RNA INTEGRITY IS NOT AFFECTED WHEN TISSUE SAMPLING IS PERFORMED POST-SCALD IN MARKET WEIGHT YORKSHIRE PIGS.

by

Amy Elizabeth Bohan

A thesis submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Master of Science

Auburn, Alabama
August, 2, 2014

Keywords: Pig, sampling, scalding, RNA integrity, meat quality

Copyright 2014 by Amy Elizabeth Bohan

Approved by

Werner G. Bergen, Co-chair, Professor of Animal Sciences
Terry D. Brandebourg, Co-chair, Assistant Professor of Animal Sciences
Cynthia McCall, Professor of Animal Sciences

ABSTRACT

Tissue samples are collected as quickly as possible following exsanguination to minimize the risk of RNA degradation and facilitate gene expression assays. However, collecting subcutaneous adipose tissue and longissimus muscle requires cutting through the hide which leaves the underlying tissue exposed during scalding-dehairing which is counter to best practice for collecting meat quality data and poses possible food safety issues. In order to test the effect of sampling pre- or post-scalding on RNA-based assays and indices of meat quality, subcutaneous adipose and *longissimus dorsi* tissues were harvested from the right tenth rib of market weight Yorkshire hogs (n=8) immediately following exsanguination and again immediately following scalding. Total RNA was extracted from all samples and RNA quality was assessed both visually by gel electrophoresis and by determining an RNA Integrity Number (RIN). The expression of adipose and muscle marker genes were then measured using real-time PCR. Ultimate pH, visual color score, and objective Hunter color scores were compared between carcasses that had been sampled prior to scalding and those carcasses that had not been sampled at all, (n=8). All RNA samples exhibited sharp ribosomal bands with a 28S to 18S ratio greater than one when visualized on a denaturing gel. All RIN values were greater than 8.8 while no differences in OD 260/280 ratio ($P > 0.71$) or RIN values ($P > 0.21$) existed between sampling times indicating that scalding did not negatively affect RNA integrity in either adipose tissue of longissimus muscle. There were no differences in the mRNA expression levels of the *ADIPOQ,*

LEP, GLUT4 or *PPARγ* genes in adipose tissue or *CKM, MYOG, GLUT4* or *TNNT1* in longissimus muscle sampled pre- or post-scalding as determined by real-time PCR.

However, sampling tissue prior to scalding resulted in greater visual color score ($P < 0.001$) and lesser L* ($P < 0.001$) and b* ($P < 0.001$) values while neither a* values ($P > 0.53$) nor 24h pH ($P > 0.41$) was affected. These data indicate that sampling post-scalding did not impair RNA quality or the ability to measure gene expression via RNA-based assays such as real-time PCR. However, sampling tissue prior to scalding and post scalding did result in darker color of the underlying muscle 24 h postmortem. Thus, if both RNA-based assays and meat quality endpoints are to be performed using the same animal, the best choice would be that tissue sampling should occur at a time point immediately following scalding.

Acknowledgements

I would like to thank my parents, my grandparents and my twin sister for supporting me throughout these last three and a half years.

I would also like to extend my gratitude to my Co Major Professors, Dr. Werner G. Bergen and Dr. Terry D. Brandebourg for taking me on as their Masters of Science student. The knowledge I have gained from this experience has been life altering and I am sincerely grateful for their mentorship. Lastly, I would like to thank Julia Bartosh, Meggan Marvin and Stephanie Perkins for the countless hours that they've spent in the lab, aiding in obtaining the data presented in my thesis

Table of Contents

Abstract....................ii

Acknowledgments....................iv

List of Tables....................vi

List of Figures....................vii

List of Abbreviations....................vii

Chapter 1....................1

Chapter 2....................4

Literature Review....................4

1. Summary of harvest procedure for pork....................4

1.1 Conversion of muscle to meat....................5

1.2 Effect of handling and gene expression analysis may have on meat quality....................5

2. Role of water holding capacity on pork quality....................6

2.1 pH on water holding capacity…………………………………………………………………7

2.2 Physical mechanisms of water holding capacity……………………………………………..8

3. Rigor Mortis and associated mechanisms……………………………………………………8

4. Consumer satisfaction and meat freshness……………………………………………… …9

4.1 Appearance quality traits……………………………………………………………………… .9

4.2 Eating quality traits……………………………………………………………………….10

5. Real time polymerase chain reactions………………………………………………………11

Materials and Methods……………………………………………………………………… 19

1. Animals and design……………………………………………………………………..20

2. Carcass fabrication……………………………………………………………………….20

3. Gene expression analysis………………………………………………………………..20

4. Statistical Analysis……………………………………………………………………….23

Results……………………………………………………………………………………….24

1. RNA purity………………………………………………………………………………….24

2. RNA integrity……………………………………………………………………………….25

3. Effect of scalding on gene expression as measured by Real-time PCR………………………26

4. Measures of pork quality…………………………………………………………………...27

Discussion……………………………………………………………………………..28

References 43

Appendix A 46

Appendix B 55

List of Tables

Table1. Ratio of 260/280 absorbance by sampling group RNA samples extracted from subcutaneous adipose tissue…………………………………………………………..……….33

Table 2. Ratio of 260/280 absorbance by sampling group in total RNA samples extracted from *longissimus dorsi* muscle tissue…………………………………………………………………34

Table 3. RNA integrity number by sampling group in total RNA samples extracted from subcutaneous adipose tissue……………………………….…………………………………38

Table 4. RNA integrity number by sampling group in total RNA samples extracted from *longissimus dorsi* muscle tissue. ……………………….……………………………………….39

Table 5. Meat quality traits by sampling group……………………………………..………..40

List of Figures

Figure 1. Assessment of RNA purity relative to water blanks from a sample harvested prior to scalding and a sample taken from the same animal immediately following scalding as judged by spectral scans encompassing absorbance readings between 200-400 nm…..32

Figure 2. Assessment of adipose tissue RNA quality as judged by visualization of 28S and 18S bands on a denaturing agarose gel. Total RNA extracted from subcutaneous adipose tissue samples harvested prior to scalding (Top Panel) and following scalding (Bottom Panel) are depicted……………………………………………………………………………..…..35

Figure 3. Assessment of muscle tissue RNA quality as judged by visualization of 28S and 18S bands on a denaturing agarose gel. Total RNA extracted from longissimus dorsi tissue samples harvested prior to scalding and following scalding are depicted………………………………..36

Figure 4. Assessment of adipose tissue RNA integrity as judged by electropherograms of total RNA isolated subcutaneous adipose tissue harvested from the tenth rib of the same animal prior to scalding and immediately following scalding…………………………………………...37

Figure 5. Messenger RNA abundance (expression) of adipose tissue marker genes, (A) adiponectin, (B) leptin, (C) peroxisome proliferator-activated receptor gamma (*PPAR*□), and (D) glucose transporter 4 (*GLUT4*) in the subcutaneous adipose tissue of market weight hogs as measured by Real-time qRT- PCR…………………………………………………………41

Figure 6. The mRNA expression of muscle tissue marker genes, (A) creatine kinase, (B) troponin, (C) myogenin, and (D) glucose transporter 4 (GLUT4) in the longissimus dorsi muscle of market weight hogs as measured by Real-time qRT- PCR……………….…………………42

List of Abbreviations

ATP Adenosine Triphosphate

ADP Adenosine Diphosphate

AQT Appearance Quality Traits

cDNA copy Deoxyribonucleic acid

cp crossing point

ct threshold cycle

DFD Dark, Firm and Dry

EQT Eating Quality Traits

IMF Intramuscular Fat

Kb kilobases

Mb Myoglobin

mRNA messenger Ribonucleic acid

Nm nanometer

PCR	Polymerase Chain Reaction
PSE	Pale, Soft and Exudative
RIN	RNA Integrity Number
RNA	Ribonucleic acid
RT- qPCR	Real Time quantitative Polymerase Chain Reaction
RT	reverse transcription
SCF	subcutaneous fat
WHC	Water Holding Capacity

CHAPTER 1

INTRODUCTION

Performing RNA extractions, isolations and assays designed to target specific genes has provided great insights of living systems to scientists. In the agricultural domain, where work is done with species that actually become food for human consumption, the timing and method of tissue sampling and the potential meat quality effects this may create has been a recurring issue for both scientists and those concerned with the quality of the meat. On one hand, obtaining tissue samples at harvest of pigs immediately after slaughter but prior to scalding may affect food safety and quality of the meat as for example the exposed longissimus muscle and subcutaneous adipose tissue are both now vulnerable to the effects of temperature and other outside influences (dust, insects, microorganisms). Conversely, delaying tissue sampling immediately after slaughter until some processing of the pig carcass has occurred to preserve the color, texture, marbling and wholesomeness of the meat as a human food may lower integrity of RNA necessary for down-stream gene expression studies. During this delay, potential RNases may be activated and result in degradation of RNA, lowering RNA integrity and quality for future experimental work.

Obtaining tissue samples either immediately after slaughter or after post initial processing (scalding-dehairing) has thus become a prevalent issue within slaughter facilities and laboratories, alike. Research described in this thesis was designed and performed to show the

impact that sampling pre versus post scalding and dehairing has on both meat quality and potential RNA degradation. This study may be impactful as its results could change our perception of the best timing of post-slaughter tissue harvest for RNA isolation. Pork that's offered on the shelves of grocery stores should be uncontaminated and contain few visible blemishes for improved consumer appeal. However, those interested in gene expression and regulation of said genes widely argue that tissue sampling should occur as quickly and as efficiently post mortem as possible. Because RNA is subject to degradation by endogenous and extrinsic RNases when influenced by initial tissue dissolution, fluctuations in temperature, or possibly by any other extrinsic, non-sterile source that potentially may introduce RNase activity and subsequent RNA degradation into the tissues. This then leads to gene expression data that may be untrustworthy. If the results cannot be trusted because of the compromised RNA that was sampled from the animal post scald, there is really no arguable point in going forward with the rest of the RNA isolation and gene expression assays as sampling procedures pre-doomed success. Thus a poor sampling strategy or approach may result low RNA Integrity Numbers (RIN) which will depress the effectiveness of the downstream applications of the RNA in the laboratory. For the purpose of gene expression assays, an RIN of seven to eight has been deemed optimal. The RIN values are based on the ratio for quantity of 28s and 18s RNA determined by electrophoresis. Values for RIN vary from one where no distinct RNA is visible to ten where undegraded 28s and 18s peaks stand out prominently. Generally some RNA degradation cannot be avoided but workers try to obtain the most undegraded RNA possible. Therefore if tissue harvests post scalding results in unsatisfactory RIN, then sampling prior to scalding could be a quick fix to this RNA degradation issue. Thus from a growth biologist's view sampling pre scalding and dehairing would be the most sensible approach.

However, on the meat quality side of the equation, it may be detrimental to the quality of the pork and food safety to let tissue samples be taken before the animal is scalded. Once the animal is sampled prior to scalding, the exposed/ damaged tissue site may negatively affect palatability, texture, color and marbling and potential consumer appeal. Thus these negative impacts on the carcass will lower market value of the meat. Therefore, from the perspective of a meat scientist, it may be intuitive to believe that the best way to preserve the meat and its quality is to not to allow any sampling be executed on the carcass until after the scalding and the dehairing process has been completed and thus presumably preserving meat quality. Generally if a sampling site is too dominant, irrespective of pre or post scald sampling the meat value will be discounted.

The testable hypothesis of this study was that RNA quality and RIN would be negatively affected in tissue samples harvested from pigs after scalding and dehairing. Scalding and dehairing are the initial processing steps in the harvest of pork.

CHAPTER 2

LITERATURE REVIEW

A question being raised in many University slaughter plants today is whether or not tissue sampling from pigs, pre versus post scald affects either the integrity of the RNA or the quality of meat. Typically, tissue samples are collected as quickly as possible and frozen in liquid nitrogen to ensure the RNA doesn't suffer any level of degradation before further RNA isolations and assays are performed. However, while this is thought to be the most effective approach from a molecular biology approach, meat scientists may disagree. When the subcutaneous adipose tissue and the longissimus muscle are cut through the hide, the tissue underlying both of these is now exposed to the outside environment, posing possible food safety issues as well as data collection difficulties for meat quality assessment. Therefore, a study was designed that would determine whether sampling pre or post scalding has a negative impact on RNA quality and/or meat quality in pigs.

1. SUMMARY OF HARVEST PROCEDURE FOR PORK

Upon arriving at the slaughter facility, the animal will be held in some type of holding pen with free access to water until the time of immobilization happens, wherein the animal is rendered unconscious. After the animal is visibly unconscious, the blood will be removed from the animal through a process known as exsanguination. Ideally, after exsanguination, the animal

should be moved and stored in the meat cooler as quickly as possible to try to avoid a very rapid decline in pH, as the postmortem pH decline has a very tremendous impact of the color of the meat, affecting its' quality, independent of whether the meat is classified as normal, dark or light.

1.1 **CONVERSION OF MUSCLE TO MEAT**

In a live animal, energy is stored in the muscle in the form of glycogen. Glycogen is then converted to glucose and finally to pyruvate. When the muscle is dying, especially seen around twenty four hours post mortem, glycogen is converted to lactic acid and subsequently, the lactic acid lowers pH in the muscle as it accumulates. An increased glycogen breakdown results in decreased pH and if this decrease is too rapid, the meat quality will be compromised. Several factors can affect the concentration of glycogen, such as the animal being exposed to cold temperatures, being exhausted, a sudden withdrawal in feed and any number of sicknesses (Aberle et al., 2010)

1.2 **EFFECT HANDLING AND GENE EXPRESSION ANALYSIS MAY HAVE ON MEAT QUALITY**

Handling the animal properly in a stress free environment as possible during slaughter can positively affect meat quality and the amount of muscle glycogen stored. When certain genetic traits that can affect meat quality and/or improper handling has occurred, less than ideal meat quality and color may be the result. One example of such meat is known as Pale Soft Exudative (PSE) pork in which the pH of the muscle is in the range of 5.6 or lower, because of the lactic acid accumulation. In this pH range, the purple color of the meat which is considered desirable, will change to a bright red or possibly will have a pink appearance. In pork, this

phenomena, termed PSE, likely leads to rapid glycolysis due to antemortem excitement or possibly holding the animal on the kill floor for an extended period of time before chilling, an issue in the handling postmortem. In these scenarios, there is a short-term glycogen depletion that occurs before the animal is immobilized and subsequently, the pH reaches 5.2 in as little as two hours postmortem. This is extremely undesirable for meat quality, resulting in visually notable changes in water holding capacity, firmness and color will be noticed. Because this issue seems to be one that has a large impact on meat quality, solutions have been examined in hopes of avoiding PSE, particularly in pigs with an inherited characteristic for this problem. Taking the utmost care to keep the animal from becoming excited and stressed before slaughter and keeping the time the carcass spends on the kill floor at a minimum is critical to meat quality. On the other hand, meat that is termed as dark, firm and dry (DFD; pH does not decline post mortem, glycogen lowered before slaughter) ought to be avoided as it will not appear desirable in color to the consumer when on the shelf and once cooked will most likely not be satisfactory to the consumer. The DFD problem is a result of environmental factors resulting in a long term depletion of glycogen. Because it has been shown that hauling and transporting pigs for an extended amount of time without providing feed depletes the glycogen in muscle, pigs should be fed and well rested 24 to 48 hours before they are slaughtered to avoid DFD. (Aberle et al., 2010).

2. ROLE OF WATER HOLDING CAPACITY ON PORK QUALITY

Because lean muscle primarily is composed of an estimated 70- 75% water, Water Holding Capacity (WHC) plays critical role on the quality of pork (Lawrie, 2002). Ideally, one would strive to aim for the highest WHC possible, because a high WHC capability is directly

correlated to reduced loss in weight during storage and retention the meat to retain a higher percentage of water during processing stages. In addition, high WHC abilities have been thought to enhance the eating quality of meat (Lawrie, 2002). Furthermore, it has been estimated that as much as 50 % of the pork that is produced has a drip loss that is so substantial that it has been thought to lead to a decline in fresh retail cuts. (Lawrie, 2002).

