber (MWG-Biotech: # 4120-000002), Array-Finder, (provided with the kit) and cover slips or microarray Gene Frames (provided with the kit).

Procedure

For hybridization we recommend the use of the microarry Gene Frames together with the salt-based hybridization. The microarray Gene Frame has been developed specifically for microarray slide hybridizations. The gas-tight sealing system can withstand temperatures of up to 97°C and prevents reagent loss due to evaporation, thus improving the hybridization reliability.In the case you already used MWG-Arrays before together with our formamide-based hybridization buffer we recommend to do the hybridization using normal cover slips.

Hybridization using Microarray Gene Frames

The Gene Frame has been designed for use with standard microscope slides and are available in two sizes for different microarray formats. The adhesives on either side of the frame are of differing strengths. The side that adheres to the glass slide has an easy release adhesive wheras the reverse, which sticks to the cover slip, forms a stronger bond. This means that when the cover slip is removed, due to the disparity in the strengths of the different adhesives, the frame is removed simultaneously with no sticky residue being left behind on the slide.

1. Preheat the hybridization buffer for 10 min at 42°C.
2. Heat your labeled cDNA in salt-based hybridization buffer for 3 min at 95°C. The hybridization volume depends on the array you are using. (MWG E.coli K12 V2 Array has a recommended hybridization volume for salt-based buffer of 120µl)
3. Incubate the hybridization mixture for maximal 3 min on ice.
4. In some cases a precipitate may occur in the hybridization mixture. In this case preheat the mixture with the precipitate to 42°C until the precipitate completely disappears.
5. Spin down the hybridization mixture briefly.
6. Use the Array-Finder to locate the spotted area on the MWG-Array.
7. Ensure tht the surface of the slide that will come in contact with the adhesive Gene

Frame is both dry and clean.

8. It is highly recommended to practice the use of the Gene Frames with "dummy" slides and frames beforehand.
9. Separate an individual frame from a strip of frames by tearing along the perforations. Each frame is sandwiched between a thin polyester sheet and a thick polyester sheet that has the center square removed. Carefully remove the thick polyester sheet (with the center square removed), ensuring that the frame remains bound to the thin polyester sheet. With the slide on a flat surface lay it over the microarray, taking care not to touch the exposed adhesive surface. Press the frame down firmly, trying not to trap any air under the adhesive.
10. Applying the frame to the microarray slide the day before it is to be used will improve the adhesion, however this will generally not be necessary for standard hybridization experiments.
11. Remove the thin polyester backing sheet from the frame.
12. Pipette the appropriate volume of probe/hybridization solution at one end of the frame.
13. Carefully place the polyester cover slip over the Gene Frame at the end where the reagent has been pipetted and slowly press the cover over the frame, applying pressure from one end and gradually moving across to the other. If care is taken, the bead of reagent will spread out evenly within the frame without trapping air. The slight reagent excess is then squeezed out between the adhesive and the cove slip preventing air entrapment. This does not affect the adhesion of the frame to the cover slip.
14. Press the polyester cover slip down with a blunt instrument on the adhesive around the edge of the frame. The Gene Frame is now ready to use.
15. Place slides into a wet hybridization chamber.
16. Transfer the hybridization chamber to a shaking incubator or water bath at 42°C. Incubate the array at an intermediate shaking frequency for 16-24 hours.
17. After the hybridization, remove the Gene Frame by holding down the slide with one hand and pulling back the tab of the cover slip along the same plane as the slide. The process is greatly facilitated by lifting one edge of the Gene Frame with a blunt instrument like a spatula or tweezers. Continue immediately with the washing procedure.

Washing steps

Perform all washing steps at 30°C.

Required reagents

Washing buffer 1 (2x SSC, 0.1% SDS), washing buffer 2 (1x SSC), washing buffer 3 (0.1x SSC), all reagents should be filter sterilized.

Required materials and equipment

Non-powdered gloves, clean washing container, conical-bottom tube, orbital shaker

Procedure

1. Place the hybridization array into a clean slide-staining jet filled with 200 ml pre-warmed (30°C) washing buffer 1. Continue washing for 5 min at room temperature with gentle agitation on an orbital shaker or equivalent.
2. Wash microarray in pre-warmed (30°C) washing buffer 2 for 5 min at room temperature as described in the previous step.
3. Wash microarray in pre-warmed (30°C) washing buffer 3 for 5 min at room temperature as described in the previous step.
4. Dry the slide in a 50ml conical-bottom tube by centrifugation at room temperature for 2 min at 500g. It is important to place the label of the slide at the bottom of the tube. **Do not touch the spotted area at any time.**

APPENDIX E

ESCHERICHIA COLI GENE LIST

Escherichia coli Gene List

Meta Row	Meta Column	Row	Column	Gene name	Description
1	1	1	1	B3734	membrane-bound ATP synthase, F1 sector, alpha-subunit; atpA
1	1	1	2	B0431	cytochrome o ubiquinol oxidase subunit I; cyoB
1	1	1	3	B2095	putative tagatose 6-phosphate kinase 1; gatZ
1	1	1	4	B2282	NADH dehydrogenase I chain H; nuoH
1	1	1	5	B0862	arginine 3rd transport system permease protein; artQ
1	1	1	6	B4259	DNA polymerase III, chi subunit; holC
1	1	1	7		Arabidopsis Control Oligo
1	1	1	8	B0660	putative ATP-binding protein in pho regulon; ybeZ
1	1	1	9	B3000	orf, hypothetical protein; b3000
1	1	1	10	B2280	NADH dehydrogenase I chain J; nuoJ
1	1	1	11	B3304	50S ribosomal subunit protein L18; rplR
1	1	1	12	B1542	putative oxidoreductase; ydfI
1	1	2	1	B0114	pyruvate dehydrogenase (decarboxylase component); aceE
1	1	2	2	B0430	cytochrome o ubiquinol oxidase subunit III; cyoC
1	1	2	3	B2551	serine hydroxymethyltransferase; glyA
1	1	2	4	B2278	NADH dehydrogenase I chain L; nuoL
1	1	2	5	B1541	orf, hypothetical protein; b1541
1	1	2	6	B1379	heat shock protein hslJ; hslJ
1	1	2	7	B0014	chaperone Hsp70; DNA biosynthesis; autoregulated heat shock proteins; dnaK
1	1	2	8	B0879	putative ATP-binding component of a transport system; ybjZ
1	1	2	9	B1216	sodium-calcium/proton antiporter; chaA
1	1	2	10	B3628	UDP-D-galactose:(glucosyl)lipopolysaccharide-1,6- D-galactosyltransferase; rfaB
1	1	2	11	B3318	50S ribosomal subunit protein L23; rplW
1	1	2	12	B1791	putative amino acid/amine transport protein; yeaN
1	1	3	1	B3732	membrane-bound ATP synthase, F1 sector, beta-subunit; atpD
1	1	3	2	B3894	formate dehydrogenase-O, major subunit; fdoG
1	1	3	3	B1136	isocitrate dehydrogenase, specific for NADP+; icdA
1	1	3	4	B2277	NADH dehydrogenase I chain M; nuoM
1	1	3	5	B2592	heat shock protein; clpB
1	1	3	6	B3931	heat shock protein hslVU, ATPase subunit, homologous to chaperones; hslU
1	1	3	7	B0439	DNA-binding, ATP-dependent protease La; heat shock K-protein; lon
1	1	3	8	B0966	orf, hypothetical protein; yccV
1	1	3	9	B2610	GTP-binding export factor binds to signal sequence, GTP and RNA; ffh
1	1	3	10	B0414	bifunctional pyrimidine deaminase/reductase in pathway of riboflavin synthesis; ribD
1	1	3	11	B3302	50S ribosomal subunit protein L30; rpmD
1	1	3	12	B2608	orf, hypothetical protein; yfjA
1	1	4	1	B3733	membrane-bound ATP synthase, F1 sector, gamma-subunit; atpG
1	1	4	2	B4152	fumarate reductase, anaerobic, membrane anchor polypeptide; frdC
1	1	4	3	B1817	PTS enzyme IIAB, mannose-specific; manX
1	1	4	4	B3403	phosphoenolpyruvate carboxykinase; pckA
1	1	4	5	B3066	DNA biosynthesis; DNA primase; dnaG

