Quantitative Real-Time RT-PCR

A Very Short Course

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I. Assay Development

- A. Sequence selection
- B. Primer & Probe Selection
- C. Quencher dye and internal reference
- D. Assay Validation

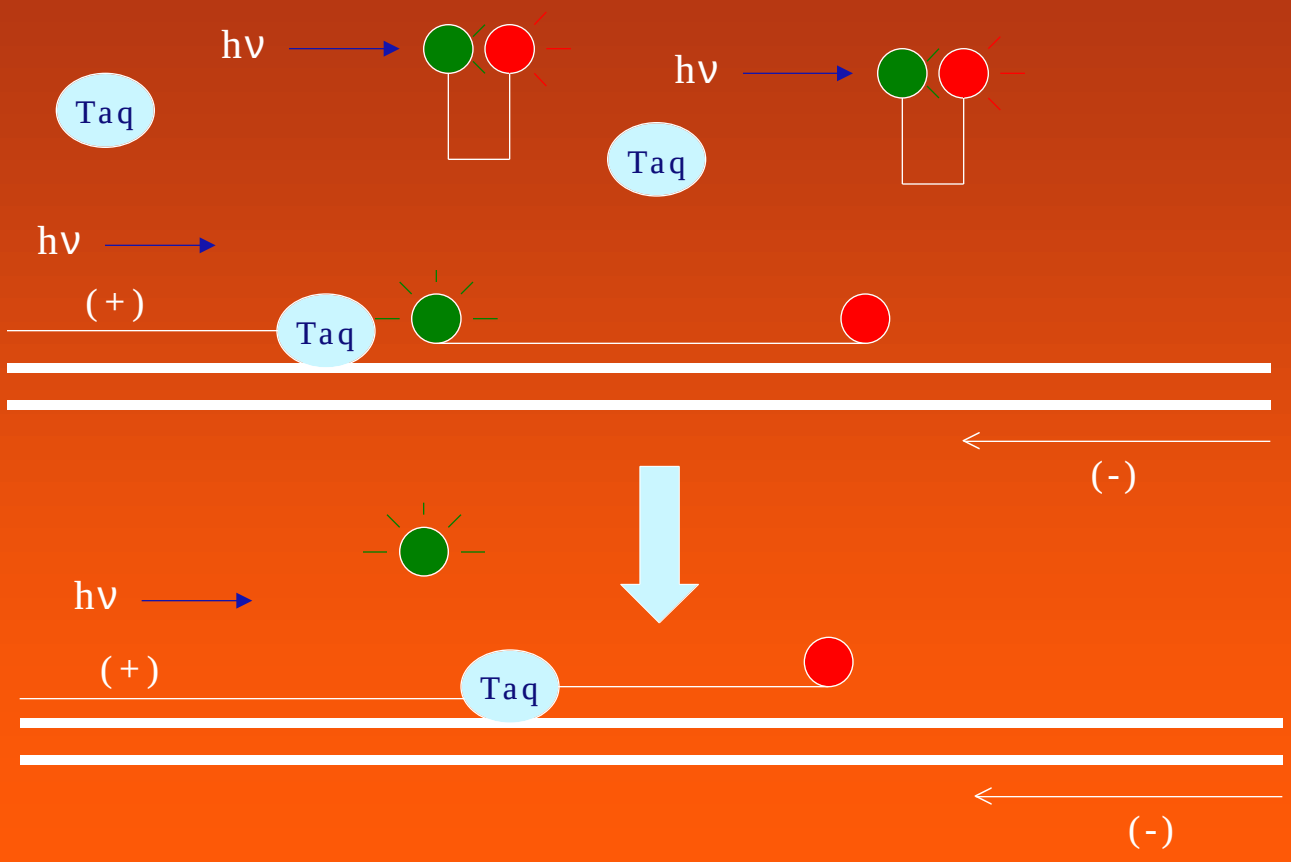
II. Assay Setup

- A. One- vs Two-Step RT-PCR
- B. Plate Setups for Robotics
- C. Thermocycler settings

III. Data Analysis

- A. Baseline and threshold settings
- B. Standard Curves
- C. Inter- vs intra-assay variability
- D. Sample normalization

Taq Exonuclease Reaction



Sequence Selection

The most important component of building an assay

Make sure the initial sequence is the correct one

Use Blast at the NCBI to find other sequences
(http://www.ncbi.nlm.nih.gov/)

Download all the unique sequences you find, including other species (human, mouse, rat)

Look particularly for the NM_ or refseqs

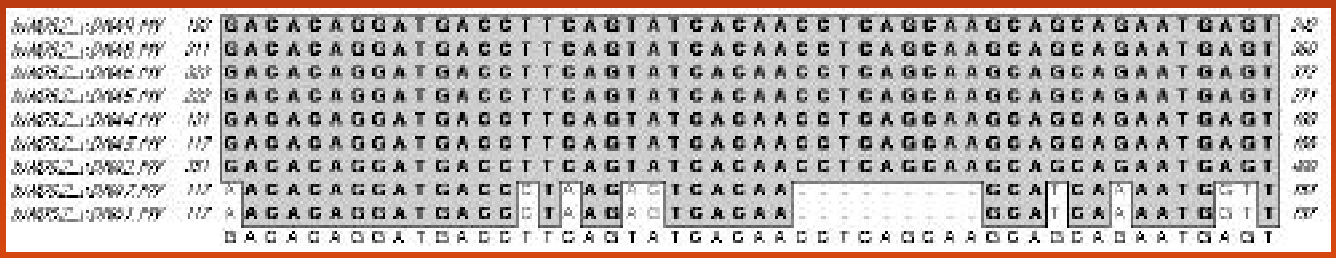
Sequence Comparison

Align the sequences by species
- Look for single base errors, insertions and deletions

