Anole IIS qPCR

qPCR Analysis of Brown Anole IGF1, IGF2, and EEF2

This protocol was developed by Abby Beatty in the TS Schwartz Lab for the quantification of gene expression of igf1 and igf2 in brown anoles. This protocol will be published with the manuscript: "Gene expression of the IGF hormones and IGF binding proteins across time and tissues in a model reptile" by Beatty and Schwartz (2020). Please use this paper to cite this protocol. Anoles have the most diverse igf1 sequences we have found in reptiles (McGaugh et al 2015; Yates et al In Prep), but with primer and probe modifications this protocol also being used in green anoles and could apply to other species. In this protocol we describe the lab work going from RNA isolation from a tissue to collection of the qPCR data. Scripts for data analysis will also be available as supplemental information. Time estimates for the lab procedures are assuming an experienced researcher

CONTENT AND ESTIMATED TIME COMMITTMENT

PART 1 **RNA Preparation**

1. RNA Isolation (1.5 hours / 24 samples)
2. Quantification (1 hour)
3. Standardization (1 hour/ 96 samples)
4. cDNA Synthesis (1.5 hours)
5. cDNA Verification (1.5 hours (Taq Dependent))

PART 2 **Primer and Probe Design** (30 minutes/gene for design)

Shipping from IDT can take 3-5 BD

PART 3 **Standard Curve Preparation**

1. Linearization of Plasmids Containing Genes
2. Determination of Copy Number
3. Creation of Pool Master Stock
4. Created 1/10 Dilution Standard Curve

PART 4 **Multiplex qPCR Analysis** (30 minutes setup/ 2 plates and 1.5 hours run/plate)

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Part 1: RNA Preparation

OBJECTIVE

RNA will be extracted from each tissue sample. The RNAwill then be used in a cDNA synthesis reaction to produce template for qPCR amplification.

MATERIALS/KITS

RNA Isolation: GE Illustra RNAspin Kit (Cat: 25-0500-70)
Manufactures link to protocol: https://cdn.gelifesciences.com/dmm3bwsv3/AssetStream.aspx?mediaformatid=10061&destinationid=10016&assetid=14733

Proteinase K: Qiagen (Cat: 19131)
Manufactures link to protocol: https://www.promega.com/-/media/files/resources/protocols/technical-manuals/101/quantifluor-rna-system-protocol.pdf

** Any proteinase K at 20mg/mL will work

RNA Quantification: Promega Quantifluor RNA System (Cat: E3310)
Manufactures link to protocol: https://www.promega.com/Products/Quantitation-and-Analysis/dna-and-rna-quantitation/QuantiFluor-RNA-System?catNum=E3310
Lysis tubes: VWR (Cat: 16466-058)
Lysis Beads: Qiagen (Cat: 69989)
96 well plates: VWR (Cat: 211-0262)
Foil Plate Seal: VWR (Cat: 60941-074)
Beta-mercaptoethanol: JT-Baker (Cat: 4049-00)
TissueLyser II: Qiagen (Cat: 85-300)
Plate Reader: BioTek Cytation 3

FIGURES

Standard	Volume of RNA Standard	Volume of 1X TE Buffer	Concentration (ng/µl)
A	50µl	50µl	50
B	50µl of Standard A	50µl	25
C	50µl of Standard B	50µl	12.5
D	50µl of Standard C	50µl	6.25
E	50µl of Standard D	50µl	3.13
F	50µl of Standard E	50µl	1.56
G	50µl of Standard F	50µl	0.78