1	1	4	6	B3932	heat shock protein hslVU, proteasome-related peptidase subunit; hslV
1	1	4	7	B1531	multiple antibiotic resistance; transcriptional activator of defense systems; marA
1	1	4	8	B1321	putative EC 2.1 enzymes; ycjX
1	1	4	9	B2436	coproporphyrinogen III oxidase; hemF
1	1	4	10	B3386	D-ribulose-5-phosphate 3-epimerase; rpe
1	1	4	11	B3341	30S ribosomal subunit protein S7, initiates assembly; rpsG
1	1	4	12	B3107	orf, hypothetical protein; yhaL
1	1	5	1	B3735	membrane-bound ATP synthase, F1 sector, delta-subunit; atpH
1	1	5	2	B4151	fumarate reductase, anaerobic, membrane anchor polypeptide; frdD
1	1	5	3	internal control	internal control
1	1	5	4	B0726	2-oxoglutarate dehydrogenase (decarboxylase component); sucA
1	1	5	5	B0015	chaperone with DnaK; heat shock protein; dnaJ
1	1	5	6	B1829	heat shock protein, integral membrane protein; htpX
1	1	5	7	B4171	delta(2)-isopentenylpyrophosphate tRNA-adenosine transferase; miaA
1	1	5	8	B3382	orf, hypothetical protein; yhfY
1	1	5	9	B2526	heat shock protein, chaperone, member of Hsp70 protein family; hscA
1	1	5	10	B3317	50S ribosomal subunit protein L2; rplB
1	1	5	11	B2609	30S ribosomal subunit protein S16; rpsP
1	1	5	12	B3377	putative transport system permease protein; yhfT
1	1	6	1	B1824	orf, hypothetical protein; b1824
1	1	6	2	B2094	galactitol-specific enzyme IIA of phosphotransferase system; gatA
1	1	6	3	B0963	methylglyoxal synthase; mgsA
1	1	6	4	B0727	2-oxoglutarate dehydrogenase (dihydrolipoyltranssuccinase E2 component); sucB
1	1	6	5	B3179	cell division protein; ftsJ
1	1	6	6	B3687	heat shock protein; ibpA
1	1	6	7	B4143	GroEL, chaperone Hsp60, peptide-dependent ATPase, heat shock protein; mopA
1	1	6	8	B0016	IS186 hypothetical protein; yi81_1
1	1	6	9	B3291	mechanosensitive channel; mscL
1	1	6	10	B3320	50S ribosomal subunit protein L3; rplC
1	1	6	11	B2183	16S pseudouridylate 516 synthase; rsuA
1	1	6	12	B3508	putative transport ATPase; yhiD
1	1	7	1	B0733	cytochrome d terminal oxidase, polypeptide subunit I; cydA
1	1	7	2	B3734	membrane-bound ATP synthase, F1 sector, alpha-subunit; atpA
1	1	7	3	B0553	outer membrane porin protein; locus of qsr prophage; nmpC
1	1	7	4	B0740	periplasmic protein involved in the tonb-independent uptake of group A colicins; tolB
1	1	7	5	B2614	phage lambda replication; host DNA synthesis; heat shock protein; protein repair; grpE
1	1	7	6	B3686	heat shock protein; ibpB
1	1	7	7	B4142	GroES, 10 Kd chaperone binds to Hsp60 in pres. Mg-ATP, suppressing its ATPase activity; mopB
1	1	7	8	B3399	putative phosphatase; yrfG
1	1	7	9	B0675	N-acetylglucosamine metabolism; nagD
1	1	7	10	B3319	50S ribosomal subunit protein L4, regulates expression of S10 operon; rplD
1	1	7	11	B1656	superoxide dismutase, iron; sodB

1	1	7	12	B4233	putative ligase; yjfG
1	1	8	1	B0432	cytochrome o ubiquinol oxidase subunit II; cyoA
1	1	8	2	B2096	tagatose-bisphosphate aldolase 1; gatY
1	1	8	3	B2284	NADH dehydrogenase I chain F; nuoF
1	1	8	4	B0742	orf, hypothetical protein; ybgF
1	1	8	5	B4172	host factor I for bacteriophage Q beta replication, a growth-related protein; hfq
1	1	8	6	B1380	fermentative D-lactate dehydrogenase, NAD-dependent; ldhA
1	1	8	7	B2193	nitrate/nitrite response regulator (sensor NarQ); narP
1	1	8	8	B0978	probable third cytochrome oxidase, subunit I; appC
1	1	8	9	B0679	PTS system, N-acetylglucosamine-specific enzyme IIABC; nagE
1	1	8	10	B3985	50S ribosomal subunit protein L10; rplJ
1	1	8	11	B3450	ATP-binding component of sn-glycerol 3-phosphate transport system; ugpC
1	1	8	12	B0014	chaperone Hsp70; DNA biosynthesis; autoregulated heat shock proteins; dnaK
2	2	1	1	B3734	membrane-bound ATP synthase, F1 sector, alpha-subunit; atpA
2	2	1	2	B0431	cytochrome o ubiquinol oxidase subunit I; cyoB
2	2	1	3	B2095	putative tagatose 6-phosphate kinase 1; gatZ
2	2	1	4	B2282	NADH dehydrogenase I chain H; nuoH
2	2	1	5	B0862	arginine 3rd transport system permease protein; artQ
2	2	1	6	B4259	DNA polymerase III, chi subunit; holC
2	2	1	7		Arabidopsis Control Oligonucleotide
2	2	1	8	B0660	putative ATP-binding protein in pho regulon; ybeZ
2	2	1	9	B3000	orf, hypothetical protein; b3000
2	2	1	10	B2280	NADH dehydrogenase I chain J; nuoJ
2	2	1	11	B3304	50S ribosomal subunit protein L18; rplR
2	2	1	12	B1542	putative oxidoreductase; ydfI
2	2	2	1	B0114	pyruvate dehydrogenase (decarboxylase component); aceE
2	2	2	2	B0430	cytochrome o ubiquinol oxidase subunit III; cyoC
2	2	2	3	B2551	serine hydroxymethyltransferase; glyA
2	2	2	4	B2278	NADH dehydrogenase I chain L; nuoL
2	2	2	5	B1541	orf, hypothetical protein; b1541
2	2	2	6	B1379	heat shock protein hslJ; hslJ
2	2	2	7	B0014	chaperone Hsp70; DNA biosynthesis; autoregulated heat shock proteins; dnaK
2	2	2	8	B0879	putative ATP-binding component of a transport system; ybjZ
2	2	2	9	B1216	sodium-calcium/proton antiporter; chaA
2	2	2	10	B3628	UDP-D-galactose:(glucosyl)lipopolysaccharide-1,6- D-galactosyltransferase; rfaB
2	2	2	11	B3318	50S ribosomal subunit protein L23; rplW
2	2	2	12	B1791	putative amino acid/amine transport protein; yeaN
2	2	3	1	B3732	membrane-bound ATP synthase, F1 sector, beta-subunit; atpD
2	2	3	2	B3894	formate dehydrogenase-O, major subunit; fdoG
2	2	3	3	B1136	isocitrate dehydrogenase, specific for NADP+; icdA
2	2	3	4	B2277	NADH dehydrogenase I chain M; nuoM
2	2	3	5	B2592	heat shock protein; clpB
2	2	3	6	B3931	heat shock protein hslVU, ATPase subunit, homologous to chaperones; hslU
2	2	3	7	B0439	DNA-binding, ATP-dependent protease La; heat shock K-protein; lon
2	2	3	8	B0966	orf, hypothetical protein; yccV
2	2	3	9	B2610	GTP-binding export factor binds to signal sequence, GTP and