Choose a ‘type’ sequence for each species
- Compare potential SNPs with SNP data in the refseq file header

Align the ‘type’ sequence for each species
- Look for potential splice variants in one species not reported in the one of interest

Splice Variants



Primer & Probe Selection

Available Software for Designing Taqman® Assays

Primer Express®, Applied Biosystems

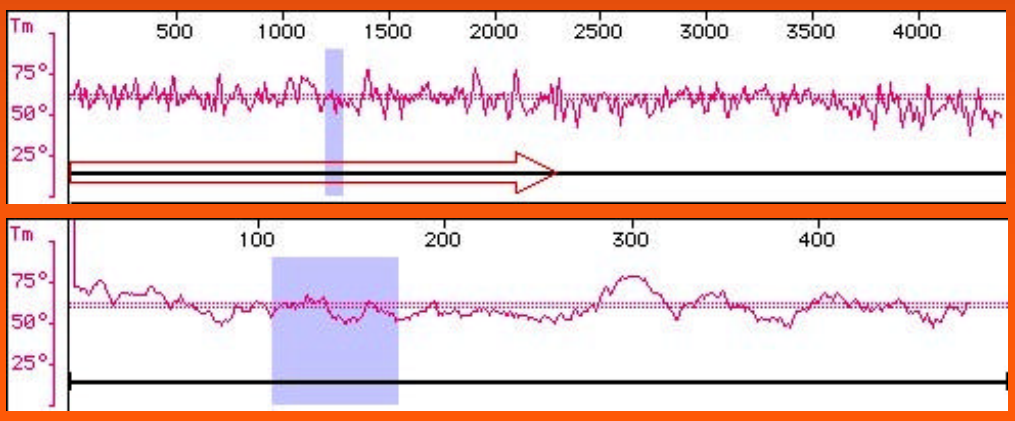
Primer3 from The Whitehead Institute @ MIT

http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi

Using Primer Express®, Personal Tricks

1- For sequences over 1 Kb, use a 500 bp subsequence

2- Choose the area based on a region with an even Tm content from the 'Map' tab



Primer & Probe Selection

Using Primer Express®, Personal Tricks

3- Under the 'Rxn Cond' tab, set the primer concentrations to 200 nM from 50 nM
4- Under the 'Params' tab, set the G/C clamp to 1 from 0
5- Keep the %G/C content of the primers and probe as close as possible during selection
6- Match the Tms of primers & probe as a set
eg., 59˚, 59˚, 69˚C
7- Keep the total number of G&Cs between 2-3 in the last 5 bases of the 3' end of the primers
8- Probes should not start with a G
9- Avoid probes with more than 3-Gs in a row or those with much higher G than C content, make the reverse probe.
10- Select 3-4 primers around a probe to test
11- Check the sequence alignment to make sure the assay is in an acceptable region

Reporter, Quencher and Internal Reference Dyes

1- The classical reporter dye is 6-FAM (fluorescein)
2- Other reporters used for multiplexing are Joe and Vic.
3- Some other real-time machines, such as the Stratagene Mx4000, can use red dyes as reporters as well
4- The classic quencher dye has been TAMRA (rhodomine)
5- Newer quenchers are the dark dyes, DABYCL and the black hole quenchers (Biosearch Technologies)
6- TAMRA-quenched probes do not require a reference dye; they can use the TAMRA itself
7- Single probe reactions quenched by dark dyes should use an internal reference dye, classically ROX (dark red)
8- Multiplex reactions usually use dark quenchers and ROX

Assay Validation

1- Test primer pairs in all combinations with the probe with a known template, eg., plasmid clone, sDNA, RNA
2- Use standard assay conditions: 300-400 nM primers; 100 nM probe, 3 mM $MgCl_2$
3- Choose the primer pair that gives the highest deltaRn and the lowest Ct
4- Make a dilution of a template, either sDNA, sRNA or total RNA for a standard curve
5- If the slope of the standard curve of the best primer pair is around -3.5 increase the $MgCl_2$ to 5 mM
6- If the slope is higher than -3.6, find another primer
7- An ideal assay will have a slope of -3.3