Figure 1: RNA Quantification Standard Curve Dilutions

PROCEDURES

RNA Isolation: Following manufactures protocol except when (*) is present

1. In a screw cap tube combine: 350 µL of kit lysis Buffer, 3.5 µL of beta-mercaptoethanol, and a bead. Lyse in TissueLyser at 30Hz, for 3 minutes.
2. (*)**add 590µL H2O and 10µL of Proteinase K and shake at 55 °C for 10 minutes**
3. Place homogenized tissue in a RNA Mini Filter (kit) & centirifuge at 11,000G for 1 minute.
4. Without disturbing the pellet, transfer the supernatant with RNA to a new tube
5. Add 350 µL of 70% ethanol and mix by vortexing
6. Pipet the mixture 2-3 times, and load lysate into a RNA Mini column
7. Centrifuge at 8,000G for 30 seconds
8. Transfer the column containing RNA to a new collection tube
9. Add 350 µL of desalting buffer to the spin column
10. Centrifuge at 11,000G for 1 minute and discard the flow through
11. Centrifuge for an additional 30 seconds to remove residual salt.
12. In a seperate 2 mL tube, for each RNA extraction being performed, add 10 µL of DNase to 90 µL of DNA Reaction Buffer and mix by flicking
13. Add 95 µL of the mixture directly to the filter and incubate for 15 minutes at room temperature.
14. Add 200uL of WB1 and spin at 11,000G for 1 minute
15. Add 600uL of WBII and spin at 11,000G for 1 minute
16. Add 250uL of WBII and spin at 11,000G for 2 minutes
17. To dry membrane, spin again for 1 minute
18. Put column in a clean 1.5mL tube
19. Elute with 100uL of sterile water (kit) and incubate for five minutes at room temp.
20. Spin at 11,000G for 1 minute
21. Store at -80°C or place on ice for use immediately

RNA Quantification and Standardization:

1. Warm all assay components to room temperature for use. Components will freeze at 4 degrees
2. Dilute 20X TE Buffer (kit) to 1X with Nuclease free water Needed: ~40mL (2mL of TE and 38mL of Water per plate)
3. Dilute Quantifluor RNA Dye 1:400 in 1X TE Buffer to make working solution
4. Prepare the standard curve materials: See Figure 1.
5. Pippet 200uL of RNA dye working solution into each well of 96 well plate.
6. Add 10uL of standards to each well
7. Take aliquot of RNA extractions and dilute 1:5 in water . Add 1-20uL of diluted sample to remaining wells. We use 1uL for our analyses.
8. Mix plate by pipetting thoroughly
9. Protect from light and incubate at room temp for 5 minutes
10. Measure at 492nm/540nm using a plate reader
11. Quantify using the Quantifluor Excel Spreadsheet. This can be downloaded here: https://www.promega.com/resources/tools/quantifluor-dye-systems-data-analysis-workbook

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Part 1 (cont.): cDNA Synthesis

OBJECTIVE

RNA that has been isolated from tissue samples will be standardized based on concentration. The cDNA reaction assumes that for each RNA template, one cDNA strand is produced. By standardizing at this step, the starting amount of template to the qPCR reaction is standardized across samples and treatments. cDNA will be produced and verified for use as a template in qPCR amplification.

MATERIALS/KITS

cDNA Synthesis: QuantBio qScript XLT cDNA Synthesis Kit (Cat: 95161-025)
Manufactures link to protocol: https://www.quantabio.com/media/contenttype/IFU-024.1_REV_02_95047_qScript_cDNA_Synthesis _Kit.pdf

96 well plates: VWR (Cat: 211-0262)
Foil Plate Seal: VWR (Cat: 60941-074)

PROCEDURES

RNA Standardization

1. Haphazardly randomize your samples to locations on a 96 well plate.
2. Dilute each RNA sample to 100ng/µL with DEPC treated water in the appropriate randomized location.

cDNA Synthesis:

1. In a separate 96 well plate, use a multichannel pipet to add 15µL of DEPC treated water to each well that will contain a sample.
2. Using the multichannel, transfer 1uL of the standardized RNA samples to their corresponding well on the new cDNA plate.
3. Add 4µL of the SuperScript XLT cDNA Supermix to each well.
4. Briefly centrifuge plate to collect contents at the bottom of the wells.
5. Seal the plate with a foil cover.
6. Incubate in a thermocycler following this protocol:

   5 minutes at 25ºC
   60 minutes at 42ºC*
   5 minutes at 85ºC
   Hold at 4ºC

6. Once the incubation protocol has concluded, maintaining your randomized plate order, make a new plate of working dilution samples diluted 1:100 in a total volume large enough to complete your analyses assuming 10µL of each sample per run (3µL of sample in triplicates). This is the sample format that will be used in qPCR analysis. Store undiluted stock cDNA at -20°C. Alternative dilutions may be recommended dependent on varying expression level of genes due to treatment and age.

Verification of cDNA production:

1. Using any set of previously validated primers, run a PCR reaction with a standard Taq to ensure cDNA was produced successfully.
   a. IGF2, and EEF2 primers are recommended.
   b. Expected results using EEF2 primers can be seen to the left (Figure 2).

