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## Quantitative analysis of leptin mRNA using competitive reverse transcription polymerase chain reaction and capillary electrophoresis with laser-induced fluorescence detection

Leptin, the protein hormone product of the obese (*ob*) gene, functions in the regulation of appetite, energy expenditure, and reproduction in animals and humans. Since changes in the level of circulating leptin can have marked physiological consequences, it is important to be able to accurately quantify leptin gene expression. Toward this goal, we have constructed a chicken leptin RNA competitor and successfully employed it as an internal standard in the development of a quantitative-competitive reverse transcription polymerase chain reaction (QC-RT-PCR) assay for leptin mRNA. Capillary electrophoresis with laser-induced fluorescence detection (CE-LIF) was utilized for the separation and analysis of chicken leptin target (261 bp) and competitor (234 bp) dsDNA products from QC-RT-PCR assay samples. Leptin amplicons were separated using a DB-1 coated capillary (27 cm × 100 µm ID) at a field strength of 300 V/cm in a replaceable sieving matrix consisting of 0.5% hydroxypropylmethyl cellulose (HPMC) in 1 × TBE (89 mM Tris-base, 89 mM boric acid, 2 mM EDTA, pH 8.3) buffer with 0.5 µg/mL Enhance™ fluorescent intercalating dye. Samples were diluted 1:100 with deionized water and introduced into the capillary by electrokinetic injection. QC-RT-PCR/CE-LIF was used to quantify leptin mRNA in liver and adipose tissue from 8-week-old male and female broiler chickens. This study is the first report of quantitative analysis of leptin gene expression using QC-RT-PCR/CE-LIF.

**Keywords:** Leptin / mRNA / Reverse transcription / Polymerase chain reaction / Capillary electrophoresis / Laser-induced fluorescence / DNA

EL 3812

### 1 Introduction

Following the initial discovery of the obese gene (*ob*) in mice and humans by positional cloning and the subsequent characterization of the protein product of this gene called leptin, there has been growing interest in studying its role in the regulation of feeding behavior, energy balance, and body weight [1–5]. Leptin, a 16 kDa cytokine-like polypeptide hormone synthesized and secreted into the bloodstream primarily by white adipose tissue in mammals, has been reported to influence a number of important physiological functions including: appetite, body temperature, energy expenditure, reproduction, angiogenesis, and immune function [3]. As a component of a newly discovered endocrine system (the leptin axis), lep-

tin is synthesized by adipocytes and released into the bloodstream in relation to the amount of energy (in the form of triglycerides) stored in adipose tissue [2, 3]. Circulating leptin is then transported across the blood-brain barrier where it binds to neuronal receptors located in discrete hypothalamic regions of the brain which, in turn, activate the specific neuroendocrine pathways that ultimately lead to decreased caloric intake and increased energy expenditure [2–5]. Thus, leptin represents an afferent signal in a negatively regulated feed-back loop between adipose tissue and the central nervous system [6]. Administration of biologically active, recombinant leptin to animals results in diminished food intake and a reduction in body weight restricted to the loss of adipose tissue while sparing lean tissue mass [6–8]. The mechanisms by which energy stores are 'sensed' and leptin gene expression is adjusted to maintain optimal energy balance are not well understood. However, the physiological responses to quantitative changes in circulating leptin levels suggest that the regulation of leptin gene expression is a critical control point [3].

An increasing number of biomedical research studies have focused on characterizing the underlying mechanisms involved in the regulated expression of leptin and

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**Abbreviations:** HPMC, hydroxypropylmethyl cellulose; *ob*, obese gene; QC-RT-PCR, quantitative-competitive reverse transcription polymerase chain reaction; TBE, Tris-boric acid-EDTA buffer

its receptor since recessive mutations in either of the two genes encoding these proteins result in morbid obesity, diabetes, and infertility [2, 3]. There is also increasing interest in understanding what role(s) leptin might play in domestic animal species since feeding behavior, energy balance, and reproduction are important factors in ensuring the success of commercial animal production [5, 9]. Among the various domestic animal species studied to date, the leptin gene has recently been identified in chickens and its complete coding sequence reported (GenBank Accession Nos. AF012727, AF082500). Chickens differ from mammals in that leptin is expressed in liver as well as in adipose tissue [10, 11]. This reflects the prominent role of the liver in regulating lipogenesis and energy metabolism in birds [12]. Thus, the chicken represents an excellent species in which to conduct comparative studies on leptin gene expression and its regulation.

