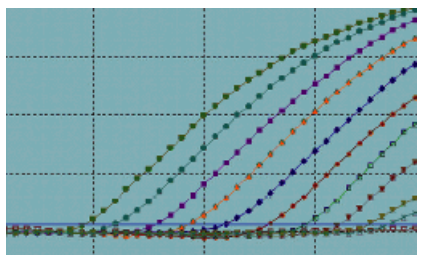
Real Time Quantitative PCR

Assay Validation, Optimization and Troubleshooting



Dr Steffen Muller

Field Applications Scientist

Stratagene Europe

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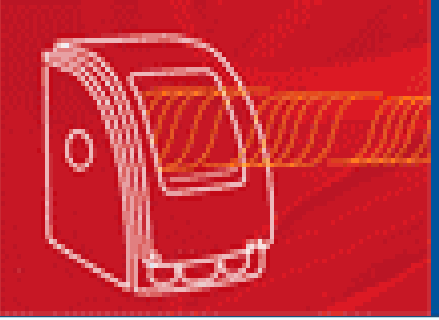

Overview

- The Importance of Controls
- Validation and Optimization
- Assay Troubleshooting
- The Quizshow ☺

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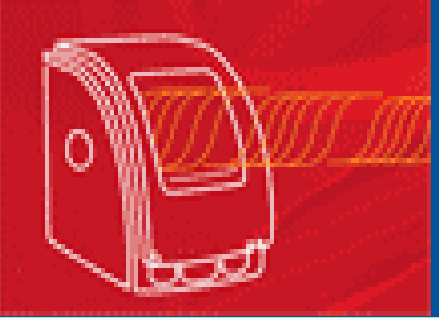

The Benefit of Controls

Controls enable you to understand unexpected results and are necessary components of assay validation:

No Template Control (NTC):
Template contamination, primer dimers, probe degradation

No Reverse Transcriptase Control (noRT):
Amplification from genomic DNA

Negative Sample:
Non-specific amplification (non-specific primer/probe binding)

Positive Controls:
Validating assay performance, control for Inhibition

Only controls will tell you which data is good or bad!

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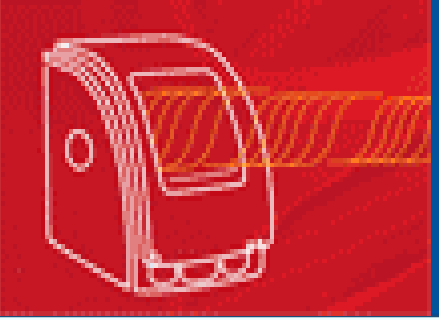

Validation and Optimization

Validating an assay generates important performance data

- The specificity of your primers and probes
  Melting curves, Negative controls

- The working range and sensitivity of your assay
  Positive controls, Standard curves

- The reproducibility of your experiments
  Replicates, Statistics

Assay validation makes it easy to avoid or understand unexpected results in future experiments

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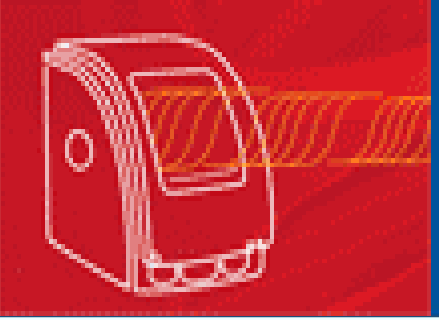

Validation and Optimization

Optimizing your assay can help you to

- Increase specificity: Get rid of non-specific amplification eg. primer dimers

- Increase sensitivity: Get earlier Ct values, detect lower concentrations

- Increase reproducibility: Lower replicate variability, high amplification efficiency

Assay optimization will improve the robustness of your assay and minimize intra- and inter-assay variability

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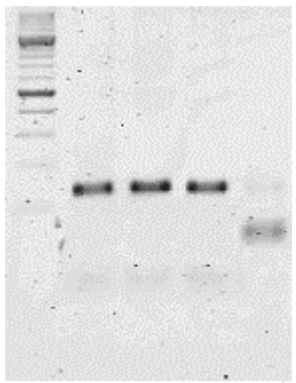
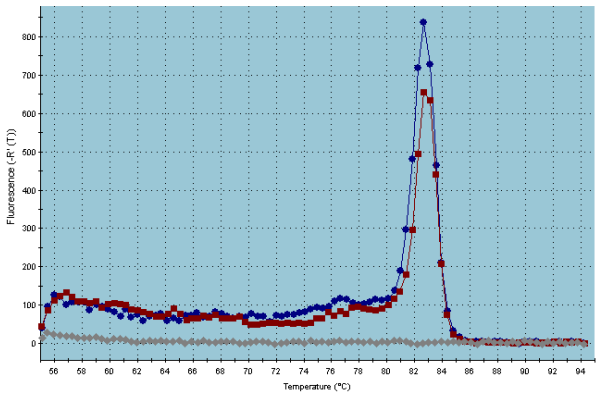
Assay Validation
Specificity