2.1 pH ON WATER HOLDING CAPACITY

While, the pH directly affects color and quality of meat, it also plays a critical role in water-holding capacity of meat. The pH is directly affected by the amount of glycogen concentration in the muscle at the time of slaughter as this ultimately determines the formation of lactic acid during postmortem conversion of muscle to meat. (Bendall and Swatland, 1988). The pH of the meat is also correlated with WHC. The more rapid of pH decline occurs, the less WHC capability the meat has. A prior study has shown that when a pH declines rapidly, the drip loss percentages are increased in the postmortem Longissimus in pigs bred for a growth efficiency as opposed to control pigs. (Lonergan et al.,, 2001).

2.2 PHYSICAL MECHANISMS OF WATER HOLDING CAPACITY

Most of the water located in the muscle is actually shown to be within the intra and the extra-myofibrillar spaces with the cells. Therefore, as rigor progresses, water loses its ability to maintain its location in the myofibrils and is forced into the extra-myofibrillar spaces. From here, the water is all too easily lost as drip. (Lonergan, 2005). Water is found in meat within one three locations. One location would be bound wherein charged hydrophilic groups in the muscle protein attracts water and forms a tightly “bound layer”. A second location containing

water or moisture is commonly known as immobilized in which there is quite a bit less orientation, molecularly, toward the charged group. In a third location, free, capillary forces are present and the orientation is completely independent of charged groups. The point in which the water-holding capacity is the lowest is at the isoelectric point, where the numbers of positively and negatively charged groups of myofibrillar proteins are found to be equal. Naturally, at this point, since the charges are canceling one another out, there is no charge available to hold the bound and immobilized water. (Aberle et al., 2010).

3. RIGOR MORTIS AND THE ASSOCIATED MECHANISMS

Once the animal is exsanguinated, rigor mortis which literally means “death stiffening” starts to occur. There are three phases associated with rigor mortis. The initial post mortem phase takes place as the muscle is still in a relaxed phase which means that no cross bridges are forming between the thick and thin myoflilaments. The reason as to why the muscle is still able to remain relaxed has to do with the fact that there is still an abundant amount of ATP within the muscle.

In the next phase, commonly referred to as the “Delay phase”, depletion of both ATP and Creatine Phosphate is occurring. Due to Creatine Phosphate being no longer readily available, ADP is no longer able to be rephosphorylated to ATP which results in the formation of rigor bonds. Naturally, muscle will lose its’ extensibility as the bonds formed between the thick and thin myofilaments continue to form.

The full effects of rigor mortis are shown in the “Completion” phase as Creatine Kinase has been depleted, there is no longer a way for ATP to be regenerated. (Aberle et al., 2010).

4. CONSUMER SATISFACTION AND MEAT FRESHNESS

Now that the muscle is well on its way to becoming meat, the main concern at this point is the freshness of the meat quality. To define “freshness” scientifically, it is a combination of composition, nutrients present, colorants, water holding capacity (WHC), tenderness, functionality, flavors, spoilage and contamination (Lawrie, 2002, Aberle et al., 2010) However, the traits desired for fresh meat and for eating quality are not the same. Color, WHC, texture and amount of fat are the most desired when looking specifically at the freshness of the meat. When primarily concerned with eating quality of cooked pork, tenderness, flavor and juiciness are the traits that are most important. (Lawrie, 2002)

4.1 APPEARANCE QUALITY TRAITS

A producer needs to be concerned about factors known as “Appearance Quality Traits” (AQT) as well as “Eating Quality Traits” (EQT) as a consumer takes both of these into consideration when evaluating quality of meat. Primarily, with AQT, meat color is the most important trait dealt with. Reasonably so, as this is the first impression the packaged meat makes on the consumer and implies that the meat is both wholesome and fresh. Meat color is dependent upon a variety of factors such as age of animal at time of slaughter, species and muscle type. Differences in muscle color can be accounted for by the content of Myoglobin (Mb) in the muscle. The more efficient Mb is in storing and delivering oxygen in the muscle, the higher the amount of Mb in type 1 muscle fibers. Unfortunately, while the Mb content is directly positively correlated with the exercise and diet of the animal, issues with storage and display can affect even the most desired amounts of Mb in the muscle. (Faustman, Sun, Mancini and Suman,

2010). The main proponent in the stability of meat color stability can be attributed to the rate of Oxy-Myoglobin oxidation. (Faustman and Cassens, 1999; Faustman, Sun, Manciniand Suman, 2010).

Texture of meat can be affected by multiple parameters such as the size of the muscle fiber, the quantity of connective tissue present and to some degree, the amount of intramuscular fat, (IMF) present. (Fernandez et al., 1999). In addition to IMF affecting meat texture, IMF also affects flavor, juiciness and visual characteristics of meat. Thus it is of the utmost importance to control the factors that affect the amount of IMF present in the meat. The factors proven to affect IMF quantity include but are not limited to animal breed and the weight of the animal at the time of slaughter. (A-Ouali et al., 2006). It has also been shown that IMF tends to increase as the animal ages, as at this point in growth and development, (muscle growth) has come to a plateau. Furthermore, animals with higher total body-fat have greater IMF deposits. IMF deposits are typically higher than in leaner animals. IMF has also shown to be highly heritable and there seems to be a highly correlated relationship with red muscle fiber and conversely, a negative correlation with white muscle. (Hwang et al., 2010).

4.2 EATING QUALITY TRAITS

As far as tenderness is concerned, it is the most desirable trait when looking at Eating Quality Traits. This is reasonably so, as tenderness will either negatively or positively affect the consumer’s opinion of the quality of the meat once cooked and ready for consumption. Things that can affect meat tenderness are the amount of connective tissue present within the muscle, the contractile strength of the muscle fiber and the amount of proteolysis that has already and or is

presently occurring in the rigor muscle. (Aberle et al, 2010). As far as eating quality is concerned, flavor is also a very important aspect as people associate flavor directly to the savoriness of the meat. Things affecting muscle flavor include things such as sex of the animal, age, the stress prior to slaughter, feeding strategy as well the muscle to adipose ratio that makes up the animal's composition.

Another very critical element when dealing with EQT is juiciness of the meat. Juiciness has a positive correlation with WHC and IMF found within the meat. When examining processes that occur during the conversion of muscle to meat, there are numerous variables affecting the quality of the meat that occur before the animal is immobilized and exsanguinated. Genetics can also play a vital role in the color of the meat and the percentage of glycogen that is stored in the muscle post slaughter. Therefore, proper handling and breeding plays a very critical role in the meat quality of any animal.

5 REAL TIME POLYMERASE CHAIN REACTIONS

The most commonly used method for measuring gene expression is the reaction known as Real Time Quantitative Polymerase Chain Reaction (RT-qPCR) for the quantification of mRNA abundance (Bustin and Nolan, 2004). While RT-qPCR is regarded as the standard to which all other assays should be measured against, it is far from flawless. Differences in RNA templates, variances in protocols, materials and methods and incorrect interpretation and normalization of data all introduce problems with this quantitative assay (Nolan, Hands and Bustin, 2006). Despite of these potential shortcomings, RT-qPCR remains the most preferred method.

The processes that make up the entire RT-qPCR method consist of three steps. The first step is to perform a reverse transcriptase (RT) reaction wherein isolated RNA is transcribed into cDNA. A problem with this RT reaction is that this is not a standardized reaction and introduces variability into RT-qPCR in the first step. Secondly, the cDNA is amplified using PCR and lastly, the products are quantified though Real Time imaging equipment (Bustin and Nolan, 2004). Real Time qPCR relies on increasing the fluorescence signal, and correlates with the amount of DNA produced at each signal. The threshold cycle, (Ct) measures when the fluorescence crosses over a parameter, and is also commonly called the crossing point (Cp). This is how the quantity of amplified product is measured.

However, quality control at the initial stages, before RT-qPCR is run, is crucial. The quality of the RNA being extracted for further use for RT-qPCR, microarrays, etc. determines the quality and whether or not the end result is a trust worthy and accurate reading of mRNA abundance/gene expression. While the PCR efficiency may not be affected by RNA integrity, the qRT-PCR performance most likely will. (Fleige and Pfaffl, 2006). RNA quality is comprised of two characteristics, integrity and purity. The purity of the RNA means that it is free of protein, inhibitors and DNA contaminants. (Tichopad et al., 2010). In the past, the two methods to examine RNA quality were to either analyze the A260/A280 ratio or to run a denaturing agarose gel and analyze the amount and sharpness rRNA bands present. However, in recent times, it seems as though most workers have switched over to a method that is an automated capillary-electrophoresis system. This system yields crucial information such as RNA concentration, approximated ratios between the mass of ribosomal sub-units and allows for a visual inspection of RNA integrity. (Fleige and Pfaffl, 2006). RNA Integrity Number (RIN), range from one to ten with ten being the highest classification rating (intact) and zero representing completely

degraded RNA. It is thought that RNA needs to score at least a five with respects to the RIN to be able to be considered useable RNA for some downstream methods. A RIN score higher than eight is thought to be sufficient total RNA quality for applications such as microarrays. (Fleige and Pfaffl, 2006). It is critical to aim for RNA of the highest RINs because the evaluation of gene expression is directly correlated to the quantity and quality of RNA from the start. The consequences of low RINs include compromising the results produced by RT-qPCR and microarrays and can be very costly. Because of this, it is of utmost importance that through every step, the time of handling samples away from cooling ice are limited and all steps are performed in a careful, timely manner in order to prevent RNA degradation. Without proper storage, handling and the methods used during sampling, RNA is susceptible to degradation. (Perz-Vova et al., 2005).

One step that is very crucial to the quality and integrity of RNA is the method by which it is extracted. In addition, fragments that are up to 10 kb are already predisposed to degradation as there can be cleavage by RNAses that are introduced by the handler/sampler during the extraction and handling process. (Bustin and Nolan, 2004; Pfaffl, 2005). During the extraction and purification process, the RNA must be free of protein, free of genomic DNA, must be undegraded with a 28S:18S spectrometric ratio in the range of 1.8 to 2.0. The RNA must be free of enzymatic inhibitors for RT and PCR reactions and this largely depends on the care and techniques used during the purification process. There must be no nucleases as these can interfere with the intactness of the RNA when being stored for an extended amount of time. Because Manganese (Mn) and Magnesium (Mg) are essential reaction co- factors, they as well as similar substances must be limited in the RNA samples. (Bustin and Nolan, 2004; Pfaffl, 2005). In addition to RNA meeting all of the aforementioned requirements, other problems arise and

affect reproducibility. For example, RNA isolation techniques can lend a hand to problems with reproducibility as well the source of RNA and the techniques used in isolating said RNA. These techniques differ between laboratories and subsequently introduce variation with results as well as the different extraction methods, i.e. those performed by hand or by the use of an automated system. (Fleige and Pfaffl, 2006). In addition to sampling methods, storage conditions as well as time spent in storage, sampling techniques in animal tissue may introduce a plethora of issues as far as the potential for RNA degradation is concerned. Sampling of animal tissues is very time sensitive and the time it takes to sample tissues from the animal and then place samples in liquid nitrogen needs to be kept to a minimum. Due to downstream laboratory procedures that are currently in place for routine use as well as experiments utilizing animals during the slaughter process, the quality of RNA may be jeopardized by overall poor handling and may be susceptible to degradation.

When RNA quality and the assessment of quality are at stake, often times, conventional assessment methods are not sensitive enough and contaminants may be introduced. In order to assess the quality of the RNA early workers adopted spectrometric UV/VIS methods to evaluate RNA quality. It was reasoned that at 240 nm one would expect to see background absorption and the possibility of contaminants. At 260 nm, nucleic acids should be present and at 280 nm, one would expect to assume proteins are present. Furthermore, at 320 nm, it is assumed that possible contaminants as well as background absorption are being read (Sambrook et al.,1989; Manchester, 1996). The classical 260/280 nm ratio is not the most appropriate but widely used measure of purity. Since the extinction coefficients are different between proteins and nucleic acids, this procedure is more suitable to identify nucleic acid contamination in protein preparations than proteins in nucleic acid preparations (Warburg and Christian, 1942). Newer

spectrometric quantitative assays of nucleic acids (RNA) may be achieved using a Nano Drop spectrophotometer in conjunction with RNA Ribo Green dye. Some believe this to be a superior method to the UV/VIS spectrophotometer, due to the fact that it only needs a mere one microliter or two in order to give an accurate reading of RNA concentration. In addition to these two methods, one can perform an additional check by pouring and running agarose gel electrophoresis with the RNA either being stained with SYBR Green dye or ethidium bromide which proves to be less sensitive than the SYBER Green. (Bustin and Nolan, 2004).

The preferred method when quantitating measurement of low abundant mRNA gene expression is undoubtedly RT-qPCR as it shows high sensitivity, good reproducibility and a wide range of quantification. (Bustin and Nolan, 2005). In order to have the RT-qPCR be successful, one must use intact RNA as this is a crucial initial step in obtaining gene expression measurements. This is easier said than done as many factors that are present in the sample as well as outside contaminants prove to inhibit the reverse transcriptase (RT) reaction as well as affecting the PCR and giving inaccurate readings. (Stahlberg et al., 2004).

Fleige and Pfaffl (2006), performed a study using tissue derived from bovine tissues as well as cell lines also derived from bovine tissue. Their objective was to examine the influence that RNA integrity, by use of the RIN classification, had on the intact transcriptome of a bovine tissue that either degraded by enzymatic digest or by ultraviolet light. Fleige and Pfaffl (2006) also used RIN analysis with bovine tissue and cell lines to examine the relationship between RNA integrity and the intact transcriptome of bovine tissue subjected to enzymatic digestion or ultraviolet light. The result of RNA degradation with an RIN classification of one to three, leads to enzymatic cutoffs, also known as breaks, in the original RNA strand. The result of these cutoffs leads to strands that are different lengths. The expression of four genes was studied using

RT-qPCR on RNA that was deemed intact as the RIN ranged from eight to ten as well as the RNA that had been deemed degraded with RIN less than three. The results of the qRT-PCR showed that with RNA with high RIN, (indicating that this RNA was of high quality as well as intact), the Cp was actually shorter than in RNA that had low RIN for the same gene. Furthermore, it was shown that the variability decreased for those genes that were run through qRT-PCR with RNA with high RINs (Hugget et al., 2005; Fliege et al., 2006). In addition, there was a correlation in some tissues between the RIN and the Cp values when the products were shorter in length. However, this correlation was also visible in WBC and corpus luteum even while these products were actually longer in length that aforementioned products.

Sometimes, even RNA with the highest of RIN doesn't ensure that one will have good results after the PCR assay is run. There are other factors that play into the integrity of the sample, such as the sample containing inhibitors that may or may not reduce amplification efficiency. (Bustin and Nolan, 2004; Wong and Medrano, 2005). Examples of such may include an array of things such as the length of the amplicon, the secondary structure and, in addition, the quality of the primer being used in the reaction. Therefore, aside from the RIN alone, one needs to consider and evaluate the efficiency as this is a marker that can easily skew results with RNA of even the highest of RINs. (Bustin et al., 2004).

Basically, the conclusion of the above reviewed studies was that the amplification of a long product that is over 400 bp is going to have the tendency to be incredibly dependent on RNA that is of high quality. Further it appears that products of qRT-PCR that are 70-250 bp in length, seem to act as if they are much less dependent of high quality RNA. When performing qRT-PCR, it is of the utmost importance that one does not use degraded RNA as this will most definitely limit the efficiency of the RT reaction and therefore the yield shall most likely be

much less than from pure and high quality RNA (Stahlberg et al., 2004). In summation, if one can only utilize RNA that has been compromised as far as the integrity, quality and RIN are concerned; one must design a set of primers that anneal to a specific internal region of interest. With that said, if one is aiming to look at the quantitative RT-PCR, using RNA that is degraded or even, at best, partially degraded, is most likely going to yield results that are not an accurate representation of the gene expression that is being studied (Bustin and Nolan, 2004).