Reverse transcription polymerase chain reaction (RT-PCR) has been widely used to study gene expression in humans and animal species because this assay is well suited to the analysis of low abundance mRNA transcripts like leptin. However, only a limited number of quantitative-competitive RT-PCR (QC-RT-PCR) assays have been developed to more accurately quantify leptin mRNA in human adipose tissue and cultured mouse adipocytes [13, 14]. The advantages of using capillary electrophoresis with laser-induced fluorescence detection (CE-LIF) for both semiquantitative and quantitative-competitive analyses of dsDNA products from PCR and RT-PCR reactions have been discussed previously [15–23]. Although, to date, analytical methods for detecting chicken leptin protein are very limited [24], two techniques involving RT-PCR have recently been reported for the analysis of chicken leptin mRNA [10, 11]. Thus, RT-PCR assay represents the best currently available method to study leptin gene expression in chickens. The objective of this study was to develop a combined QC-RT-PCR/CE-LIF assay technique for the quantitative analysis of leptin mRNA in total RNA isolated from chicken liver and adipose tissues.

## 2 Materials and methods

### 2.1 Materials

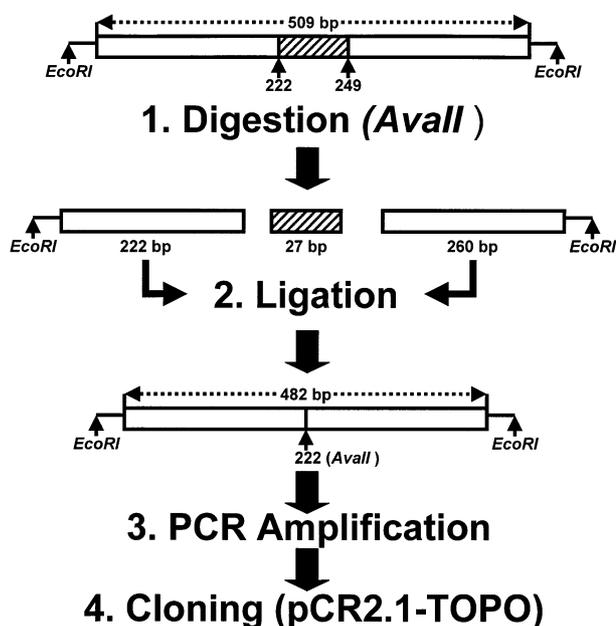
Hydroxypropylmethyl cellulose (HPMC, Cat. No. H-7509) was obtained from Sigma (St. Louis, MO). LIFluor (Enhance™, Cat. No. 477409) and  $\phi$ X174 DNA, *Hae*III digest (Cat. No. 477414) were from Beckman Coulter (Fullerton, CA). All other chemicals used in the preparation of buffers and reagent solutions were of the highest purity grade commercially available.

### 2.2 Animals, tissue collection, and RNA isolation

All studies involving the use of animals were conducted in accordance with research protocols approved by the Beltsville Animal Care and Use Committee. Day-old broiler chicks (Shaver Poultry Breeding Farms, Cambridge, Ontario, Canada) were raised in brooder batteries until three weeks of age, at which time they were transferred to individual cages. At 6–8 weeks of age, male and female birds were sacrificed, and samples of liver, abdominal fat pad, and subcutaneous fat were obtained and immediately frozen in liquid nitrogen. Tissues were stored frozen at  $-80^{\circ}\text{C}$  prior to RNA isolation. Total RNA was isolated from tissue samples using the Tri-Reagent procedure according to the manufacturer's instructions (Life Technologies, Gaithersburg, MD). Isolated RNA was quantified using a UV spectrophotometer (Genequant II; Pharmacia Biotech, Piscataway, NJ).