Electrophoresis



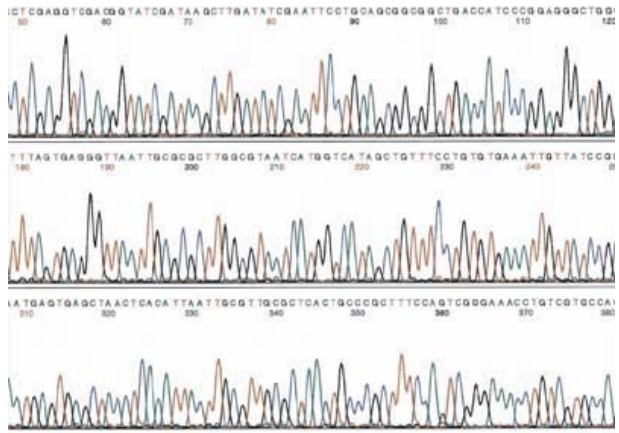
- Size information
- Primer dimers
- Non-specific products

SYBR Dissociation Curve



- T_m information
- Amplicon heterogeneity
- Primer dimers
- Non-specific products

Sequencing



- Only necessary in specific cases
- Subtle amplicon variations

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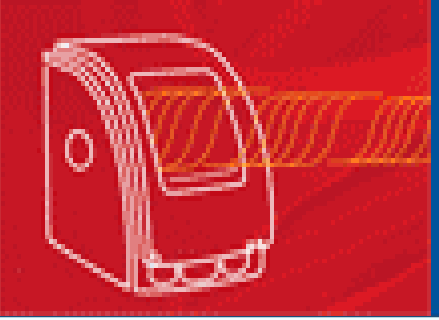

Assay Validation
Dynamic Range and Sensitivity

A standard curve enables you to identify the linear working range and the efficiency of your assay

- Perform a serial dilution series over a wide range of concentrations using a positive sample/control

- Use replicates for your standard curve
  → enables outlier detection and statistics

Properties of a good standard curve:

- high efficiency (80%<85%<90%-105%<110%<115%)
- good R^2 (>0.98)
- low replicate variability for individual standards

  ($SD_{rep}/mean_{rep}*100 = \%CV < 1\%$)

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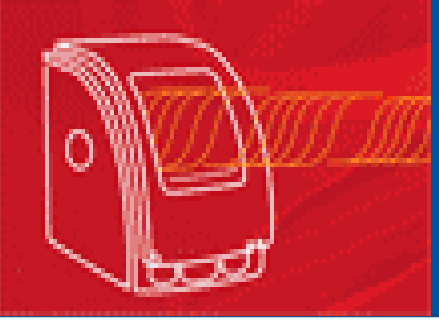

Assay Validation
Standard curves

- Use concentrations that will reflect your unknowns
- Select Cts in the middle of the range, 15-30
- Select points that have lowest Variability (%CV)
- Range with highest R^2 and efficiency close to 100%

Well Name	Ct Avg.	Ct SD	%CV
std01	7.78	0.12	1.54
std02	10.91	0.07	0.64
std03	14.17	0.12	0.85
std04	17.4	0.06	0.34
std05	20.55	0.06	0.29
std06	23.58	0.05	0.21
std07	26.79	0.12	0.45
std08	30.29	0.19	0.63
std09	33.64	1.19	3.54
std10	42.95	3.27	7.61
std11	35.54	3.75	10.55