Another problem with RNA that is partially degraded presents itself in the reaction, known as Reverse Transcriptase (RT), before one even attempts to run qRT-PCR on the sample. The RNA must first be converted to cDNA before any gene expression assays can be performed. As the RT reaction transcribes RNA into cDNA and because the cDNAs directly reflect the amount of mRNAs produced, thus possibly affecting the accuracy of the quantification, one must ensure the RNA is as intact and pure as possible (Stahlberg et al., 2004 a). While the RT reaction can happen without any addition of a primer, employing three basic priming strategies will raise the level of efficiency. These three main strategies are based on an oligo (dT) primer, random sequence primers and primers that are specific to the gene being quantified. Once the oligo (dT) primers are added to the reaction, it will begin to hybridize to the poly (A) tail that is found in nearly all eukaryotic mRNAs and thus, reverse transcription will be initiated beginning with the (3’end) of the mRNA. While individuals seeking to clone a gene find this approach very effective, those whose objective is to do gene expression analysis, seemingly disagree. Reason being, that if the mRNA is not intact due to degradation, it is possible that the transcription will not actually reach the end of the PCR target sequence. This potential problem becomes magnified when the samples of mRNA are varying in quality. In an effort to eliminate this issue altogether, random oligomers are the primer strategy of choice as they tend to produce transcripts

that extend past the PCR target sequences. (Stahlberg et al., 2004; Bustin, 2002). Sampling methods, RNA extraction and the preparation methods also introduce variety into the quantification of gene expression when using qRT-PCR. (Stahlberg et al., 2004).

Thus even if the RT and qRT-PCR are both executed flawlessly, if the start product was RNA that happened to be degraded, the end result, ie, gene expression quantity, is going to be an inaccurate and untrustworthy representation of the level of gene expressed. In that case, one needs to reexamine the RNA extraction method used as well as the manner in which the samples, especially when taking tissue samples, were obtained.

MATERIALS AND METHODS

All procedures involving animals were approved by the Auburn University Animal Care and Use Committee (PRN 2012-2122).

1. ANIMALS AND DESIGN

Yorkshire hogs (n = 16), at a BW of 120 kg were selected from the resident herd at the Auburn University Swine Education and Research Center (SERC) and were humanely harvested at the Auburn University Lambert Powell Meats Laboratory under USDA-FSIS inspection in groups of 8 on July 7, and August 23, 2013. Immediately following exsanguinations on July 7, 2013, samples of subcutaneous adipose tissue and longissimus muscle were collected from 8 pigs by cutting a plug of tissue from the carcass approximately 4 inches square and extending from the skin through the loin muscle at the tenth rib behind the right shoulder. Carcasses were then subjected to scalding and dehairing (10-15 minutes) and immediately following, tissue was again sampled as described above from the tenth rib behind the left shoulder. Carcasses were then processed routinely according to AU abattoir standard operating procedures and indices of meat quality were then measured as described below. Another group of 8 pigs, serving as a non-sampling control for meat quality measures were harvested on August 23, 2013. These 8 pigs were not sampled and processed normally. Indices of meat quality were measured on these carcasses 24 hour postmortem. Irrespective of the scalding regime subcutaneous adipose tissues and muscle were immediately collected, snap frozen in liquid nitrogen, and then stored at -80

degrees C until mRNA analysis. To facilitate this, a plug of SCF and longissimus muscle was removed by cutting through the hide immediately upon verifying that the animal had expired and later after scalding. Tissue samples were then processed immediately in a wet lab adjacent to the harvest floor. Then, all the processed samples were thoroughly snap-frozen by immersion in liquid nitrogen and buried in dry ice within 5-10 minutes of exsanguination.

2. CARCASS FABRICATION

Yorkshires were harvested at the Auburn University Lambert- Powell Meats Lab under USDA-FSIS inspection. Hot carcass weight was recorded after exsanguination and carcasses were chilled at 2 degrees C for 24 hours, at which point, carcasses were weighed again. At both 1 h and 24 h postmortem, carcass pH was recorded in the round on left side using a pH Spear probe) Oakton Instruments, Vernon Hills, Il). At the 24 h period, carcasses were split between the 12th and the 13th ribs for evaluation of back fat (BF); loin eye area (REA); and marbling. Longissimus muscles were also evaluated for objective color measurements at the 12th and 13th rib interface using a Hunter Miniscan XE Plus (Hunter Lab, Reston, Virginia) for Hunter L*, a*, and b* values. The Miniscan was calibrated according to manufacturer's recommendations and utilized a D 65 light source, a 10 degree viewing angle, and a 35mm viewing area.

3. GENE EXPRESSION ANALYSIS

Total RNA for both the pre and post scalding samples was extracted from adipose tissue using a two-step purification protocol with total RNA first being extracted from whole tissue using RNAzol® RT (MRC, Inc, Cincinnati, OH) followed by purification using RNAeasy spin columns (QIAGEN, Inc., Valencia, CA) according to the manufacturers' recommendations. This second isolation procedure improves the purity (much lower protein and contaminants) of the isolated RNA. RNA was quantified using a BioTek Synergy 4 plate reader utilizing the Take3

system (BioTek U.S., Winooski, VT) with all samples exhibiting an OD 260/280 between 1.8 and 2.0 and an OD 260/230 value between 1.8 and 2.2. Spectral scans ranging from 200 to 400 nm further verified sample purity as all RNA samples produced smooth curves exhibiting one peak at 260 nm. Total RNA integrity was accessed both visually by resolving 2 μg of RNA on a denaturing agarose-formaldehyde gel containing ethidium bromide and by determining an RNA Integrity Number (RIN) using an Agilent 2100 bioanalyzer (Agilent Technologies, Inc., Clara, CA). All samples demonstrated sharp ribosomal bands with a 28S to 18S ratio greater than one and RIN values greater than 7.0 and were thus judged intact and non-degraded. Total RNA was then reverse transcribed using Superscript II reverse transcriptase (Promega Inc, Madison, WI) and oligo-dT primers. Real-time PCR was performed on the resultant cDNA using a Roche Lightcycler® 480 Real-time PCR machine and LightCycler® 480 SYBR Green I Master Mix (Roche Applied Science, Indianapolis, IN) according to manufacturer's recommendations. All PCR reactions were performed using intron-spanning primers under optimized conditions with primer efficiencies ranging between 90-101 % as verified with standard curves. In the *longissimus dorsi* muscle, four genes were chosen to examine their levels of expression. Myogenin was one of the key markers as it is specific to muscle tissue and it also regulates myogenesis. Creatine Kinase was measured as it is an enzyme that catalyzes the phosphorylation of creatine utilizing of ATP. Glut 4 was examined in both the adipose tissue and the longissimus dorsi tissue as it is found in both tissues and is a glucose transport protein. Troponin which is a muscle (regulatory) protein involved with muscle contractions was the last of the four marker genes. Four genes were also utilized to measure expression in subcutaneous adipose tissue. Adiponectin was chosen as it modulates a variety of metabolic processes and is secreted

primarily from adipose tissue. Leptin was chosen as one of the key marker genes expressed in adipose tissue, stored in fat cells and is directly associated with sending signals to the brain associated with hunger and feed intake. (Spurlock et al., 2006). PPARy2 and Glut 4 were genes of interest because they both are developmental markers for preadipocyte differentiation and a reflection of active lipogenesis. (Fernyhough et al., 2007). All PCR reactions were performed using intron-spanning primers for porcine adiponectin (forward: 5'-TCTCGGCCAGGAAACCACCGA-3'; reverse: 5'-CGGCCTGGGGTACCGTTGTG-3'), porcine creatine kinase (forward: 5'-GCTCCAAGACACTCATCCAC-3'; reverse: 5'-TTGCGTAGATCAGGGTAGTC-3'), GLUT4 (forward: 5'-ATCCTGATGACTGTGGCTCTGCTT-3'; reverse: 5'-AGAAGGCCACAAAGCCAAAGATGG-3'), porcine leptin (forward: 5'-ACGATTGTCACCAGGATCAG-3'; reverse: 5'-ACAAACTCAGGACAGGATGG-3'), myogenin (forward: 5'-GTAAGAGGAAGTCCGTGTCTG-3'; reverse: 5'-TGTGGGAACTGCATTCACTG-3'), PPARγ (forward: 5'-AATTAGATGACAGCGACCTGGCGA-3'; reverse: 5'-TGTCTTGAATGTCCTCGATGGGCT-3'), porcine S15 (forward: 5'-GGTAGGTGTCTACAATGGCAAGG-3'; reverse: 5'-GGCCGGCCATGCTTC-3'), and troponin (forward: 5'-GAGATGATCGCTGAGTTCAAGG-3'; reverse: 5'-GATGTAGCCGTCCATGTTCC-3') under optimized conditions. Primer efficiencies were calculated using standard curves and were between 93% and 100% for all reactions. Product purity was assessed by melting curve analysis and expected amplicon sizes were verified on a 2 % agarose gel stained with ethidium bromide. Values were normalized to S 15 mRNA

expression. The *S15* mRNA levels represent an appropriate control as the efficiency of the *S15* primers was 100% and *S15* mRNA expression was not different between any groups tested (*P* >.05). Data are expressed as fold change relative to baseline and calculated according to Tichopad et al. (2010).

4. STATISTICAL ANALYSIS

Gene expression was measured in both the pre and post scald samples via polymerase Chain Reaction (PCR) to determine whether or not time of scalding had any effect on the amount of gene expressed in both subcutaneous adipose tissue and longissimus muscle in pre and post samples. Changes in gene expression were calculated from the cycle threshold, after correction using S15 expression and analyzed using the Pair Wise Fixed Reallocation Randomization Test of REST-MCS v2.0 (http://rest.gene-quantification.info/). Carcass traits and RNA quality data were analyzed as a completely randomized block design using a mixed linear model of SAS v9.2 with individual animal serving as the experimental unit (individual block). (SAS Institute, Inc., Cary, NC).

RESULTS

1. RNA PURITY.

The sensitivity of RNA-based assays such as real-time PCR is limited by RNA quality which is a function of both RNA purity and RNA integrity. In the present study RNA quality was assessed by spectrophotometry. The 260/280 nm absorbance ratios for samples harvested from subcutaneous adipose tissue, pre-scald and post-scald, respectively, are shown in Table 1. Similarly in Table 2, the absorbance ratios in *longissimus dorsi* muscle tissue are presented. Across all samples, the 260/280 nm ratios fell between 1.8 and 2.25 indicating low contaminations by other molecules. As expected 260/280 values not statistically significant different between pre-scald and post-scald total RNA samples extracted from either subcutaneous adipose or the *longissimus dorsi* muscle tissue respectively. Consistent with these results, spectral scans encompassing absorbance between 200-400nm were indicative of pure RNA samples in both tissues. Representative spectral scans for water and total RNA are indicated in Figure 1 for pre- and post-scald samples from adipose tissue. All samples displayed very similar profiles and were judged to be pure as evidenced by the presence of a smooth curve with a single peak at 260 nm exhibiting an absorbance roughly twice that associated with the trough (230 nm). These measures taken together support the conclusion that total RNA samples exhibited a high degree of purity regardless of sampling (considering the primary and secondary RNA isolation steps).

2. RNA INTEGRITY.

Non-degraded RNA that reflects the native transcript size and expression profile is described as having high RNA integrity. This parameter was first assessed by visualizing the 28s and 18s rRNA bands using denaturing agarose gel electrophoresis. The resulting gels depicting all total RNA samples isolated from subcutaneous adipose and *longissimus dorsi* muscle in the study are shown in Figure 2 and Figure 3 respectively. All total RNA samples were judged to be intact based upon the presence of sharp rRNA bands with 28s rRNA being roughly twice as prominent as 18s rRNA in conjunction with the absence of smearing.

Next, RNA integrity was assessed using capillary electrophoresis to determine the RNA integrity number (RIN) using matrices derived from area under the curve analysis of electropherograms to predict structural integrity ribosomal RNA (28s, 18s, 5s) and messenger RNA of various sizes (I to 100 kb). Previous work has shown that sample collection conditions may affect the integrity of isolated RNA (Bustin et al., 2009). Assessment of adipose tissue RNA integrity as judged by electropherograms of total RNA isolated subcutaneous adipose tissue harvested from the tenth rib of the same animal prior to scalding is depicted in Figure 4. Samples from adipose tissue are shown as it is often more difficult to extract RNA from this tissue. The quality of the displayed samples is representative of samples from muscle (see appendices). Results for RIN assessment of pre-scald and post-scald RNA samples from subcutaneous adipose tissue and *longissimus dorsi* muscle respectively are presented in Tables 3 and 4. The RIN values were not significantly different between RNA samples extracted from tissue harvested from the same animal either prior to or immediately after scalding in any tissue examined. Furthermore RIN were in the 8.9 to 9.7 range, values indicating high structural integrity- quality RNA for downstream applications such as gene expression studies. Collectively, visualization by

denaturing gel electrophoresis and assessment of RIN by capillary electrophoresis indicate that scalding did not impact RNA integrity.

3. EFFECT OF SCALDING ON GENE EXPRESSION AS MEASURED BY REAL-TIME PCR.

A first objective was to determine of scalding impacted RNA quality of samples extracted from subcutaneous and longissimus muscle tissue. Results of RNA integrity analysis indicates that scalding does not impact RNA integrity. However, that does not preclude an impact of scalding upon transcript expression as sampling post-scalding could conceivably affect transcript levels independent of effects upon RNA quality. This second approach was to determine expression of key marker genes from subcutaneous adipose tissue and *longissimus dorsi* muscle using the RNA-based assay, real-time PCR. Figure 5 shows the relative abundances of Adiponectin, Leptin, PPAR gamma2 and Glut 4 mRNA in pre- scald and post-scald adipose tissue, respectively. As the gene expression results depict, there were no statistically significant differences in the expression of the genes tested between the pre- and post-scald adipose tissue samples.

The relative mRNA abundance (expression) values of muscle genes Creatine Kinase, Myogenin, Troponin and Glut 4 are presented for both prescald and postscald samples in Figure 6. For longissimus dorsi muscles no statistical differences were found in gene expression values between sampling groups.

4. MEASURES OF PORK QUALITY.

Lastly, the impact of sampling tissue prior to scalding or immediately following scalding was evaluated on important pork quality attributes such as ultimate pH and color. These results are displayed in Table 5. As expected, there were no differences in hot carcass weight or marbling score indicating pigs in both sampling groups were evaluated at similar physiological and compositional maturities. Interestingly, there was no difference between ultimate pH between pigs that had samples harvested prior to scalding and the no sample controls. However, sampling prior to scalding did result in darker color of longissimus dorsi muscle as judged both visually ($P < 0.0001$) and by Minolta Hunter color scores ($P < 0.0001$). The effects of sampling prior to scalding upon color was sufficient to change the assessment of chop color from "pale" which is generally considered suboptimal to "medium" color which is considered ideal. These results suggest that though ultimate pH was not different between sampling groups, sampling a plug of subcutaneous adipose and loin muscle prior to scalding could alter the assessment of pork color and thus potentially confound pork quality studies.

DISCUSSION

For this study, the working hypothesis was that poorer quality, more degraded RNA would be isolated from the post scald tissue samples. Overall results showed that when rigorous protocols for tissue harvest and RNA isolation were followed, RNA purity, RIN and capillary electropherograms did not differ between pre and post scold tissue samples. In addition, expression of four marker genes each for muscle and subcutaneous adipose tissues obtained using real time qRT-PCR resulted in practically identical mRNA abundance (gene expression) values for all the marker genes tested. In the design of this study, tissue samples were obtained pre and post scalding from the same pigs within the time required for completion of scalding. Collectively these results provide no evidence that pre vs post scalding tissue sampling had differential effects on RNA and our hypothesis must be rejected.

Degradation of ribosomal and messenger RNA has always been a concern in mRNA abundance studies. In the Northern blot system (Sambrook et al, 1989) purity of RNA preparations were assessed with 260/280 nm (UV wavelength) using a spectrometer. The size of mRNA (expressed as kilobases or kb) was determined by length of travel and location of the mRNA during denaturing electrophoresis (using marker-kb standards) as evaluated by specific binding (Watson-Crick) of a radiolabelled cDNA matching probe. The location of the blot would verify the mRNA size or whether mRNA was degraded. A poor Northern blot would show probe binding with smaller mRNA pieces and such a Northern would be rejected. Since then methods for assessment of degradation have been developed (240-400 nm spectrophotometric

scans and capillary, denaturing electrophoresis; Agilent Bioanalyzer) and using these newer tools in this work confirmed the high quality of the isolated RNA.