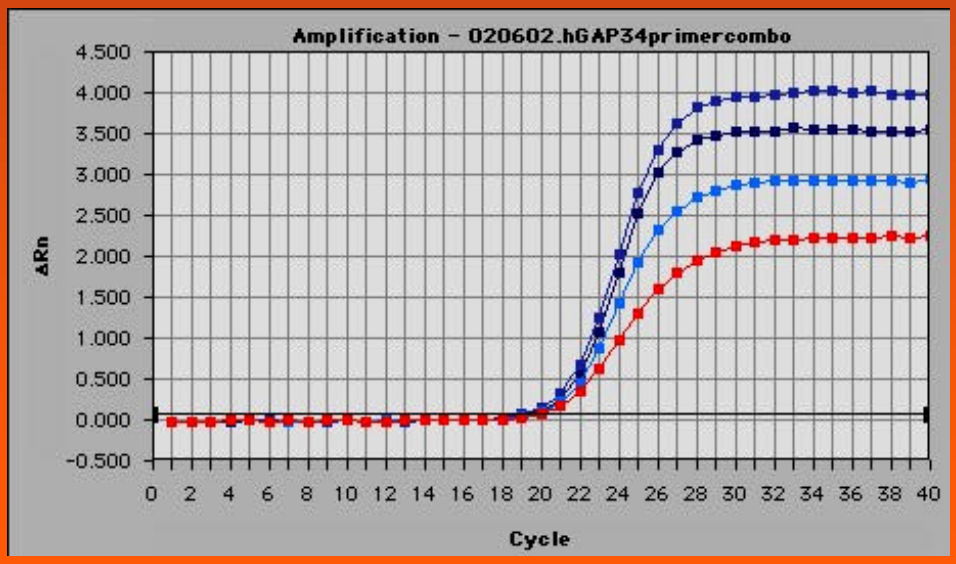
Primer Combinations - hGAP43

520(+)-CACTAAAGCTTCCACTGATAACTCG + 591(-)-TGTTTAGGCTCCTCCTTGGC — 71

521(+)-ACTAAAGCTTCCACTGATAACTCGC + 591(-)-TGTTTAGGCTCCTCCTTGGC — 70

520(+)-CACTAAAGCTTCCACTGATAACTCG + 593(-)-CTTGTTTAGGCTCCTCCTTGG — 73

521(+)-ACTAAAGCTTCCACTGATAACTCGC + 593(-)-CTTGTTTAGGCTCCTCCTTGG — 72



One vs Two-Step RT-PCR

Early experiments comparing SSII RT and Taq Gold in one- and two-step RT-PCR showed two-step reactions were superior in detecting low abundance transcripts

New one-step kits may give better results

One-step method
- Easier and faster to setup
- May be best method when only a few assays are run repeatedly
- Costs are higher (kits) and sensitivity still an issue

Two-step method
- Requires an second thermocycler for RT reactions
- More time, RT and PCR master mixes made separately
- Less costly, not dependant on kits
- Greater sensitivity, best when new assays with unknown abundance transcripts are run often

Sample Layout for Robotics

20 Unknowns + Std Curve

	1	2	3	4	5	6	7	8	9	10	11	12
A	STND Std #1 2.0e+07	STND Std #1 2.0e+07	STND Std #2 2.0e+06	STND Std #2 2.0e+06	STND Std #2 2.0e+06	STND Std #3 2.0e+05	STND Std #3 2.0e+05	STND Std #3 2.0e+05	STND Std #4 2.0e+04	STND Std #4 2.0e+04	STND Std #4 2.0e+04	NTC A12
B	STND Std #5 2.0e+03	STND Std #5 2.0e+03	STND Std #5 2.0e+03	NTC B4	UNKN Smpl #7 2.6e+05	UNKN Smpl #7 2.1e+05	UNKN Smpl #7 1.8e+05	NAC Smpl #7	UNKN Smpl #14 2.2e+05	UNKN Smpl #14 2.9e+05	UNKN Smpl #14 1.8e+05	NAC Smpl #14
C	UNKN Smpl #1 2.7e+05	UNKN Smpl #1 2.1e+05	UNKN Smpl #1 1.9e+05	NAC Smpl #1	UNKN Smpl #8 2.7e+05	UNKN Smpl #8 2.0e+05	UNKN Smpl #8 2.3e+05	NAC Smpl #8	UNKN Smpl #15 2.1e+05	UNKN Smpl #15 2.2e+05	UNKN Smpl #15 1.7e+05	NAC Smpl #15
D	UNKN Smpl #2 2.3e+05	UNKN Smpl #2 2.1e+05	UNKN Smpl #2 1.9e+05	NAC Smpl #2	UNKN Smpl #9 2.2e+05	UNKN Smpl #9 2.3e+05	UNKN Smpl #9 2.4e+05	NAC Smpl #9	UNKN Smpl #16 2.3e+05	UNKN Smpl #16 2.2e+05	UNKN Smpl #16 2.1e+05	NAC Smpl #16
E	UNKN Smpl #3 2.9e+05	UNKN Smpl #3 2.2e+05	UNKN Smpl #3 2.4e+05	NAC Smpl #3	UNKN Smpl #10 2.6e+05	UNKN Smpl #10 2.8e+05	UNKN Smpl #10 1.8e+05	NAC Smpl #10	UNKN Smpl #17 2.5e+05	UNKN Smpl #17 2.1e+05	UNKN Smpl #17 2.1e+05	NAC Smpl #17
F	UNKN Smpl #4 2.2e+05	UNKN Smpl #4 2.3e+05	UNKN Smpl #4 2.4e+05	NAC Smpl #4	UNKN Smpl #11 2.2e+05	UNKN Smpl #11 1.9e+05	UNKN Smpl #11 2.8e+05	NAC Smpl #11	UNKN Smpl #18 2.2e+05	UNKN Smpl #18 2.3e+05	UNKN Smpl #18 2.1e+05	NAC Smpl #18
G	UNKN Smpl #5 3.0e+05	UNKN Smpl #5 2.5e+05	UNKN Smpl #5 2.7e+05	NAC Smpl #5	UNKN Smpl #12 2.5e+05	UNKN Smpl #12 2.1e+05	UNKN Smpl #12 2.9e+05	NAC Smpl #12	UNKN Smpl #19 2.8e+05	UNKN Smpl #19 2.0e+05	UNKN Smpl #19 2.5e+05	NAC Smpl #19
H	UNKN Smpl #6 3.2e+05	UNKN Smpl #6 2.7e+05	UNKN Smpl #6 2.0e+05	NAC Smpl #6	UNKN Smpl #13 2.9e+05	UNKN Smpl #13 2.3e+05	UNKN Smpl #13 2.2e+05	NAC Smpl #13	UNKN Smpl #20 2.3e+05	UNKN Smpl #20 2.3e+05	UNKN Smpl #20 2.7e+05	NAC Smpl #20

