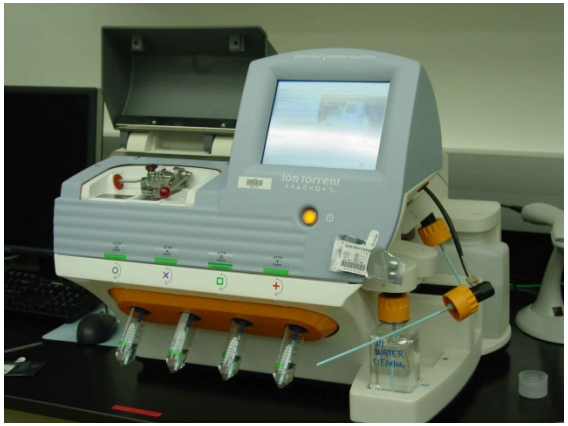
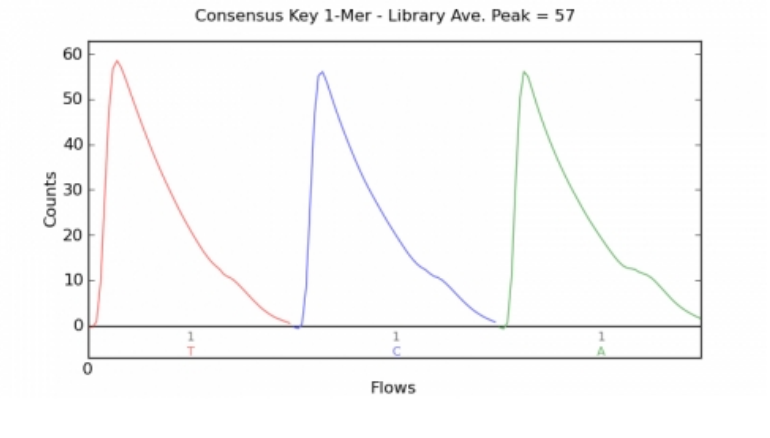
BINF 6350 ITSC 8350
Fall 2011
Biotechnology & Genomics Lab
qPCR – pt 2

http://webpages.uncc.edu/~jweller2





Topics

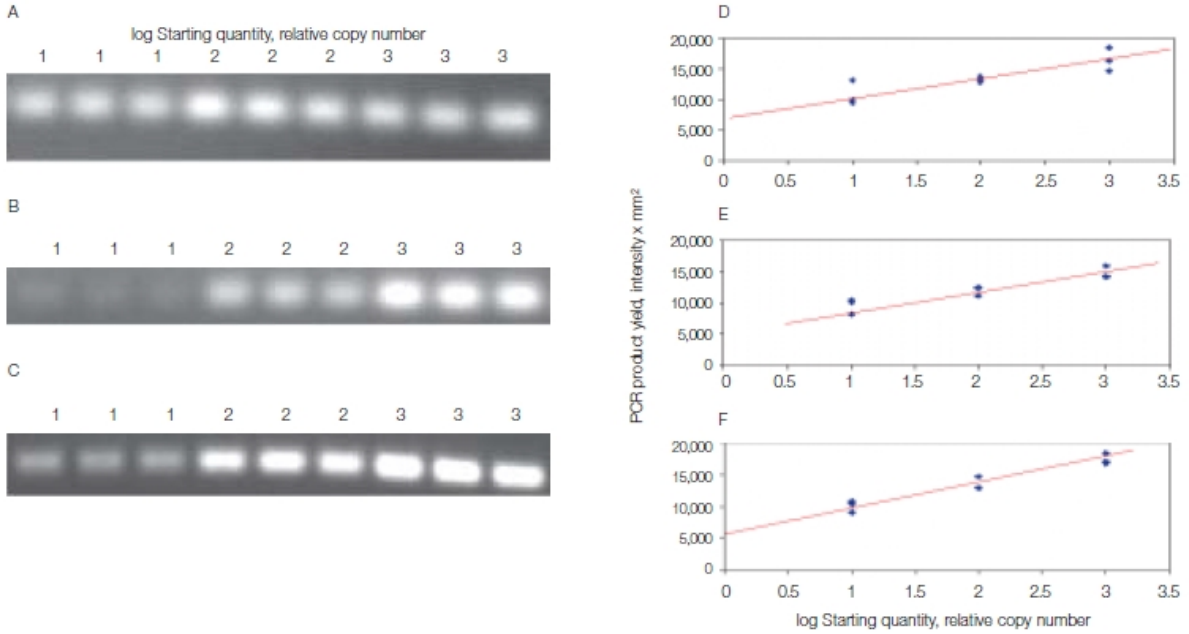
- End-point PCR
- Real-time PCR
- Detection in qRT-PCR reactions
- Quantification methods for qPCR

End-point PCR can be relative or comparative, where the comparison uses a competicon..

- Relative quantification uses a reference sample containing the genes of interest.
  - A dilution of a reference sample is used for calibration.
  - Yields are the ratio of the genes in the unknown sample to the genes in the reference sample
- Comparative quantification uses an amplicon present in every sample – by having distinguishable lengths the known and unknown cab ne distinguished.
  - The comparator can be a reference gene present in every sample, or an artificial sequence doped into the sample.
  - The targets compete for reagents in the same sample pool.
- Absolute quantification requires a calibrated competicon – it measures the number of copies of the unknown gene calibrated against a synthetic sequence *with the same efficiency* that is doped into the sample and then co-amplified.
  - Yields are the ratio of the Gene-of-Interest:Competicon

Details for relative RT-PCR

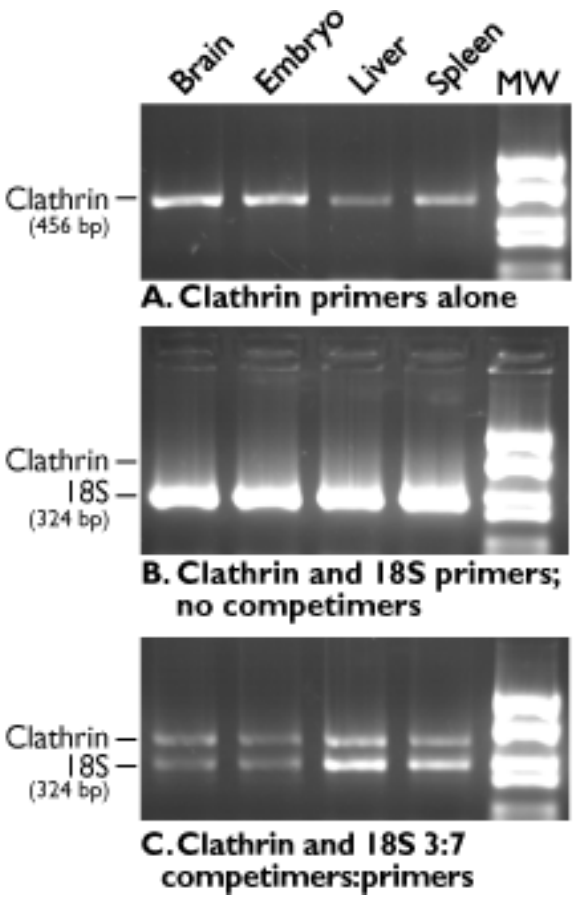
- The reaction is inherently complex and multiplex, which requires significant optimization to understand whether the samples are comparable.
  - Ideally the reactions are independent – no change in yield and no new products over the entire amplification range.
  - If this is true, variation will be due to sample characteristics, not PCR characteristics.
  - Ideally



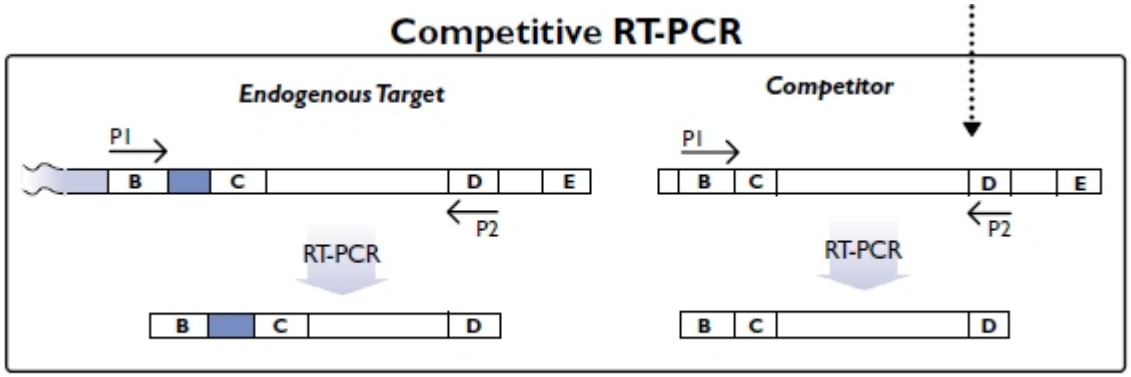
Comparative RT-PCR

- A synthetic target is doped into the sample at a known concentration, then its dilution is automatic
  - Use the same primers as the target, but a different length and internal sequence
    - usually slightly shorter (or longer - a tag may be added)
    - Calibrating the efficiency is important
    - To monitor the RT+PCR reaction, an RNA template is desirable.
  - A competicon must be created and tested for every species of interest, and checked any time a variant appears
    - Ambion provides a kit to produce an RNAase resistant competicon (the C and U are 2' modified)