Based on the evidence obtained, I conclude that harvesting tissue samples after a scalding step will not lower the quality of RNA for downstream gene expression and micro-array analysis. Other laboratories likely should try to confirm these results (this will be somewhat costly if carcasses cannot proceed into the human food chain) with their procedures; however we feel confident that post-scalding tissue samplings in pigs can be profitably utilized to combine growth studies, carcass analyses, human food production and study of molecular regulation mechanisms in protein and lipid metabolism.

Carcass merit is an important determinant of profitability in the pork industry because seventy five percent of hogs marketed by packers are sold on a carcass merit basis. The issue of tissue sampling during the slaughter procedure before scalding may have a negative effect on carcass quality. Thus with agricultural large animal research generally being underfunded, often times budget constraints make it necessary to keep animal used as low as possible in trials; however trials should be designed with an appropriate power analysis to know exactly how many degrees of freedom are needed for a satisfactory statistical analysis.

Due to food safety concerns if tissue samples must be obtained pre-scalding, then often such carcasses cannot enter the human food stream. This could result in substantial additional research costs. Thus animal science researchers at land grant agricultural universities as Auburn University are sometimes forced to use the same carcass for both tissue sampling for later molecular endpoint measures, meat quality endpoints and consequent sale for human

consumption. This posed a constant problem with obtaining adequate samples and protecting the carcass from untoward contamination effects, but the present research indicates that a single, post scald tissue sample can be used to obtain high quality RNA.

In this study, the effect of sampling time upon ultimate pH, visual color and Minolta color was assessed. Despite ultimate pH being the same in carcasses that had been sampled prior to scalding and non-sampled controls, both subjective and objective color scores were shifted toward darker profiles. The effect of only harvesting tissue samples after scalding has not yet been done. Based on data in this thesis, we may suggest that sampling prior to scalding may alter the assessment of pork color and potentially confound the ability to grade carcasses. In this case, the difference in color between the sampled and non-sampled groups was significant enough to shift the assessment of color from pale (less desirable) to medium (optimal) which would be interpreted as a significant improvement in meat quality. Thus sampling adipose and muscle at the tenth rib prior to scalding may confound the ability to study meat quality parameters on the same carcass. Given this and the fact that sampling immediately following scalding did not negatively impact RNA quality or the results of gene expression studies, we conclude that if both RNA-based molecular endpoints and meat quality endpoints are to be measured from the same carcass, tissue sampling to facilitate RNA extraction and molecular endpoints should occur post scalding. Furthermore, sampling soft tissues from the carcass prior to scalding poses food safety issues. Such issues can be completely avoided by sampling immediately following scalding. This work should form the basis for better standard operating procedures in land grant university abattoirs that must facilitate researchers in ways that

maximize data quality and food safety while so that cost recovery can be utilized and ultimate animal numbers reduced to minimize the cost of large animal research.

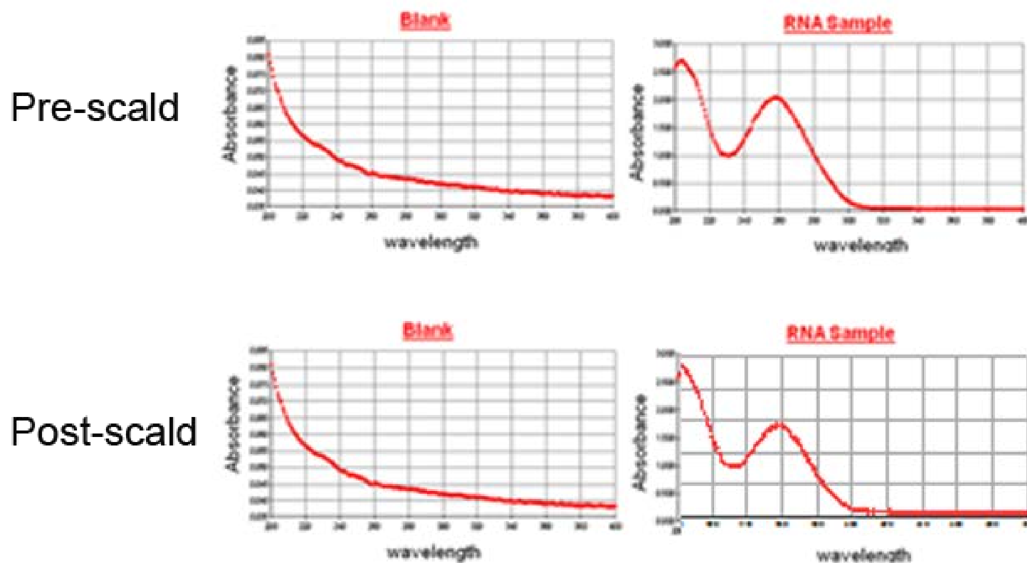


Figure 1. Assessment of RNA purity relative to water blanks from a sample harvested prior to scalding (*Top Panel*) and a sample taken from the same animal immediately following scalding (*Bottom Panel*) as judged by spectral scans encompassing absorbance readings between 200-400 nm. Samples from adipose tissue are shown as it is often more difficult to extract pure RNA from this tissue. Samples were judged to be pure as evidenced by the presence of a smooth curve with a single peak at 260 nm exhibiting an absorbance roughly twice that associated with the trough (230 nm). Quality of the depicted samples is representative of all samples from adipose tissue and muscle.

Table 1. Ratio of 260/280 absorbance by sampling group in total RNA samples extracted from subcutaneous adipose tissue

Animal	Prescald	Postscald
0609	**2.15**	**2.15**
0706	**2.16**	**2.18**
0904	**2.17**	**2.19**
0909	**2.16**	**2.19**
0911	**2.18**	**2.17**
1103	**2.15**	**2.14**
1104	**NA**[1]	**2.06**
1206	**2.19**	**2.19**
Mean[2]	**2.17 ± 0.01**[a]	**2.16 ± 0.01**[a]
***P*-value**		**0.69**

[1]Not available, sample was lost during extraction

[2]Values are means ± SEM; group means within a row with different superscripts differ (P < 0.05), n=8.

Table 2. Ratio of 260/280 absorbance by sampling group in total RNA samples extracted from *longissiumus dorsi* muscle tissue

Animal	Prescald	Postscald
0609	**2.23**	**2.18**
0706	**2.18**	**2.21**
0904	**2.20**	**2.23**
0909	**2.21**	**2.21**
0911	**2.21**	**2.19**
1103	**2.21**	**2.21**
1104	**2.20**	**2.22**
1206	**2.20**	**2.19**
Mean[1]	**2.21 ± 0.01**[a]	**2.21 ± 0.01**[a]
***P*-value**		**0.60**

[1]Values are means ± SEM; group means within a row with different superscripts differ (P < 0.05), n=8.

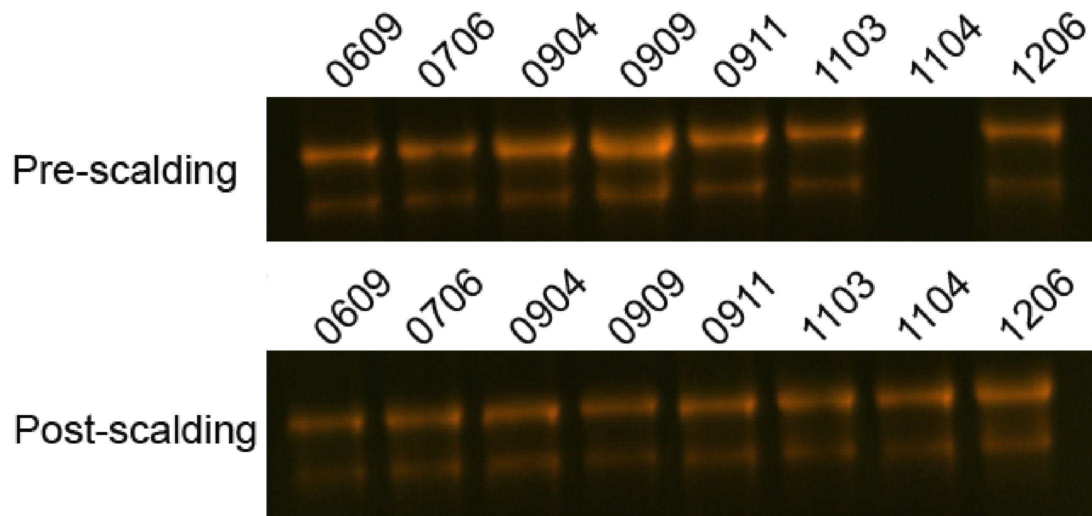


Figure 2. Assessment of adipose tissue RNA quality as judged by visualization of 28S and 18S bands on a denaturing agarose gel. Total RNA extracted from subcutaneous adipose tissue samples harvested prior to scalding (*Top Panel*) and following scalding (*Bottom Panel*) are depicted. Sample 1104 was lost during extraction from the pre-scalding cohort. Total RNA was judged to be intact based upon the presence of sharp rRNA bands with 28s rRNA being roughly twice as prominent as 18s rRNA in conjunction with the absence of smearing. Samples were harvested by cutting through the hide at the tenth rib and dissecting a 4 x 4 inch plug containing subcutaneous adipose and underlying longissimus dorsi tissue. Tissue was rapidly processed and snap-frozen in liquid nitrogen and subsequently stored at -80° C.

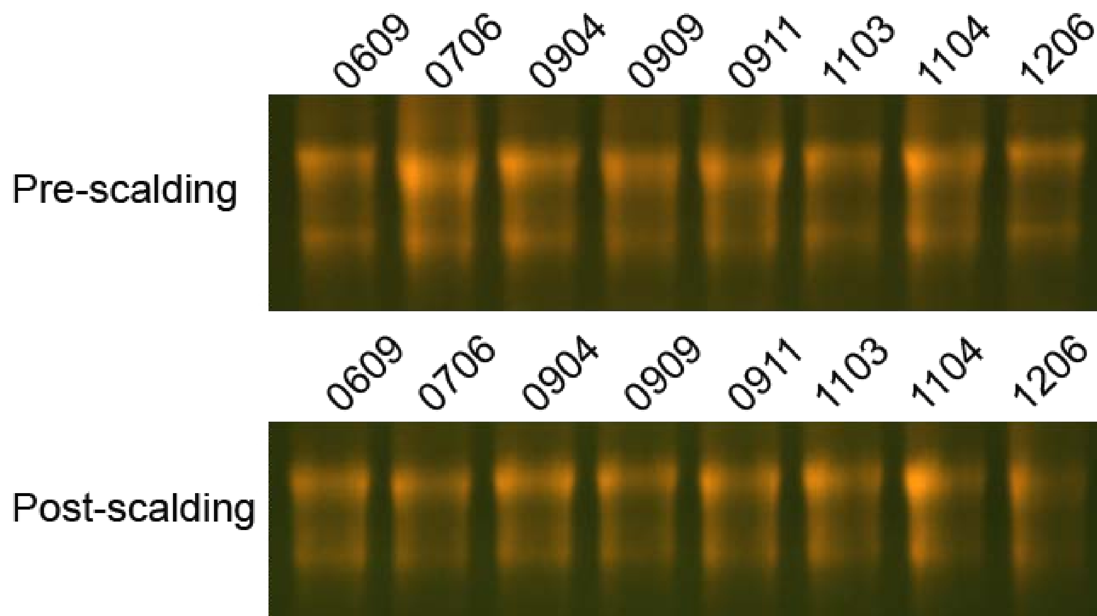
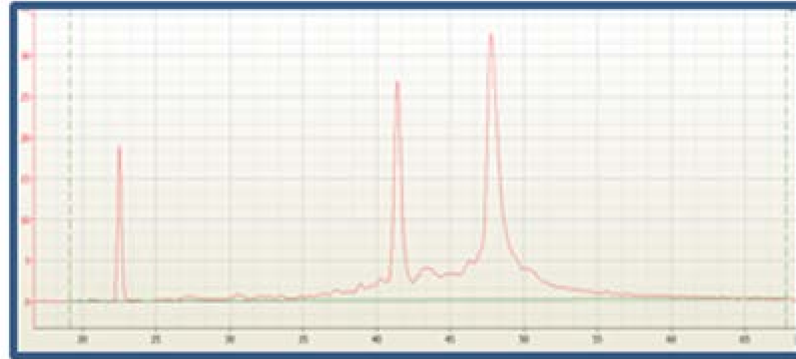


Figure 3. Assessment of muscle tissue RNA quality as judged by visualization of 28S and 18S bands on a denaturing agarose gel. Total RNA extracted from longissimus dorsi tissue samples harvested prior to scalding (*Top Panel*) and following scalding (*Bottom Panel*) are depicted. Total RNA was judged to be intact based upon the presence of sharp rRNA bands with 28s rRNA being roughly twice as prominent as 18s rRNA in conjunction with the absence of smearing. Samples were harvested by cutting through the hide at the tenth rib and dissecting a 4 x 4 inch plug containing subcutaneous adipose and underlying longissimus dorsi tissue. Tissue was rapidly processed and snap-frozen in liquid nitrogen and subsequently stored at -80° C.

Pre-scald



Post-scald

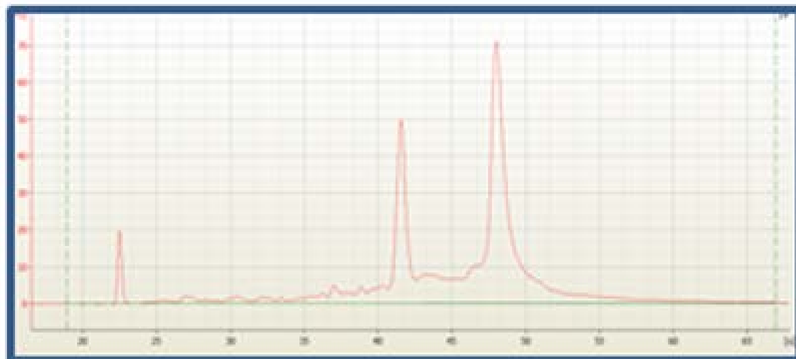


Figure 4. Assessment of adipose tissue RNA integrity as judged by electropherograms of total RNA isolated subcutaneous adipose tissue harvested from the tenth rib of the same animal prior to scalding (*Top Panel*) and immediately following scalding (*Bottom Panel*). Samples from adipose tissue are shown as it is often more difficult to extract RNA from this tissue. Quality of the depicted samples is representative of samples from muscle.

Table 3. RNA integrity number by sampling group in total RNA samples extracted from subcutaneous adipose tissue

Animal	Prescald	Postscald
0609	**9.4**	**9.2**
0706	**9.4**	**9.7**
0904	**9.6**	**9.3**
0909	**7.9**	**9.5**
0911	**8.4**	**9.5**
1103	**9.3**	**9.4**
1104	**NA**[2]	**9.5**
1206	**9.4**	**9.5**
Mean[1]	**9.1 ± 0.2**[a]	**9.5 ± 0.2**[a]
***P*-value**		**0.14**

[1]Values are means ± SEM; group means within a row with different superscripts differ (P < 0.05), n=8.
[2]Not available, sample was lost during extraction

Table 4. RNA integrity number by sampling group in total RNA samples extracted from *longissiumus dorsi* muscle tissue

Animal	Prescald	Postscald
0609	**9.1**	**8.6**
0706	**8.8**	**9.3**
0904	**8.6**	**9.8**
0909	**9.0**	**9.0**
0911	**8.6**	**9.3**
1103	**9.2**	**9.2**
1104	**8.9**	**9.0**
1206	**8.7**	**8.7**
Mean[1]	**8.9 ± 0.11**[a]	**9.1 ± 0.11**[a]
***P*-value**		**0.13**

[1]Values are means ± SEM; group means within a row with different superscripts differ (P < 0.05), n=8.