### 2.3 Primers and leptin competitor design

Forward (sense, 5'-CGTCGGTATCCGCCAAGCAGAGGG) and reverse (antisense, 5'-CCAGGACGCCATCCAGGCTCTCTGGC) primers were designed for chicken leptin as described previously [11, 23]. These primers correspond to bases 134–157 and 369–394, respectively, in the coding sequence reported for chicken leptin (GenBank Accession No. AF082500). A synthetic RNA competitor (internal standard) capable of using the same primer set designed for the leptin target was constructed from a plasmid containing a full-length chicken leptin coding sequence [11, 23]. This plasmid can be used to transcribe either sense or anti-sense RNAs. The leptin coding region (509 bp) was excised from the plasmid by *Eco*RI digestion and isolated by agarose slab gel electrophoresis. The recovered fragment, excised from the agarose gel, was then digested with the restriction enzyme *Av*all which produced three fragments 260, 222 and 27 bp in size (Fig. 1). The 260 and 222 bp fragments were isolated by agarose slab gel electrophoresis and ligated using T4 DNA ligase. The resulting ligation product was amplified by PCR using primers for the 5'- and 3'-ends of the chicken leptin coding sequence (sense, 5'-ATGGAATTCGCCTTCCATATGCCG; antisense, 5'-TCAGCATTCGGGCTAATATCC) and subjected to cloning into the pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA). The orientation of the coding sequence and the 27 bp deletion were confirmed by automated fluorescent DNA sequencing (ABI 377; Perkin Elmer Applied Biosystems, Foster City, CA). The competitor construct was designed to continue the open reading frame, deleting the codons for amino acids 71–80 in the leptin protein sequence [23]. Competitor and full-length (target) leptin synthetic RNAs



**Figure 1.** Procedure used for the construction of a chicken leptin internal competitor by excision of a 27 bp segment from a cloned DNA fragment (509 bp) containing the full length coding region for chicken leptin. To generate the competitor construct, the following steps were performed: (1) restriction enzyme (*AvaII*) digestion, (2) ligation of the resulting 222 bp and 260 bp restriction fragments, (3) PCR amplification of the 482 bp product of the ligation step, and (4) cloning of the PCR-amplified 482 bp dsDNA product into an appropriate expression vector (pCR 2.1-TOPO).

were transcribed *in vitro* from *Bam*HI-digested plasmids using T7 RNA polymerase and a MAXIscript kit (Ambion, Austin, TX). DNA template was removed following synthesis of the RNA by treatment with RNase-free DNase I for 15 min at 37°C according to the protocol included with the MAXIscript kit (Ambion). RNA produced from these reactions was isolated by ethanol precipitation and quantified in multiple dilutions using a UV spectrophotometer (Genequant II; Pharmacia Biotech). The synthetic RNAs were diluted to appropriate concentrations in nuclease-free water and stored frozen at –80°C in multiple aliquots until used. To minimize potential variability in quantitation, aliquots from the same stock of synthetic competitor RNA were used for all comparative analyses of experimental samples.

#### 2.4 Competitive RT-PCR assay for leptin mRNA

RT reactions (50 µL) contained 5 µL of synthetic competitor RNA (diluted to contain 5–300 amol in 5 µL) and 5 µg total RNA. A series of four reverse transcriptase (RT) reaction tubes, each containing a different amount of syn-

thetic competitor RNA in addition to a constant (5 µg) amount of total RNA, were prepared for each tissue sample analyzed. First-strand cDNA synthesis was performed using 5 µg of total RNA and 5–300 amol of added synthetic competitor RNA in the presence of 100 pmol of random hexamer primers. RNA samples were denatured at 65°C for 5 min and the primers were allowed to anneal while slowly cooling the reaction mixture at a rate of 2°C/min over 20 min to a final temperature of 25°C. Extension of the first strand was completed in RT buffer (Promega, Madison, WI) containing: 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl<sub>2</sub>, 10 mM DTT, 100 U Moloney murine leukemia virus RT (M-MLV RT; Promega), 40 U RNAsin (Promega), 1.0 mM dNTPs. The RT reaction tubes were incubated at 37°C for 1 h. After completion of first-strand synthesis, the M-MLV RT enzyme was inactivated by incubating the reaction tubes at 90°C for 5 min and an aliquot (5 µL) of the RT reaction mixture was then subjected to PCR.