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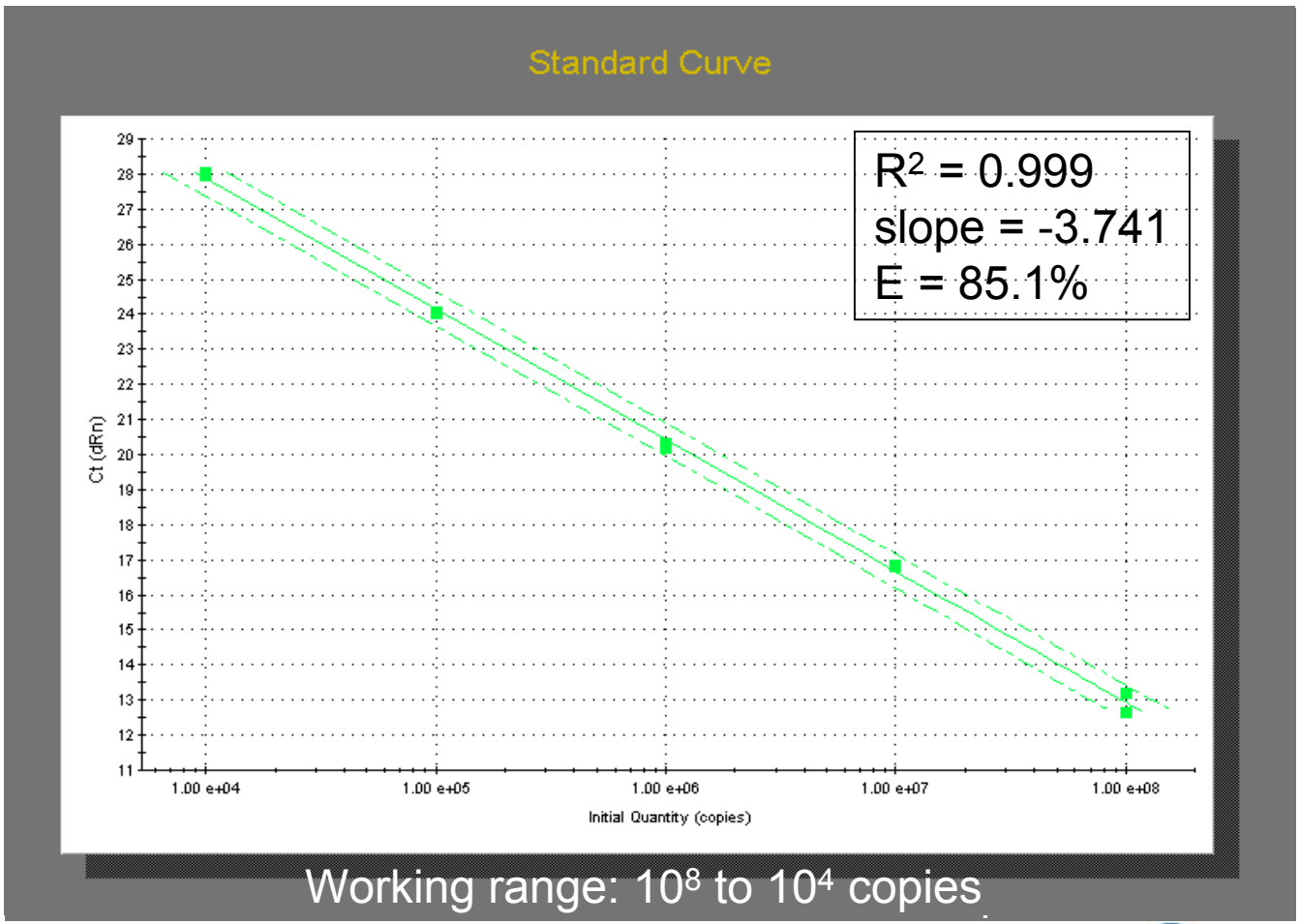
Assay Validation
Dynamic Range and Sensitivity



Working range: 10^8 to 10^4 copies

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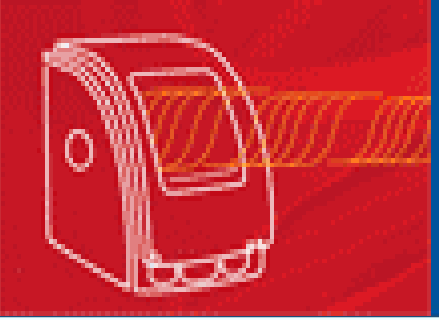

Assay Optimization
Reverse Transcription

Reverse Transcription is the main source of error in qRT-PCR

➲ Therefore optimizing the RT step improves your PCR results

➲ High quality RNA gives the most reproducible and robust results

➲Try different enzymes and priming combinations to find the one that gives the most optimal results

➲ MMLV based RT enzymes usually work up to 50-55°C
- higher temperatures improve resolution of RNA structures for full length cDNA

➲ Usually gene-specific priming works better with low expressors but this seems to be sequence specific
→ also try oligo-dT and random priming

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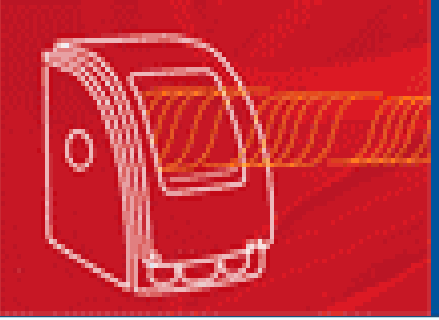

Assay Optimization
Primers and Probes

Why optimize forward and reverse primer concentrations?

- It is difficult to design a primer pair with identical T_m.
  Even with a theoretical identical T_m in real life differences exist.

- Changing annealing temperature or Mg^{2+} concentration affects both primers at the same time.

Example: T_m forward primer is 58°C and T_m reverse primer is 61°C

→ PCR at 58°C annealing: Reverse primer may bind non specifically.
Competition by amplification of unspecific products.

→ PCR at 61°C annealing: Forward primer doesn't bind efficiently.
Inefficient overall priming.

Effect: Low efficiency of amplification and high variability!

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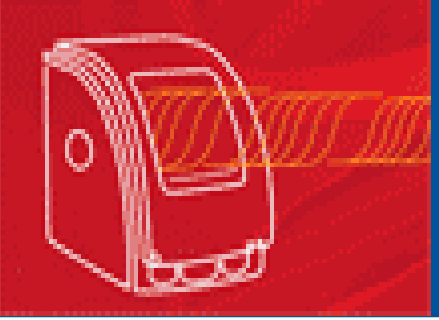

Assay Optimization
Primers and Probes

T_m of primers depends on concentration:
perform a primer matrix test to identify optimal concentration using SYBR chemistry

SYBR based

	50 nM	100 nM	200 nM	300 nM	600 nM
50 nM					
100 nM					
200 nM					
300 nM					
600 nM					

probe based

	50 nM	100 nM	200 nM	300 nM	600 nM
50 nM					
100 nM					
200 nM					
300 nM					
600 nM					