Table 5. Meat quality traits by sampling group[1]

Variable	No Sample Control	Prescald Sampling	*P*-value
Number of pigs	**8**	**8**	**NA[2]**
Hot carcass wt, kg	**78.5 ± 2.6**	**74.8 ± 2.6**	**0.33**
Ultimate pH[3]	**5.83 ± 0.05**	**5.89 ± 0.05**	**0.41**
Color[4]	**1.69 ± 0.16**	**3.09 ± 0.16**	**0.0001**
Marbling score[5]	**1.19 ± 0.09**	**1.03 ± 0.09**	**0.24**
L^*, lightness	**66.0 ± 0.8**	**55.4 ± 0.8**	**0.001**
a^*, redness	**10.4 ± 0.2**	**10.3 ± 0.2**	**0.53**
b^*, yellowness	**18.8 ± 0.2**	**16.1 ± 0.2**	**0.0001**

[1]Values are means ± SEM
[2] NA=not applicable;
[3]Ulimate pH: measured 24 h post-harvest on chilled carcasses
[4] Visual (subjective) color score: five point scale where 1= very light and pale; 5= dark red etc.
[5] Subjective Marbling Score: 1 to 2.4= Devoid; 2.5 to 4= Traces; 4 to 5 = Slight;

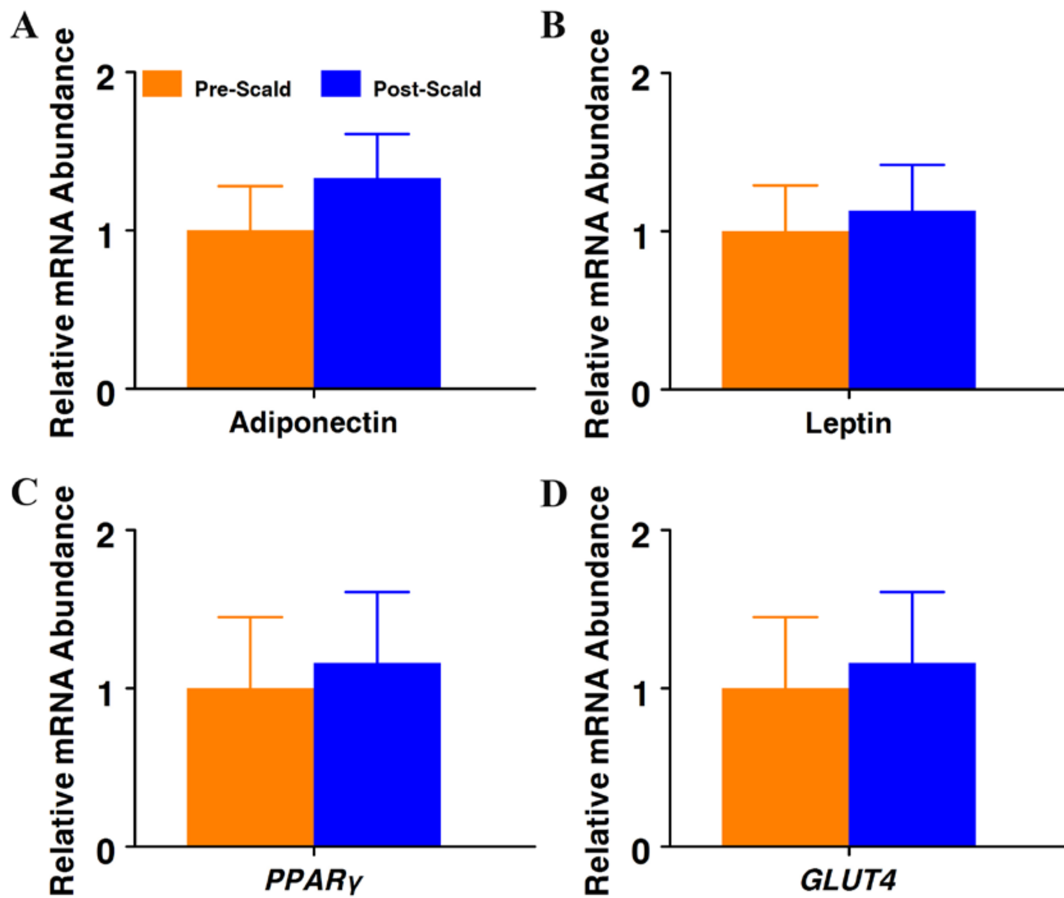


Figure 5. Messenger RNA abundance (expression) of adipose tissue marker genes, (A) adiponectin, (B) leptin, (C) peroxisome proliferator-activated receptor gamma (*PPAR*□), and (D) glucose transporter 4 (*GLUT4*) in the subcutaneous adipose tissue of market weight hogs as measured by Real-time qRT- PCR. Data were normalized to S15 mRNA abundance and are expressed as fold change relative to Pre-scald samples and calculated according to Pfaffl (2010). Bars denoted by * differ ($P < 0.05$), Pre-Scald, n=8, Post-Scald, n=8.

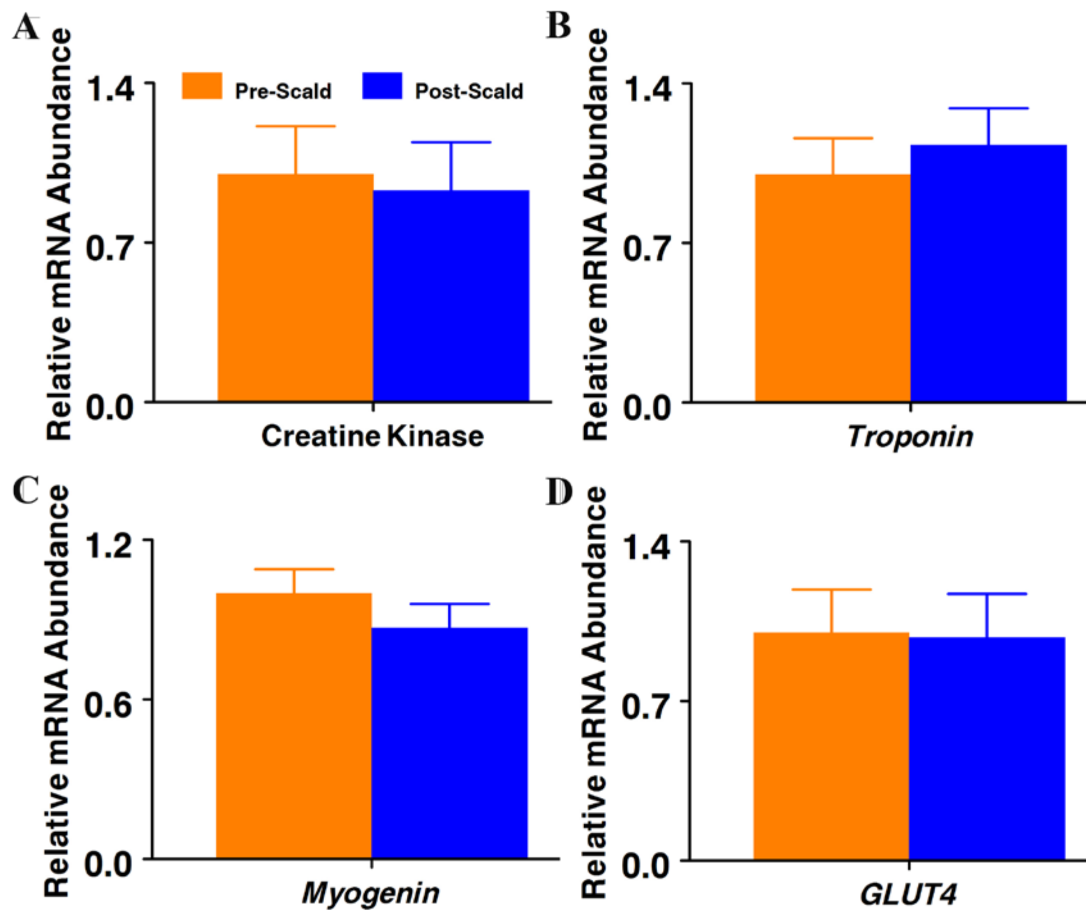


Figure 6. The mRNA expression of muscle tissue marker genes, (A) creatine kinase, (B) troponin, (C) myogenin, and (D) glucose transporter 4 (*GLUT4*) in the *longissimus dorsi* muscle of market weight hogs as measured by Real-time qRT- PCR. Data are normalized to S15 mRNA abundance and expressed as fold change relative to Pre-scald samples and calculated according to Pfaffl (2010). Bars denoted by * differ ($P < 0.05$), Pre-Scald, n=8, Post-Scald, n=8.

REFERENCES

A- Ouali, C. Hernan, H, Mendez, G. Coulis et al. Revisiting the conversion of muscle into meat and underlying mechanisms. Meat Science (2006)74:44-58

Bustin, SA. Absolute quanitification of mRNA using real-time reverse transcription polymerase chain reaction assays.Journal of Molecular Endocrinology (2000) 25: 169-19325

Bustin, SA, V. Benes, J. Garson, J Hellemans et al. The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. Clinical Chemistry (2009) 4:611-62255

Bustin SA, V Benes, T Nolan, and MW Pfaffl. Quantitative real-time RT-PCR-a perspective Journal of Molecular Endocrinology (2005) 34:597-601

Bustin SA and R Mueller. Real-time reverse transcription PCR (qRT-PCR) and its potential use in clinical diagnosis. Clinical Science (2005)109:365-379

Bustin, SA, and T Nolan. Pitfalls of Quantitative Real-TIme Reverse-Transcription Polymerase Chain Reaction. Journal of Bioimolecular Techniques. (2004) 15:155-166

England, ME, TL Scheffler, SC Kasten, SK Materneh and DE Gerrard. Exploring the unknowns involoved in the transformation of muscle to meat. Meat Science (2013) 837-84395

Faustman C, Q Sun, R Mancini and SP Suman. Myoglobin and lipid oxidation interactions: mechanistic bases and control. Meat Sci. (2010) 86:86-94. Review

Faustman, C., and R G Cassens. 1990. The biochemical basis for discoloration in fresh meat: A review. J. Muscle Foods 1:217–243.

.Fernyhough, ME, E. Okine, G. Hausman, G L Vierck and MV Dodson. PPARg and Glut 4 expression as developmental regulators/markers for preadipocyte differentiation into an adipocyte.Domestic Animal Endocrynology (2014) 33:367-378

Fernandez X, G Monin, A Talmant, J Mourot and B Lebret. Influence of intramuscular fat content on the quality of pig meat - 2. Consumer acceptability of m. longissimus lumborum. Meat Sci. (1999).1:67-72.

Fleige S and MW Pfaffl. RNA integrity and the effect on the real-time qRT-PCR performance. (2006) 19:1601-13

Fleige S, V Walf, S Huch, C Progmet, J Sehm and MW Pfaffl. Comparison of relative mRNA quantification models and the impact of RNA integrity in quantitative real-time RT-PCR Biotechnol Lett.(2006) 28:1601-1613.

Glasel, JA. Validity of nucleic acid monitored by 260/280nm absorbance ratios. Biotechniques (1995) 18:208-209.

Hugget J, K Dheda, S Bustin and A Zumla. Real-time PCR normalisation; strategies and considerations. Genes and Immunity (2005) 6:279-284.

Jacobi SK, NK Gabler, KM Ajuwon, JE Davis and ME Spurlock. Adipocytes, myofibers, and cytokine biology: new horizons in the regulation of growth and body composition. (2006). Journal of Animal Science 84:supplE140-149.

Lawrie's Meat Science, RA Lawrie. 6th Edition. (2002) Woodhead Publishers Ltd, Cambridge. UK.

Lonergan, SM and E Huff-Lonergan. Mechanisms of water-holding capacity of meat: The role of postmortem biochemical and structural changes.Meat Science (2005)71:194-204.

Manchester KL. Exploring the gene with X-rays. Trends Genet. (1996) 12:515-518.

Murphy J, and SA Bustin. Reliability of real-time reverse-transcription PCR in clinical diagnostics: gold standard or substandard? Expert Review Molecular Diagnosis (2009)2:187-1979.

Nolan T, Hands RE, Bustin SA. Quantification of mRNA using real-time RT-PCR. Nat Protoc. 2006;1:1559-1582.

Oksberg N, PM Nissen, M Therkildsen, HS Moller, LBLarsen, M Andersen and JF Young. Meat Science and Muscle Biology Symposium: In utero nutrition related to fetal development, postnatal performance, and meat quality of pork. Journal of Animal Science. (2013) 91: 1443-1453.

Principles of Meat Science.4th Edition.(2010) Aberle ED, Forrest JC, DE Gerrard and SE Mills. Published by Kendall Hunt Pub Co, pg 83-98.

Sambrook J, EF Fritsch and T. Maniatis. (2000). Molecular Cloning: A Laboratory Manual (Second Edition)

Stahlberg A, J Hakansson, X Xian, H Semb. Properties of the Reverse Transciption Reaction in mRNA Quantification. Clinical Chemistry (2004). 50:3 509-515.

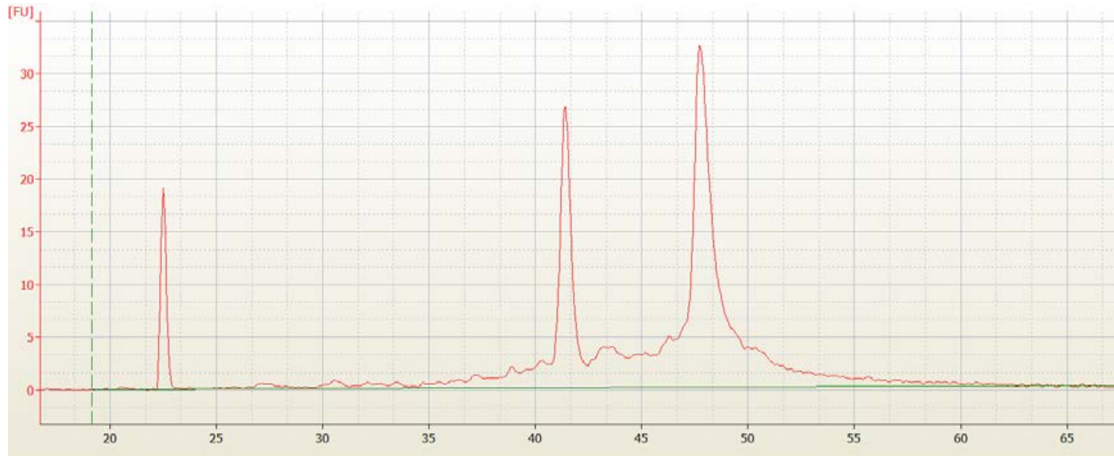
Tichopad A[1], Bar T, Pecen L, Kitchen RR, Kubista M, Pfaffl MW. Quality control for quantitative PCR based on amplification compatibility test (2010) Methods 50: 308–312

Warburg O and W. Christian. Isolation and crystallisation of enolase. Biochem. Z. 310: 384-421

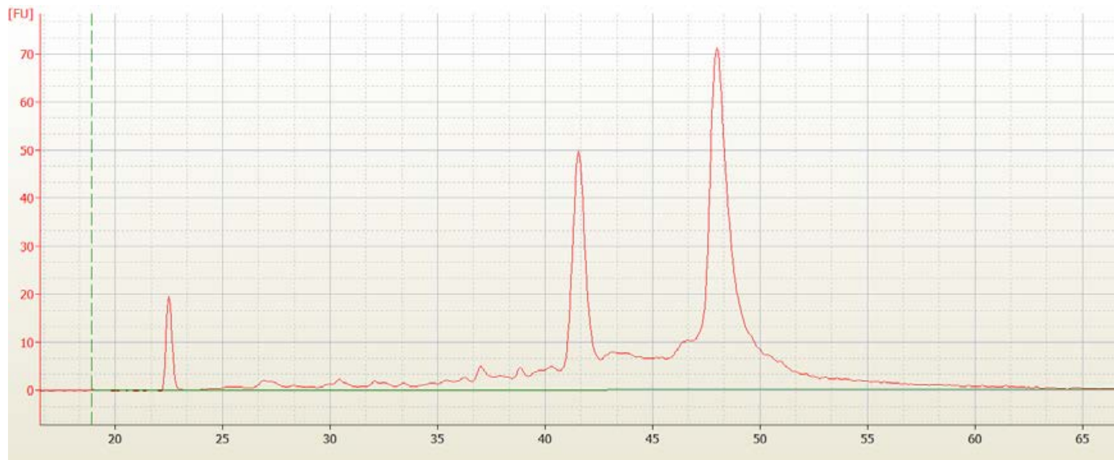
Appendix A

Capillary electrophoresis of isolated total RNA samples from the subcutaneous adipose tissue of Yorkshires harvested above the tenth rib either prior to scalding (pre-scald) or immediately following scalding (post-scald) was conducted to asses RNA integrity numbers for each sample. Assessment of adipose tissue RNA integrity as judged by electropherograms.

