Supplementary Appendix

Insulin Receptor Isoform and IGF-1R Q-PCR Assay Development and Optimization

Due to the small sequence difference between IR-A and IR-B and the high degree of sequence homology between the IGF-1R and IR mRNAs, primer design was critical to developing a Q-PCR assay to accurately detect all three receptors. Previous isoform specific Q-PCR assays have been reported for several other gene products (1-7). Vandenbroucke and colleagues determined the most accurate method was to use a primer that spans the exon junction specific to each isoform, particularly if the isoforms differ greatly in abundance (1, 8). We adopted this strategy for the IR isoform-specific assays and designed a common sense primer that anneals within exon 10 to reduce inter-assay variability and obtain similar PCR kinetics. Specific anti-sense primers were designed against exon/exon junctions unique to each isoform (Fig. 1A) and IGF-1R primers were targeted to a region of low IR homology.

For primer sensitivity tests, 10-fold serial dilutions of plasmid standards were used as template with each Q-PCR primer pair. Sample amplification plots of IR-A standards demonstrate these primers are accurate over six orders of magnitude down to 100 copies (Fig. 1B). IR-B and IGF-1R assays were also accurate over six orders of magnitude to 10^2 copies (graphs not shown).

Competition assays were used to test the capability of our primers to specifically amplify their target in the presence of excess non-specific, homologous target, as may be the case when analyzing total RNA from a biological sample. Serial dilution of standards was repeated in a fixed excess of 10^7 copies of competitor plasmid and standard curves were generated (1, 2). The standard curve from IR-A amplification plots (Fig. 1B) is shown in Figure 1E (■, blue line). Amplification plots were generated from serial dilution of IR-A standards in a fixed concentration of IR-B plasmid competitor (Fig. 1C) or IGF-1R plasmid competitor (Fig. 1D). There was no significant change in IR-A amplification curves with addition of either competitor. Thus, there was no change in the IR-A standard curve (Fig. 1E, compare ■ with ● or ▲) resulting from this data indicating specific amplification of IR-A product. Similarly, the efficiency of IR-B and IGF-1R assays was not significantly changed in the presence of competition. These data demonstrate that the primers designed here are sensitive and precise to detect 100 copies of their target in the presence or absence of 10^5 excess copies of highly homologous targets.

It is critical that efficiency of all primer pairs be approximately equivalent to allow accurate comparison of gene expression using the $\Delta\Delta C_t$ method of relative quantification (9)(Applied Biosystems User Bulletin 2 P/N 4303859), therefore each primer pair designed for these studies was validated based on efficiency as well as sensitivity, and specificity. Primer efficiency is determined by the following equation: $E = [10^{-(1/slope)}]-1$; where slope is the slope of the standard curve when Ct value is plotted versus known amount of template, and E=1.0 when slope is approximately -3.33 (i.e. 100% reaction efficiency). The efficiency of β-actin primers using cDNA as template is 97±3, and the efficiencies of IR-A, IR-B and IGF-1R primers are 99±3, 102±2 and 104±5, respectively. An additional test to determine if the efficiencies of the endogenous control primers and IR isoform and IGF-1R primers are suitably equivalent for the $\Delta\Delta C_t$ method of relative quantification is to plot the log cDNA dilution versus the ΔC_t value (C_t of target – C_t of endogenous control) and fit the data with a linear regression curve (Sample graph, Fig.2). According to Livak and Schmittgen (9), if the absolute value of the slope of the

linear regression is close to zero, then the reaction efficiencies of the target and endogenous control genes are similar and the $\Delta\Delta C_t$ method of calculation can be applied. We have performed this analysis at least three times for each receptor primer pair using two-fold serial dilution of 200 ng -12.5 ng cDNA and the absolute values of the slope of the linear regression lines are 0.013 (Fig. 2), 0.03 and 0.039, for IR-A, IR-B and IGF-1R respectively. These values are well below the value of 0.1 recommended in Applied Biosystems User Bulletin 2, indicating that the primers designed here for quantification of IR isoform and IGF-1R mRNA are suitable for use in the $\Delta\Delta C_t$ method of R.Q.