					RNA; ffh
2	2	3	10	B0414	bifunctional pyrimidine deaminase/reductase in pathway of riboflavin synthesis; ribD
2	2	3	11	B3302	50S ribosomal subunit protein L30; rpmD
2	2	3	12	B2608	orf, hypothetical protein; yfjA
2	2	4	1	B3733	membrane-bound ATP synthase, F1 sector, gamma-subunit; atpG
2	2	4	2	B4152	fumarate reductase, anaerobic, membrane anchor polypeptide; frdC
2	2	4	3	B1817	PTS enzyme IIAB, mannose-specific; manX
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2	2	4	10	B3386	D-ribulose-5-phosphate 3-epimerase; rpe
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2	2	4	12	B3107	orf, hypothetical protein; yhaL
2	2	5	1	B3735	membrane-bound ATP synthase, F1 sector, delta-subunit; atpH
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2	2	5	5	B0015	chaperone with DnaK; heat shock protein; dnaJ
2	2	5	6	B1829	heat shock protein, integral membrane protein; htpX
2	2	5	7	B4171	delta(2)-isopentenylpyrophosphate tRNA-adenosine transferase; miaA
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2	2	5	12	B3377	putative transport system permease protein; yhfT
2	2	6	1	B1824	orf, hypothetical protein; b1824
2	2	6	2	B2094	galactitol-specific enzyme IIA of phosphotransferase system; gatA
2	2	6	3	B0963	methylglyoxal synthase; mgsA
2	2	6	4	B0727	2-oxoglutarate dehydrogenase (dihydrolipoyltranssuccinase E2 component); sucB
2	2	6	5	B3179	cell division protein; ftsJ
2	2	6	6	B3687	heat shock protein; ibpA
2	2	6	7	B4143	GroEL, chaperone Hsp60, peptide-dependent ATPase, heat shock protein; mopA
2	2	6	8	B0016	IS186 hypothetical protein; yi81_1
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2	2	6	11	B2183	16S pseudouridylate 516 synthase; rsuA
2	2	6	12	B3508	putative transport ATPase; yhiD
2	2	7	1	B0733	cytochrome d terminal oxidase, polypeptide subunit I; cydA
2	2	7	2	B3734	membrane-bound ATP synthase, F1 sector, alpha-subunit; atpA
2	2	7	3	B0553	outer membrane porin protein; locus of qsr prophage; nmpC

2	2	7	4	B0740	periplasmic protein involved in the tonb-independent uptake of group A colicins; tolB
2	2	7	5	B2614	phage lambda replication; host DNA synthesis; heat shock protein; protein repair; grpE
2	2	7	6	B3686	heat shock protein; ibpB
2	2	7	7	B4142	GroES, 10 Kd chaperone binds to Hsp60 in pres. Mg-ATP, suppressing its ATPase activity; mopB
2	2	7	8	B3399	putative phosphatase; yrfG
2	2	7	9	B0675	N-acetylglucosamine metabolism; nagD
2	2	7	10	B3319	50S ribosomal subunit protein L4, regulates expression of S10 operon; rplD
2	2	7	11	B1656	superoxide dismutase, iron; sodB
2	2	7	12	B4233	putative ligase; yjfG
2	2	8	1	B0432	cytochrome o ubiquinol oxidase subunit II; cyoA
2	2	8	2	B2096	tagatose-bisphosphate aldolase 1; gatY
2	2	8	3	B2284	NADH dehydrogenase I chain F; nuoF
2	2	8	4	B0742	orf, hypothetical protein; ybgF
2	2	8	5	B4172	host factor I for bacteriophage Q beta replication, a growth-related protein; hfq
2	2	8	6	B1380	fermentative D-lactate dehydrogenase, NAD-dependent; ldhA
2	2	8	7	B2193	nitrate/nitrite response regulator (sensor NarQ); narP
2	2	8	8	B0978	probable third cytochrome oxidase, subunit I; appC
2	2	8	9	B0679	PTS system, N-acetylglucosamine-specific enzyme IIABC; nagE
2	2	8	10	B3985	50S ribosomal subunit protein L10; rplJ
2	2	8	11	B3450	ATP-binding component of sn-glycerol 3-phosphate transport system; ugpC
2	2	8	12	B0014	chaperone Hsp70; DNA biosynthesis; autoregulated heat shock proteins; dnaK

APPENDIX F

RAW EXPRESSION DATA

Raw Expression Data

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Ascension Reference	Cy3 Signal Mean	Cy3 Background Mean	Cy5 Signal Mean	Cy5 Background Mean
B3734	389.6889	82.20738	130.63725	60.993828
B0431	332.2043	88.22998	274.42856	89.159805
B2095	207.40625	69.336426	143.49515	72.45144
B2282	117.63461	65.91233	71.86139	48.360336
B0862	241.48387	77.32145	140.03261	59.991184
B4259	101.75701	72.898094	66.08491	52.778755
2_ara5	78.30769	67.97647	57.32353	47.27723
B0660	66.05825	52.29786	68.14851	50.019497
B2999M	374.81177	90.474075	144.54369	73.7081
B2280	196.71642	61.90563	94.15842	54.64643
B3304	434.05884	66.41328	578.8618	96.51216
B1542	60.563107	48.864643	55.40777	46.61301
B0114	503.57446	90.14263	362.23404	84.76352
B0430	285.79245	86.655945	233.07767	78.372
B2551	93.13	69.30176	61.76923	50.58085
B2278	127.729164	68.6759	87.088234	52.994225
B1541	73.59804	63.789444	62.39806	44.877937
B1379	76.50485	61.364147	50.08738	41.78139
B0014	122.360466	63.94495	77.51515	52.63684
B0879	81.35922	58.212055	55.235294	42.227272
B1216	132.95238	57.99312	131.03703	54.64148
B3628	60.47059	46.7313	54.12871	43.318695
B3318	761.7344	83.696175	646.11676	108.17345
B1791	56.339622	46.01793	49.04762	41.789032
B3732	240.32394	79.79528	89.303925	57.23113
B3894	226.01987	74.59424	93.89	57.351154
B1136	146.62204	68.23444	99.99039	57.495953
B2277	151.85611	70.15673	175.47273	65.20081
B2592	88.69811	70.15871	59.259617	48.161697
B3931	87.36633	69.2192	68.83673	51.764503
B0439	156.85811	73.436386	190.03798	68.709274
B0966	66.485435	54.93579	59.242718	47.256046
B2610	192.71739	68.39764	177.48454	66.810326
B0414	78.81554	52.138645	71.83495	46.581757

B3302	395.50568	71.62145	451.3471	104.565346
B2608	242.57031	55.391605	214.71765	63.14449
B3733	282.29932	77.33053	214.63889	68.94827
B4152	92.20388	71.40868	64.61386	48.042107
B1817	80.67619	62.881725	60.161903	46.111782
B3403	125.2844	63.672226	129.14493	55.363544
B3066	83.82524	61.365974	64	48.29768
B3932	161.66667	74.08253	154.21782	66.659546
B1531	79.52427	65.5087	71.84158	52.35119
B1321	72.81373	57.979637	63.039215	47.063366
B2436	95.36144	54.10073	62.721153	46.70264
B3386	117.30121	53.454365	67.28846	46.448956
B3341	344.0531	63.295383	364.05356	82.51875
B3107	65.35238	48.248764	57.182693	45.72071
B3735	606.7895	84.925026	288.59433	78.15468
B4151	97.78788	76.03144	69.7	49.020683
Control	95.194176	67.4837	72.36	50.49098
B0726	83.74039	63.74947	56.38384	45.09334
B0015	100.90385	65.67334	62	46.226032
B1829	106.94175	67.93601	104.20192	60.276165
B4171	401.42764	79.573616	493.44296	109.18117
B3382	108.01923	76.42849	64.55769	51.618717
B2526	129.97122	57.146946	119.74039	60.732574
B3317	312.1077	63.606674	254.50435	73.130806
B2609	254.90698	62.00496	261.6092	72.28857
B3377	54.4	46.15344	51.92157	42.516384
B1824	105.84466	67.55046	76.7	52.518898
B2094	107.48039	75.80305	63.666668	48.890186
B0963	68.36364	63.402523	64.57576	49.243393
B0727	86.252525	65.03491	72.34	52.22359
B3179	199.12038	70.99738	170.47368	60.825115
B3687	88.36275	64.55847	67.11651	49.48724
B4143	124.83168	73.5447	89.81373	57.05275
B0582	247.59616	68.67029	72.09901	49.310665
B3291	72.460785	51.62652	87.68627	54.37715
B3320	308.8062	59.927658	223.2384	66.65281
B2183	77.12381	49.80504	72.0202	46.186058
B3508	67.56731	52.161392	60.264706	45.322544
B0733	105.26852	68.33857	56.009434	46.361145