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Part 2: Primer and Probe Design

OBJECTIVE

Design primer and probe pairs that can be used to amplify IGF1, IGF2, and EEF2 as as housekeeping gene in the brown anole through qPCR. Primers should be between 100 and 130 base pairs, create minimal primer dimer, and work efficiently at an annealing temperature of 60 °C.

MATERIALS/KITS

EEF2 Accession: NM_001961
IGF1 Accession: NM_008110526.2
IGF2 Accession: NM_009107927.2

Brown anole gene sequences can be found in FASTA format in Appendix A.

Geneious software 11.0.5 was used for all design purposes.

Primers are ordered through IDT as Custom DNA oligos, using the default settings.

Probes dyes are selected using the IDT PrimeTime Multiplex Dyes Selection Tool and ordered under the default settings. These may need adjusted for other machines.

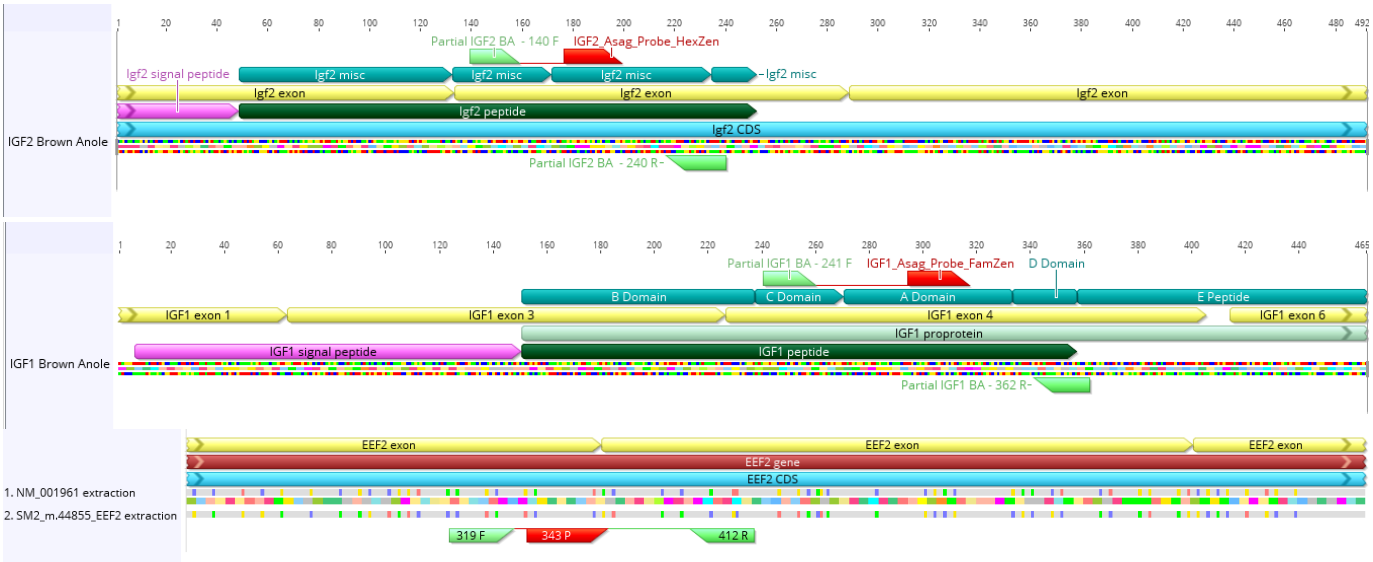
IDT Multiplex Tool: https://www.idtdna.com/site/order/qpcr/primetimeprobes/multiplex

PROCEDURES

Primer and Probe Design

1. The Green Anole IGF1, IGF2, and EEF2 genes were downloaded from NCBI. Accession numbers can be found on the right.
2. The genes were then blasted against brown anole transcriptomic data (McGaugh et al., 2015) to isolate the brown anole sequences.
3. Geneious was then used to design primer probe pairs for each gene.

Primers can be seen in green below, while probes are annotated in red. Alignments of primers and probes to brown anole gene can be seen at the bottom of the page.



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Part 3: Standard Curve Preparation

OBJECTIVE

See Appendix B for editable excel sheet to complete these calculations.