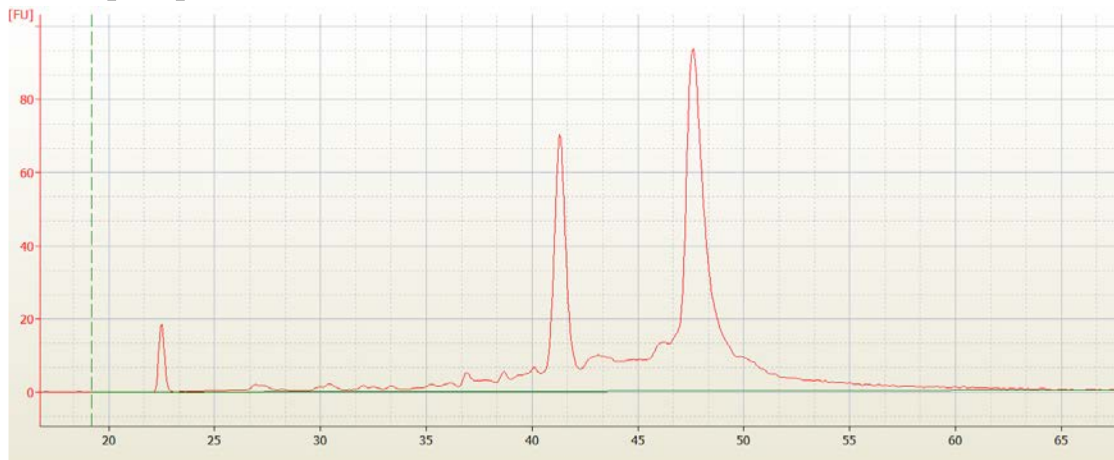
609 adipose pre-scald



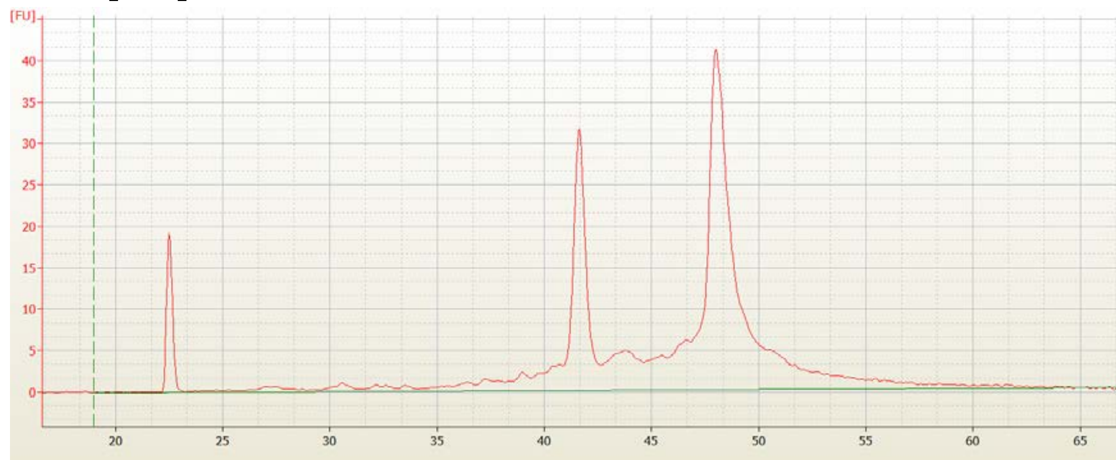
609 adipose post-scald



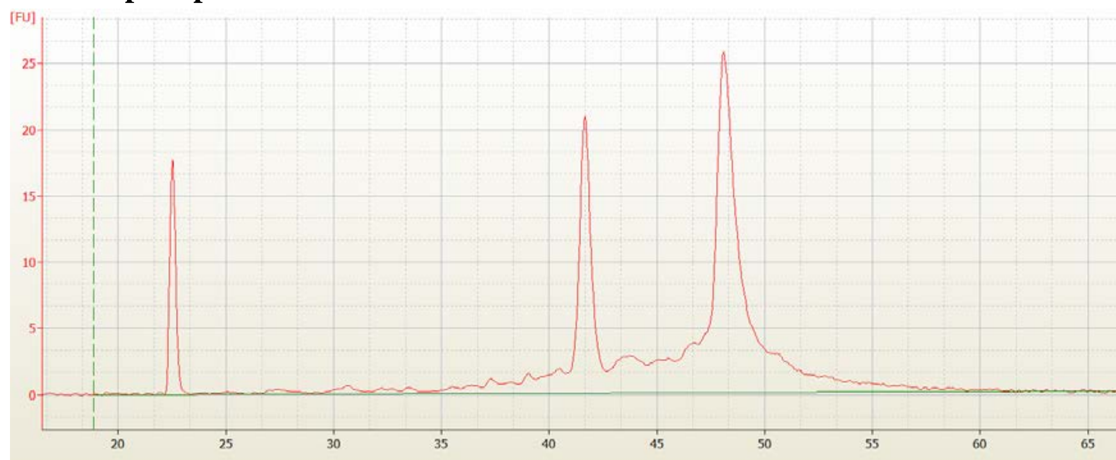
706 adipose pre-scald



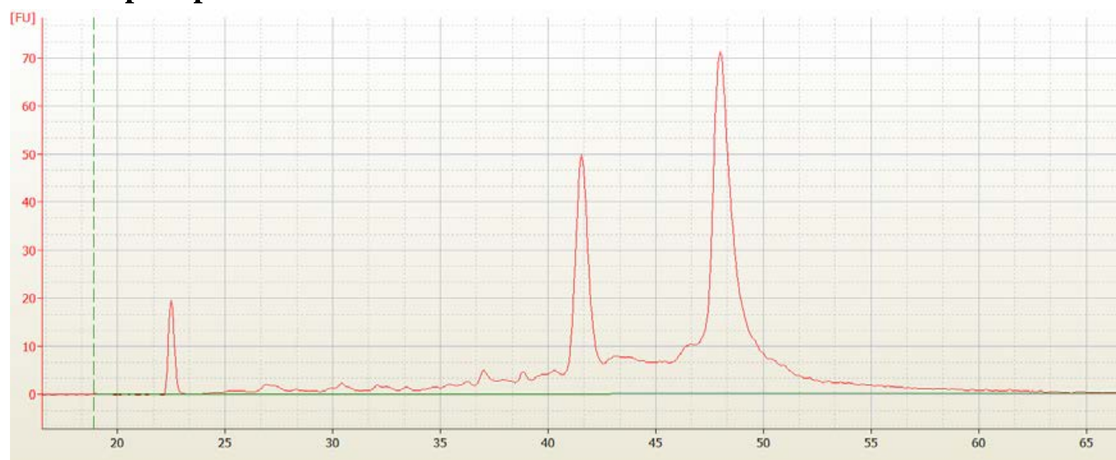
706 adipose post-scald



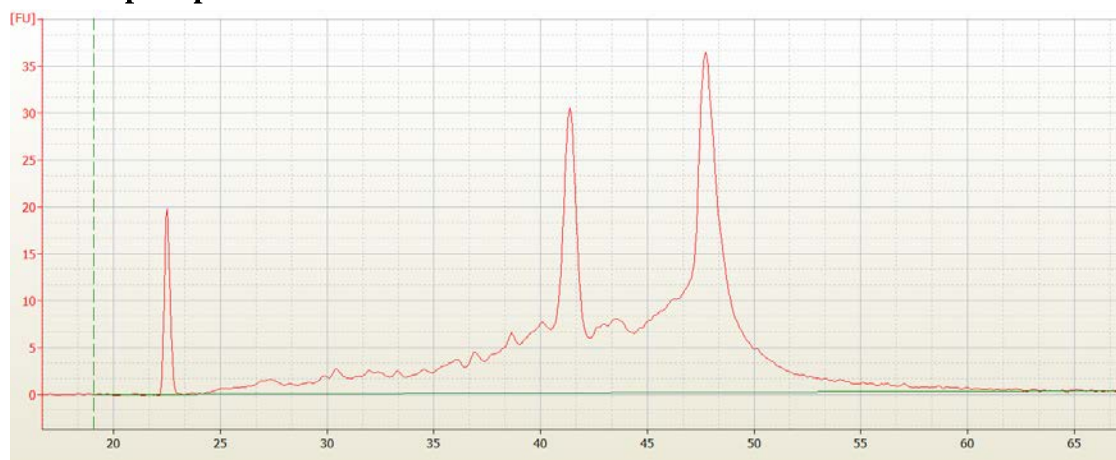
0904 adipose pre-scald



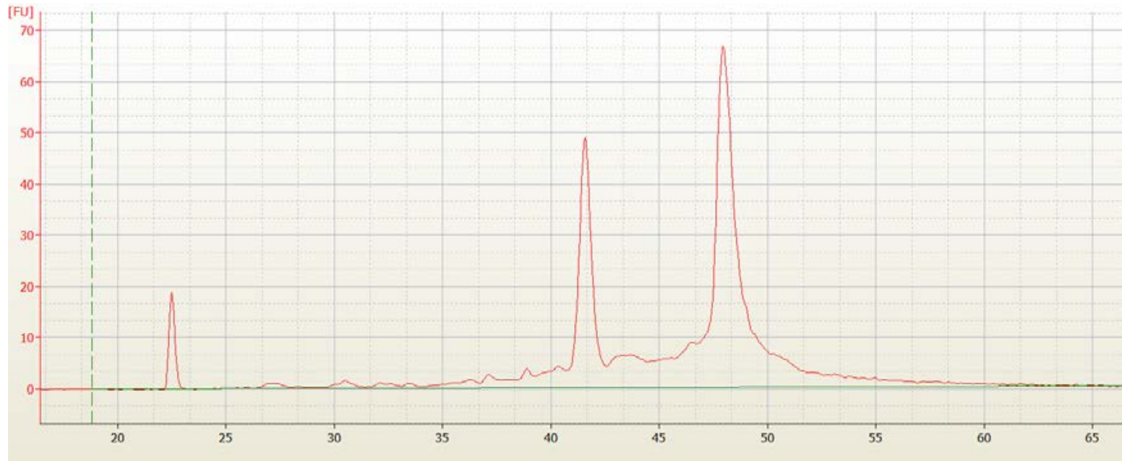
0904 adipose post-scald



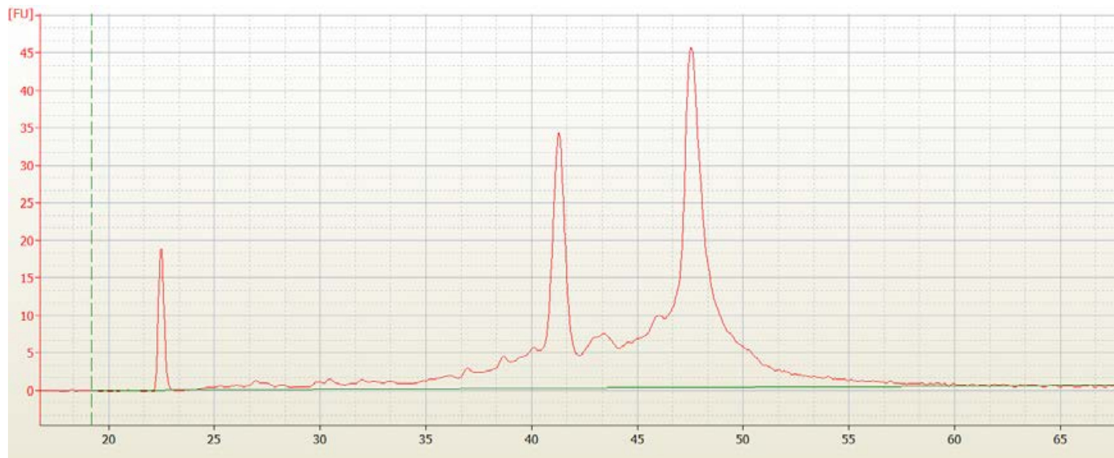
0909 adipose pre-scald



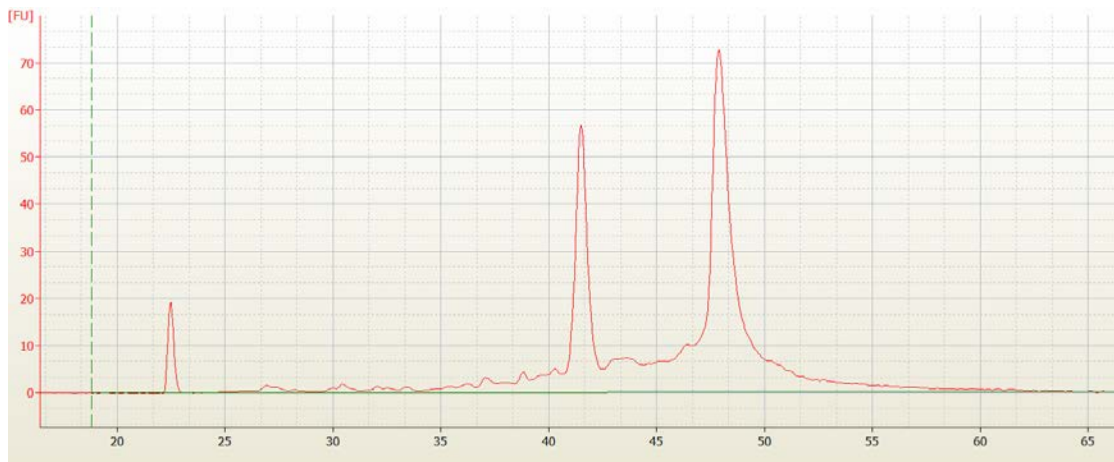
0909 adipose post-scald



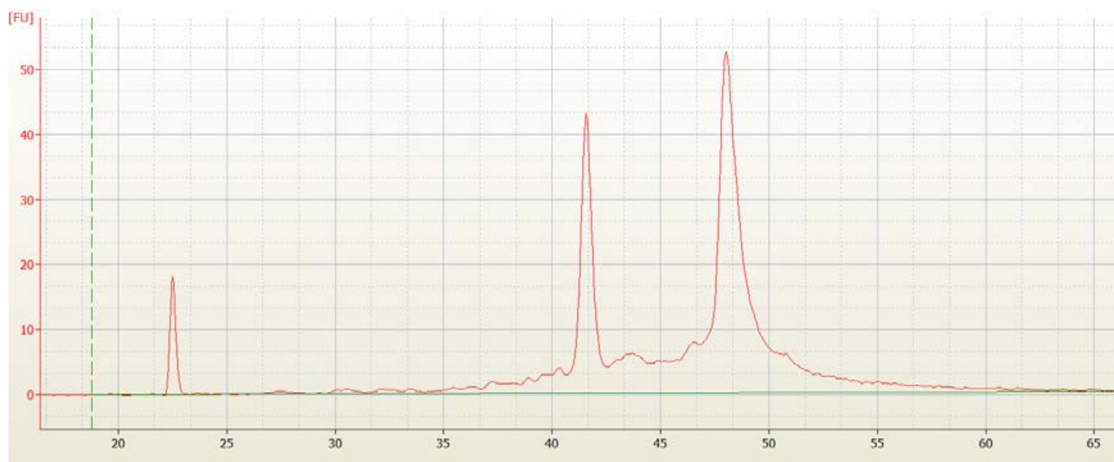
0911 adipose pre-scald



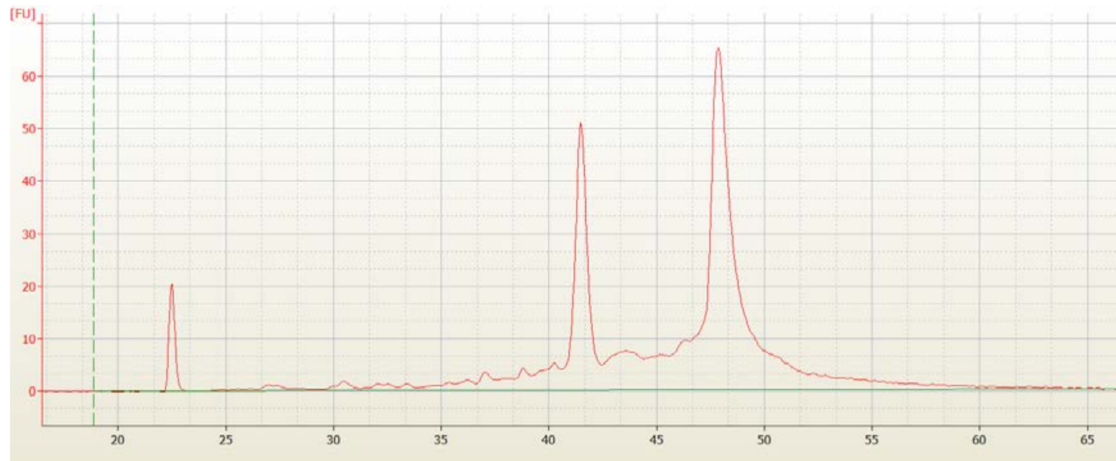
0911 adipose post-scald



1103 adipose pre-scald



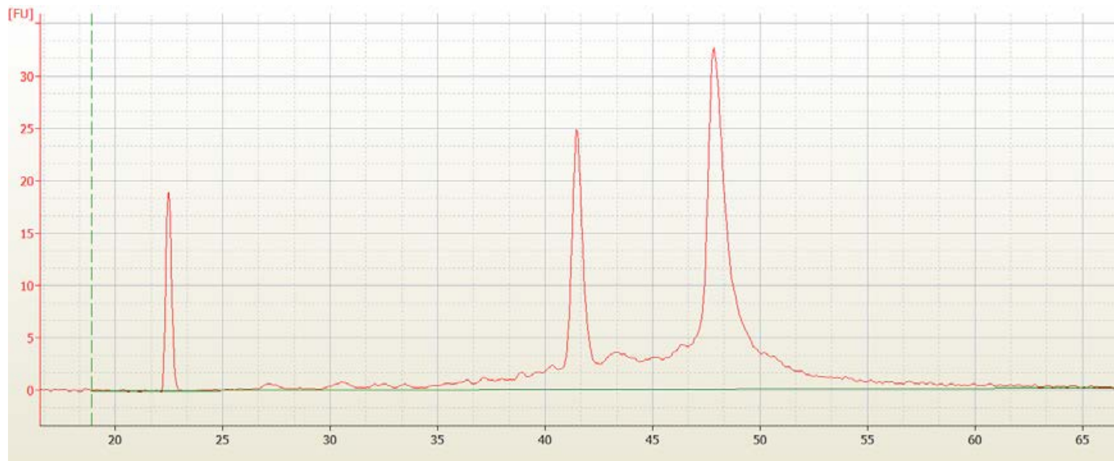
1103 adipose post-scald



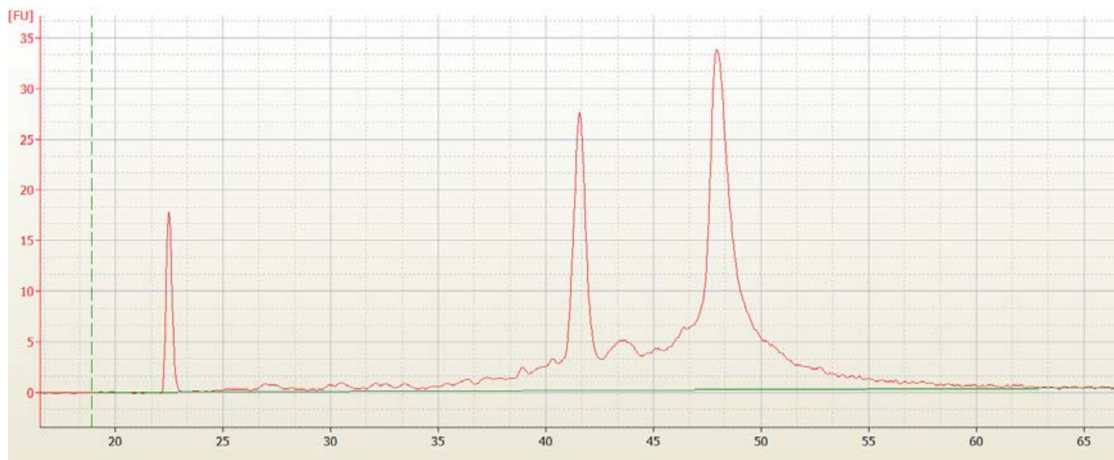
1104 adipose pre-scald

Not available.

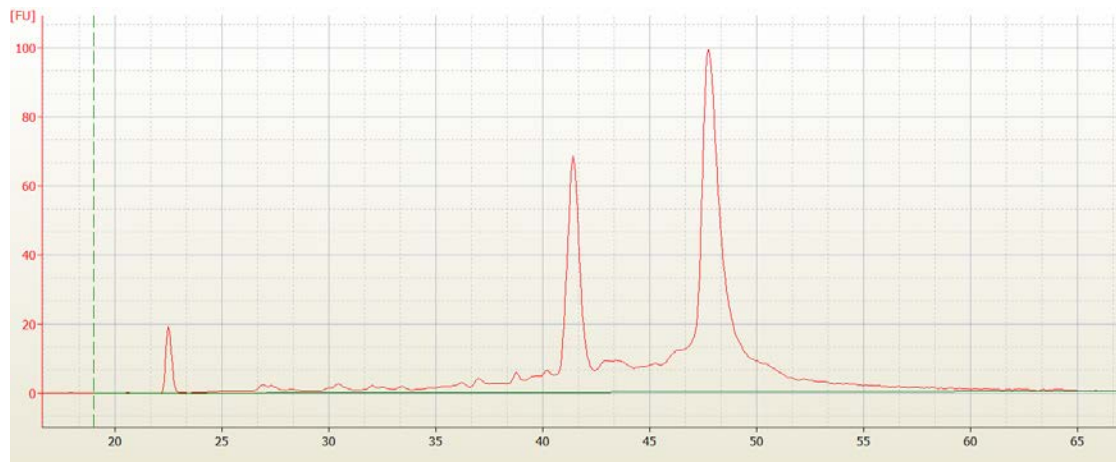