PCR reactions were performed in a separate 25 µL reaction volume containing: 15 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.1% Triton X-100, 5 µL of each RT reaction (see above), 1.25 U of *Taq* polymerase (Promega), 1.0 mM dNTPs, and 10 pmol each of chicken leptin gene-specific primers [11, 23]. PCR was performed using a commercial thermal cycler (RoboCycler; Stratagene, La Jolla, CA). PCR thermal cycling parameters were as follows: one cycle at 94°C for 4 min, followed by 30 cycles at 94°C for 30 s, 63°C for 30 s, and 72°C for 30 s with a final extension at 72°C for 8 min. The products of RT-PCR were 261 bp (57.5% GC,  $T_m$  = 86°C) and 234 bp (58% GC,  $T_m$  = 86°C) for the leptin target and competitor amplicons, respectively.

#### 2.5 CE-LIF analysis of RT-PCR dsDNA products

Aliquots (2 µL) of RT-PCR samples were diluted 1:100 with deionized water prior to analysis by CE-LIF. A P/ACE 5510 (Beckman Coulter) equipped with an argon ion LIF detector and controlled by P/ACE Station software (V. 1.0) was used for the analysis of the RT-PCR samples. Excitation of the intercalating dye was at 488 nm and emission was detected at 520 nm. The instrument was configured for reversed polarity (*i.e.*, cathode on the inlet side). A µSIL-DB-1 coated capillary (Cat. No. 197-1002, J&W Scientific, Folsom, CA) with a 100 µm ID, 0.1 µm film thickness, and length of 27 cm (20 cm to the detector) was housed in a P/ACE cartridge (Beckman Coulter) and maintained at 25°C. The sieving buffer consisted of 0.5% (w/v) HPMC in 1 × TBE (89 mM Tris-base, 89 mM boric acid, 2 mM EDTA, pH 8.3). Immediately prior to use, an appropriate amount of intercalating dye (10 µL of Enhance<sup>TM</sup>, 1 mg/mL stock in methanol) was added to

20 mL of sieving buffer, which was mixed for 15–30 min and degassed by sonication for 1 min. The sample injection/run routine was as follows: (i) Prior to the run the capillary was rinsed at high pressure (20 psi) for 1 min with sieving buffer (not subjected to prior electrophoresis); (ii) a small plug of water was injected (5 s at 0.5 psi) into the capillary prior to loading the sample; (iii) RT-PCR samples (diluted 1:100 in deionized water, 200  $\mu$ L total volume) were subjected to electrokinetic loading at 3.0 kV for 10 s; (iv) separations were performed at a field strength of 300 V/cm (8.1 kV) for 5 min. At the beginning and end of each day of use, the capillary was regenerated by rinsing consecutively with water, methanol and water again for 5 min each. A dsDNA standard (*Hae*III digest of  $\phi$ X174 DNA) was used to evaluate capillary and instrument performance prior to sample analysis.

## 2.6 Data analysis

Peak areas were calculated by P/ACE Station software (V. 1.0, Beckman Coulter). To compensate for differences in the amount of dye bound [23], the area of the competitor peak was multiplied by a factor (261/234) to reflect the size difference (in bp) between target and competitor amplicons. The log of the ratio of the integrated peak areas for leptin competitor and target amplicons was then calculated. Linear regression analysis of the log of the ratio of corrected competitor/target integrated peak areas vs. the log of the amount (amol) of competitor added to each reaction mixture was used to calculate the amount (amol) of leptin mRNA (where  $\log \text{ competitor/target} = 0$ ) present in the total RNA sample (5  $\mu$ g).