Previously cloned or ordered target genes are in a plasmid. Plasmid DNA needs to be linearized in order to amplify properly. Once linearized, the templates are mixed to make a standard stock pool containing all three genes. This is then used to create a standard dilution series. This method allows for absolute quantification rather than relative.

MATERIALS/KITS

Lambda DNA: New England Biolabs Lambda DNA (Cat: N3011S)
Link to product information: https://www.neb.com/products/n3011-lambda-dna#Product%20Information

Restriction Enzyme: New England Biolabs ApaI (Cat: R0114S)
Manufactures link to protocol: https://www.neb.com/products/r0114-apai#Product%20Information

FIGURES

Steps

1. Set up reaction as follows:

COMPONENT	50 µl REACTION
DNA	1 µg
10X CutSmart Buffer	5 µl (1X)
ApaI	1.0 µl (or 10 units)
Nuclease-free Water	to 50 µl

2. Incubate at 25°C for 5–15 minutes as ApaI is Time-Saver qualified.

Figure 3: Plasmid single digest protocol with ApaI to linearize self-cloned inserts for use as qPCR standards.

PROCEDURES

Linearization of Plasmid DNA Containing Insert:

1. Regardless of source of standard DNA (synthesized gene or self cloned), if your gene is in a plasmid, it needs to be linearized with a restriction enzyme prior to use in amplification.
2. Choose a restriction enzyme within the plasmid that is a minimum of 100bp from your insert. For our clones, this enzyme was ApaI from NEB and the entire plasmid prep was digested at 25°C for 25 minutes.
3. The digest procedure can be seen in Figure 3.

Determine the Number of Copies in Linearized Plasmid Prep:

1. Go to: https://cels.uri.edu/gsc/cndna.html
2. Enter the concentration of your linearized plasmid in ng/uL
3. Enter the length of the template DNA. This is the entire plasmid length, not the length of your insert. See appendix B for our plasmid information.
4. This will return the number of insert/plasmid (1:1 ratio) copies in each microliter of linearized plasmid DNA.

Create One Stock with Each Target at a 10^8 concentration:

1. Calculate the volume of linearized plasmid DNA stock needed to make a stock containing IGF1, IGF2, and EEF2 at a concentration of 10^8 copies per µL .
2. Bring the total volume to 1mL with DEPC treated water.

Preparation of Lambda DNA Water Stock:

1. Create a 2ng/µL stock of Lambda DNA by adding 64.52 µL of 310ng/µL stock Lambda DNA to 9.925 mL of DEPC treated water.
2. Use this water to create the standard dilution series. Lambda DNA is added at a constant concentration to protect the highly diluted standards.

Create a 1/10 Dilution Series Ranging from 10^7 to 10^1 copies

1. Setup a series of eight 2mL tubes and add 900µL of the 2ng/µL Labmda DNA to each tube. The use of water + Lambda DNA
2. To the first tube, add 100µL of the 10^8 IGF2/IGF1/EEF2 standard stock. This is standard 1.
3. Mix thoroughly, and take 100µL of standard one to add to the second tube. This is standard 2.
4. Continue through standard 7.
5. The eight tube should contain Lambda DNA with no plasmid DNA. This is the negative control.
6. Aliquot these standards in 8-strip tubes that can be used for the number of plates you plan to run in a short period of time, assuming use of 10uL per plate. This reduces freeze-thaw cycles of the standards that may effect quality and efficiency of the reaction.

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Part 4: Multiplex qPCR Amplification

OBJECTIVE

An editable excel spreadsheet for the reaction setup shown on this page are available in Appendix C.

Samples will be amplified in triplicate using qPCR taq, primer, and probe pairs to get quantitative expression data.

MATERIALS/INSTRUMENTS

PrimeTime qPCR Matermix: Integrated DNA Technologies (Cat: 1055772)
Manufactures link to protocol: https://sfvideo.blob.core.windows.net/sitefinity/docs/default-source/protocol/primetime-gene-expression-master-mix.pdf?sfvrsn=a4ef3707_8

Armadillo qPCR 96-well Plates: Thermo Scientific (Cat: AB-3496)
Link to product: https://www.thermofisher.com/order/catalog/product/AB3496