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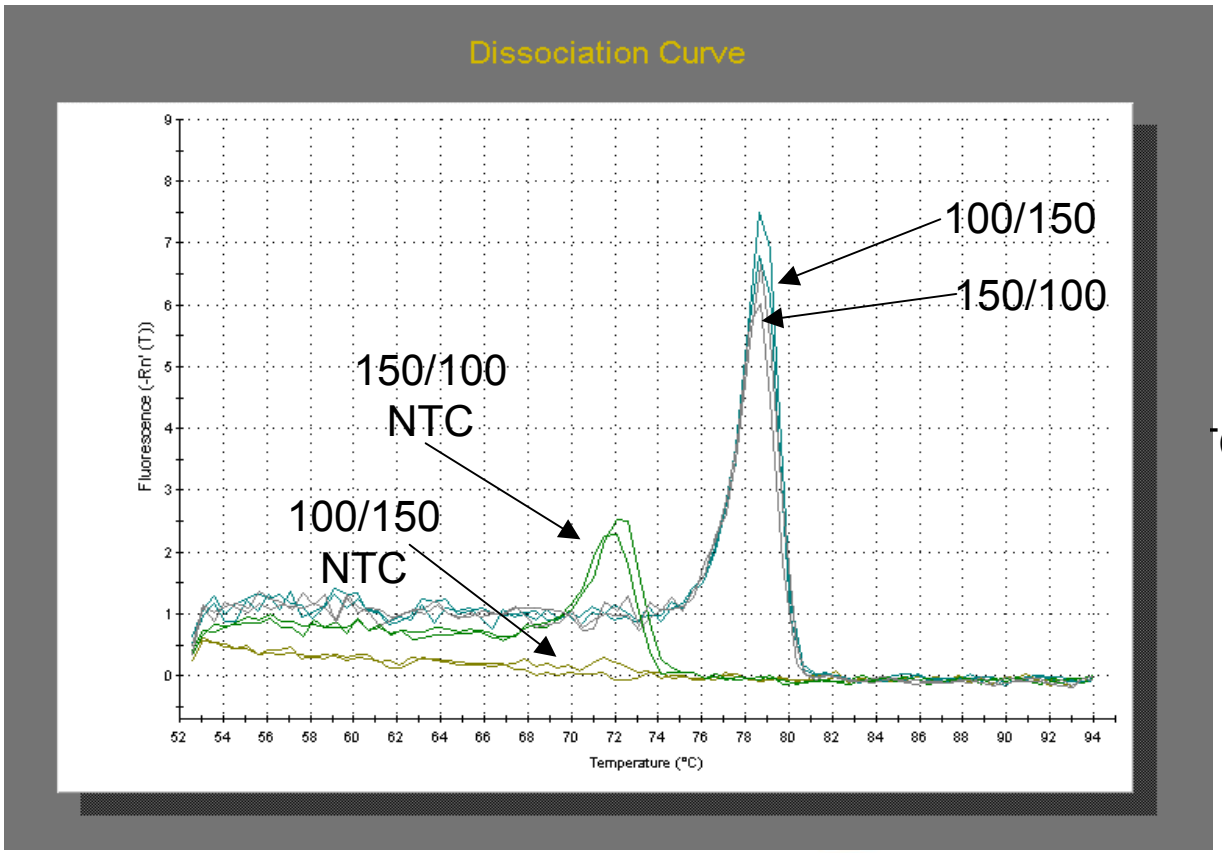
Assay Optimization
Primers and Probes

Primer titration 50 nM – 200 nM
duplicates for pos. Control & NTC

Aims:

- low Ct values
  → sensitivity
- no unspecific amplification or primer dimers
  → specificity
- Low interreplicate variability
- high efficiency of Amplification
  → separate run



Cs

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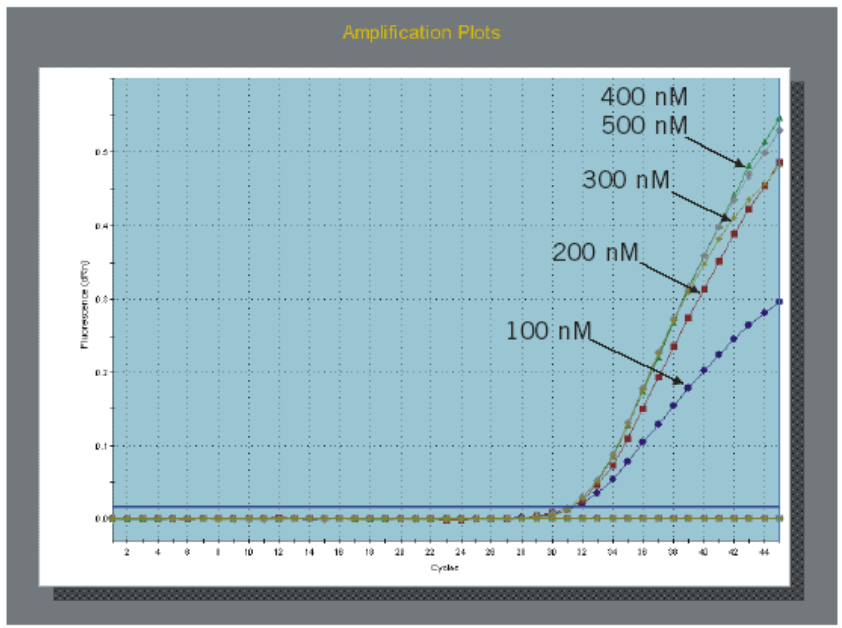
Assay Optimization
Primers and Probes