B3734	321.936	71.91817	120.09615	58.72837
B0553	83.83495	65.99496	61.757282	47.024227
B0740	177.14	73.15874	183.62886	68.62926
B2614	75.71154	65.38734	75.36539	53.87867
B3686	82.902916	62.040215	53.9703	43.721676
B4142	186.5	73.08432	198.07777	65.61026
B3399	107.76699	71.65342	104.882355	56.287346
B0675	64.88461	49.352837	52.271843	43.95573
B3319	125	57.598087	103.875	54.15647
B1656	69.048546	49.261337	61.73077	42.39268
B4233	53.81132	49.396687	52.278847	42.301975
B0432	311.11038	80.01982	217.43103	76.95248
B2096	106.83654	68.41804	93.71154	54.048996
B2284	101.49515	67.01586	53.833332	43.79522
B0742	131.96078	80.655365	104.79612	62.44025
B4172	114.52525	75.592575	136.49039	62.395535
B1380	68.72816	57.5464	59.27451	47.89215
B2193	65.50495	59.427094	52.708736	44.226875
B0978	60.961166	52.795	49.927834	40.752804
B0679	133.56604	59.787174	69.42857	47.345356
B3985	403.72174	68.387215	523.03876	95.53203
B3450	46.441177	46.704906	52.70297	40.912743
B0014	52.247524	46.676594	50.737865	42.276447
B3734	316.93332	76.449265	160.23529	58.896744
B0431	166.71765	67.40566	143.80583	63.768673
B2095	98.15888	69.24661	59.32039	47.061752
B2282	81.78095	70.36244	63.48077	48.086903
B0862	128.11688	62.367672	66.33	48.242542
B4259	77.28713	58.564117	56.33663	43.828396
2_ara5	74.245094	59.558228	44.33663	39.32131
B0660	80.19	63.975437	53.757282	44.923706
B2999M	224.98877	66.90949	94.14286	51.945156
B2280	59.566036	49.67363	56.91589	41.64582
B3304	134.46317	55.12347	189.4507	57.8369
B1542	55.873787	48.896748	60.568626	45.655647
B0114	232.45882	71.01856	147.85849	63.775093
B0430	126.35238	68.425224	154.89706	56.529736
B2551	73.49056	67.18068	49.26923	43.951286
B2278	69.99039	60.281067	53.627453	43.83839

B1541	64.31373	57.6361	56.019608	41.969532
B1379	78.05102	61.965446	49.203884	40.434727
B0014	65.64286	55.101288	56.32	42.036354
B0879	64.237625	59.759495	54.67	46.164375
B1216	64.069305	53.43117	57.127453	45.229027
B3628	65.666664	50.835342	67.29126	46.15687
B3318	89.962265	60.422417	90.78846	54.347664
B1791	46.771427	46.6398	56.057144	44.27424
B3732	91.72115	65.26844	68.35294	49.852978
B3894	146.52336	65.94118	86.96	56.793247
B1136	74.21212	62.344204	59.53398	44.875854
B2277	154.17778	69.787544	75.04951	54.655384
B2592	61.41905	56.684307	53.106796	42.766495
B3931	71.70874	59.163174	48.10784	39.84235
B0439	71.55882	63.580166	69.71287	45.90381
B0966	87.97059	68.14838	54.509804	48.674038
B2610	78.01923	58.689507	63.262627	45.05045
B0414	53.00971	46.516388	53.13592	42.52207
B3302	59.91346	50.21693	52.52381	45.26928
B2608	59.0991	49.69355	65.19266	49.611908
B3733	174.73119	68.910385	163.17757	65.63872
B4152	80.24	61.69101	61.098038	44.527924
B1817	71.20388	66.53971	54.79208	44.06383
B3403	63.883495	60.19388	68.52941	47.385944
B3066	61.932037	54.76588	45.757282	39.900337
B3932	81.64078	59.304108	57.00971	44.137127
B1531	58.346535	55.31057	61.19802	46.535885
B1321	69.088234	60.840157	61.14706	47.452774
B2436	58.00971	58.140656	51.475246	41.06605
B3386	56.436893	50.132206	56.485435	47.27086
B3341	76.00952	54.726276	59.514565	44.464558
B3107	52.932037	46.92886	48.893204	42.32624
B3735	141.04616	61.35294	68.9898	52.115982
B4151	83.40404	63.78383	43.089108	39.903404
Control	70.696075	60.773727	61.21212	44.585995
B0726	108.85437	75.38714	68.37736	52.75485
B0015	122.68317	84.89742	163.7027	63.29871
B1829	61.932693	58.582737	54.31068	43.28105
B4171	86.9406	67.13564	58.153847	45.924152

B3382	56.23301	50.321648	49.86	41.227913
B2526	53.235294	49.22847	64.87255	45.713287
B3317	181.39394	60.487206	161.1718	64.32061
B2609	55.71287	50.17754	70.38614	49.968147
B3377	79.902916	58.63622	59.846153	42.23979
B1824	61.17647	56.015144	53.019608	42.602394
B2094	74.339806	66.61352	53.294117	45.041775
B0963	67.88776	55.928127	57	42.600986
B0727	62.166668	59.378246	42.359222	38.017212
B3179	65.60577	61.097206	36.432693	37.222992
B3687	72.53398	59.472237	49.82353	43.196922
B4143	75.69474	61.558113	58.142857	45.298462
B0582	79.06061	64.49682	45.474747	41.13927
B3291	87.86139	70.4343	73.50516	51.72316
B3320	60.10891	46.70909	63.138615	43.624226
B2183	53.205883	53.798172	58.33663	43.828316
B3508	54.735294	52.458496	61.58	43.907608
B0733	72.40196	59.34583	54.058823	45.339214
B3734	74.20388	59.373135	54.313725	41.966923
B0553	62.86	56.02001	43.48	38.27455
B0740	68.36735	56.732525	52.58416	42.789574
B2614	60.951458	56.119884	42.62136	38.37815
B3686	66.79808	55.67022	43.252426	40.233902
B4142	64.05769	52.560535	54.08738	42.38248
B3399	91.87619	57.821526	62.417477	48.25716
B0675	62.475727	60.929935	56.911766	45.47028
B3319	110.77193	56.491802	72.181816	47.604664
B1656	65.435646	50.63546	53.14	40.93316
B4233	62.240383	52.04875	47.137257	42.121017
B0432	99.20388	88.21082	76.20192	67.53738
B2096	77.68627	64.223526	49.490383	43.351494
B2284	54.209522	51.550133	51.817307	43.08872
B0742	68.78723	60.233578	61.32353	46.841705
B4172	70.53465	59.588306	60.05	44.485573
B1380	65.419044	57.958237	81.40952	51.67472
B2193	63.66346	53.94649	51.881187	41.97413
B0978	64.765305	51.74111	48.427185	40.401417
B0679	189.56842	59.07954	57.718445	42.63751
B3985	514.9489	75.01168	673.27405	105.508255

B3450	95.10577	58.06462	42.45631	39.043636
B0014	63.903847	55.661167	48.89216	43.51216

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Accension Reference	Cy3 Signal Mean	Cy3 Background Mean	Cy5 Signal Mean	Cy5 Background Mean
B3734	145.73077	47.13227	129.67677	66.37596
B0431	231.68687	37.149876	211.46956	57.311943
B2095	92.49451	31.00977	135.57281	51.15558
B2282	49.2549	26.120634	49.07	29.088041
B0862	95.43299	23.987219	77.37255	34.347427
B4259	44.45098	21.174603	39.265305	25.08425
2_ara5	16.913462	13.12692	41.35577	20.544674
B0660	28.411215	18.302769	48.57843	22.146374
B2999M	70.70707	22.20141	93.08911	33.73188
B2280	72.85135	21.505835	100.65	36.393974
B3304	349.90985	31.19074	664.3364	79.76146
B1542	21.843138	18.230476	38.57843	22.935806
B0114	254.80374	37.291687	330.61447	72.92245
B0430	190.33636	36.728874	152.8077	59.372948
B2551	36.861385	26.028942	42.969387	34.876057
B2278	40.19608	24.253334	42.636364	24.841564
B1541	24.5	17.550165	37.878788	22.63292
B1379	22.807692	16.994875	36.86	19.389843
B0014	38.68182	21.595448	60.73148	25.856255
B0879	25.361904	16.288363	45.74	19.42682
B1216	46.096153	20.945227	75.96	27.2153
B3628	33.08738	18.718786	51.70707	23.452477
B3318	462.51562	38.44791	572.5833	90.156555
B1791	19.336634	15.436268	42.303032	22.993935
B3732	108.139244	32.725765	63.683674	41.104553
B3894	75.73333	31.75827	87.31633	47.589073
B1136	99.76786	30.229807	67.91	39.264744
B2277	95.78322	25.485123	78.50961	33.936157
B2592	30.897196	19.832754	51.42157	27.531595
B3931	29.619047	18.910902	34.387756	20.59799
B0439	67.23881	23.253565	89.33663	32.650333
B0966	26.538462	18.54614	43.51485	22.622416
B2610	79.14179	22.425554	78.28571	32.33021
B0414	34.557693	19.890503	44.357143	22.119175