Specific Methods for Assay Development

Q-PCR Standards

The following primers to the region encompassing the 3' end of exon eight to the 5' end of exon 11 (exon 12 in IR-B) of the IR and IGF-1R were used to synthesize plasmid standards for Q-PCR: IR standard sense 5'AGGTCCAACGACCCCAAGTCTCAGACCCC3' and antisense 5'AATGGTAGAGGAGACGTTGGGGAAATCTGGAAGTG3', product size 672/636 bp; IGF-IR standard sense 5'CCCTCACCATGGTGGAAAACGACCATATCCG 3' and antisense 5'TGTGATATTGTAGGTGTCAGCTACCGTGGTGTTCC3', product size 545. One microgram of Calibrator cDNA (for definition of Calibrator cDNA, see "RNA Isolation Quantification and cDNA Synthesis" section of Materials and Methods), 45 μL of Platinum PCR SuperMix High Fidelity (Invitrogen, Carlsbad, CA), and 0.2 μM each of sense and antisense primers were used in the following PCR reaction: 94°C for 2 minutes to denature cDNA followed by 35 cycles of 94°C for 30s, 55°C for 30s and 68°C for 1m. Products were analyzed on a 2% agarose gel run at 100V for 3.75 hours in order to separate products that represent IR-A and IR-B. Appropriate bands were subsequently gel purified using the MinElute Gel Extraction Kit, microcentrifuge protocol (Qiagen, Valencia, CA).

Two microliters of each purified product was cloned into the pCRII-TOPO vector (Invitrogen, Carlsbad, CA) using the protocol suggested for Chemical Transformation of *E. coli*. Two microliters of the ligated vector was used to chemically transform One Shot TOP10 competent cells (Invitrogen, Carlsbad, CA). Transformed cells were spread on 50 μg/ml ampicillin LB plates and incubated overnight at 37°C. Five positive colonies from each transformation were used to seed 3 ml LB+50 μg/ml ampicillin cultures. Cultures were grown overnight at 37°C with shaking at 225 rpm. Plasmid DNA was isolated from three cultures per transformation using QIAprep Spin Miniprep Kit, microcentrifuge protocol (Qiagen, Valencia, Ca). Plasmid DNA from insert-positive colonies was sequenced by the Molecular Resource Facility of UMDNJ using the SP6 primer. IR-A and IGF-1R plasmid sequences were compared for fidelity to NCBI sequences,gi:157057177 and gi:112983655, respectively. The previously unpublished mouse IR-B sequence was compared to the mouse IR-A sequence and the Rhesus monkey exon 11 sequence (accession number L42997) and submitted to NCBI under the accession number gi:170177902.

One microgram of plasmid DNA was linearized using the BglII restriction enzyme (Promega, Madison, WI) and analyzed on 1% agarose gel as above. Linear DNA was purified using the MinElute Reaction Cleanup Kit, microcentrifuge protocol (Qiagen, Valencia, CA) and quantified by spectrophotometry. Copy number was calculated using the following formula, [DNA concentration (g/μl) /(plasmid length (bp)*660)]*6.022×10^{23} = molecules/μl. Linear plasmid DNA is referred to as standards for the purposes of this manuscript.

Q-PCR Primer Design and Optimization

IR-A, IR-B and IGF-1R Q-PCR primers were designed against the sequences listed above using Primer Premier v.5 Software (Premier Biosoft International, Palo Alto, CA) with attention to standard criteria for Q-PCR primer design (i.e. - all primers either span exon/exon junctions or anneal to two different exons to prevent interference from contaminating genomic DNA). Primer sequences and product sizes are as follows: IR-A and IR-B sense 5'TCCTGAAGGAGCTGGAGGAGT3'; IR-A antisense 5'CTTTCGGGATGGCCTGG3', product size 89; IR-B antisense 5'TTCGGGATGGCCTACTGTC3', product size 123; IGF-1R sense 5'GGCACAACTACTGCTCCAAAGAC3' and antisense 5'CTTTATCACCACCACACACTTCTG3', product size 114. β-actin primers were published previously (10). All primers listed above, were purchased from Integrated DNA Technologies, Inc. (Coralville, IA). Cytokeratin-18 (CK18), E-cadherin (E-cad), N-cadherin (N-cad) and β-casein pre-optimized QuantiTect Primer Assays were purchased from Qiagen (Valencia, CA).