Titration of probes

- usually only necessary for multiplex of 2-4 target sequences
- low Ct values, minimal replicate variability and robust signal

	100nM	200nM	300nM	400nM	500nM
Optimal [primer]					



In this example: 100-500 nM probe result in approx. same Ct but 200-500 nM give better signal to noise ratio.

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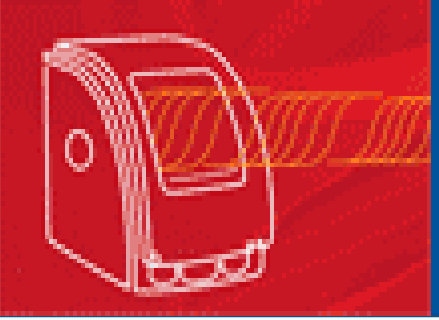

Assay Optimization
Multiplexing

Optimization:

Optimization of primers and probes for each target separately.
lower primer and probe concentrations are better.

The first run:

Serial dilution of a positive control as target.
Run each target separate and in multiplex.
Increase Taq concentration (50-100%).
Increase dNTP concentration (50-100%).
Increase Mg^{++} concentration (0.25-0.50 mM).
In some cases: Increase buffer concentration (1.5x).

} Ready-made multiplex qPCR mastermixes

Quality criteria:

For each target multiplex data and singleplex data should look as similar as possible.
No signal crosstalk contamination.

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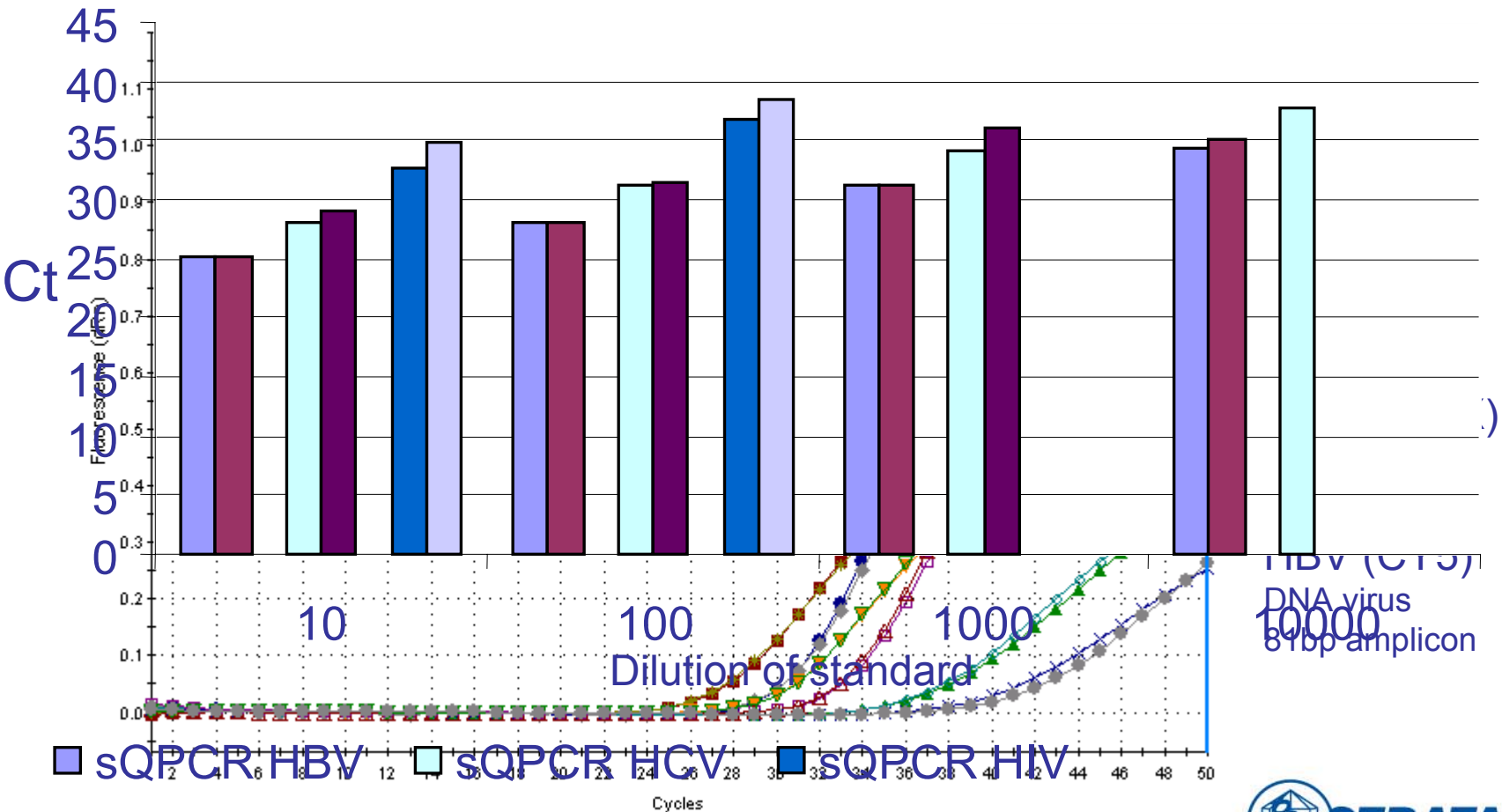
Assay Optimization
Multiplexing