1104 adipose post-scald



1206 adipose pre-scald



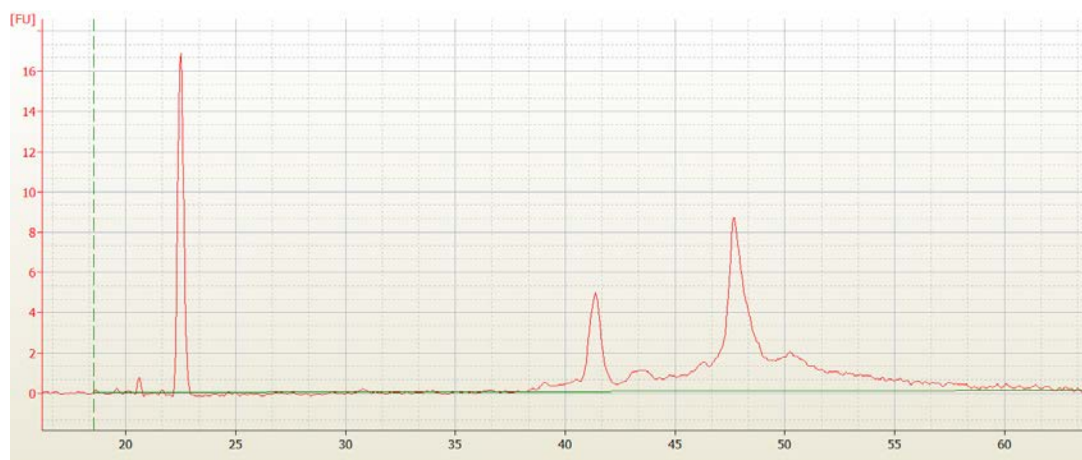
1206 adipose post-scald



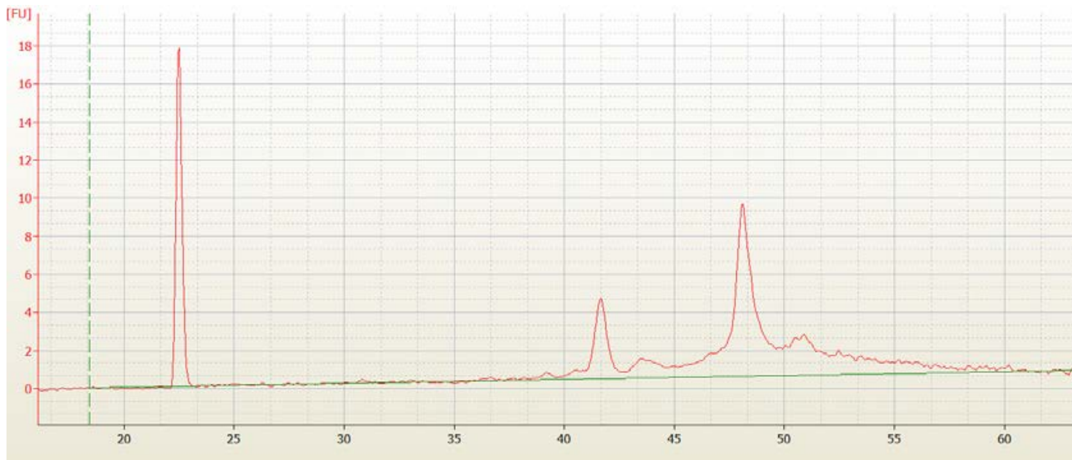
Appendix B

Capillary electrophoresis of isolated total RNA samples from the *longissimus dorsi* tissue of Yorkshires harvested above the tenth rib either prior to scalding (pre-scald) or immediately following scalding (post-scald) was conducted to asses RNA integrity numbers for each sample. Assessment of adipose tissue RNA integrity as judged by electropherograms.

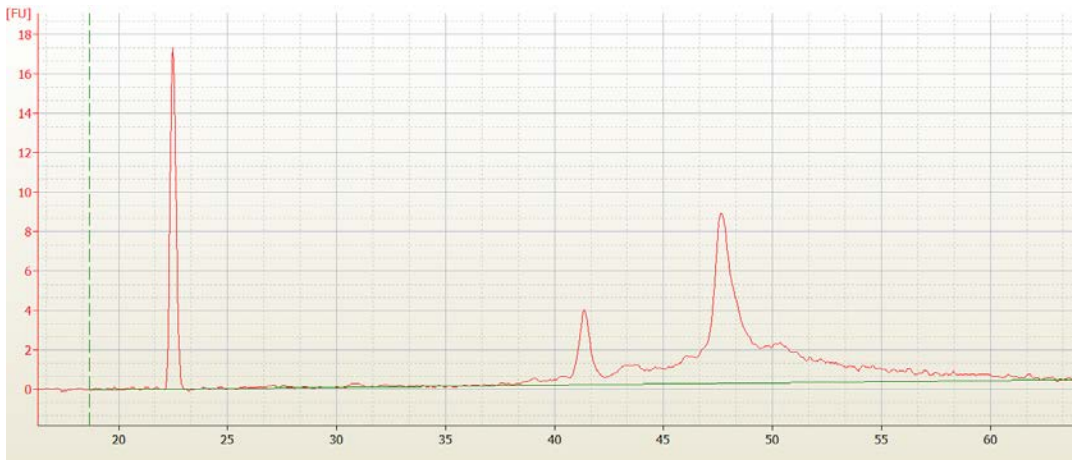
609 *longissimus dorsi* total RNA pre-scald



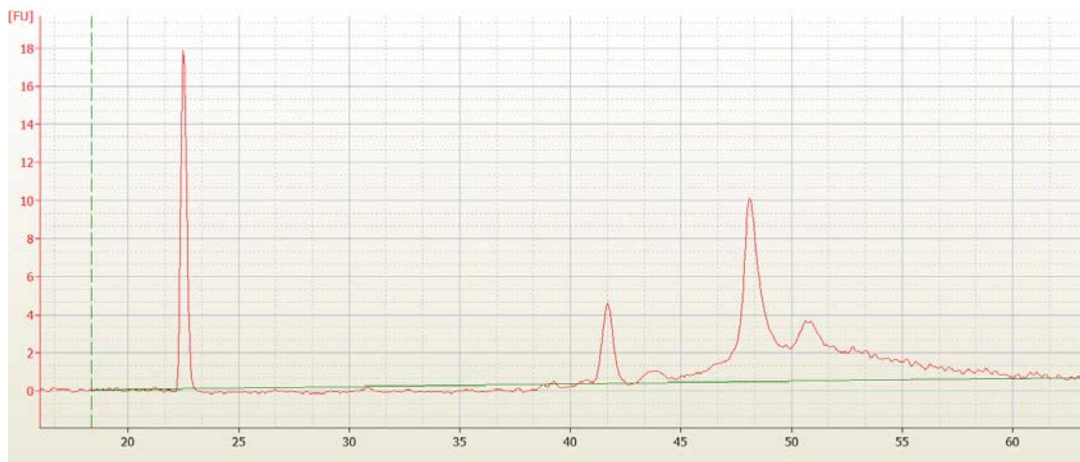
609 *longissimus dorsi* total RNA post-scald



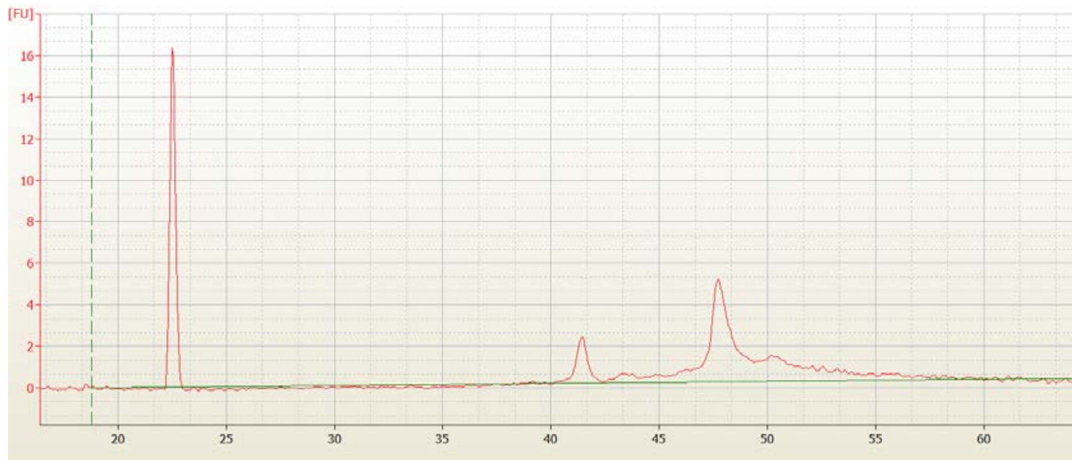
706 *longissimus dorsi* total RNA pre-scald



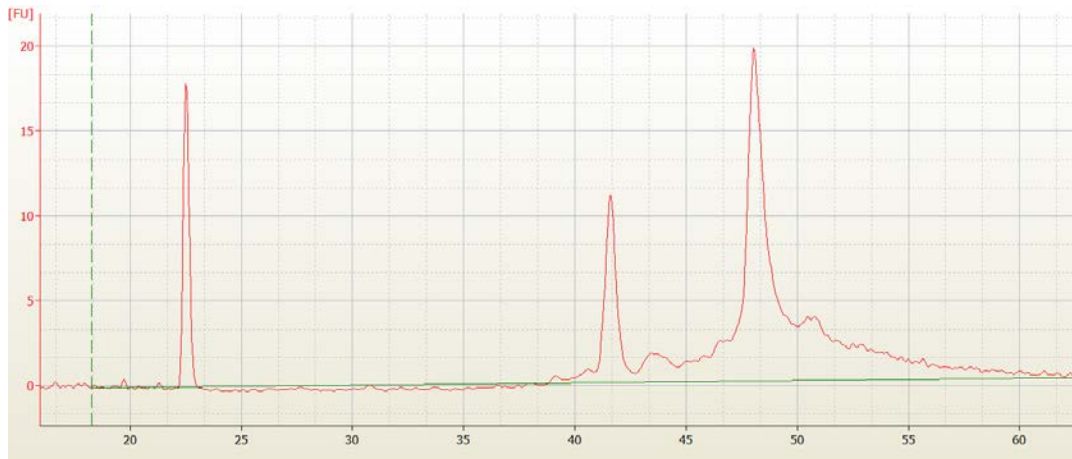
706 *longissimus dorsi* total RNA post-scald