To test the detection limit and efficiency of each primer pair, 10^7 molecules/μl of each plasmid standard was serially diluted and used as template in Q-PCR assays with a dissociation stage and reaction conditions as described below to generate a standard curve of threshold cycle (Ct) value versus Log copy number. Efficiency was also evaluated from five independent reaction plates using two-fold dilutions of 200 ng Calibrator cDNA (see RNA isolation, quantification and cDNA synthesis section for description) as template.

For competition assays, 10^7 copies of a non-specific plasmid DNA target was mixed with each standard and used as template in Q-PCR assays (e.g. to test IR-A primer specificity, 10^7 copies of IR-B standard, or IGF-1R standard was mixed with each of the IR-A standard) and standard curves were generated as described above.

Further tests of assay specificity were performed on post-amplification Q-PCR products. Q-PCR products were taken directly from a post-amplification assay plate and analyzed by gel electrophoresis, a single band of size consistent with dissociation curve analysis was present for each assay (data not shown). Post-amplification products were also cloned, and sequences were confirmed against NCBI published sequences.

Q-PCR

The $\Delta\Delta C_t$ method of relative quantification and SYBR green chemistry were used to measure expression of genes reported herein. β-actin was used previously as an endogenous control for analysis of kinase gene expression in mammary gland mRNA (11) and was used here because it displayed minimal variability between primary MEC samples.

All reactions were performed on the Applied Biosystems (ABI) 7900HT Fast Real-time PCR system using associated Sequence Detection Systems Software, Version 2.2.2 (ABI, Foster City, CA) or on the Applied Biosystems 7300 Real-time PCR system using SDS Software version 1.2.3. The thermal profile for all reactions was as follows: 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds and 58°C (annealing temperature) for 1 minute. Unless otherwise indicated, IR-A, IR-B, IGF-1R, and β-actin reactions contained final concentrations of the following: 1X QuantiTect SYBR Green PCR Master Mix (Qiagen, Valencia, CA), 0.25 μM both sense and antisense primers and 50 ng cDNA. β-casein, CK18, E-cad and N-cad reactions contained 1X SYBR Green master mix, 1X QuantiTect primer assay mix and 50 ng cDNA. Q-PCR reactions performed to generate standard curves (i.e. for primer sensitivity tests and competition assays which used plasmid standards as template) were run using the absolute quantification assay and included a dissociation stage. Q-PCR reactions

performed to quantify target gene expression were analyzed using the SDS relative quantification (ΔΔCt) assay and were combined into an SDS relative quantification (ΔΔCt) study to determine the expression of each target mRNA in primary MEC samples. All absolute quantification and ΔΔCt assays (except β-casein) were carried out at least twice and each sample was run in quadruplicate. Universal Mouse Reference RNA (Stratagene, La Jolla, CA) was the calibrator sample for all Q-PCR relative quantification studies except β-casein.