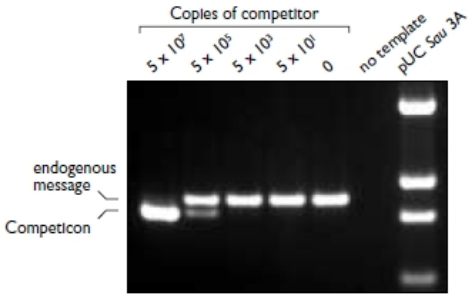
Using an internal gene as a comparison

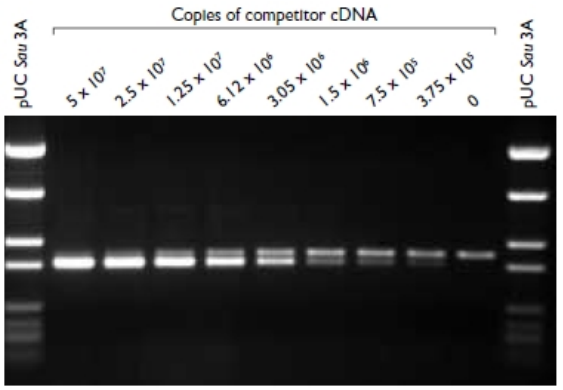


Competicon Construction and Results

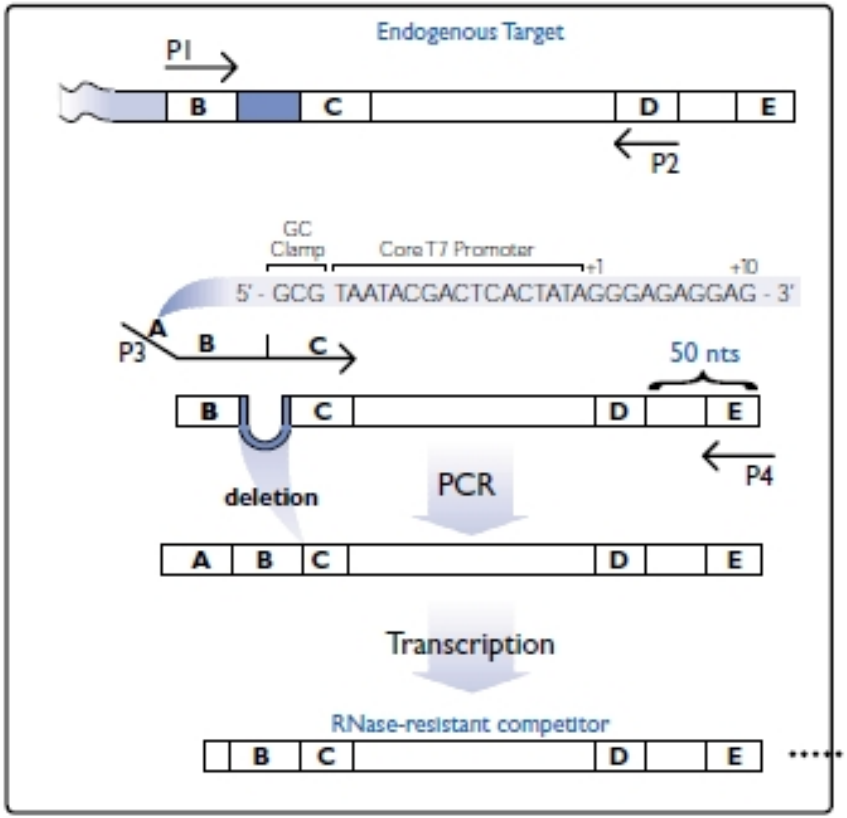


See Ambion Bulletin on Competicon Construction

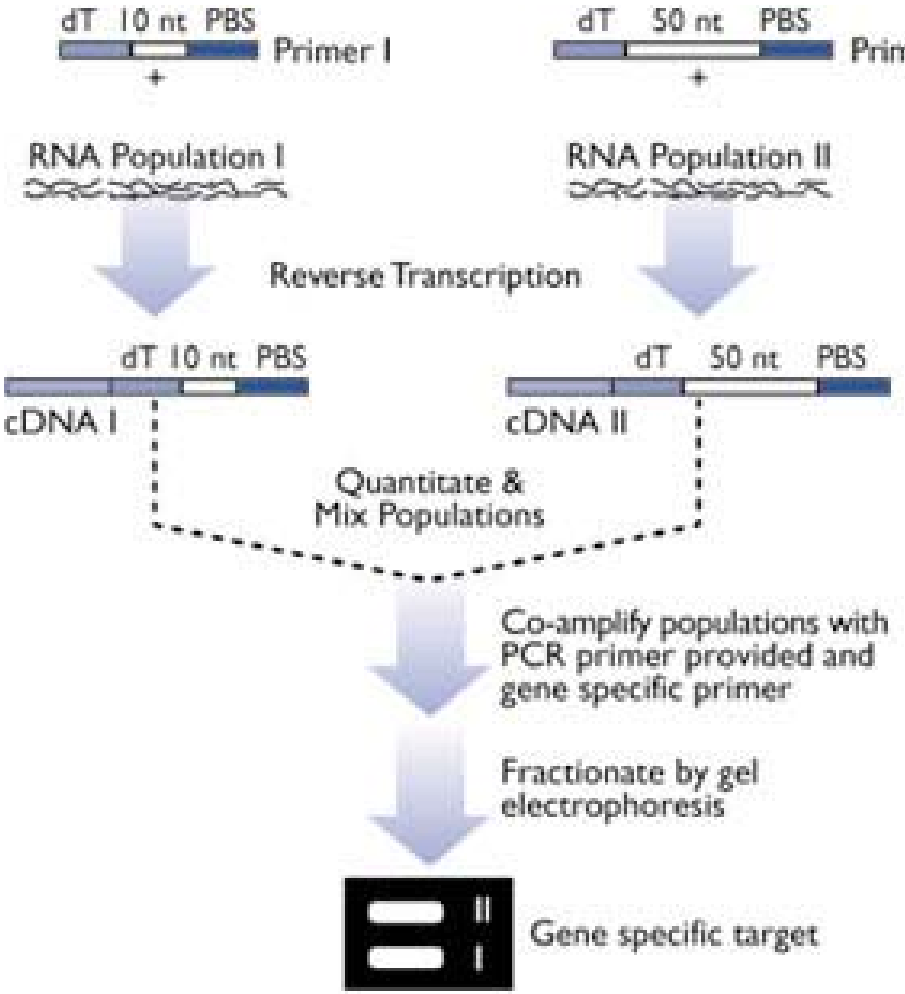




Making the RNA competicon



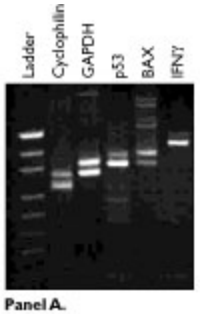
Comparative RT-PCR



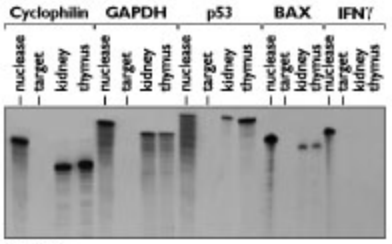
In creating the sample, the tags internal to the primer binding site are of different lengths.

Now you can mix the targets and co-amplify them – all reagents and dilutions will have been identical

You can do this for calibration sequences also.



Panel A.



Panel B.

End-point analysis is useful for pairwise comparisons of closely related samples.

- The limitations of end-point analysis include
  - handling/contamination issues
  - sensitivity/saturation issues.

Real-time PCR detects the amount of dsDNA at each cycle.