Sample Layout for Robotics

21 Unknowns + Std Curve

	1	2	3	4	5	6	7	8	9	10	11	12
A	STND Std #1 2.0e+08	STND Std #1 2.0e+08	STND Std #2 2.0e+07	STND Std #2 2.0e+07	STND Std #3 2.0e+06	STND Std #3 2.0e+06	STND Std #4 2.0e+05	STND Std #4 2.0e+05	STND Std #5 2.0e+04	STND Std #5 2.0e+04	STND Std #5 2.0e+04	NTC A12
B	UNKN Smpl #21 4.3e+04	UNKN Smpl #21 3.7e+04	UNKN Smpl #21 3.4e+04	NAC Smpl #21	UNKN Smpl #7 3.2e+04	UNKN Smpl #7 3.8e+04	UNKN Smpl #7 2.3e+04	NAC Smpl #7	UNKN Smpl #14 2.4e+04	UNKN Smpl #14 2.7e+04	UNKN Smpl #14 3.1e+04	NAC Smpl #14
C	UNKN Smpl #1 5.3e+04	UNKN Smpl #1 5.7e+04	UNKN Smpl #1 3.9e+04	NAC Smpl #1	UNKN Smpl #8 3.3e+04	UNKN Smpl #8 2.9e+04	UNKN Smpl #8 3.2e+04	NAC Smpl #8	UNKN Smpl #15 4.4e+04	UNKN Smpl #15 4.0e+04	UNKN Smpl #15 3.5e+04	NAC Smpl #15
D	UNKN Smpl #2 2.4e+04	UNKN Smpl #2 2.5e+04	UNKN Smpl #2 2.1e+04	NAC Smpl #2	UNKN Smpl #9 6.4e+04	UNKN Smpl #9 4.8e+04	UNKN Smpl #9 4.3e+04	NAC Smpl #9	UNKN Smpl #16 5.2e+04	UNKN Smpl #16 5.6e+04	UNKN Smpl #16 7.8e+04	NAC Smpl #16
E	UNKN Smpl #3 7.7e+04	UNKN Smpl #3 6.8e+04	UNKN Smpl #3 6.3e+04	NAC Smpl #3	UNKN Smpl #10 4.0e+04	UNKN Smpl #10 2.9e+04	UNKN Smpl #10 4.4e+04	NAC Smpl #10	UNKN Smpl #17 5.7e+04	UNKN Smpl #17 5.3e+04	UNKN Smpl #17 3.8e+04	NAC Smpl #17
F	UNKN Smpl #4 8.3e+04	UNKN Smpl #4 7.6e+04	UNKN Smpl #4 7.6e+04	NAC Smpl #4	UNKN Smpl #11 4.1e+04	UNKN Smpl #11 2.7e+04	UNKN Smpl #11 3.1e+04	NAC Smpl #11	UNKN Smpl #18 4.5e+04	UNKN Smpl #18 3.7e+04	UNKN Smpl #18 4.3e+04	NAC Smpl #18
G	UNKN Smpl #5 4.5e+04	UNKN Smpl #5 3.4e+04	UNKN Smpl #5 2.8e+04	NAC Smpl #5	UNKN Smpl #12 7.6e+04	UNKN Smpl #12 7.0e+04	UNKN Smpl #12 6.1e+04	NAC Smpl #12	UNKN Smpl #19 7.0e+04	UNKN Smpl #19 7.3e+04	UNKN Smpl #19 7.3e+04	NAC Smpl #19
H	UNKN Smpl #6 1.3e+05	UNKN Smpl #6 1.1e+05	UNKN Smpl #6 8.7e+04	NAC Smpl #6	UNKN Smpl #13 2.6e+04	UNKN Smpl #13 2.8e+04	UNKN Smpl #13 3.1e+04	NAC Smpl #13	UNKN Smpl #20 4.3e+04	UNKN Smpl #20 5.1e+04	UNKN Smpl #20 5.2e+04	NAC Smpl #20

Thermocycler Settings

Applied Biosystems SDS software defaults to one thermocycler setting to match the contents of their kits

Standard cycle is: 50˚C-2 min; 95˚C-10 min; 40 cycles of 95˚C-15 sec; 60˚C-60 sec = a full 2 hr run time
Assumes use of Amperase-like enzyme and hotstart Taq

Amperase and hotstart Taq are not necessary for RT-PCR

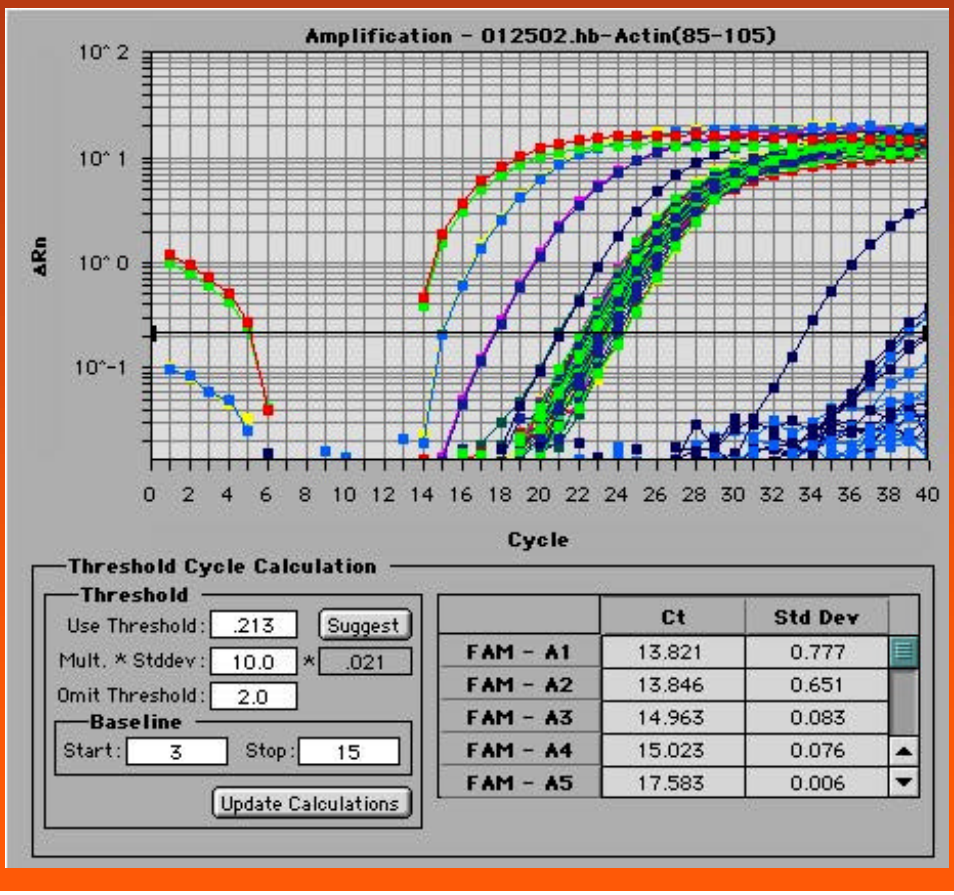
Can cut cycle time down to 95˚C-1 min and 40 cycles of 95˚C-12 sec; 60˚C-60 sec = 1 hr 36 min run time