B3302	208.07207	27.75188	191.34285	49.449974
B2608	155.65834	26.099434	301.02298	52.08079
B3733	174.02362	34.085873	217.20312	55.974194
B4152	48.91262	32.529774	51.05	38.331593
B1817	33.17476	26.131466	56.804123	36.808243
B3403	52.638096	27.863518	46.408165	29.351078
B3066	48.95098	24.39197	76.35644	34.594757
B3932	89.336205	22.777271	70.4898	29.711308
B1531	31.826923	19.12393	44.99	24.765146
B1321	33.076923	21.811869	46.07921	22.889587
B2436	29.647058	18.260921	64.5098	24.470861
B3386	53.481014	19.585684	60.637257	27.695482
B3341	193.39655	27.837688	261.875	60.018078
B3107	26.69608	17.57976	50.990196	25.13807
B3735	297.53278	35.671898	321.07446	74.22701
B4151	35.699028	28.883602	66.376236	40.858307
Control	31.215687	23.452055	48.077778	35.655884
B0726	26.185568	18.8812	41.206184	26.304733
B0015	44.873787	21.208899	44.215687	25.508892
B1829	44.451923	21.806486	61.673267	29.157106
B4171	308.8387	35.293365	193.0097	58.5727
B3382	21.747572	18.141888	44.19	23.871943
B2526	72.92126	25.83049	88.19	37.515476
B3317	496.65973	37.498985	933.45	142.65959
B2609	129.32433	24.754658	138.7255	45.0307
B3377	17.320755	15.094313	31.445545	24.3326
B1824	87.039215	31.024391	65.42268	43.250618
B2094	53.333332	30.971294	60.242104	39.817192
B0963	39.53	27.806175	55.09375	36.710476
B0727	36.29	22.282572	34.526318	25.983994
B3179	114.333336	24.79248	98.3	36.734867
B3687	27.54369	18.030941	34.978493	19.904444
B4143	77.88	23.739243	80.57692	30.694979
B0582	40.298077	20.473747	41.886597	24.128134
B3291	87.099236	26.322495	110.74258	38.789352
B3320	223.30508	28.08613	198.77142	59.327568
B2183	41.911766	19.799276	58.757576	26.216322
B3508	34.60194	20.358656	72.79	29.117825
B0733	47.737865	32.788925	71.4433	43.11333

B3734	149.79797	32.64978	159.63235	50.779964
B0553	34.35577	25.910755	57.356434	37.899303
B0740	127.962616	28.568012	113.84762	34.703243
B2614	33.484848	20.9743	51.081635	22.830406
B3686	29.942308	18.77699	26.928572	18.791601
B4142	151.83571	26.484638	183.19801	52.01911
B3399	93.07477	24.223984	93.55	32.633335
B0675	33.135136	20.534883	49.918182	20.822094
B3319	443.7857	37.86202	648.0585	113.243034
B1656	45.615383	21.275494	75.43	27.931221
B4233	31.049505	19.717865	53.388348	27.439384
B0432	168.71297	36.188957	149.64706	60.097954
B2096	70.17461	30.238806	106.660194	49.67517
B2284	48.31068	29.838997	43.01	34.88865
B0742	85.62921	26.173697	65.83168	33.3607
B4172	211.14503	29.190428	185.80583	55.2681
B1380	27.524752	16.790169	39.82828	25.414036
B2193	37.59406	20.708841	36.19608	20.77475
B0978	19.163462	14.475979	44.009804	23.074867
B0679	58.74286	24.166925	91.94118	38.03079
B3985	551.00653	39.597046	587.9259	94.53964
B3450	27.097088	19.601204	36.694736	23.706171
B0014	38.1875	16.284012	70.97143	31.071753
B3734	136.37646	34.70181	94.19	47.244957
B0431	195.34906	34.736263	215.44304	52.758045
B2095	85.2375	29.495161	88.544556	42.315533
B2282	48.73077	23.405031	41.226803	25.969261
B0862	87.6	22.158295	71.55769	26.293104
B4259	35.443398	18.432505	41.24	23.94248
2_ara5	19.951923	14.751001	30.050505	18.30511
B0660	28.14	17.489988	36.121212	22.057306
B2999M	78.056335	21.091095	102.10101	33.739468
B2280	52.813725	20.663061	102.21568	31.708643
B3304	275.76428	29.061956	350.22858	63.516163
B1542	23.14423	16.490322	25.638096	21.272795
B0114	241.21649	35.726902	351.0241	79.68369
B0430	183.52942	34.21917	218.52632	52.96084
B2551	30.871286	24.604017	53.98969	36.22059
B2278	40.32039	23.026123	37.82178	23.793203

B1541	24.866667	17.851076	35.319588	20.174126
B1379	30.038095	16.953074	38.603962	21.818783
B0014	37.66346	21.992546	70.21359	29.496752
B0879	20.621359	13.678287	45.61616	24.165628
B1216	39.18	19.566324	50.267326	23.843792
B3628	32.04902	17.64262	50.646465	25.002052
B3318	427.68796	37.93893	469.55356	85.554016
B1791	23.453608	15.89419	28.113401	20.493883
B3732	108.68687	32.661655	93.04951	44.488644
B3894	83.57692	29.868353	88.52475	42.5929
B1136	75.603775	29.953537	61.9375	40.077065
B2277	102.12613	25.81713	79.26733	33.44692
B2592	33.452633	21.09091	51.642857	24.349775
B3931	30.396227	19.637129	36.980198	21.515924
B0439	49.650486	23.3808	89.18446	34.06813
B0966	31.097088	18.156742	54.24	24.648464
B2610	87.32758	25.231045	70.623764	29.755543
B0414	35.59223	20.220108	38.561226	21.211056
B3302	175.35088	25.038616	241.71288	57.255573
B2608	160.16667	23.34666	295.56097	53.586765
B3733	180.69444	34.539352	148.86139	62.659447
B4152	34.09901	27.173065	61.217823	38.2127
B1817	39.00971	26.326738	50.99	33.99938
B3403	44.740383	23.132408	72.38461	31.562353
B3066	39.096153	20.683933	44.09	22.056301
B3932	87.31746	24.066393	83.61165	31.33661
B1531	27.209524	18.42497	39.18182	21.404762
B1321	24.553398	16.067345	45.60194	24.940607
B2436	25.262136	17.070414	45.216496	22.972185
B3386	38.980392	20.216085	46.843136	26.915953
B3341	188.89815	26.622934	218.92157	61.481964
B3107	35.574257	21.045338	34.336735	21.286337
B3735	253.14174	36.006046	334.81732	68.74352
B4151	42.705883	30.481157	47.05263	37.376736
	35.33654	24.524244	48.063156	32.627724
B0726	29.190475	20.248432	52.51923	21.828938
B0015	32.75	20.473896	49.39	24.005222
B1829	58.87143	21.27258	24.145632	15.487676
B4171	267.26352	31.28368	154.56863	45.403584

B3382	39.068626	23.107775	36.57143	18.96811
B2526	71.57	23.223671	77.42157	28.313406
B3317	272.922	30.635765	322.93268	80.29119
B2609	128.46535	25.639486	173.36539	47.424442
B3377	21.27451	17.814934	57.019608	28.181593
B1824	99.51219	32.409794	61.408604	40.073437
B2094	55.47059	29.76935	57.46875	38.85121
B0963	37.300972	26.585524	39.081635	34.008953
B0727	41.35	21.866213	48.80612	27.450855
B3179	104.947365	21.88736	114.32692	36.026115
B3687	27.76923	18.037603	38.226803	18.44488
B4143	73.42	23.44901	107.82524	38.2884
B0582	33.21698	19.536264	61.708736	23.153698
B3291	81.07143	24.055996	78.1619	32.90479
B3320	163.54109	27.489513	258.80234	49.454067
B2183	39.20202	20.526733	51.535355	25.354624
B3508	30.884615	20.244137	58.660194	25.181017
B0733	48.343136	31.377695	53.948452	37.979843
B3734	156.86792	34.781166	130.73077	49.61194
B0553	38.23301	25.596209	43.88421	34.1952
B0740	136.55789	28.780933	88.78218	34.09597
B2614	115.84869	32.811806	72.0101	28.190447
B3686	44.74	24.916061	67.13725	24.60707
B4142	153.84285	29.073753	294.4189	56.74845
B3399	104.94068	26.26768	134.40384	36.748302
B0675	45.72549	22.428795	38.020203	20.379929
B3319	403.19608	39.318108	672.86957	113
B1656	36.221153	19.502398	67.54902	26.181992
B4233	31.368933	21.71213	49.82178	23.55989
B0432	156.52542	33.793102	159.38835	55.61757
B2096	62.425	29.87961	70.408165	40.870567
B2284	72.611115	28.716055	90.67647	38.549908
B0742	77.19	25.548132	81.52	33.41438
B4172	244.27966	29.76622	262.7553	45.081375
B1380	30.621359	19.206995	48.01	19.118294
B2193	47.838097	23.283123	37.951458	23.804655
B0978	23.06	15.690755	27.41237	17.20913
B0679	94.86719	26.528952	99.32039	35.701763
B3985	377.8024	34.034393	488	75.619484