ThermaSeal RT Sealing Films: EXCEL Scientific (Cat: TS-RT2-100)
Link to product: http://www.excelscientific.com/thermalseal_content.html

qPCR Machine: BioRad CFX96 (Cat: 1855195)
Manufactures link to protocol: https://www.bio-rad.com/en-us/product/cfx96-touch-real-time-pcr-detection-system?ID=LJB1YU15

qPCR Data Analysis Software: BioRad CFX Maestro Software
Manufactures link to user guide: https://www.bio-rad.com/webroot/web/pdf/lsr/literature/10000068703.pdf

PROCEDURES

1. Combine PrimeTime Master Mix, primers, and water in a total volume large enough to run all samples (up to two plates can be prepared at one time).
    a. Add a couple reactions to account for pipetting error. Below is the template we used to complete 2 plates.

			Volume of	Reactions
Reagent	**Stock concentration**	**Final concentration**	**20**	**192**
2X IDT qPCR Mix	2	1	10	1920
IGF1: F	10	0.3	0.6	115.2
IGF1: R	10	0.3	0.6	115.2
IGF1: Probe	**10**	**0.2**	**0.4**	76.8
IGF2: F	10	0.3	0.6	115.2
IGF2: R	10	0.3	0.6	115.2
IGF2: Probe	**10**	**0.2**	**0.4**	76.8
EEF: F	10	0.3	0.6	115.2
EEF: R	10	0.3	0.6	115.2
EEF: Probe	**10**	**0.2**	**0.4**	76.8
			DNA	
Template cDNA	50 Dilution		3	
Water			2.2	422.4
NOTE: Primer vol are sufficient for a 300nM rxn conc.				**17 Aliqote**

2. Aliquot 17 µL of the mix into each well of the qPCR plate.
3. Add the standards and samples (3µL each) to the plate in triplicate.

** The use of a multichannel pipet is recommended for mastermix and sample aliquot.

WELL	1	2	3	4	5	6	7	8	9	10	11	12
				Column 1			Column 2			Column 3		
A	STD1	STD1	STD1	EDBP23	EDBP23	EDBP23	St2_2	St2_2	St2_2	01L	01L	01L
B	STD2	STD2	STD2	33L	33L	33L	286	286	286	Adult L3	Adult L3	Adult L3
C	STD3	STD3	STD3	ST_4 2	ST_4 2	ST_4 2	38L	38L	38L	67L	67L	67L
D	STD4	STD4	STD4	289	289	289	EDBP26	EDBP26	EDBP26	03L	03L	03L
E	STD5	STD5	STD5	Adult L4	Adult L4	Adult L4	EDBP15	EDBP15	EDBP15	Adult L7	Adult L7	Adult L7
F	STD6	STD6	STD6	35L	35L	35L	Adult L2	Adult L2	Adult L2	56L	56L	56L
G	STD7	STD7	STD7	EDBP14	EDBP14	EDBP14	290	290	290	08L	08L	08L
H	NTC	NTC	NTC	37L	37L	37L	65L	65L	65L	EDBP29	EDBP29	EDBP29

WELL	1	2	3	4	5	6	7	8	9	10	11	12
				Column 4			Column 5			Column 6		
A	STD1	STD1	STD1	EDBP39	EDBP39	EDBP39	Adult L5	Adult L5	Adult L5	02L	02L	02L
B	STD2	STD2	STD2	54L	54L	54L	60L	60L	60L	St_2 1	St_2 1	St_2 1
C	STD3	STD3	STD3	50L	50L	50L	EDBP24	EDBP24	EDBP24	27L	27L	27L
D	STD4	STD4	STD4	St_4 1	St_4 1	St_4 1	04L	04L	04L	Adult L1	Adult L1	Adult L1
E	STD5	STD5	STD5	293	293	293	Adult L8	Adult L8	Adult L8			
F	STD6	STD6	STD6	EDBP43	EDBP43	EDBP43	24L	24L	24L			
G	STD7	STD7	STD7	11L	11L	11L	52L	52L	52L			
H	NTC	NTC	NTC	42L	42L	42L	Adult L6	Adult L6	Adult L6			

4. Seal the plate with a plate sealing film. Be sure it is firmly pressed to the top of the plate and that there are no fingerprints on the film.
5. Briefly centrifuge the contents to the bottom of the plate.
6. Run the following protocol, and store second plate (optional) in the fridge covered with foil to protect from light.