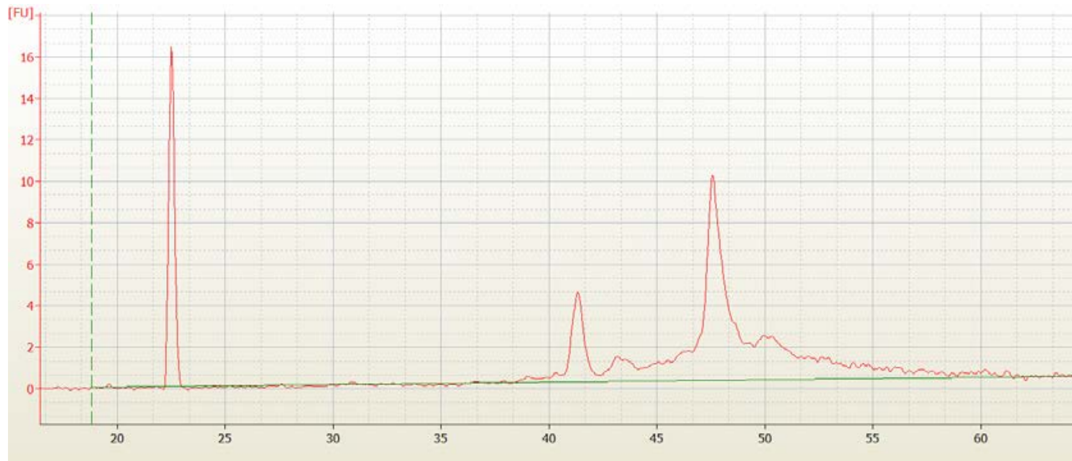
0904 *longissimus dorsi* total RNA pre-scald



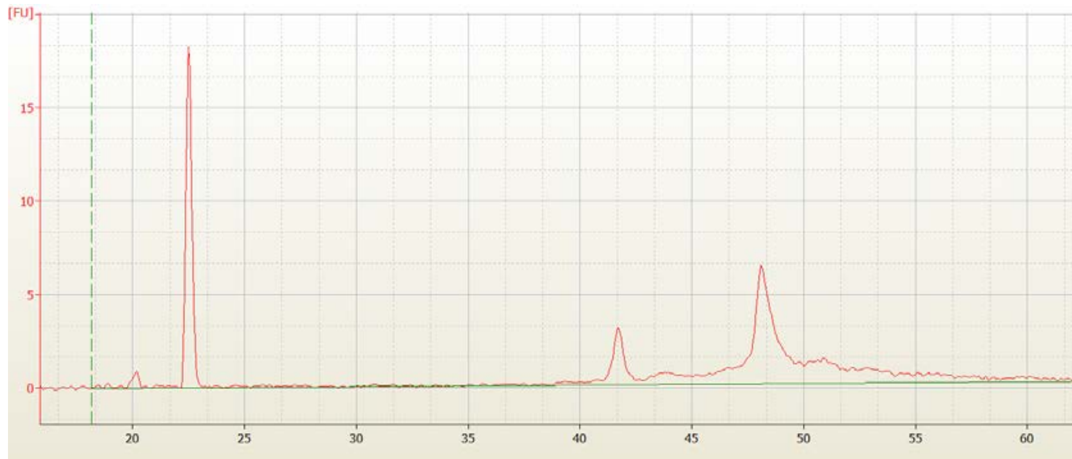
0904 *longissimus dorsi* total RNA post-scald



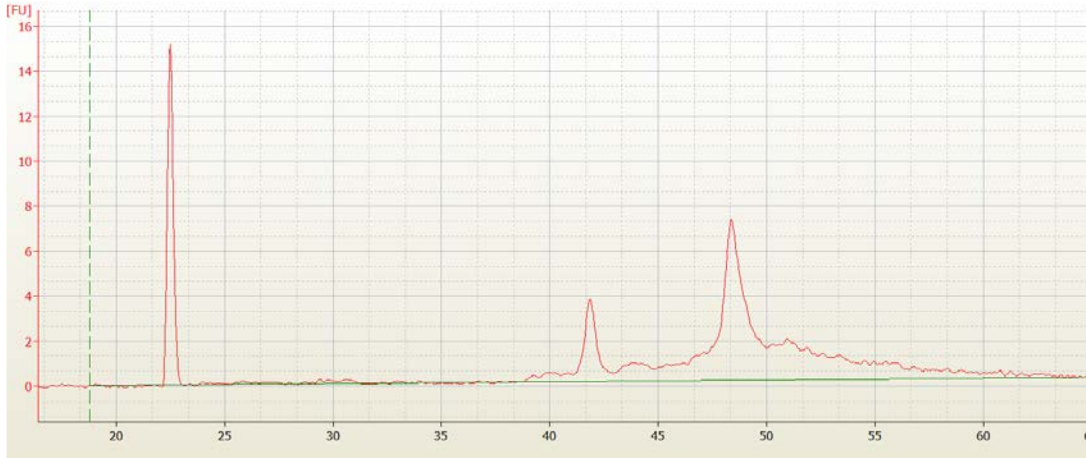
0909 *longissimus dorsi* total RNA pre-scald



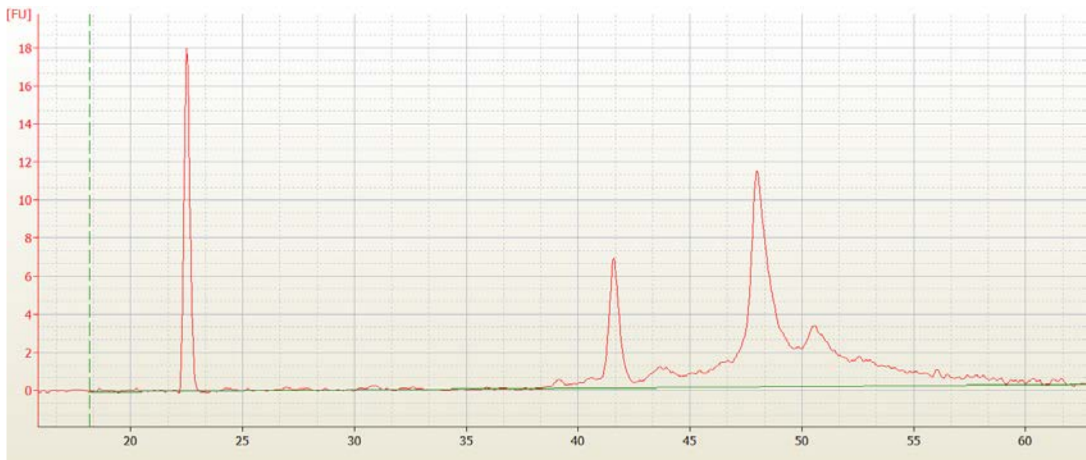
0909 *longissimus dorsi* total RNA post-scald



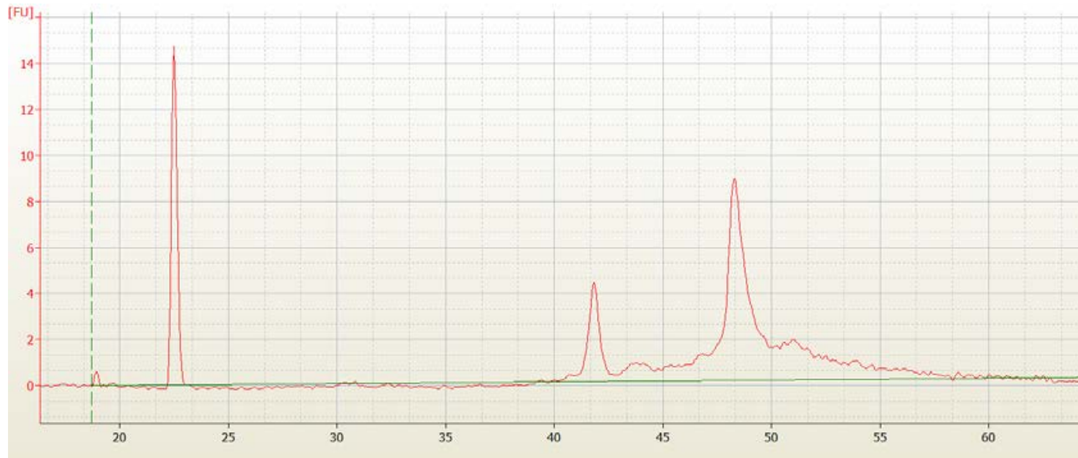
0911 *longissimus dorsi* total RNA pre-scald



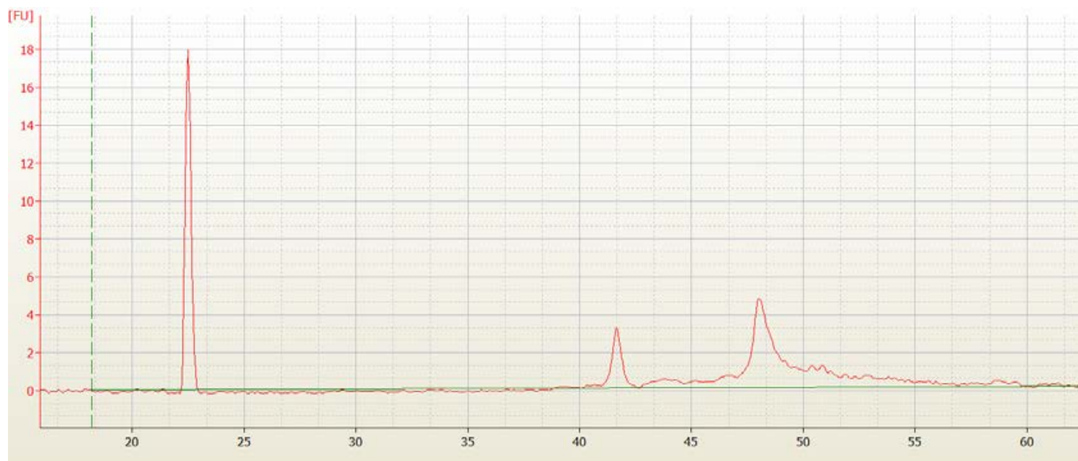
0911 *longissimus dorsi* total RNA post-scald



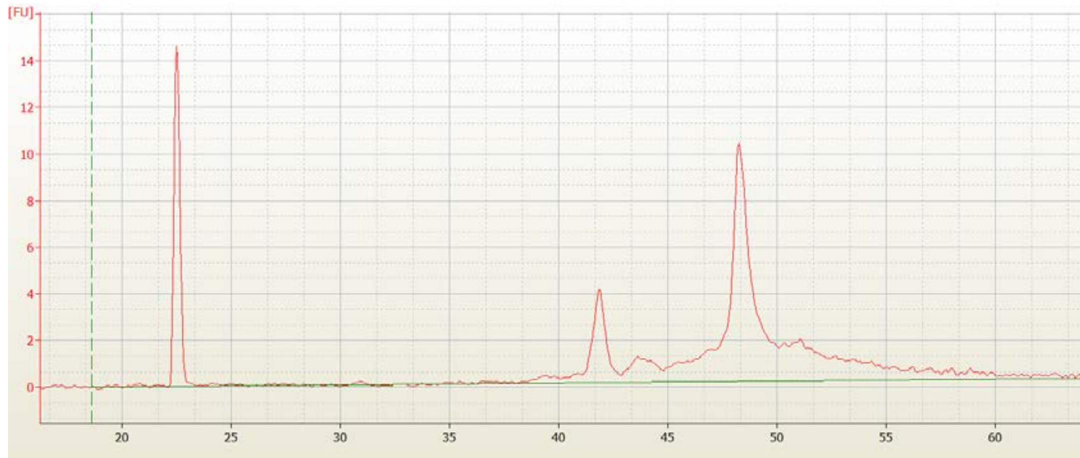
1103 *longissimus dorsi* total RNA pre-scald



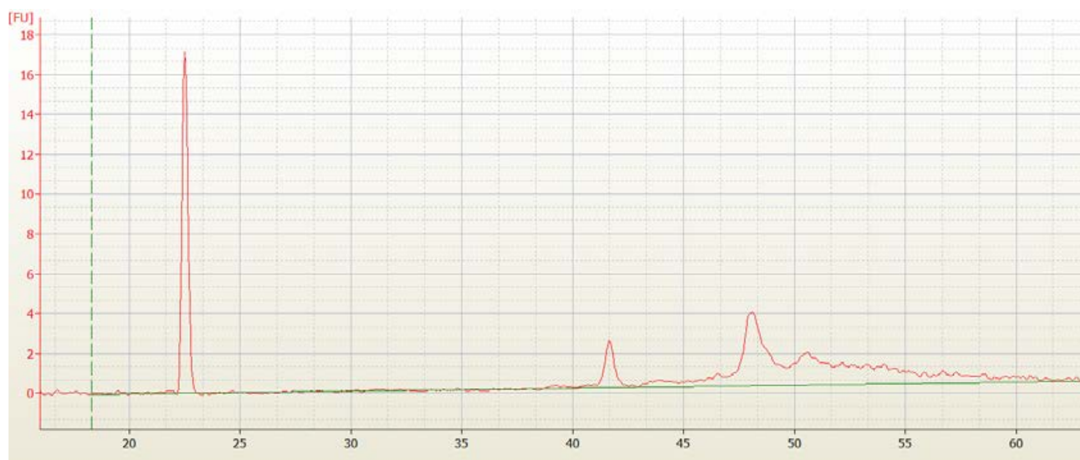
1103 *longissimus dorsi* total RNA post-scald



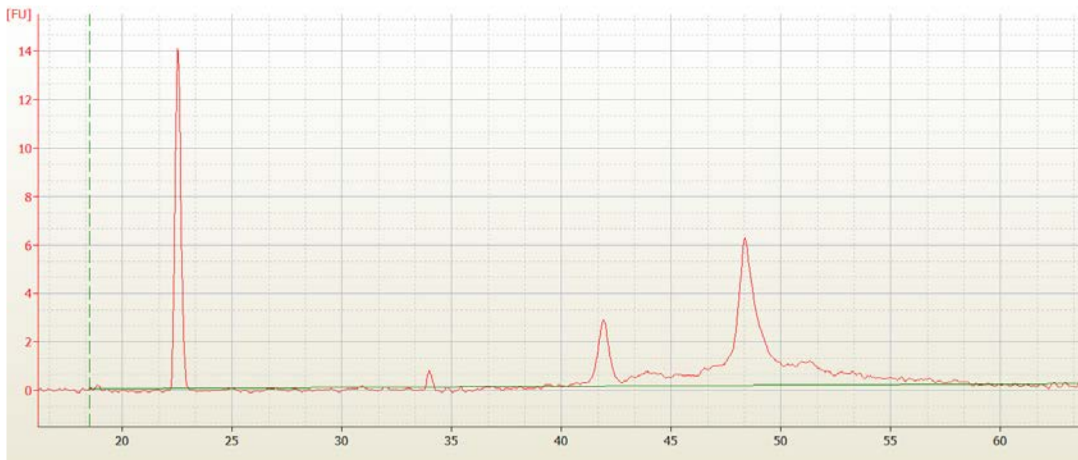
1104 *longissimus dorsi* total RNA pre-scald



1104 *longissimus dorsi* total RNA post-scald



1206 *longissimus dorsi* total RNA pre-scald



1206 *longissimus dorsi* total RNA post-scald