At 95˚C- 12 sec; 60˚C-30 sec = 1 hr 15 min run time

At 94˚C-12 sec; 60˚C-20 sec = 1 hr 05 min

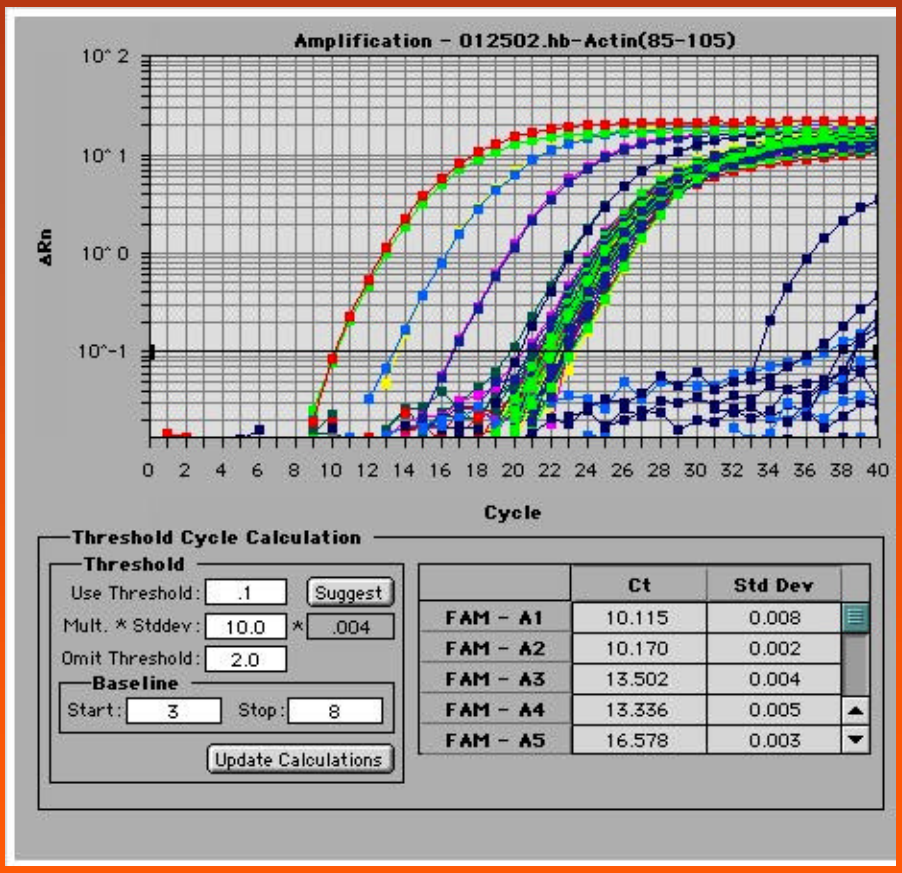
Setting the Baseline & Threshold

Pre-adjustment



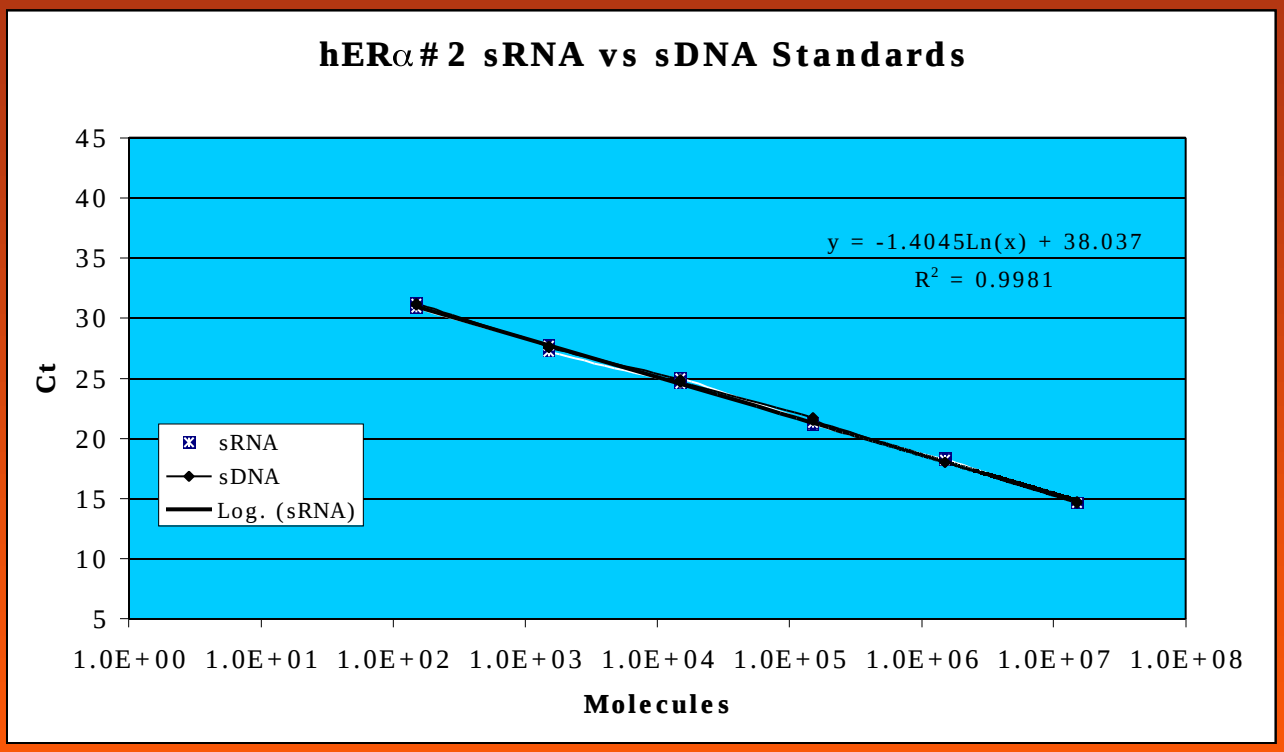
Setting the Baseline & Threshold

Post-adjustment



Final Standard Curve

sRNA vs sDNA Standards