## 3 Results and discussion

### 3.1 Leptin competitor design

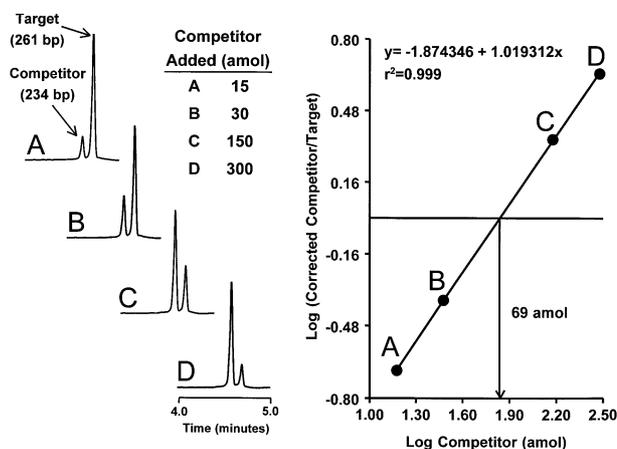
Previous estimates of chicken leptin mRNA levels have been made by relative quantitation methods that involve normalizing the amount of leptin PCR amplicon to a coamplified reference template ( $\beta$ -actin). Such assays have been successfully applied to investigate the effects of different hormones (*e.g.*, insulin, dexamethasone, estrogen, growth hormone) on leptin gene expression in chicken liver and adipose tissues [11, 23, 27]. This approach, while yielding valuable estimates of relatively large changes in gene expression, may not be able to precisely quantify more subtle shifts in the level of a low abundance transcript like leptin mRNA. In fact, a number of investigators have noted the potential for inaccurate estimation of mRNA levels using relative or semiquantitative RT-PCR analysis methods [14, 22, 25, 26]. Instead, the use of competitive PCR or RT-PCR assays is recommended for more precise mRNA quantitation because

such assays adjust for the inherent variability in efficiency of the RT and/or PCR steps through the use of a coamplified competitor or internal standard. A major reason why competitive-based PCR and RT-PCR assays are not routinely used is that the design and production of a suitable competitor (either RNA or DNA) can be technically difficult to achieve. There are two basic types of internal standards/competitors. Homologous competitors have nearly identical sequence to that of the target with the exception of a small addition, deletion, or modification of sequence to produce a new restriction site. A heterologous standard contains a mainly unrelated sequence and only shares sequences common to primer recognition sites with the target. Both homologous [14] and heterologous [13] competitor standards have been designed and used in QC-RT-PCR analyses of human and mouse leptin gene expression.

We have designed and constructed a specific homologous competitor from the complete coding sequence for chicken leptin (509 bp) and cloned it into an appropriate expression vector for the production of synthetic RNA. Figure 1 describes the general steps in the design process including: (i) restriction endonuclease digestion, (ii) ligation, (iii) PCR amplification, and (iv) cloning into a suitable expression vector (pCR2.1-TOPO). By taking advantage of two naturally occurring restriction endonuclease (*Av*all) sites at positions 222 and 249, we were able to delete 27 bp from the original coding sequence, resulting in an approximate 5% reduction in size of the construct (482 bp). RNA, transcribed *in vitro* from the vector containing the competitor construct, produces a 234 bp amplicon that is 27 bp smaller than the leptin target amplicon (261 bp) when subjected to RT-PCR. The same gene-specific primer set can be utilized to generate both the chicken leptin target and competitor amplicons in the RT-PCR assay.

### 3.2 QC-RT-PCR/CE-LIF assay for leptin mRNA

Figure 2 depicts a typical QC-RT-PCR/CE-LIF analysis of a total RNA sample from the liver of a 6-week-old female broiler chicken. Typically, four quantities of competitor (Fig. 2A–D) are chosen that will bracket the amount of mRNA anticipated to be present in the total RNA sample isolated from a specific tissue. In the case of a liver RNA sample, the appropriate values were determined to range from 15–300 amol with two of the competitor amounts higher and two lower than the expected amount of leptin mRNA. Although this bracketing approach must be established experimentally by trial and error for each new sample type, it results in the most precise estimate of the target mRNA. Others have also found that four levels of competitor are sufficient for an accurate determination of