B3450	28.38835	18.353249	42.484535	22.115797
B0014	35.568626	18.442896	93.666664	33.619076

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Accension Reference	Cy3 Signal Mean	Cy3 Background Mean	Cy5 Signal Mean	Cy5 Background Mean
B3734	281.1402	67.616516	102.84314	47.6218
B0431	373.18332	63.85722	93.78846	46.24701
B2095	291.7736	61.49625	133.25	51.88479
B2282	142.98718	55.34139	64.805824	39.602108
B0862	231.64423	62.33267	91.06796	44.911472
B4259	92.55882	57.197506	42.54	35.546925
2_ara5	55.7451	50.368633	48.742573	35.037636
B0660	64.58253	55.79682	47.382355	35.371834
B2999M	201.27957	59.66919	92.05825	42.960632
B2280	152.02	55.722366	78.30693	41.355778
B3304	586.63043	77.58734	228.83168	56.576057
B1542	62.03846	51.978	43.980583	38.35211
B0114	648.3496	72.84959	262.18918	59.645958
B0430	447.14667	70.13123	124.26733	47.089046
B2551	86.22115	56.80935	47.17822	34.70303
B2278	98.19178	53.583187	40.598038	31.667334
B1541	65.19231	51.43046	52.096153	34.951653
B1379	65.5098	51.65678	41.38614	32.533127
B0014	129.11765	57.32326	55.7549	35.567272
B0879	44.356434	47.64199	32.784946	30.684616
B1216	140.20192	58.28811	52.28	36.310425
B3628	70.63461	57.33535	49.57843	35.660576
B3318	711.5241	80.722984	253.12903	62.354595
B1791	55.182693	47.6748	41.04902	34.970997
B3732	172.44048	60.261917	64.95098	40.988476
B3894	166.88608	63.05219	60.48077	38.468822
B1136	171.54082	60.104916	63.843136	40.730587
B2277	218.21739	74.34641	129.20221	51.15394
B2592	64.68932	54.518284	48.576923	38.980984
B3931	67.38614	51.233997	39.656864	33.263092
B0439	165.66176	60.714798	65.4902	42.755615
B0966	84.16	58.281902	41.958763	33.361958
B2610	206.48	65.33606	91.871284	47.523315
B0414	87.66991	53.33454	52.834953	34.83983

B3302	265.16083	65.01437	94.864075	46.111053
B2608	233.20535	58.55695	117.58947	43.476776
B3733	334.80508	70.44838	135.96841	48.92716
B4152	91.70874	64.91052	51.168316	35.967213
B1817	72.70588	54.4566	49.2	33.93313
B3403	154.63971	66.45114	80.55446	49.260143
B3066	117.795456	54.257847	64.223305	42.05094
B3932	231.77216	65.93357	87.94118	48.26688
B1531	78.40594	56.511375	46.186275	35.31566
B1321	71.89216	59.329697	49.752476	34.82707
B2436	78.43137	56.66308	46.205883	34.85477
B3386	90.048546	56.110714	53.737865	38.706665
B3341	355.69168	81.18259	186.88422	60.490093
B3107	58.48	51.90625	46.2549	36.249256
B3735	536.8684	68.04195	192.88461	52.472366
B4151	64.42453	58.131805	45.403847	34.925667
Control	66.29126	59.3359	52.548077	37.284824
B0726	74.31132	54.386227	49.427185	37.40915
B0015	120.43363	56.212887	44.747574	34.492737
B1829	126.52525	54.92509	54.47059	37.221043
B4171	658.67834	83.41057	226.20721	60.191273
B3382	55.89216	52.23601	51.441177	36.505978
B2526	159.61429	63.120796	104.72857	43.173088
B3317	591.0137	81.460915	260.45206	68.384895
B2609	156.6909	58.290066	79.80769	43.4528
B3377	49.271843	48.751526	43.134617	32.854465
B1824	64.71154	56.601154	49.656864	36.38407
B2094	76.66346	55.72967	47.40566	35.85207
B0963	73.0991	58.60226	40.625	33.788425
B0727	89.41122	54.34879	54.839622	37.040546
B3179	136.65517	55.41916	47.857143	34.46857
B3687	67.81554	53.835114	47.69307	34.536514
B4143	185	60.265625	66.81554	39.33941
B0582	60.50476	53.12223	44.679245	32.963108
B3291	243.91112	70.15653	95.5	50.935898
B3320	252.92381	67.80111	123.48	45.95198
B2183	78.69903	53.602875	47.930695	35.998116
B3508	70.88349	52.477024	41.923077	36.342804
B0733	76.8932	61.448814	49.359222	34.52366

B3734	198.70526	60.58388	57.495144	40.873314
B0553	70.09524	56.224243	42.40777	33.703358
B0740	259.8	61.92904	79.73585	45.272446
B2614	74.99056	54.02003	37.50476	31.87024
B3686	66.51961	52.127373	38.911766	31.592396
B4142	301.74698	66.57193	92.281555	48.023907
B3399	248.45378	62.466297	98.631065	45.292473
B0675	80.125	58.332817	52.42157	37.888435
B3319	936.01324	93.37405	362.49588	81.45038
B1656	79.10577	59.369957	68.13461	44.022366
B4233	64.67961	53.73955	40.48077	36.333538
B0432	393.57516	72.73272	158.67021	51.338116
B2096	159.69748	64.302155	55.03	38.234562
B2284	117.96154	60.58401	63.640778	39.919292
B0742	199.7653	65.24376	63.235294	42.390285
B4172	782.38934	76.67087	279.97458	66.35333
B1380	64.398056	50.536667	47.485435	35.66971
B2193	110.66936	55.2686	53.490196	37.287163
B0978	69.03883	53.8347	46.66	34.141346
B0679	146.5923	56.90452	79.54808	43.258
B3985	982.1823	93.887856	437.90756	80.13122
B3450	71.00961	59.565315	44.563107	35.1041
B0014	108.289856	51.23971	48.462265	37.511177
B3734	264.9684	67.685265	116.38202	45.881428
B0431	398.52475	66.99286	96.84466	46.197563
B2095	314.17172	65.63498	138.9367	47.49203
B2282	120.31035	54.29643	56.22549	35.981033
B0862	253.28282	60.948795	77.21154	42.90083
B4259	119.630135	52.758377	50.215687	34.989426
2_ara5	65.066666	50.446888	38.138615	30.892925
B0660	59.705883	49.18704	36.530613	30.453798
B2999M	181.70589	57.44327	101.84906	39.496483
B2280	118.13725	57.397385	64.37374	38.879673
B3304	413.28845	70.85139	190.42105	50.087307
B1542	56.6	48.478832	42.875	34.0263
B0114	603.28455	71.742165	217.0238	57.83275
B0430	381.7762	66.94512	154.04054	49.57855
B2551	68.07843	55.690983	42.40404	34.019596
B2278	81.46939	58.937534	41.728157	32.75192