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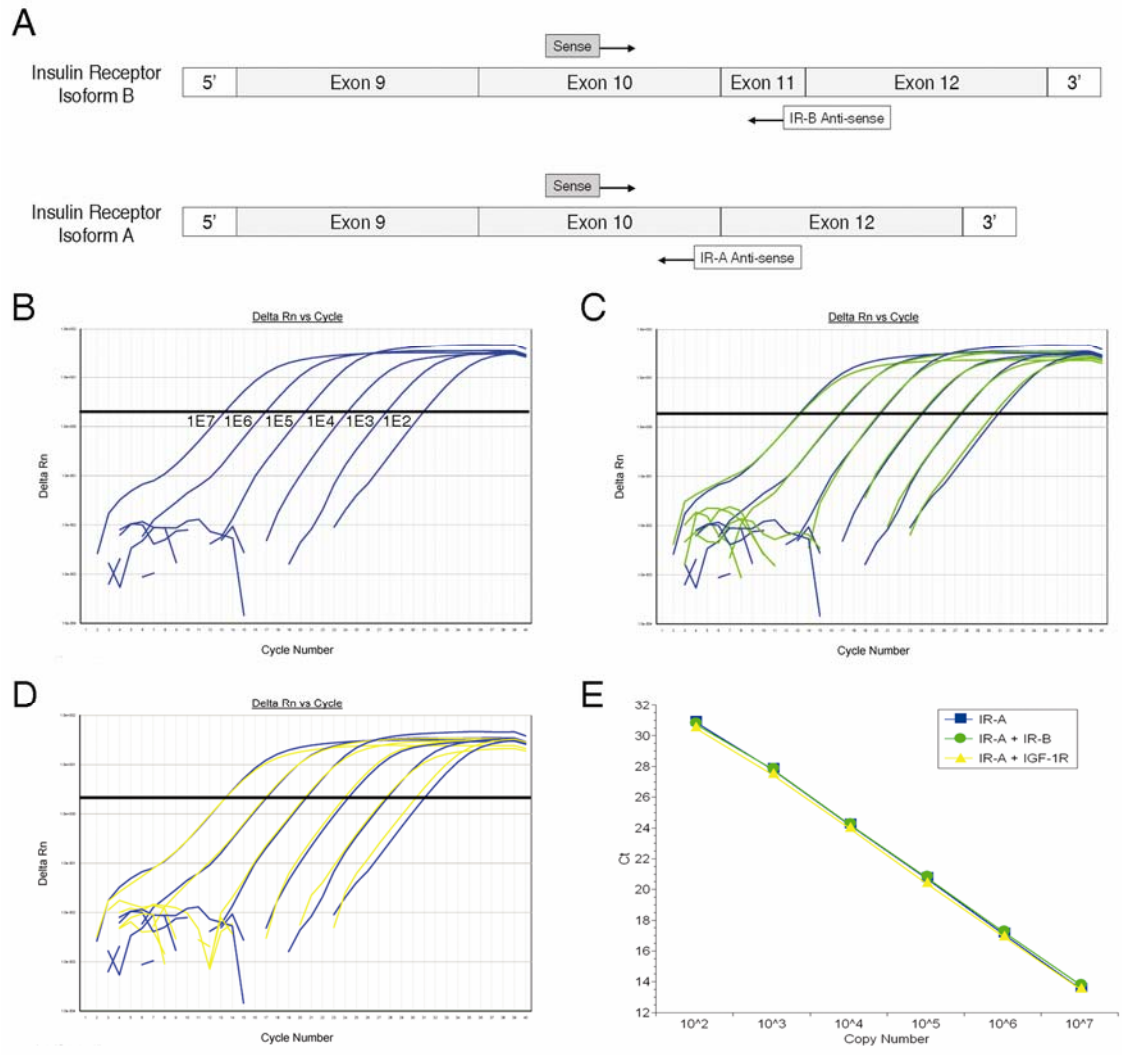
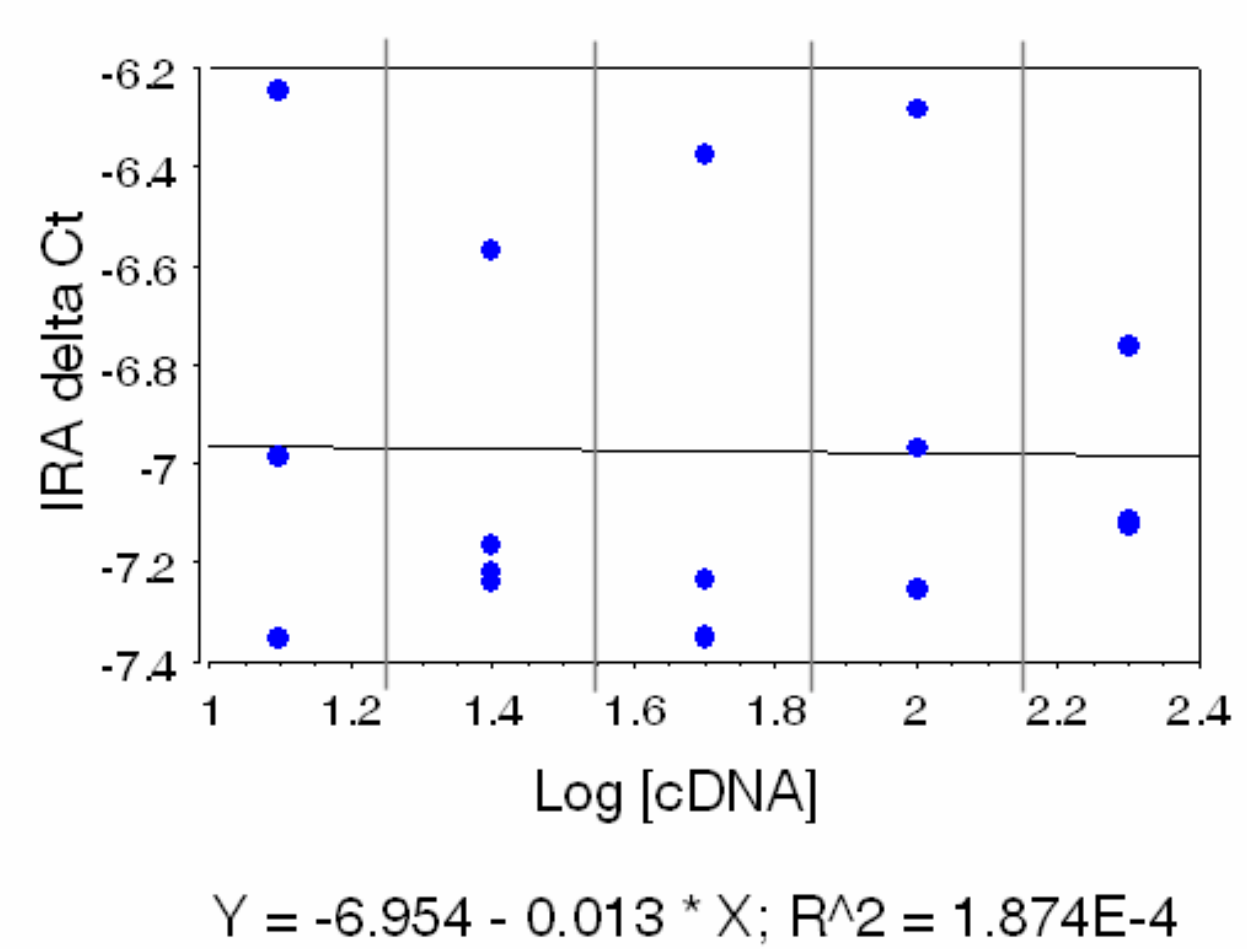