- A reporter molecule is incorporated into the assay. The following are the common fluorescent probes:
  - SYBR Green I
  - TaqMan probes
  - Molecular Beacon probes
  - Scorpion probes
- The platform will detect emitted photons of the correct wavelength, capturing the signal and reporting it at the end of each cycle
  - Background levels must be determined
  - Signal increases as the concentration of product increases
- How do you know that you aren't amplifying space junk?

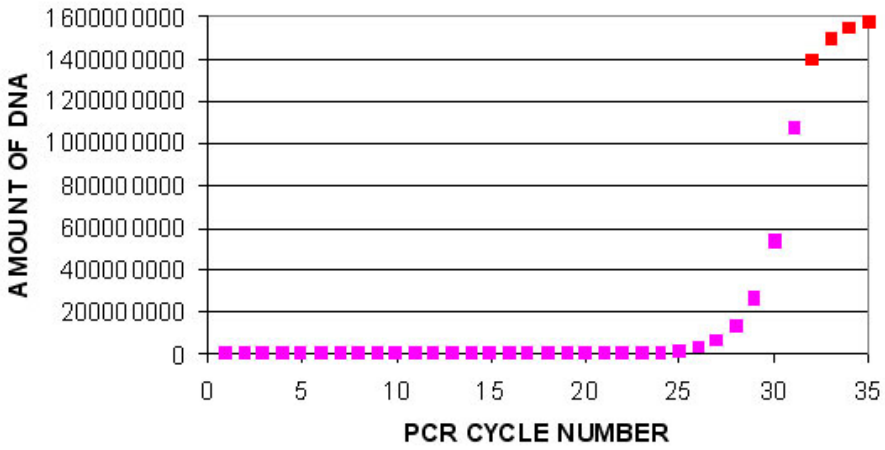
Instrumentation

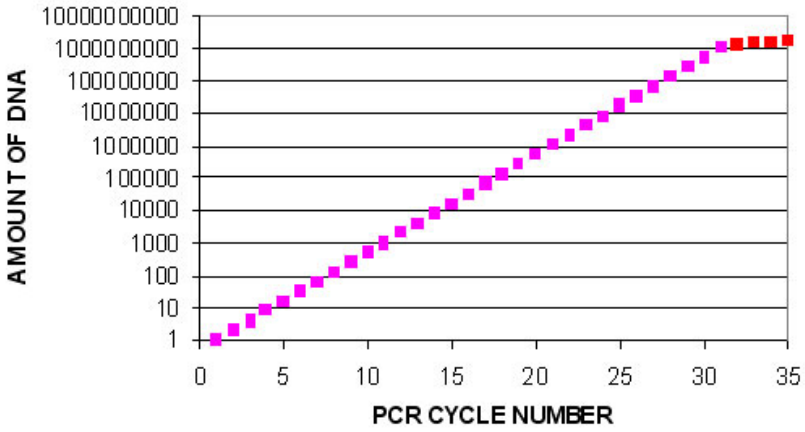
- Thermal cycler
  - 96-well or specialized glass tubes
- Optics for fluorescence excitation and emission
  - Lasers or tunable filter with broad-spectrum sources
- Photon capture hardware
- Computer with signal processing capability (data acquisition and processing)
  - Some present results as they occur, others when the run is complete
- Software for data analysis
  - Some allow raw data access allowing use of third-party software, others only produce transformed output.

What part of the PCR cycle should you try to quantify?

- Here is what happens in PCR:
  - Start: reagents are in excess of template
  - Early on: reagents are in excess of product so rate of production is exponential
    - The exponent varies by template/primer
    - This is the only reproducible part of the reaction
      - End-point relative PCR has 2-3 logs of dynamic range
      - Extend by performing dilutions of samples that plateau early
      - Real-time PCR capture up to 7-logs by capturing data from every cycle.
  - Later: the reaction becomes linear when product is as likely to anneal with itself as with primer
  - Terminal: the plateau phase – no product is made

CYCLE NUMBER	AMOUNT OF DNA
0	1
1	2
2	4
3	8
4	16
5	32
6	64
7	128
8	256
9	512
10	1,024
11	2,048
12	4,096
13	8,192
14	16,384
15	32,768
16	65,536
17	131,072
18	262,144
19	524,288
20	1,048,576
21	2,097,152
22	4,194,304
23	8,388,608
24	16,777,216
25	33,554,432
26	67,108,864
27	134,217,728
28	268,435,456
29	536,870,912
30	1,073,741,824
31	1,400,000,000
32	1,500,000,000
33	1,550,000,000
34	1,580,000,000





http://pathmicro.med.sc.edu/pcr/realtime-home.htm

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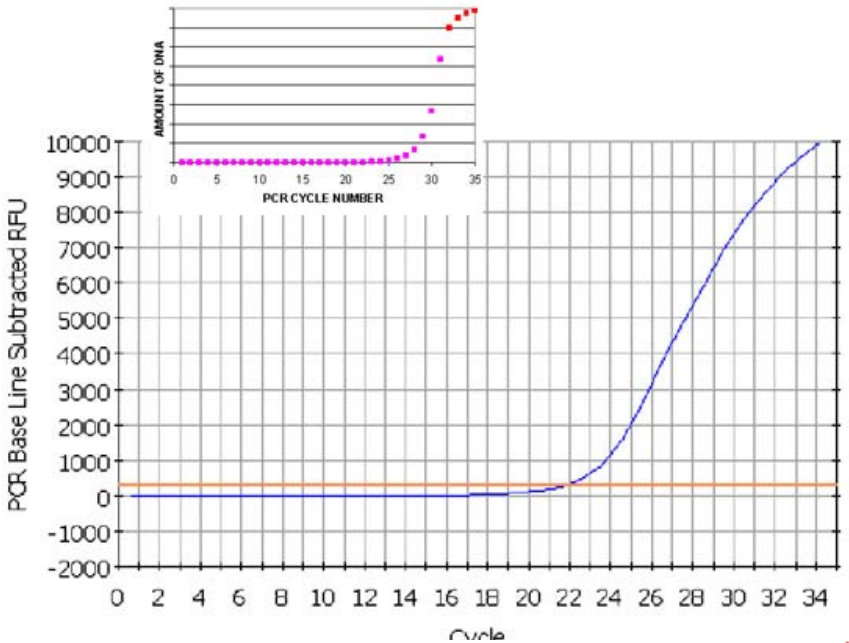
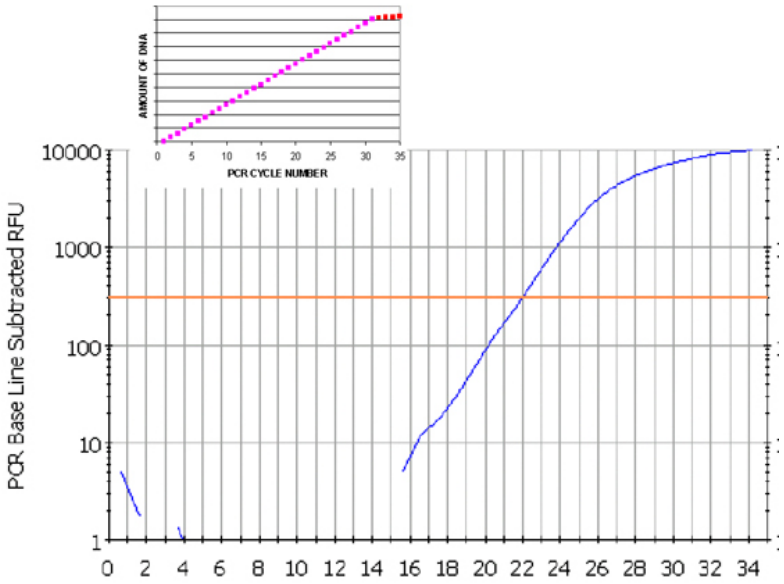
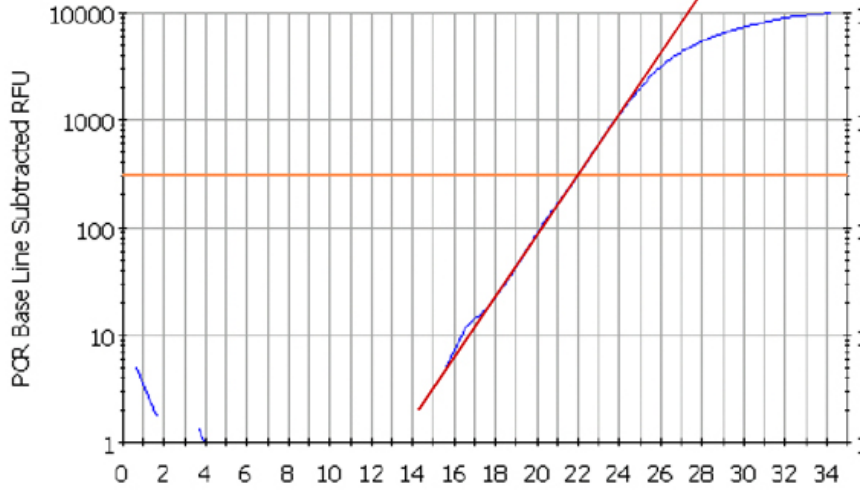
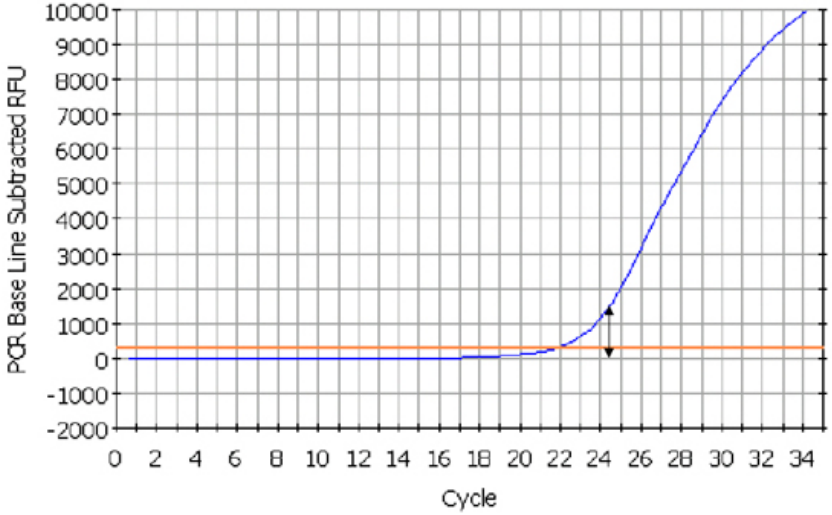