**Figure 2.** A typical QC-RT-PCR assay for leptin mRNA. Total RNA was isolated from liver tissue obtained from a 6-week-old female broiler chicken and analyzed by QC-RT-PCR as described in Section 2.4. Four levels of a synthetic RNA competitor (A–D; 15, 30, 150, 300 amol) were added to four separate assay tubes, each containing 5  $\mu$ g of total RNA. The dsDNA amplicons produced by PCR were separated and detected by CE-LIF. A portion (1 min, corresponding to 4–5 min of the 5 min run) of each of the four electropherograms is depicted on the left side of the figure with the target (261 bp) and competitor (234 bp) amplicons denoted. Integrated peak areas for both amplicons were determined and a competitor/target peak area ratio of was determined after correcting the competitor peak to compensate for less dye binding due to its slightly smaller size. The log of the corrected peak area ratio was plotted against the log of the quantity of added synthetic competitor RNA (right) and the quantity of leptin mRNA in the sample (69 amol) determined from the regression equation shown.

specific mRNA targets [14, 22]. However, it is important to carefully choose the amounts of competitor used to bracket the samples to be analyzed because it has been reported that inaccurate quantification can occur if the amounts of standard and target differ greatly [26]. Thus, for each sample of total RNA to be analyzed for leptin mRNA by QC-RT-PCR, four individual assay samples containing differing amounts of the leptin competitor are required to be run.

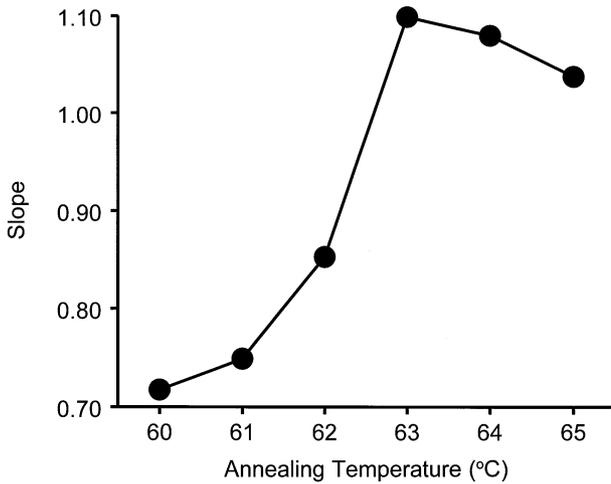
Because each sample of total RNA must be assayed by QC-RT-PCR in quadruplicate, a rapid method for separation and detection of the dsDNA amplicons is essential. The standard method of agarose gel electrophoresis with ethidium bromide staining, while adequate, suffers from a limited range of quantitation due to dye saturation and requires the added time-consuming steps of gel imaging and image analysis to derive quantitative data. A number of investigators have described various CE-LIF techniques for the analysis of dsDNA products from PCR and

RT-PCR samples [15–23], although some of these methods involve relatively lengthy (*i.e.*, > 10 min) separation times. We have developed a rapid separation technique involving CE-LIF in which the two leptin amplicons (target and competitor) are resolved in under 5 min. With an additional 1 min of time required for rinsing the capillary prior to each run, the total time required to run all four assay samples is 24 min.