Daniel Candotti - Cambridge, UK



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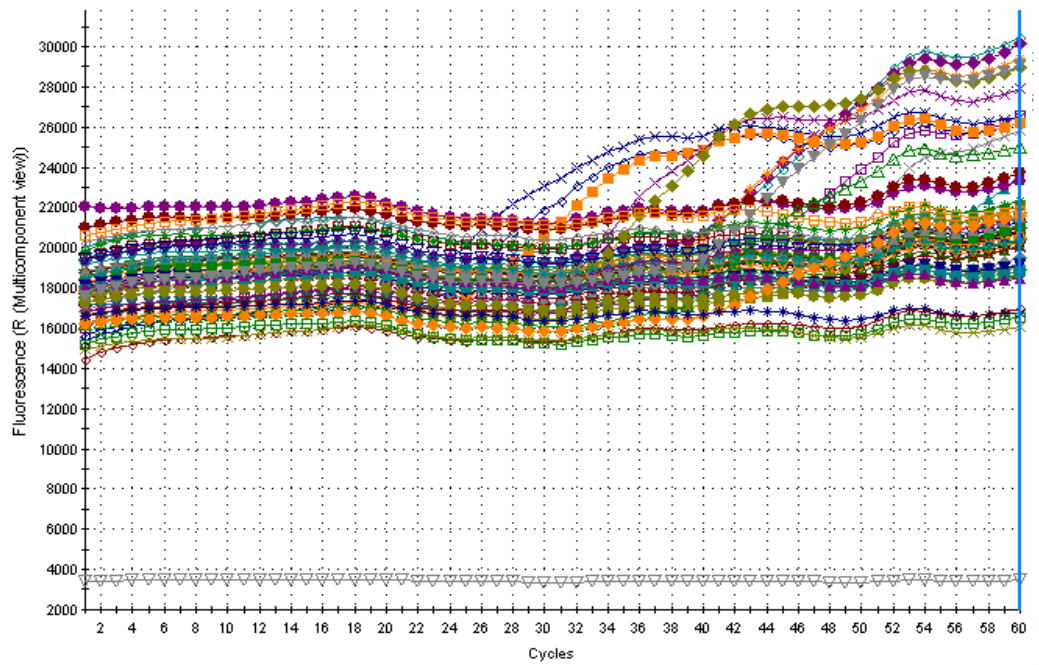
Basic Protocol for Troubleshooting

Look at your raw data (R multicomponent view).
Does it look good:
Background below 30000 for all used dyes?
Nice signal to noise?
Do amplification plots look normal?



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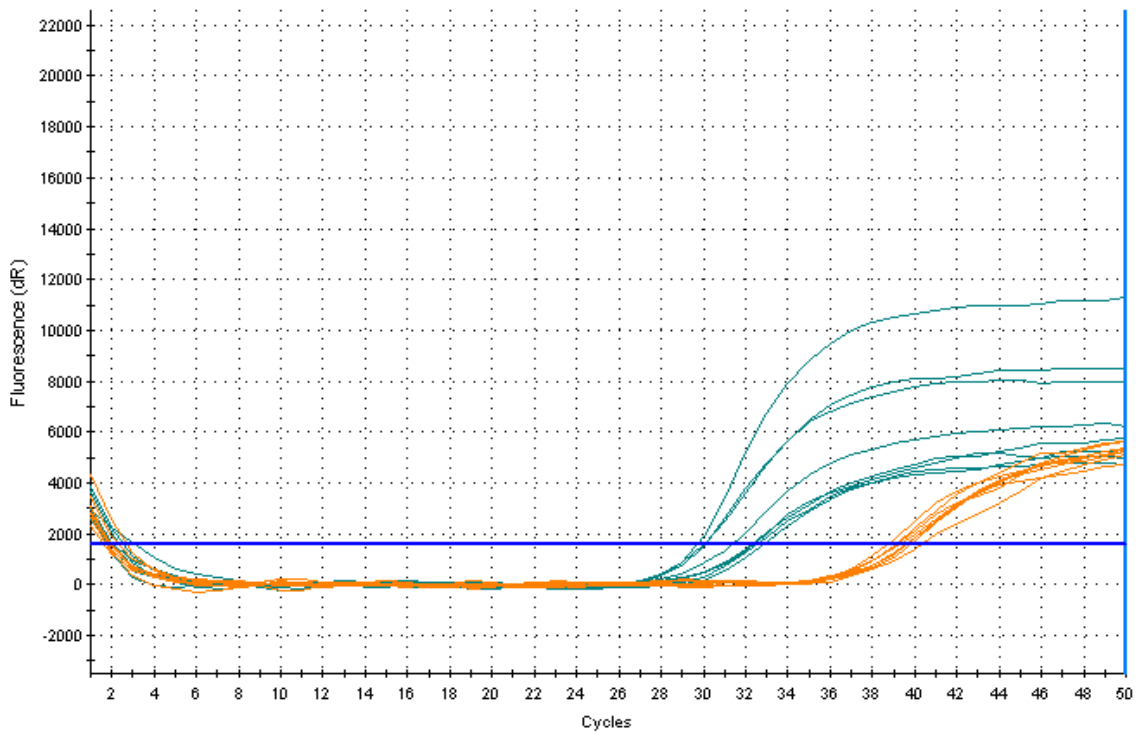
Basic Protocol for Troubleshooting