Linear ~20 to ~1500



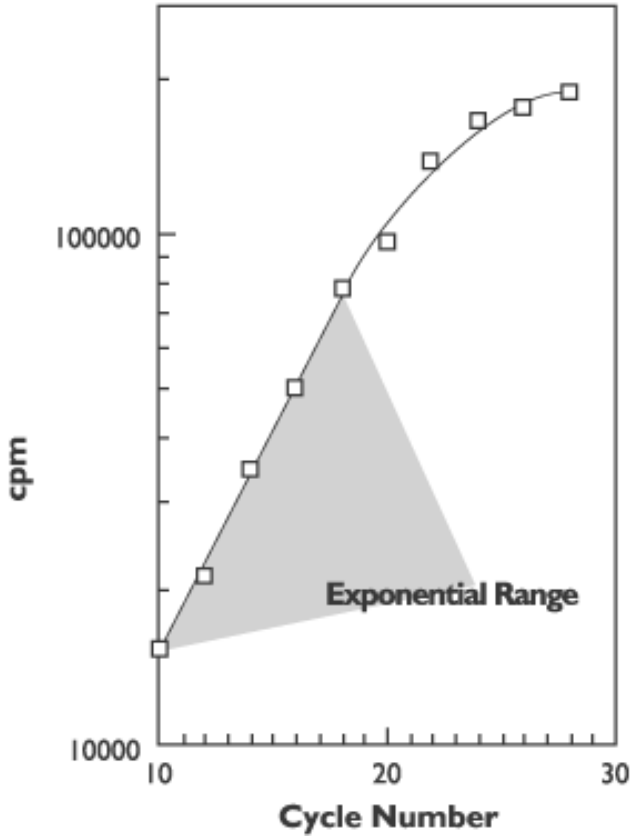
Linear ~20 to ~1500

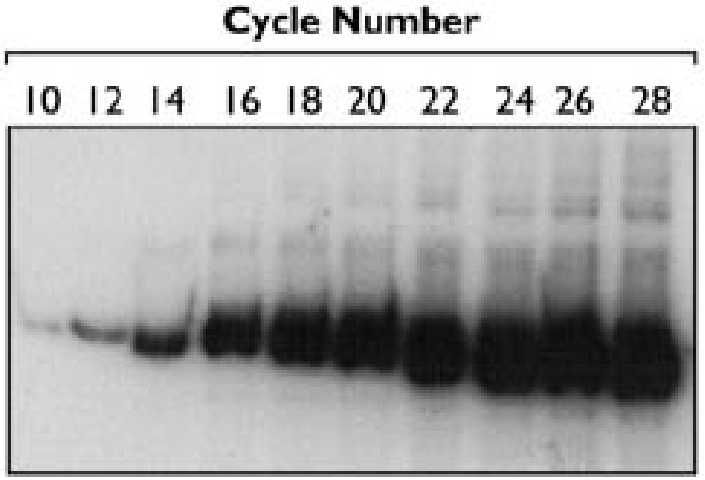


Estimating yields from RT-PCR is based on the threshold cycle.

- At the lower detection limit enough amplicon is made to produce a signal – the cycle where that occurs is called the threshold cycle (Ct).
  - Fewer cycles means more starting material
  - The efficiency of each amplicon varies so optimization is very important
  - This is very sensitive (as few as 10 copies in one cell).

Exponential range and Ct



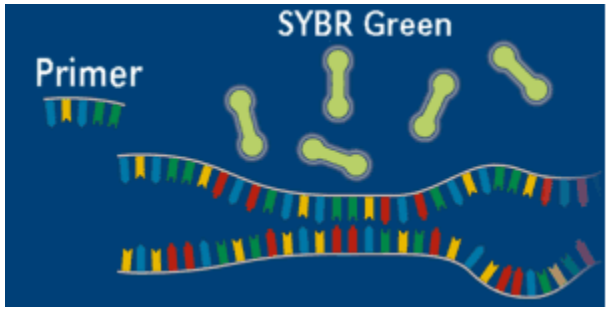


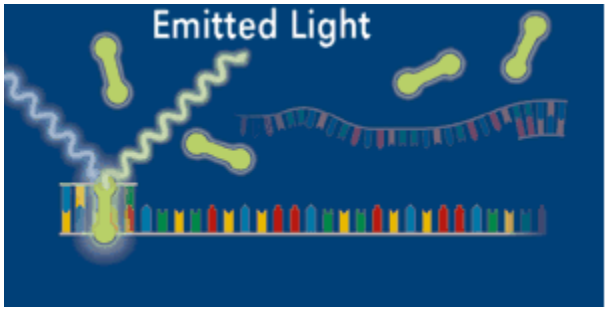
5% urea-PAGE

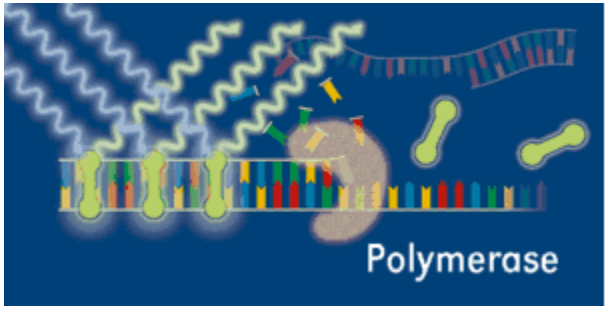
SYBR Green Chemistry

- SYBR Green is the simplest, least expensive but least accurate detection method.
  - SYBR Green binds dsDNA and emits light upon excitation.
    - What about primer-dimers and unsuspected mis-primed targets? Controls are very important!
    - Follow-up may include running gels to make sure single products appear, and photospectrometry to verify yields.