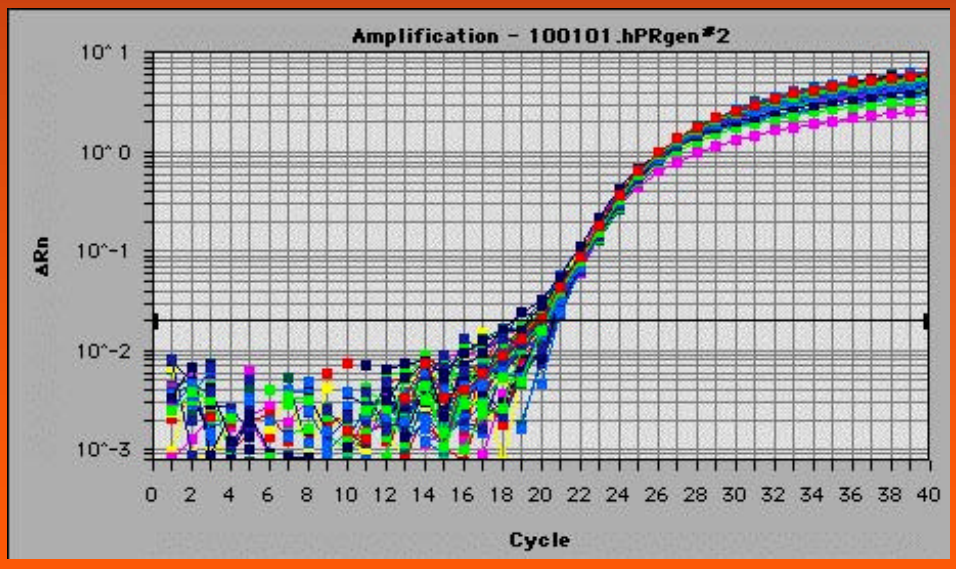


Value of unknown (mean of 3 values):
sRNA - 17477
sDNA - 18574

Intra-assay Variability

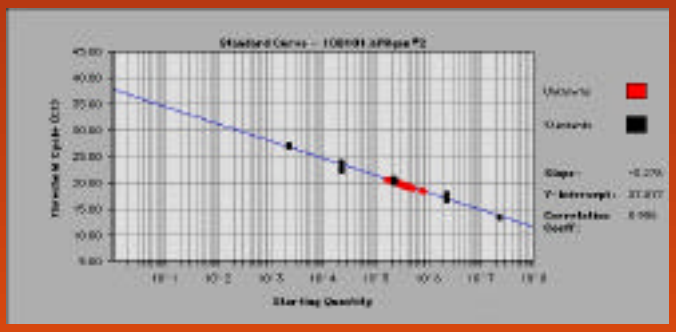
Effect of Increasing Threshold Value on PCR Efficiency

96-well plate set up robotically
60 wells with +RT master mix
All with the same RNA sample