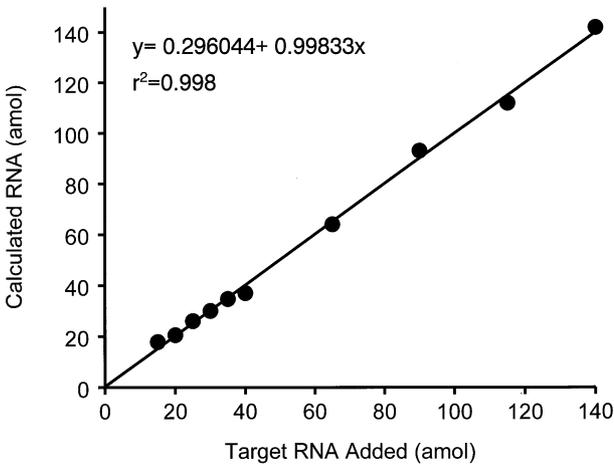
The 1 min portions (4–5 min) of actual electropherograms shown in the left-hand portion of Fig. 2 depict changes in leptin target and competitor dsDNA peaks separated by CE-LIF and demonstrate the competition between target and competitor. For the QC-RT-PCR assay results to be valid, a true competition of competitor and target for reaction components (*i.e.*, primers, enzymes, dNTPs, *etc.*) must exist to assure a constant competitor/target ratio at any given level of added competitor [26]. The right-hand portion of Fig. 2 depicts the results of a linear regression analysis of the corrected competitor/target peak area ratios plotted against the amount of added competitor RNA. The point at which the ratio is equal to unity (*i.e.*, competitor = target) defines the quantity of leptin mRNA in the original 5  $\mu$ g total RNA sample. Another important consideration in determining the validity of QC-RT-PCR results involves ensuring equivalence in the efficiency of amplification for both the competitor and the target [26]. This can be determined experimentally from the slope of the regression line. A slope of 1 indicates equal efficiencies, whereas a slope < 1 indicates target amplification with a greater efficiency than that of competitor and a slope > 1 indicates a greater amplification efficiency for the competitor. From the results depicted in Fig. 2, it is clear from the calculated slope value of 1.02 that the amplification efficiencies are essentially equivalent. Moreover, an  $r^2$  value of 0.999 indicates excellent response linearity, which suggests constant amplification efficiencies for all four assay samples [26].

During the course of optimizing the QC-RT-PCR assay for leptin mRNA, we investigated the effect of changing the annealing temperature on the slope of the regression line. In a previous study, 60°C was used for the annealing temperature step in the PCR protocol [23]. This resulted in slopes of the linear regression lines less than unity, which was indicative of PCR amplification favoring target over competitor. Figure 3 clearly shows that at temperatures  $\leq 62^\circ\text{C}$  target amplification is favored. Between 61°C and 63°C there is a sharp transition and improvement toward equivalent amplification efficiencies. This undoubtedly reflects secondary structure effects on the annealing of the gene-specific primers resulting in different amplification efficiencies for target and competitor. Thus, annealing temperature is an important factor to

consider and must be optimized experimentally for each mRNA analyzed.



**Figure 3.** A plot of the slope of the regression line determined from a series of QC-RT-PCR/CE-LIF analyses of total RNA vs. the annealing temperature used in the PCR step. Replicate samples of total RNA isolated from 8-week-old male broiler fat pad tissue were used to generate the data that was analyzed as described in the legend to Fig. 2.

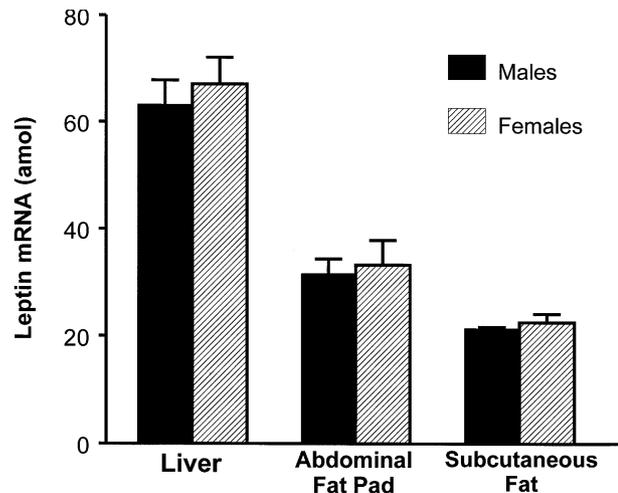


**Figure 4.** A plot of calculated vs. added amounts of a synthetic target RNA produced by *in vitro* transcription of an expression vector containing full-length chicken leptin coding sequence. A series of synthetic target RNA samples ranging from 15–140 amol (determined spectrophotometrically) were subjected to QC-RT-PCR using two separate competitor RNA brackets consisting of 5, 10, 40, 100 amol for target RNA samples added at < 40 amol and 15, 30, 150, 300 amol for target RNA samples added at > 40 amol. The amount of target RNA was calculated as described in the legend to Fig. 2. The line shown was derived from linear regression analysis and exhibited a slope of 0.998 and a coefficient of variation ( $r^2$ ) of 0.998.