SYBR Green

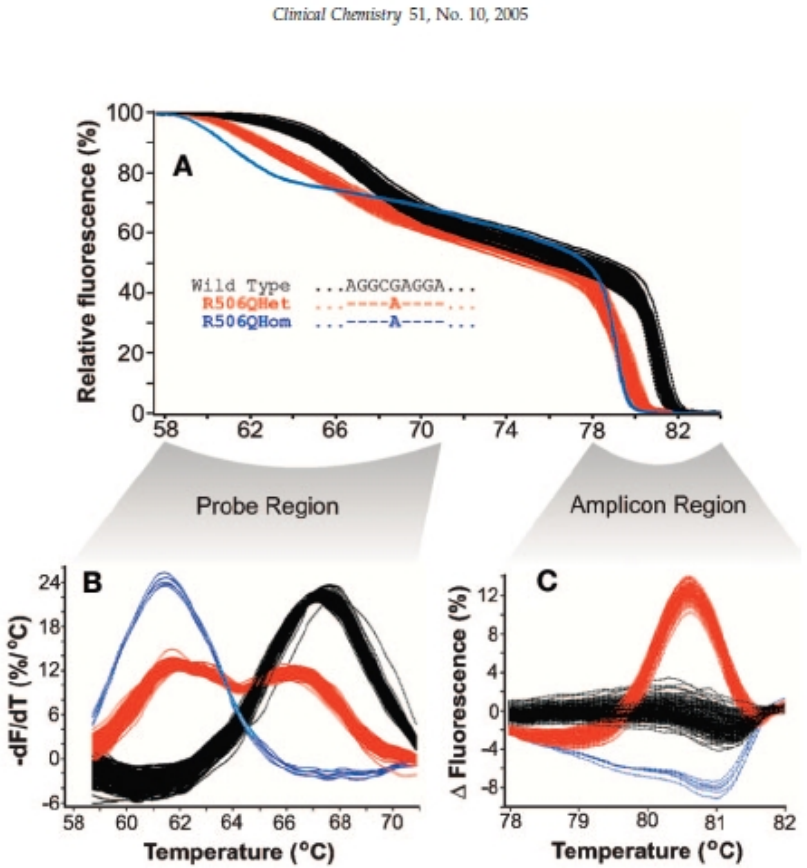






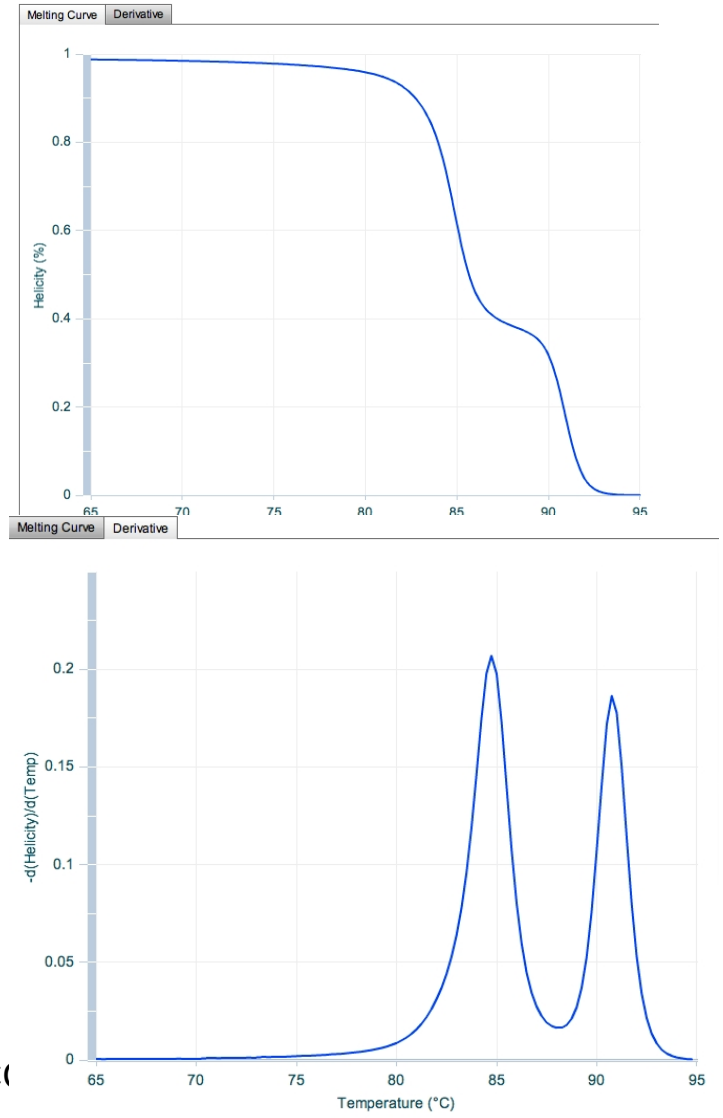
Melt Curve Analysis

See papers from the Wittwer group (Utah) for more details.
The Tm is not as useful as the shape of the melting curve (not at equilibrium, and dye intercalation stabilizes the duplex)
The rate of temperature rise should be 0.1-0.3°C/sec, with 20-100 sampling points taken per degree (e.g. 300ms frame interval with 15ms exposure gives 25points/°C at 0.1°C/sec).
A normal curve would start at 50C and proceed to 90C, slope 0.3C
Background is fit to an exponential function.
Derivatives of the melting transitions were obtained with the Salvitsky-Golay polynomial, and then difference poits were compared



Melt curve analysis software

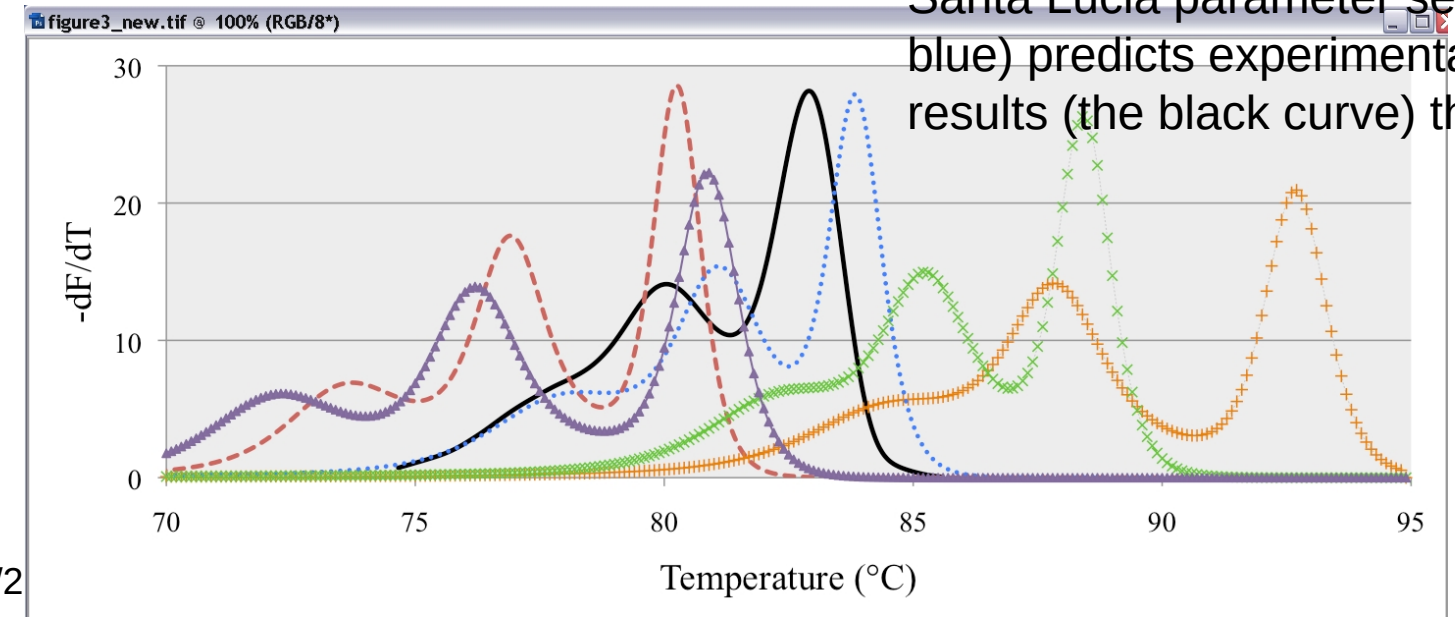
- Wittwers group has a project called uMelt (Dwight et al, Bioinformatics 27(7), 1019:1020, 2010.)
  - To predict melting curves of amplicons in specific environments
    - Parameters: nearest-neighbor stacking energies, loop entropies, monovalent and divalent cation concentrations, temperature range.
    - Profiles are presented as direct or first-derivative plots.
      - The temp for 50% helicity or %H at a stated temperature



Melt curve analysis methods

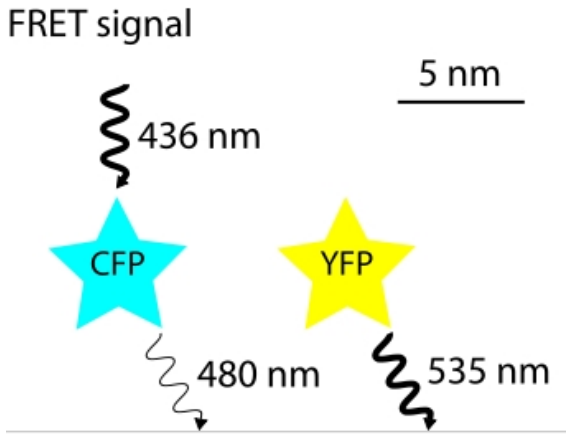
- If a fragment has several stable folded forms there may be several melt peaks associated with the output, even though there is only one sequence present (especially in GC-rich domains).
- Axes on melt curves: helicity versus temperature.
- Axes on derivative plots: -derivative of helicity versus temperature.
- Polymer domain melting has to account for multiple states (oligomers are 2-state).