Look at the baseline corrected view (dR).
Amplification plots still look perfect?
Tilted plots hint at baseline correction problem!



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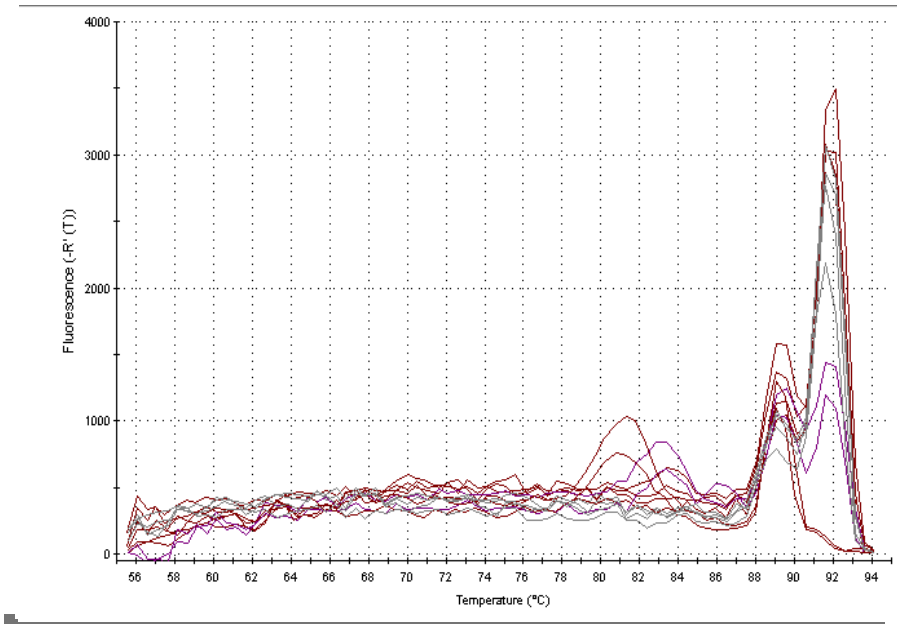
Check your Controls

There are several reasons for positive negative controls:

- Primer dimers seen in SYBR
- Probe degradation during PCR process
- Template contamination
- non-specific amplification due to mispriming or non-specific probe binding



SYBR meltcurves are a good tool to find out about the reasons for positive negative controls

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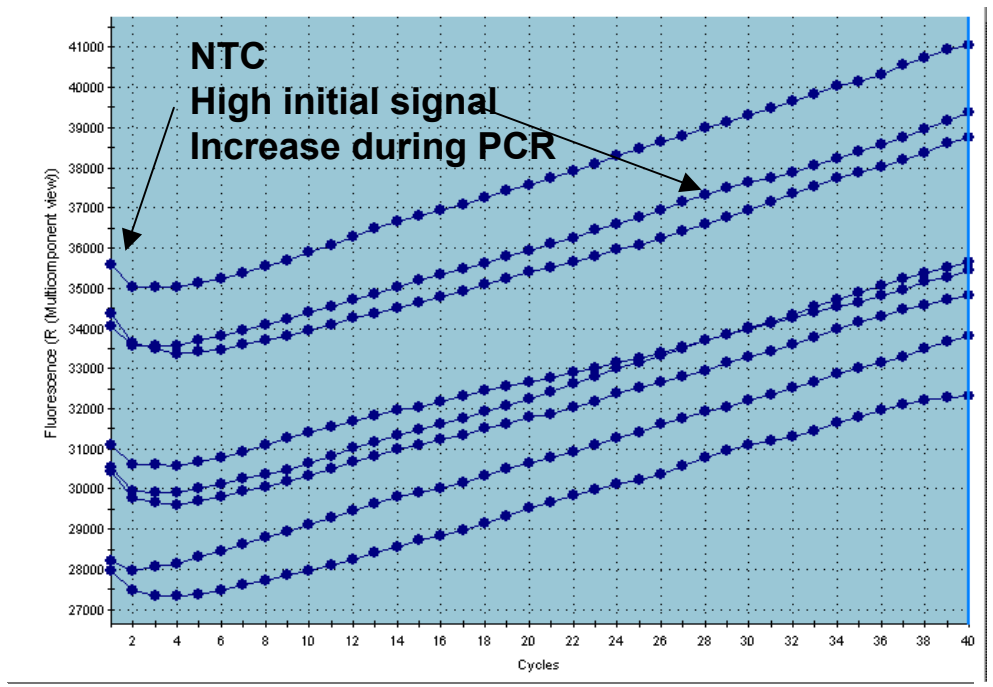
High Background