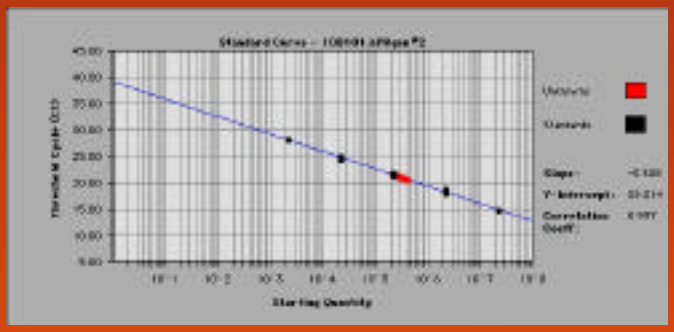


Increasing Thresholds vs Efficiency

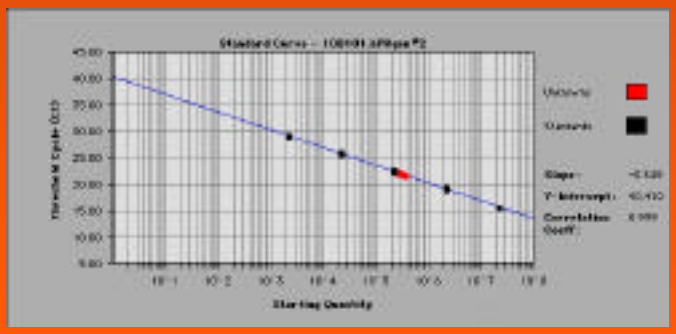
Human Progesterone Receptor A



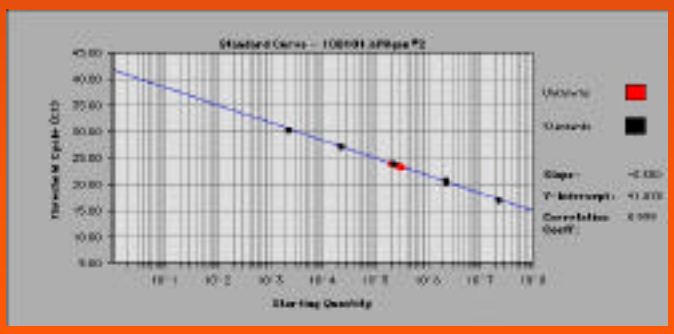
0.02 Th/%CV=24.2



0.05 Th/%CV=8.2



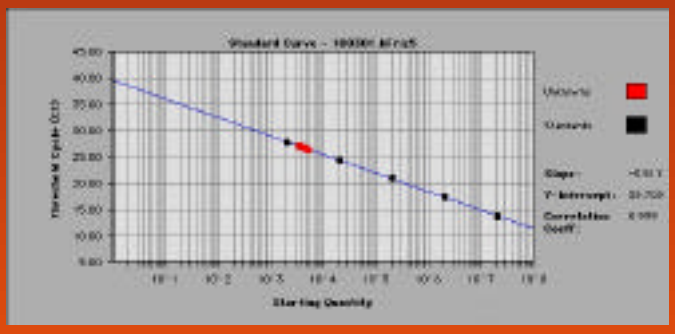
0.1 Th/%CV=7.7



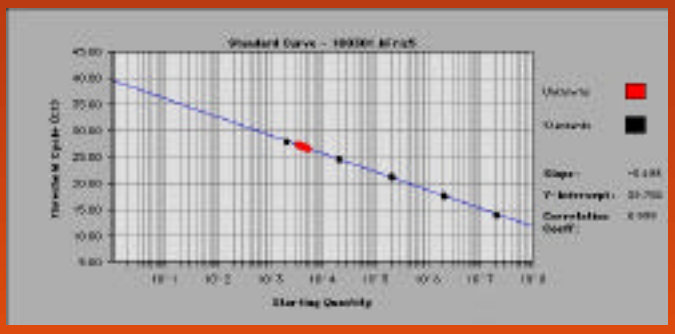
0.3 Th/%CV=7.5

Increasing Thresholds vs Efficiency

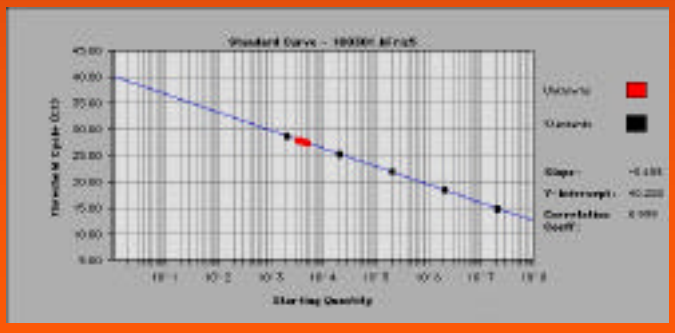
Human Frizzled 5



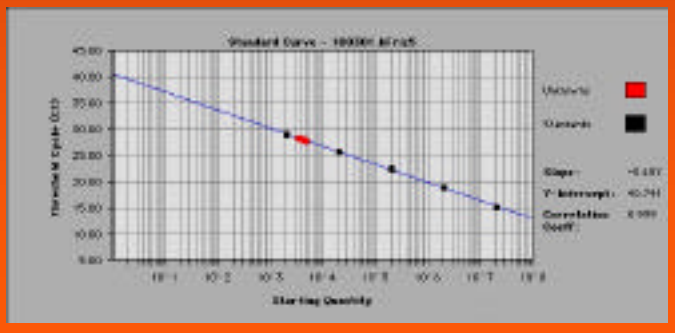
0.05 Th/%CV=7.3



0.1 Th/%CV=6.2



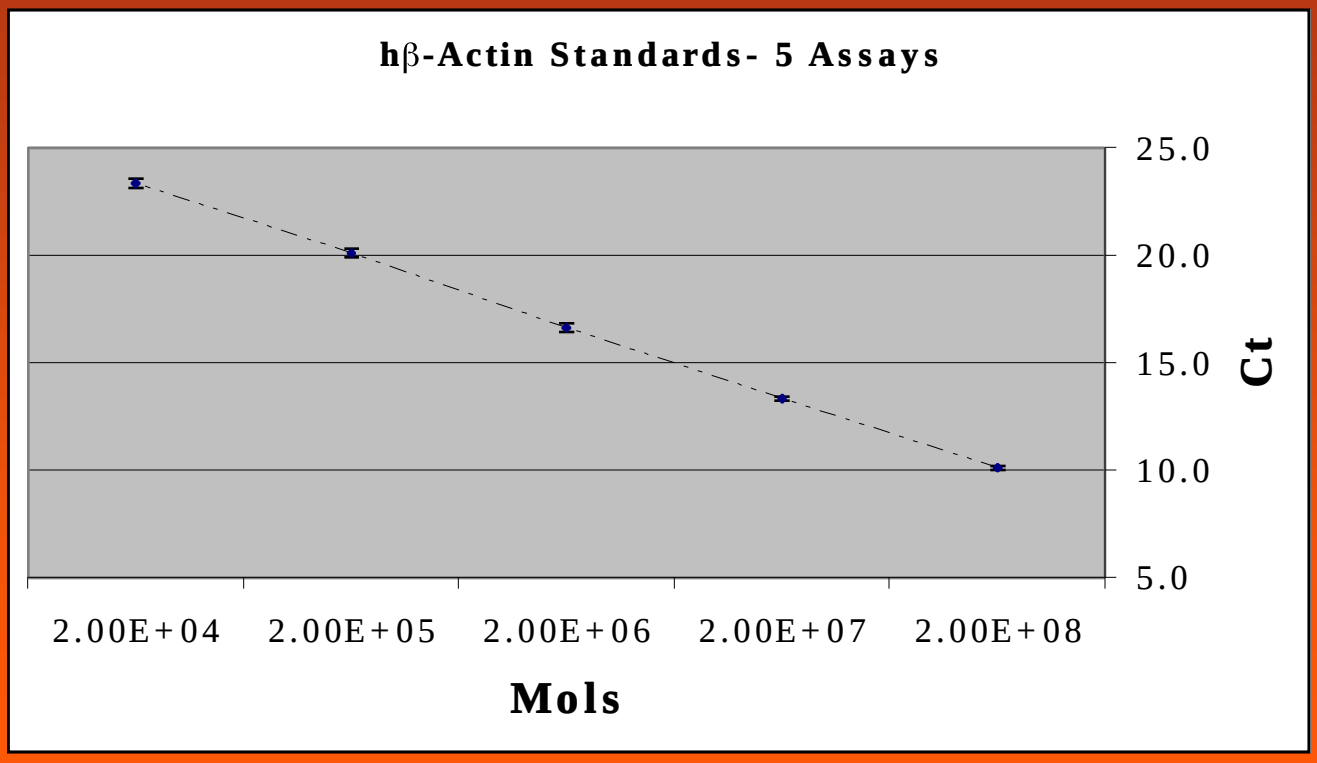
0.2 Th/%CV=7.4



0.3 Th/%CV=7.4

Inter-assay Variability

Standard Curves



$R^2 = 0.9999$

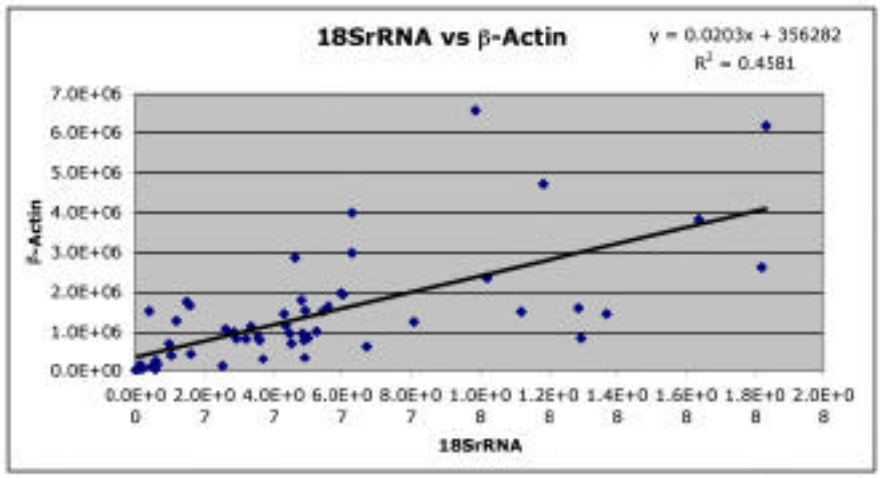