Santa Lucia parameter set (in blue) predicts experimental results (the black curve) the best

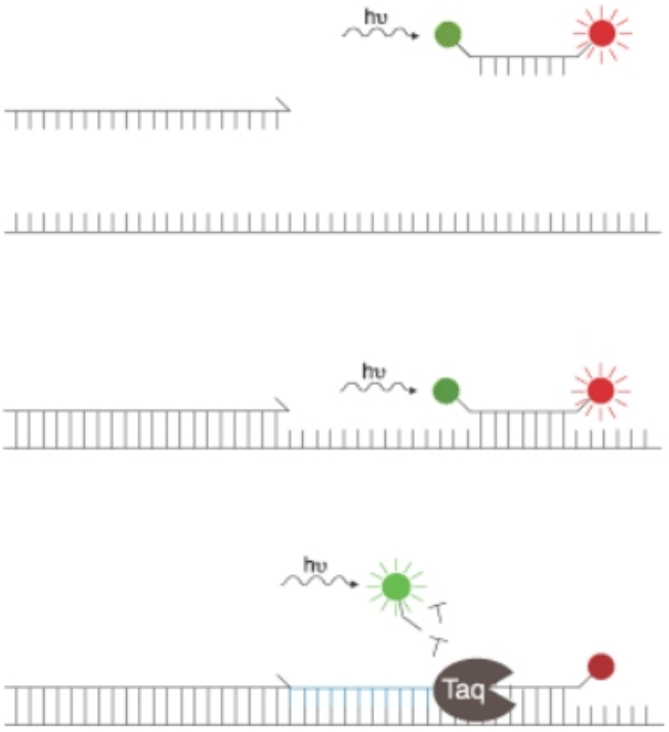


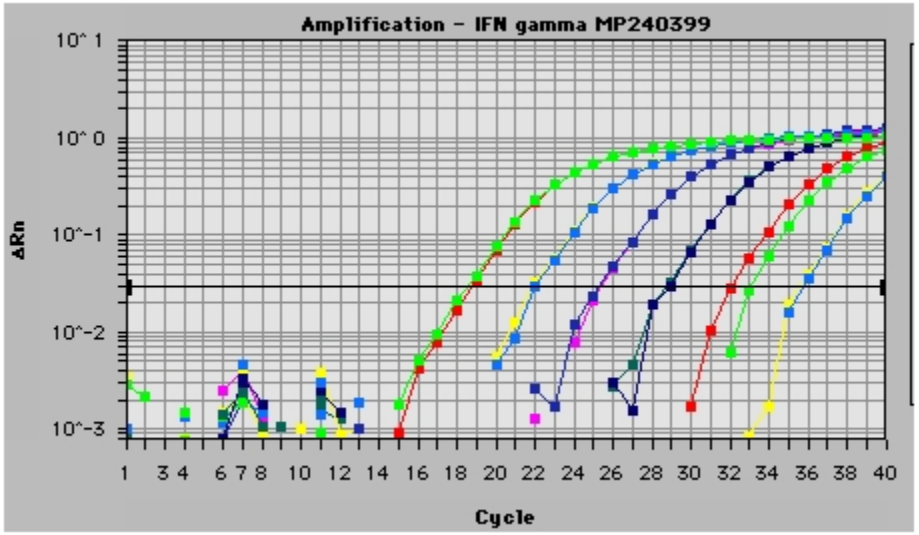
TaqMan Chemistry

- Taqman uses the 5’ nuclease activity of Taq polymerase
  - An oligo complementary to a site internal to the target contains a 5’reporter-3’quencher dye pair
    - Free in solution they line up and quench (Förster Resonance Energy transfer = FRET).
    - When oligo is bound to the ss target the dyes line up and quench
    - Hydrolyzed they cannot line up so the reporter is not quenched – this happens when the polymerase uses the ss target to make a copy. More copies means more hydrolysis and more signal.
  - The rules for probe design are well worked out – use the Applied Biosystems site tools.
    - You can multiplex by using different dyes
    - This is expensive
      - 2 dyes per oligo
      - One oligo per unit to be detected
    - This is very accurate



TaqMan

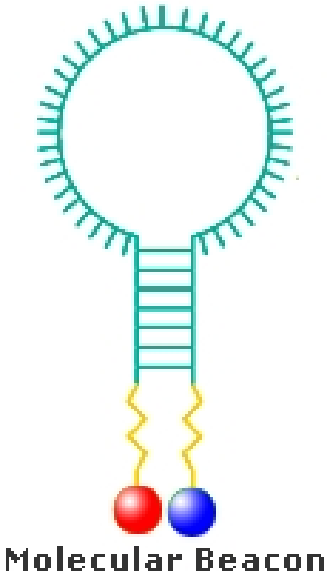

hυ
Denature
hυ
Anneal
hυ
Taq
Extend


Amplification - IFN gamma MP240399
10^ 1
10^ 0
10^-1
10^-2
10^-3
ΔRn
1 3 4 6 7 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40
Cycle

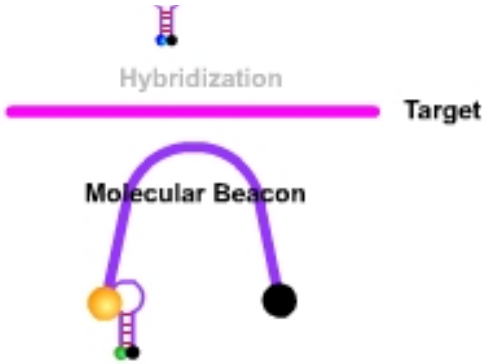
Molecular Beacon Chemistry

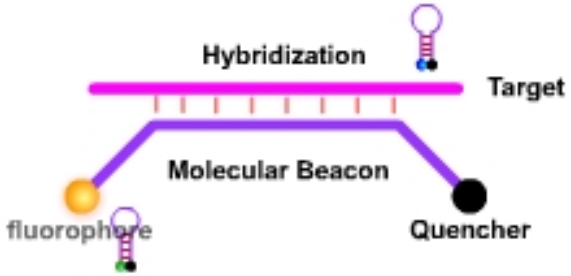
- Molecular Beacons also use FRET, with fluors on the 5' and 3' ends of a probe.
  - Uses a stem-loop structure to keep R-Q in proximity when not bound to DNA.
  - The probe is not degraded during the assay.
  - Advantages: sensitive and specific
  - Disadvatages: expensive and require one per species detected.

Molecular Beacons



There are 4 parts:
The loop is 18-30bp and complements the target.
The stem is 5-7nt on the ends of the loop – complementary
5’ fluor is on the 3’ end of the probe
3’ quencher is on the 5’ end of the probe





Scorpion Chemistry

- Scorpions also use FRET, with fluors on the 5' and 3' ends of a probe.
  - Uses a stem-loop structure to keep R-Q in proximity when not bound.
  - The 3' part of the stem contains the sequence that extends from the primer into the target.
  - The sequence is linked to the 5' end of a specific primer using a non-amplifiable monomer.
  - After extension of the primer, the specific sequence binds its complement in the extended amplicon, which opens up the hairpin loop so that quenching cannot occur.
  - Disadvantages: expensive and require one per species detected.

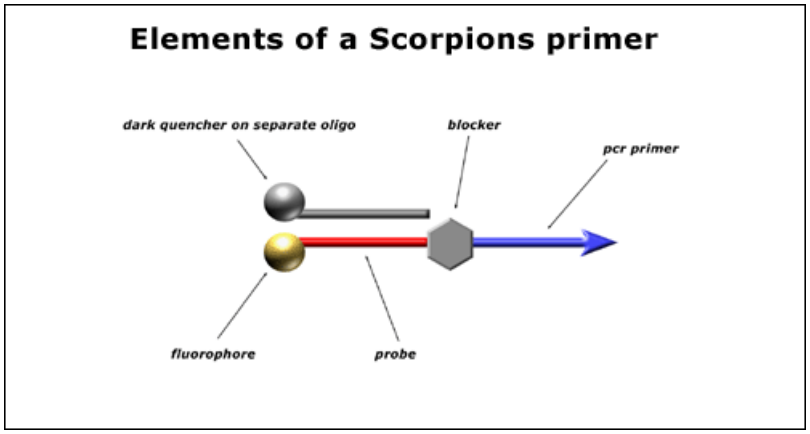
The Scorpions reaction

Step 1 - the Scorpions primer is extended on target DNA.