Appendix Figure 1: Insulin receptor isoform-specific Q-PCR assays. (A) Schematic representation of IR-B (exon 11^{+}) and IR-A (exon 11^{-}) primer design strategy. Both assays use a common sense primer that anneals within exon 10 and isoform-specific anti-sense primers that anneal across exon-exon junctions unique to each isoform. (B) Sample amplification plot for IR-A assay using ten-fold serial dilution of IR-A standard as template. Horizontal line indicates threshold determined by Applied Biosystems Sequence Detection Systems Software. Numbers to the left of each amplification curve indicate amount of standard used as template in each reaction. (C, D) Competition assays. Amplification plot shown in (B, blue) overlaid with amplification plot using IR-A standards plus 10^7 copies of IR-B (C, green) or of IGF-1R (D, yellow) standard at each data point. Graphs in (B-D) were generated using Applied Biosystems Sequence Detection Systems Software. (E) IR-A standard curves from data generated in (B-D). Plots demonstrate C_t value v. IR-A copy number. ■, blue: data generated in (B); ●, green: data generated in (C); ▲, yellow: data generated in (D).



Appendix Figure 2: IR-A primers are suitable for the comparative Ct method of quantitation. Two-fold serial dilutions of Calibrator cDNA (200 ng- 12.5 ng) were analyzed by Q-PCR for IR-A and β-actin amplification. For each blue data point, the log [cDNA dilution] was plotted against the ΔC_t value (average C_t β-actin – average C_t IR-A, from three replicates). Data at each cDNA concentration are from four independent serial dilutions of cDNA. Vertical lines have been added to separate data at each concentration. A linear regression (horizontal line, equation below graph) was fit to data. The absolute value of the slope of the regression must be <0.1 for the comparative Ct method to be valid. As shown, this value for IR-A is 0.013.