Data Normalization

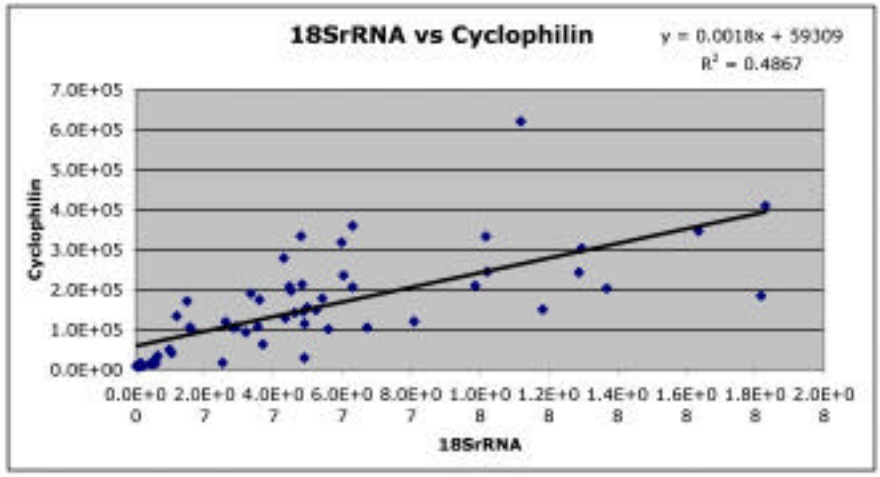
Normalizing measured values for sample load is essential for any quantiative technique

Methods used for qRT-PCR:

- Total RNA-
  - A_{260}: Very convenient but not specific
  - ribogreen: sensitive, fairly specific, costly
  - 18SrRNA qPCR: the Cadillac, cost
- Housekeeping Transcripts-
  - β-Actin: works for most projects, not all
  - Cyclophilin & 36B4: works well if protein synthesis is not elevated

18SrRNA Testing for Pol II Transcripts





Samples from formaldehyde fixed and embedded human heart tissue pre- and post-LVAD implantation.

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