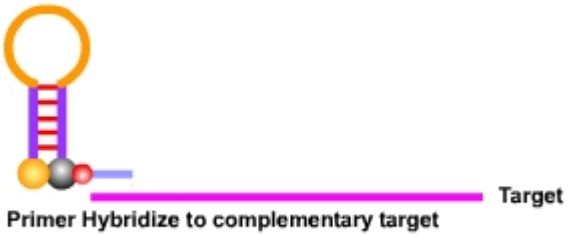
Step 2 - the extended primer is heat denatured - the quencher disassociates.

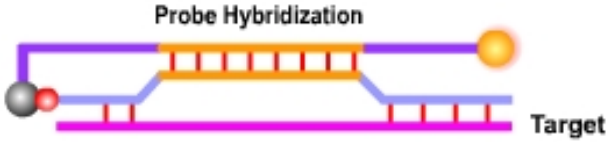
Step 3 - as it cools the extended Scorpion rearranges and begins to fluoresce in a target specific manner; unextended primer is quenched.

Scorpion Primer

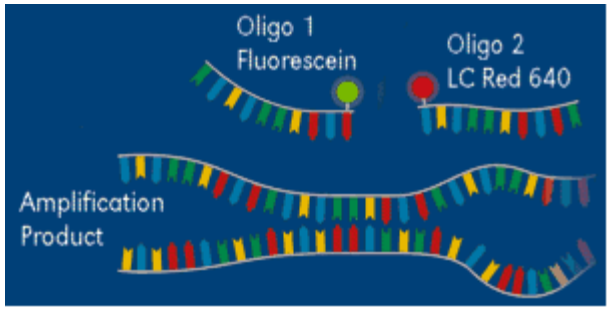


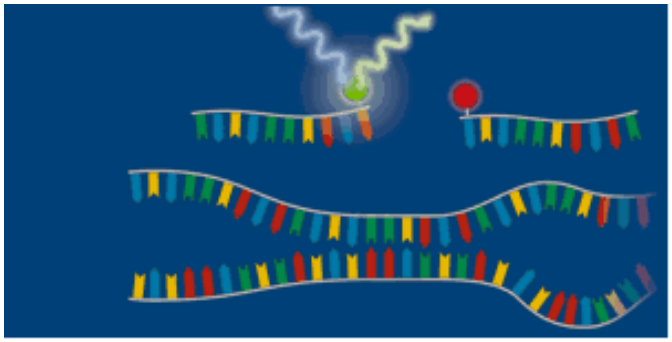
The probe is attached to the primer (unimolecular reaction).
As one PCR cycle complete, the new target strand is attached to the prone, so in the second cycle the probe hybridizes to the target, the hairpin on the probe melting before the duplex target, so the hairpin hybridizes to the target.

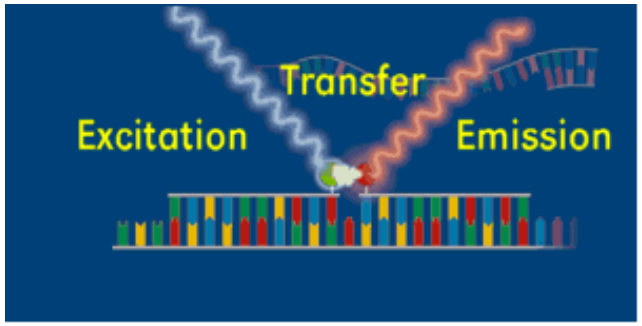




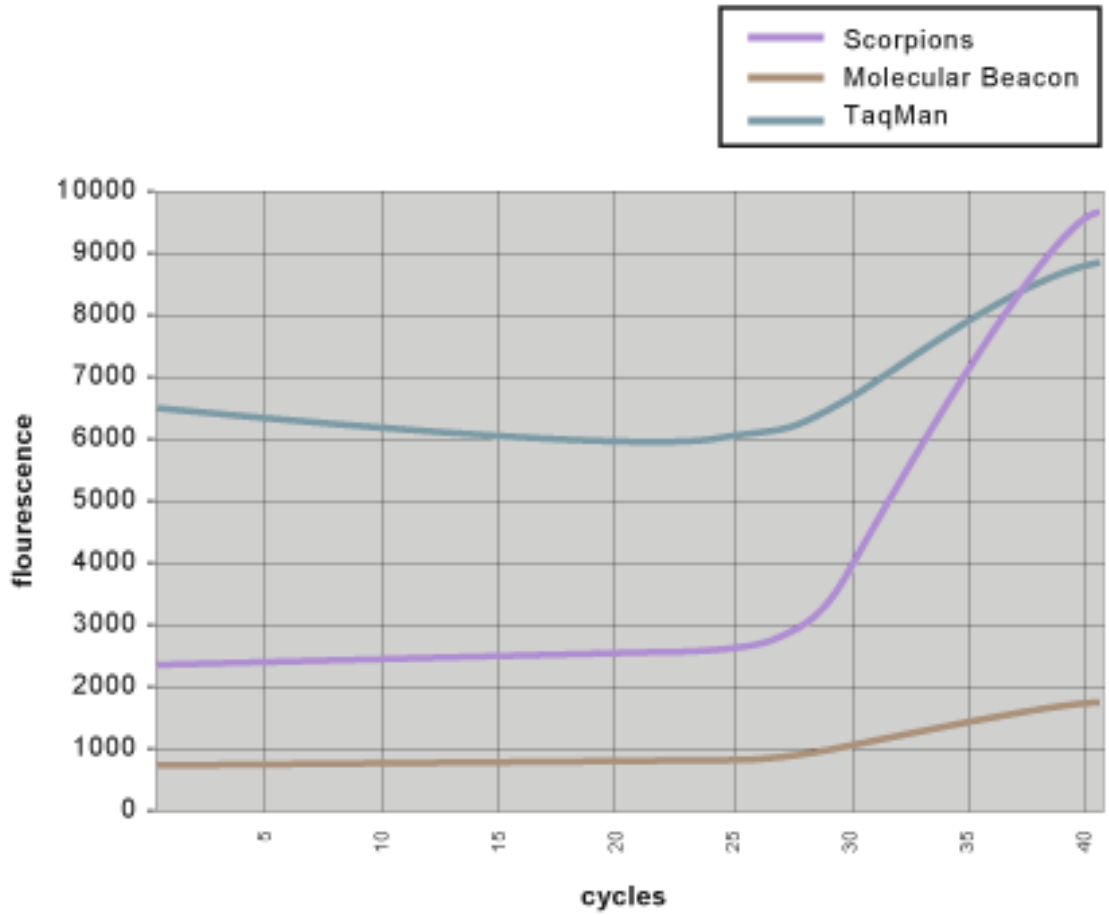
FRET Transfer in the Light Cycler







http://www.gene-quantification.de/chemistry.html#sybrgreen


Scorpions
Molecular Beacon
TaqMan
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
flourescence
5
10
15
20
25
30
35
40
cycles

The space junk problem

- Run a gel of the final product
- Use a method that incorporates a probe (a third-check on the sequence).

Quantifying results

- Densitometry of the plateau-level product
- Cycle-by-cycle detection of the product
  - What constitutes the background?
  - What constitutes a false positive?
  - What part of the response is most reliable and accurate?
  - What type of function best represents the response?