Step	Cycles	Temperature	Standard cycling (min:sec)
Polymerase activation	1	95°C	3:00
Amplification:	35–45		
Denaturation		95°C	0:15
Annealing/Extension*		60°C	1:00
Hold, if needed	1	4°C	Up to 24 hr

See next page for expected view of qPCR results.

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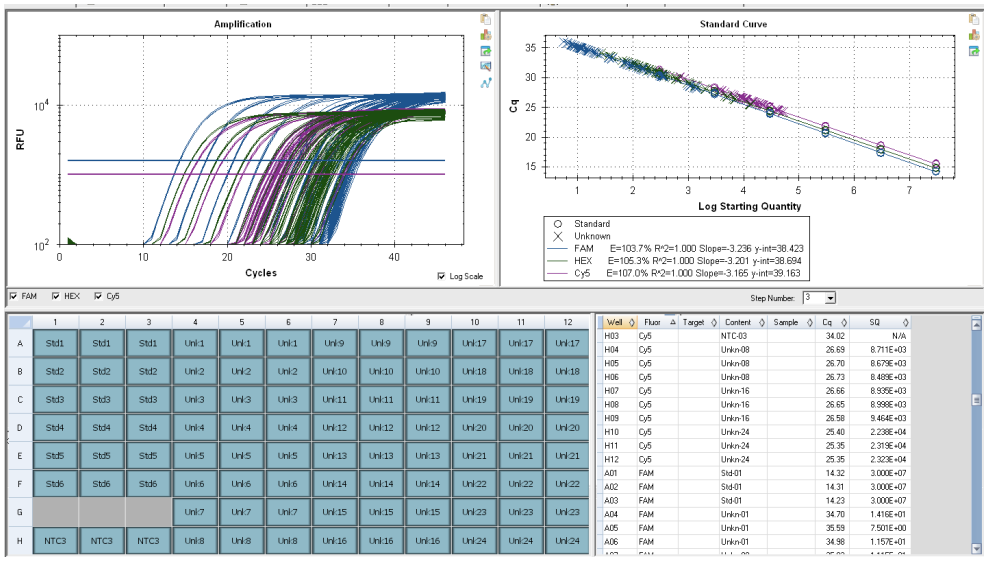
Part 4 (cont.): Multiplex qPCR Results

When the run is finished, the data will originally appear in this format. All three genes will be present with samples and standard curves. You should see three separate standard curves to the right, with an efficiency and an R2 value for each gene.

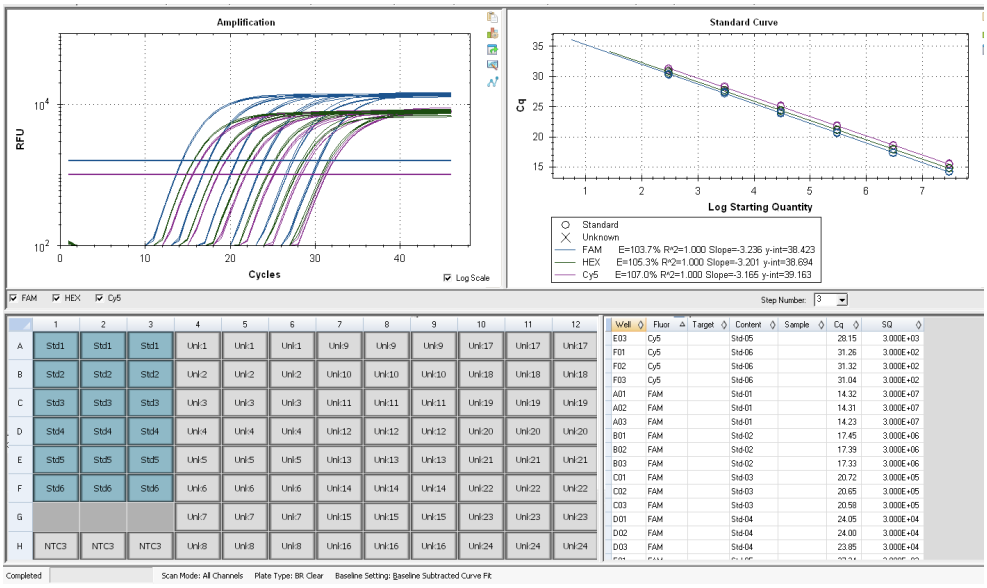
In this case IGF1 (FAM; Blue) had an efficiency of 103.7%. IGF2 (HEX; Green) had an efficiency of 105.3% and EEF2 (Cy5; Purple) had an efficiency of 107%.