B1541	54.460785	49.36643	42.544556	31.152042
B1379	71.64762	53.167282	44.970875	32.277306
B0014	78.754715	58.24447	48.46602	34.705772
B0879	60.45631	50.58155	45.009804	32.581337
B1216	88.4902	57.352184	41.838383	33.20261
B3628	58.83	48.112602	43.9901	33.026676
B3318	574.4046	72.28311	217.37634	52.71255
B1791	53.60784	47.230083	38.14563	33.040867
B3732	134.60318	58.143406	45.271843	33.66313
B3894	86.25961	56.68999	45.673077	32.07905
B1136	129.58824	57.4503	46.747574	33.874073
B2277	144.10378	58.594433	64.94175	39.664997
B2592	63.72277	51.53953	42.990383	33.01063
B3931	77.211006	57.037903	49.17857	35.179424
B0439	116.056816	57.004322	54.74286	37.871025
B0966	79.00944	53.837456	37.60194	31.658236
B2610	214.5298	70.58793	92.84314	50.36406
B0414	72.411766	50.51017	63.235294	36.833332
B3302	182.83133	55.507343	59.79798	38.99364
B2608	254.80612	57.285854	129.14285	47.399487
B3733	269.33636	65.12854	105.81554	49.438057
B4152	74.254715	58.114017	42.561905	32.675114
B1817	58.971428	51.500298	32.660194	29.404852
B3403	69.76923	54.902157	42.563107	31.453218
B3066	97.940475	50.29472	41.77	33.053104
B3932	109.90678	53.75122	50.114285	35.09539
B1531	74.4466	55.72017	47.10784	35.17297
B1321	71.02941	53.35627	38.89423	33.025154
B2436	75.00971	52.325092	49.68932	35.634617
B3386	105.753624	52.497944	57.786407	41.030594
B3341	349.85577	63.002983	128.86407	58.133194
B3107	76.91262	53.932343	42.11	34.80993
B3735	548.3	70.73653	197.49474	57.721493
B4151	79.79808	59.716602	39.372547	32.326984
Control	66.93269	53.09086	44.471153	33.371517
B0726	74.26923	54.261806	36.451923	31.508486
B0015	86.63461	53.511665	45.294117	33.112133
B1829	138.54878	61.517136	46.388348	36.188377
B4171	671.1233	79.98579	230.83505	65.59384

B3382	87.68932	56.81178	49.884617	35.412754
B2526	172.1	59.30609	66.460785	40.588806
B3317	692.1042	76.271545	290.14816	70.45779
B2609	224.4253	65.772514	94.66991	50
B3377	56.46602	50.189022	46.14706	34.555103
B1824	144.71	59.14054	71.25714	40.450817
B2094	98.57843	60.671276	52.24	37.039883
B0963	79.59615	58.781033	47.436893	33.67995
B0727	88.81554	57.616943	58.39423	39.06119
B3179	180.37209	55.9849	63.904762	40.676796
B3687	72.15	57.04	35.068626	29.585348
B4143	166.21	62.521694	61.902912	38.027027
B0582	73.14706	53.1632	37.62136	31.02737
B3291	233.86667	64.86564	94.73786	43.981953
B3320	322.74756	68.307495	117.02885	50.82398
B2183	88.40777	55.040104	60.194176	37.020443
B3508	72.75961	54.86635	53.10577	37.378296
B0733	85.06731	61.66518	58.62136	38.708015
B3734	269.60205	67.28531	74.93204	43.72492
B0553	65.207924	54.002472	41.484848	33.966793
B0740	280.04504	63.56663	118.46535	45.08523
B2614	83.15238	55.729004	33.941177	31.280396
B3686	92.75247	66.9247	78.70408	46.27896
B4142	317.55554	69.00617	81.80769	45.64111
B3399	238.992	60.901054	77.38835	43.778942
B0675	175.39743	59.03108	44.575756	34.01323
B3319	666.7655	82.11771	280.1282	63.941772
B1656	72.660194	50.802998	51.068626	33.178528
B4233	62.326923	52.151237	45.653847	34.72722
B0432	413.0811	68.91523	113.6	48.18912
B2096	143.94737	60.202454	65.11881	39.837738
B2284	149.91	60.24962	55.19802	35.435448
B0742	154.25961	68.244385	69.47525	41.110126
B4172	639.8973	74.36983	236.64865	61.71911
B1380	83.78302	58.178673	44.6	31.905207
B2193	130.39131	61.50791	57.32353	35.828327
B0978	68.0098	55.04608	40.901962	32.263523
B0679	208.66386	72.664085	61.778847	39.193493
B3985	765.4133	79.37405	361.75	68.02227

B3450	52.673267	45.98383	40.382355	30.749352
B0014	75.29808	53.50077	44.235294	35.17647

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Accension Reference	Cy3 Signal Mean	Cy3 Background Mean	Cy5 Signal Mean	Cy5 Background Mean
B3734	325.53845	90.008606	93.7619	47.461937
B0431	347.44873	79.47403	120.65048	51.901043
B2095	365.79022	82.90459	102.864075	50.722935
B2282	159.24039	104.03099	57.784313	43.494846
B0862	1604.0901	430.09576	140.60785	97.866066
B4259	134.25961	111.08615	57.76923	46.67962
2_ara5	77.745094	66.3145	45.11	35.978245
B0660	94.40777	75.60739	44.89216	34.362526
B2999M	316.33334	68.72106	77.98039	42.616226
B2280	149.01334	63.196163	48.807693	35.181
B3304	583.8919	80.10707	258.67307	60.739326
B1542	60.313725	54.845707	48.862743	32.80845
B0114	573.9245	103.53564	220.01076	60.73581
B0430	319.83762	82.613396	119.84314	45.52009
B2551	101.32692	66.90731	49.78846	35.150578
B2278	100.66346	87.28836	50.961906	36.086395
B1541	288.55768	275.6317	104.5534	92.75033
B1379	246.2549	153.30374	81.20588	56.978493
B0014	136.09558	63.482277	38.89	31.581255
B0879	77.260414	61.153805	33.326733	27.990694
B1216	109.53398	67.35773	52.173077	35.691956
B3628	68.93137	55.97311	41.82653	31.631231
B3318	797.61487	96.68953	327.1316	68.06602
B1791	70.09434	58.71366	39.142857	33.310474
B3732	193.6762	74.943985	63.356434	40.00588
B3894	184.02963	66.33282	71.666664	42.46769
B1136	148.16037	79.39089	64.50476	43.99317
B2277	167.60127	81.334694	62.00971	41.275345
B2592	225.04465	179.5724	72.16964	58.488506
B3931	472.33673	206.32451	88.2381	64.88639
B0439	136.35239	83.991234	59.333332	41.27376
B0966	117.747574	70.89145	44.930695	32.978058
B2610	130.17476	72.35306	67.93204	40.42524
B0414	78.86139	56.313614	36.455444	29.11047

B3302	326.3526	90.39451	141.92	65.802025
B2608	328.48343	70.82389	130.62627	56.301212
B3733	303.5678	85.13989	69.696075	45.15478
B4152	101.95	66.92241	42.39216	31.49776
B1817	114.47059	78.60169	48.201923	36.357475
B3403	131.16924	74.770004	51.699028	38.775463
B3066	145.375	115.2082	53.735294	43.61139
B3932	247.03773	146.8006	70.252426	49.396584
B1531	94.55238	69.41879	37.72381	33.53311
B1321	81.20388	62.721554	51.784313	33.41055
B2436	92.357796	66.34492	48.49091	34.295666
B3386	87.790474	63.035183	41.761906	32.35013
B3341	254.0955	69.2667	105.427185	49.045063
B3107	60.50476	52.615795	47.650944	35.15172
B3735	664.07916	99.919815	213.55933	61.52546
B4151	106.5534	77.95583	49.02941	35.265045
Control	114.853935	73.013435	53.284313	35.69581
B0726	86.29703	73.755844	44.617645	34.90903
B0015	177.0726	84.40197	66.79	46.787586
B1829	161.28156	76.26759	51.843136	37.787476
B4171	334.18292	81.620705	101.80198	50.70911
B3382	71.930695	62.311184	55.961166	35.28532
B2526	166.59494	70.709946	55.28846	37.332375
B3317	919.88196	103.76162	326.54285	76.1992
B2609	233.48485	64.39707	101.30097	44.652466
B3377	58.019802	50.462532	49.764706	37.73756
B1824	188.64151	80.46572	65.460785	42.479465
B2094	140.41176	83.6048	53.30392	38.15297
B0963	214.88461	72.5999	54.57843	38.77804
B0727	101.33009	70.33132	51.87	39.141155
B3179	780.35	185.31053	201.48454	72.386536
B3687	95.69903	91.039825	46.740383	37.47859
B4143	144.24193	64.80256	52.514286	33.889297
B0582	162.08333	73.03482	54.970875	36.660957
B3291	130.26923	74.00876	54.884617	36.800594
B3320	233.66667	67.65394	64.22115	38.424294
B2183	100.58253	59.111565	49.186275	33.695976
B3508	72.84466	55.291065	61.9703	37.045628
B0733	128.82022	68.56355	44	32.94529