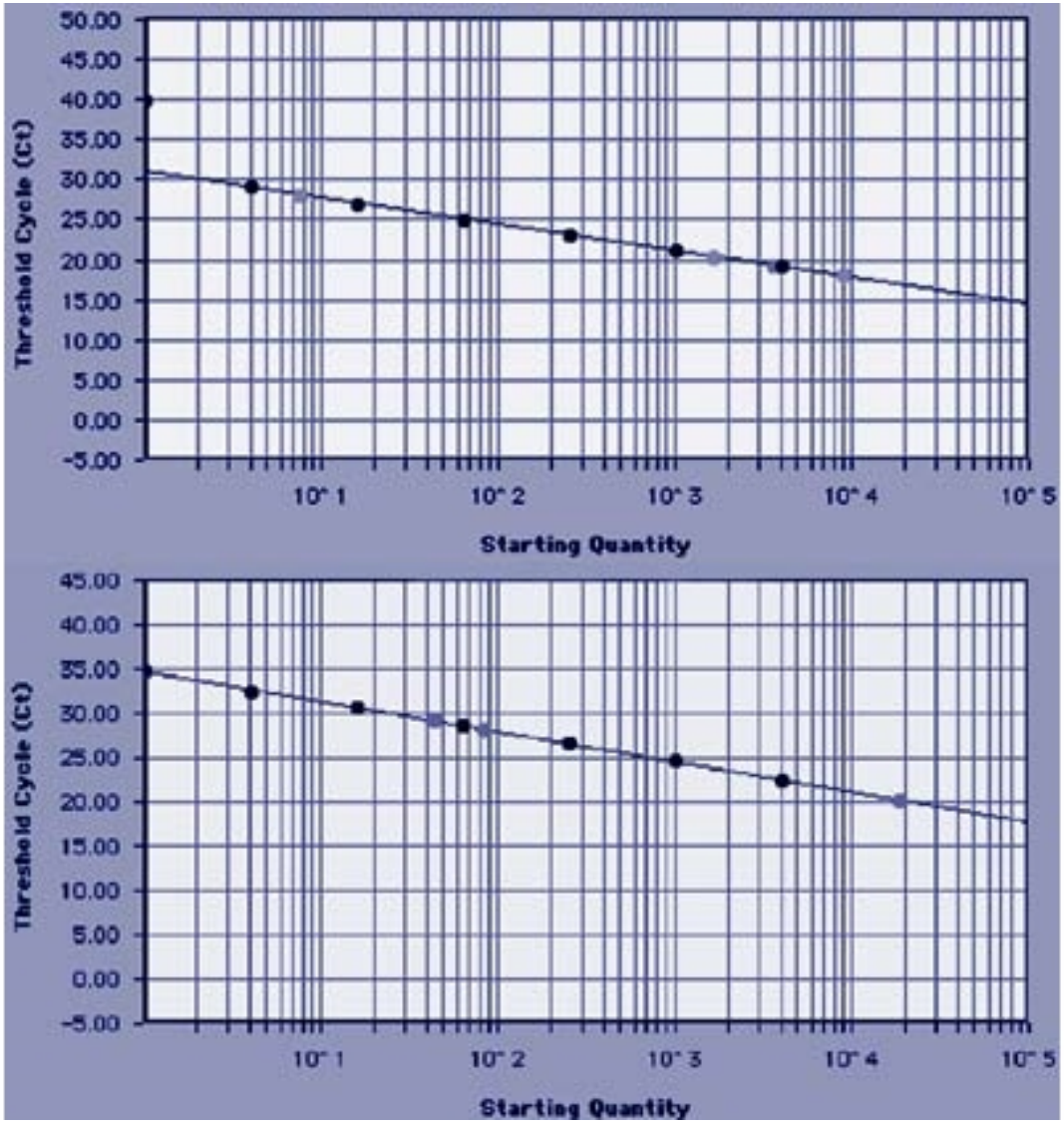
Quantification by standard curve

- One way to calibrate the response is to use an external target with a known concentration to set up a response curve
  - If you are doing RT-RT+PCR use an RNA since the reverse transcriptase variation must be included in the process.
  - Construct a titration series, usually 10-fold serial dilutions, in triplicate.
  - Convert the mass to a copy-number per sample for reporting purposes.
- Ideally the calibration molecule has the same length and efficiency as your target
  - Should you use a solution of the exact target?

Relative PCR setup

- The standard curve comes from the dilution of a reference sample, thus the relative rather than the absolute quantification – everything is with respect to that reference.
  - 18S rRNA is often used as an endogenous control across both reference and experimental samples

Relative PCR example



Top is alpha macroglobulin, bottom is 18S.

Kidney was designated the reference (so it is '1X').

	A	B	C	D
	Raw Values		Normalized	Relative
	a2 M	18S	a2 M/18S	Value
Sample 1: kidney	82	3592	0.023	1.0
Sample 2: liver	18351	8966	2.05	90.0
Sample 3: ovary	44	1669	0.03	1.1
Sample 4: spleen	1	8	0.13	5.6

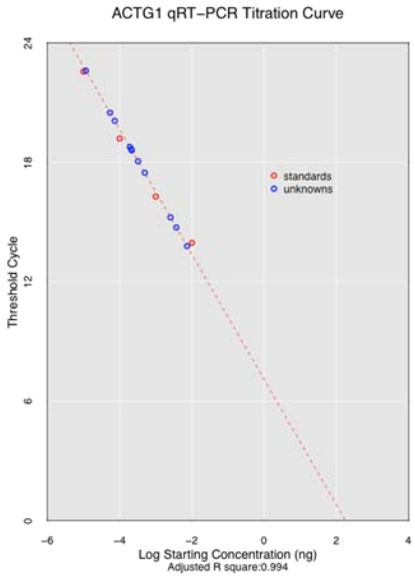
Quantification by comparative threshold or $\Delta\Delta C_t$

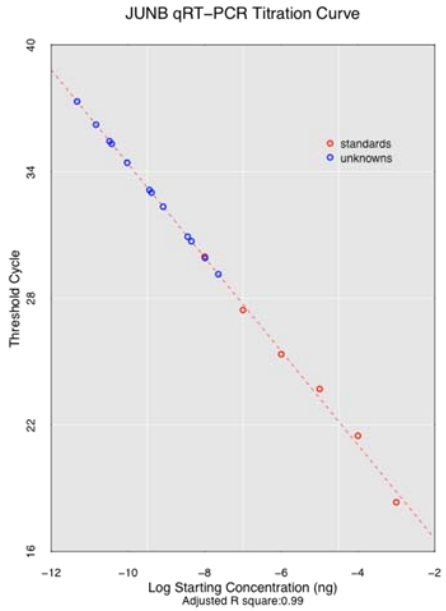
- Another approach to calibration is to use an internal control – a gene that should always be present and in consistent levels.
  - A characterized sample is assayed
  - The calibrator gene in the sample of interest is assayed
  - The gene of interest in the sample of interest is assayed.
- To analyze this: the control gene is used to normalize the response of the gene of interest in the unknown sample to its expression in the characterized samples
  - So $\Delta\Delta C_t = \Delta C_{t,sample} - \Delta C_{t.reference}$ where ΔC_t indicates the normalized values.
  - The validity of the comparison of the two genes depends on having similar efficiencies.

More elegant solutions have been developed for unequal reactions

- Pfaffl method: when reaction efficiencies are different.

$$Ratio = \frac{(E_{target})^{\Delta Ct_{target}(control-sample)}}{(E_{ref})^{\Delta Ct_{ref}(control-sample)}}$$





qRT-PCR Rules

- Primer-probe design is key with amplicons of 70-250bp
- Efficiency is important:
  - Eff= $10^{(-1/slope)}$ -1
  - with 3.6>slope>3.1 which means 90-110%
- RNA quality must be good and master mixes are essential
- Avoid contamination! Use the PCR station and always include a no-template control
- If you are doing RT+PCR then one control leaves out the RT (tests for gDNA contamination)
- Use an endogenous or spiked-in control to correct for handling variation.
- Perform melting analysis (slowly increase the temperature of the PCR reaction past the Tm of the amplicon – with SYBR Green you should suddenly lose all of the signal).
- Baseline threshold is actully 2 cycles prior to the appearance of the most concentrated amplicon (ensures exponential phase for all amplicons).
- Standard curves are used for interpolation only, never for extrapolation.

The relevance of the method to the lab lies in the use of qRT-PCR to calibrate the bead loadings.

- We will use quantitative real-time PCR to quantify the amount of PCR product attached to our sequencing beads after emulsion PCR and post-enrichment.
- We will use a BioRad iCycler equipped for multi-dye detection.
  - The kit detects the presence of the primer attached to the bead and the primer that is part of the adaptor on the sequence.
  - The kit contains a 173bp control sequence as a calibration standard.

Kapa Biosystems

- Library quantification for template-to-bead ratios.
- Beads have adaptor-primers sequences, some of which have the target. The goal is to have 10-30% of the beads with targets, but when a bead does have a target there will be thousands of copies.
- The targets have an adaptor that serves as the emulsion PCR (emPCR) primer – only beads with two primers will give qPCR signals.
- A standard fragment is provided to make the serial dilutions.
- This is a SYBR Green based assay, so it is not very expensive.
  - WT Taq polymerase is inhibited by SYBR Green, so an engineered version is used.
  - Note: they provide a qPCR efficiency calculator app that you might want to play with.

Designing Degenerate Primers

- The homework assignment asked that you use a multiple-alignment of imperfectly matched sequences (from one of the tubulin genes in plants) and design primers to amplify the cognate region in at least 2 and less than 10 variants. This would require that some primers have positions with mixed bases (‘degenerate’ bases) in order to preserve specificity.
- One software program that handles this design problem specifically is HYDEN (http://acgt.cs.tau.ac.il/hyden/) from the Shamir group.