All three genes had an R2 of 1.00.



This can be better seen on the bottom image, where only the standard curve is displayed. Without the data points, it is easy to see the how the standard curve is formed.

Note: The seventh standard was removed from the data set. The extremely low copy number introduced a large amount of error.



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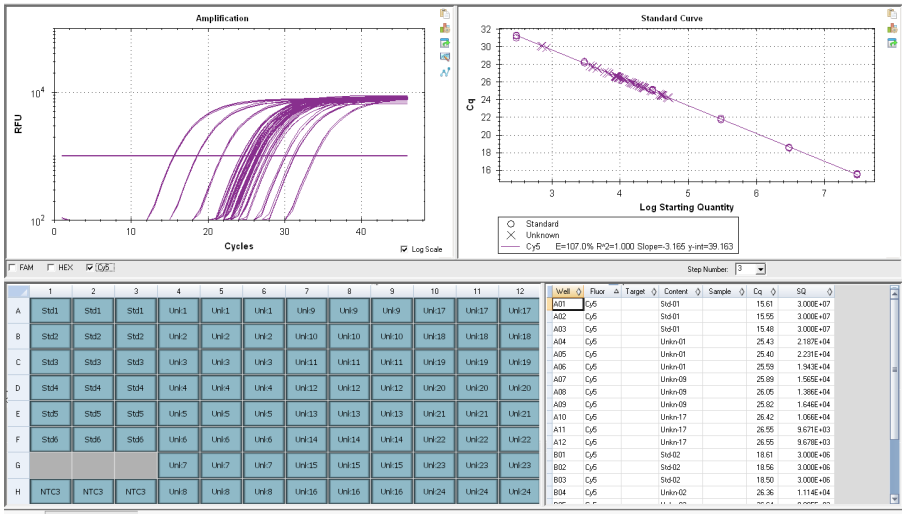
Part 4 (cont.): Multiplex qPCR Results

Each gene can be seen below separately.

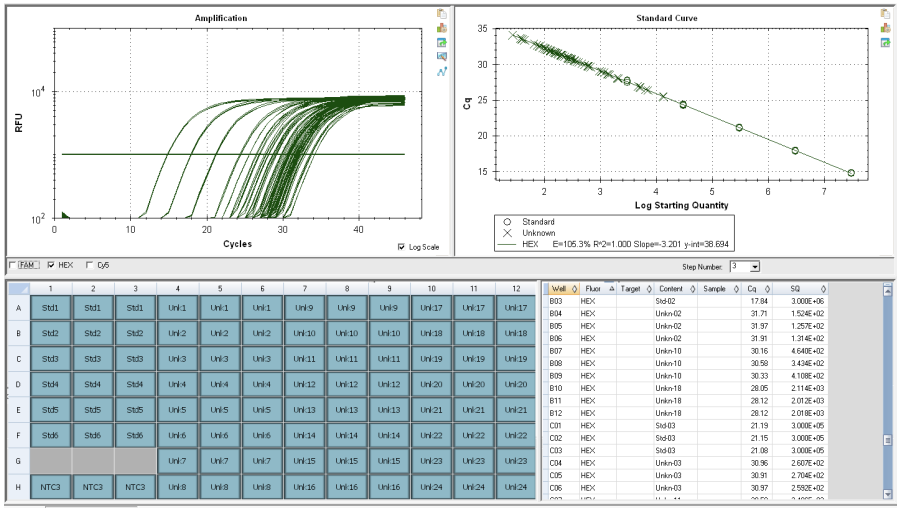
Note: Adjusting the cDNA concentration may be benifical depending on project. Due to the wide range of expression between age groups, we tried multiple dilutions, including 1:50, 1:100, and IGF1 at 1:2 and undiluted cDNA concentraions. Regardless of dilution factor, quanitifcation value relationships did not change. For simplicity we chose to keep dilution factors consistant at 1:100.

Ideally, samples would have come up between the 3rd and 5th standard. While many of the samples displayed this pattern, some juvenile and embryonic samples expressed IGF1 at such a low level, that it could not be accomplished with our study design.

EEF2



IGF2



IGF1