A final step in validating the QC-RT-PCR assay for chicken leptin was to demonstrate linear response of the assay over a range of RNA amounts encompassing the quantities of leptin mRNA likely to be found in tissues. Figure 4 demonstrates the linear response for differing amounts of synthetic target RNA (synthesized by *in vitro* transcription of the expression vector) ranging from 15–140 amol. Each of the values was calculated using an appropriate four-level competitor bracket. From the slope (0.998) and  $r^2$  (0.998) values, excellent agreement between the calculated amount and the amount added (determined spectrophotometrically) is seen. Although we show linear response over two log unit amounts of added RNA in Fig. 4, it is likely that the linear dynamic range for the assay extends beyond that point. Thus, this QC-RT-PCR assay for chicken leptin mRNA demonstrates an excellent linear response over the complete range of values likely to be encountered in typical tissue of total RNA samples. Four independent determinations made on a single synthetic target RNA sample gave a value of  $20.5 \pm 1.0$  (mean  $\pm$  SD, 20 amol expected) with a relative standard deviation of 4.9%. This result is typical of the reproducibility and reliability we routinely achieve using this technique.

### 3.3 Application of QC-RT-PCR/CE-LIF to quantify tissue leptin mRNA levels

Figure 5 depicts the results from QC-RT-PCR/CE-LIF analyses of total RNA samples isolated from liver and adipose tissue (abdominal fat pad and subcutaneous fat) ob-



**Figure 5.** Leptin mRNA levels determined by QC-RT-PCR/CE-LIF for total RNA (5  $\mu$ g) samples isolated from liver and adipose (abdominal fat pad and subcutaneous fat) tissue from male ( $n = 3$ ) and female ( $n = 3$ ) broiler chickens at 8 weeks of age. The values shown represent the mean  $\pm$  SD.

tained from male and female broiler chickens at eight weeks of age. Although there is a tendency toward higher leptin mRNA in female vs. male tissues, this gender-related trend was not statistically significant ( $p > 0.05$ ). One finding demonstrated by the current results that has been shown repeatedly in previous studies using semi-quantitative RT-PCR assay methods [11, 23, 27] is that leptin mRNA levels in chicken liver tend to be twice as high as those found in abdominal fat. This holds true for both males and females at this age. It appears to indicate more active expression of the leptin gene by liver as compared to adipose tissue in chickens, which would be consistent with an important role of the liver in energy metabolism [12]. An additional finding in this study is that subcutaneous fat obtained from 8-week-old birds demonstrated the lowest level of leptin expression of the three tissues analyzed. Currently, we are investigating the effects of age and gender on leptin gene expression in tissues obtained at different times during embryonic and post-hatching development.

#### 4 Concluding remarks

This and a previous study [23] are the first reports of the application of CE-LIF to the analysis of leptin gene expression as determined by RT-PCR. In this study, we employed a rapid (6 min/sample) CE-LIF method to separate and quantify leptin amplicons produced by a QC-RT-PCR assay of total RNA samples from chicken liver and adipose tissues. We have also developed and evaluated a specific internal competitor for chicken leptin and successfully applied it in a QC-RT-PCR assay. Since no techniques currently exist for the quantitative measurement of chicken leptin protein, QC-RT-PCR/CE-LIF represents a promising method to accurately quantify leptin gene expression in chickens. The utility of this assay was clearly demonstrated by quantifying leptin mRNA in total RNA samples isolated from liver and adipose tissues collected from 8-week-old male and female broiler chickens.

*The authors wish to acknowledge Dr. James M. Hempe (LSU, School of Medicine, Department of Pediatrics, New Orleans, LA) for his helpful advice and many practical suggestions which were instrumental in the development of the CE-LIF technique used in this study. The authors also wish to acknowledge contributions made by Dr. Susan M. Czerwinski during the initial stages of this work and Dr. Melissa S. Ashwell for her assistance with DNA sequencing. Mention of a trade name, proprietary product, or specific equipment does not constitute a guarantee or warranty by USDA and does not imply its approval to the exclusion of other suitable products.*

Received August 2, 1999

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