B3734	289.6143	91.01856	78.821785	46.593887
B0553	100.79612	73.414734	54.623764	38.611694
B0740	191.35135	79.06752	55.7	41.463852
B2614	105.81188	96.75498	44.262135	37.246376
B3686	99.6	85.06268	58.47059	44.650215
B4142	222.12102	75.05485	103.65263	43.11591
B3399	233.07071	77.14159	59.85294	35.84151
B0675	79.14019	61.82169	49.125	35.285545
B3319	656.12164	85.800896	250.33058	60.937344
B1656	79.7619	57.40155	50.819046	33.44121
B4233	68.78095	55.57881	43.96154	33.97564
B0432	351.10318	86.21902	115.34615	51.69975
B2096	191.02083	82.03312	51.834953	40.864307
B2284	330.94968	101.25727	83.06731	49.762062
B0742	117.67619	77.271965	56.153847	36.93803
B4172	365.9737	95.66472	101.875	53.229424
B1380	148.62857	69.7572	41.660194	34.307392
B2193	73.820755	59.743504	40	33.22619
B0978	103.01905	71	37.633663	31.137817
B0679	153.11539	78.93957	71.31068	43.274277
B3985	447.20493	78.630394	171.88776	53.362755
B3450	54.990196	48.481083	41.71287	31.162758
B0014	67.82353	54.95025	43.04	35.560467
B3734	386.78632	120.13266	81.396225	49.32753
B0431	397.7159	103.74836	95.78	53.93789
B2095	295.894	101.66647	81.23077	49.926296
B2282	90.89423	64.17618	44.366337	35.608055
B0862	236.03125	77.913994	56.56	38.94553
B4259	79.57843	55.24268	53.623764	37.184517
2_ara5	57.815533	52.18089	46.534653	32.795254
B0660	79.26923	60.552536	44.51923	32.86804
B2999M	221.93549	63.62589	64.166664	40.6033
B2280	145.60089	61.51694	50.271843	36.69316
B3304	441.38806	76.02647	203.88889	52.841785
B1542	52.12381	48.379536	43.125	34.193695
B0114	563.5289	141.64899	205.51648	64.11608
B0430	251.74675	95.48458	77.747574	48.151463
B2551	293.1979	143.49701	92.00944	56.31757
B2278	85.37255	64.87042	53.019417	34.877068

B1541	96.02913	77.5706	44.300972	34.31032
B1379	64.34314	53.876896	41.77778	30.830456
B0014	80.84158	54.53541	43.137257	32.838245
B0879	57.8	52.21809	35.727272	28.810404
B1216	86.56863	55.737373	52.80198	36.577816
B3628	61.72277	52.966007	47.048542	32.44163
B3318	505.44156	73.15647	211.01834	57.60306
B1791	57.327103	49.979317	47.30841	35.363857
B3732	276.62402	119.488304	64.71429	44.470005
B3894	169.30096	126.647194	63.990196	48.161503
B1136	314.3188	120.98999	73.02913	54.28547
B2277	110.81554	77.95444	59.19608	41.87356
B2592	76.96191	61.443005	39.62136	31.889376
B3931	64.411766	53.733852	44.72381	31.745098
B0439	87.18627	58.03557	51.59406	37.070694
B0966	65.62136	51.98501	34.19	29.700632
B2610	92.38461	57.717705	48.80392	34.02395
B0414	80.58654	56.28135	52.705883	34.872074
B3302	223.96297	69.67284	104.84	49.85662
B2608	296.5224	64.18315	120.754906	52.506855
B3733	398.40186	143.18999	78.33654	52.753845
B4152	136.7864	122.95646	49.813725	41.427082
B1817	196.42223	139.91559	80.57143	53.964874
B3403	164.53622	69.686005	41.53846	34.098
B3066	75.57843	55.18398	54.19608	35.395893
B3932	125.70886	59.016148	56.875	37.89527
B1531	75.15686	57.507866	44.142857	35.342705
B1321	66.15385	53.17941	42.471153	33.03123
B2436	81.75238	58.553894	51.50476	36.633144
B3386	88.38461	59.955673	54.66337	37.672085
B3341	220.26389	65.676155	80.27451	44.657696
B3107	62.388348	50.915756	44.735294	34.691612
B3735	655.63715	162.88852	157.15842	66.773155
B4151	378.71133	183.0148	77.68269	53.223503
Control	188.97087	125.15462	50.737373	42.702396
B0726	71.35294	58.887478	55.33	35.971443
B0015	72.67	55.56557	45.816326	34.233414
B1829	88.80198	58.5211	51.07767	32.98771
B4171	323.87598	67.581726	87.617645	50.546257

B3382	59.820755	50.36313	38.903847	30.932732
B2526	106.04082	60.05744	40.291264	31.993877
B3317	719.46765	94.36201	177.68932	63.984856
B2609	210.3211	59.058163	81.62264	42.20258
B3377	61.2549	49.160084	40.460785	34.18103
B1824	180.91878	87.91899	61.7549	45.81541
B2094	129.47525	66.252045	66.47475	43.251923
B0963	147.0084	72.89097	52.14706	37.452843
B0727	78.89109	56.762512	44.28866	32.9207
B3179	188.78572	60.95992	67.05882	38.96393
B3687	64.39423	52.695236	42.826923	30.191696
B4143	149.26666	61.81519	61.443398	37.15833
B0582	79.10577	59.516598	43.235294	32.086555
B3291	140.64844	61.682606	56.650486	36.675972
B3320	208.50467	64.994514	86.99039	54.449722
B2183	92.66346	55.429356	49.757282	37.597546
B3508	108.26724	54.068928	44.33654	35.37695
B0733	89.24039	62.87921	56.432693	36.70105
B3734	277.89795	69.3909	74.747574	41.739628
B0553	108.83654	72.33893	45.572815	33.29075
B0740	164.9619	62.2388	57.85437	37.438255
B2614	66.56731	53.544235	43	31.68553
B3686	62.504856	54.2951	51.903847	30.823786
B4142	241.92169	65.991516	94.79798	47.354584
B3399	182.86905	68.27249	61.48515	39.702763
B0675	69.776695	52.613354	49.221153	33.686855
B3319	546.5583	78.07121	206.94286	61.034325
B1656	83.54717	60.980892	58.048542	36.421658
B4233	63.330097	52.30682	48.298077	34.212383
B0432	308.229	67.29578	135.56818	44.673355
B2096	141.2069	65.62841	64.15151	40.25562
B2284	117.27723	62.741203	51.04	33.21765
B0742	130.38158	57.99382	51.425743	35.481956
B4172	320.90976	65.90881	155.50526	49.907143
B1380	70.01923	56.373608	40.388348	31.074482
B2193	74.34906	56.928535	47.826923	34.86865
B0978	66	51.568233	44.80357	32.899673
B0679	126.45192	62.86121	47.361904	35.134533
B3985	507.43182	73.883514	186.82474	55.88845

B3450	62.634617	49.526405	42.17822	32.170837
B0014	122.98781	58.984203	57.951458	40.072144

APPENDIX G

TROUBLESHOOTING

Troubleshooting

Problems Encountered	**Conceivable Cause and Remedy**
Faint Signal	**Hybridization time too short.** Prolong hybridization time up to 48-72 hours. **Low concentration of cDNA** The concentration of cDNA needed to hybridize may have been too low. Determining a more effective purification of cDNA after its creation is suggested **Insufficient shaking during hybridization** Since the cDNA concentration was on the low end, more vigorous shaking or oscillation would have been helpful to ensure hybridization mixture touched every part of the array surface. Hybridization chamber should be firmly attached to water bath and not allowed to freely float in the shaking water bath
High background noise and distortion of spots in Cy5 channel	**Scanner problem.** Maintenance on the scanner may be necessary to scan slides properly.
Uneven Hybridization	**Insufficient shaking during hybridization** Uneven distribution of hybridization solution during hybridization. **Uneven surface during hybridization** Ensure surface is flat so that gravity isn't working against hybridization.