There are several potential reasons for high background:

- SYBR based:
  - Too much SYBR
  - Too much template
- Probe based:
  - Insufficient quenching
    - → quencher doesn't fit to dye
    - → quencher too far from dye
  - probe concentration too high
  - probe degraded
  - free dye in your probe



High background leads to higher variability and lower sensitivity!

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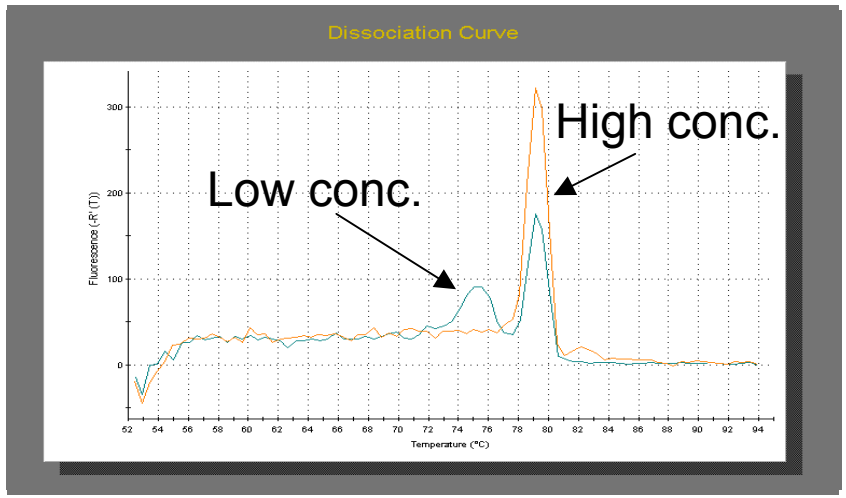
Primer Dimers

- Detection of primer dimers is only possible with SYBR melt curves. They tend to have a T_m between 72-78°C depending on sequence
- Primer dimers tend to occur in low concentrated samples or NTCs at late Cts
- In SYBR they contribute to the overall signal and may make accurate quantification impossible.



- Ignoring primer dimers by using a 4th plateau at higher temperature or in a probe based assay increases variability of the assay due to competition in the reaction
- **Try to get rid of them by primer titration, decreasing annealing time, or redesign**

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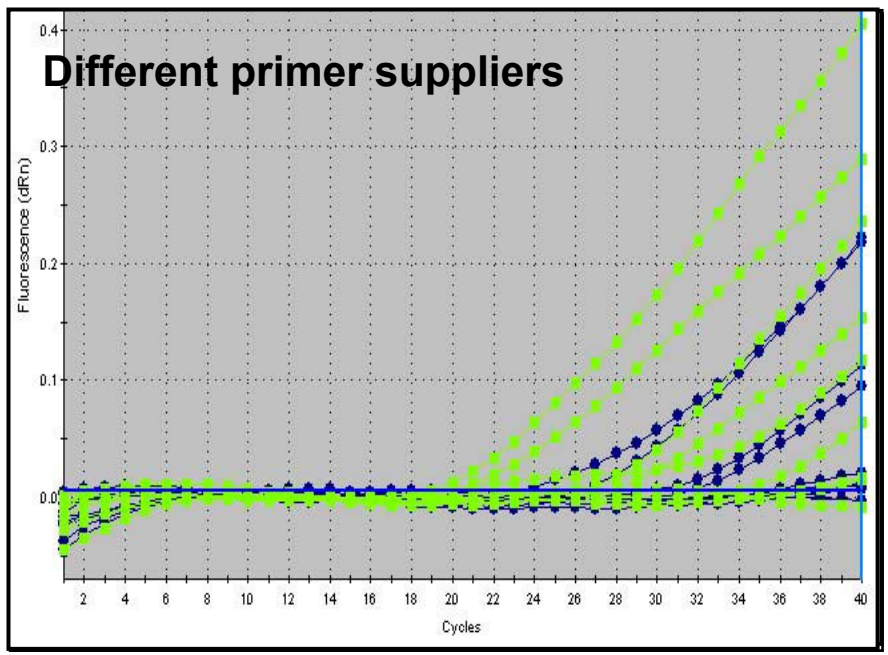
Probes and Primers

Varying primer and probe quality may lead to unexpected results

- You can't expect to get the same results with new lots of already tested primers and probes
- It is beneficial to quality control every new lot of primers and probes:
  - Test your primers and probes with a standard curve
  - Quality control new probe lots with quantitative plate reads and DNase I digest



pDNA with copy numbers from 10^7 to 10^1
Stoverock, v. Samson, Hannover (Germany)

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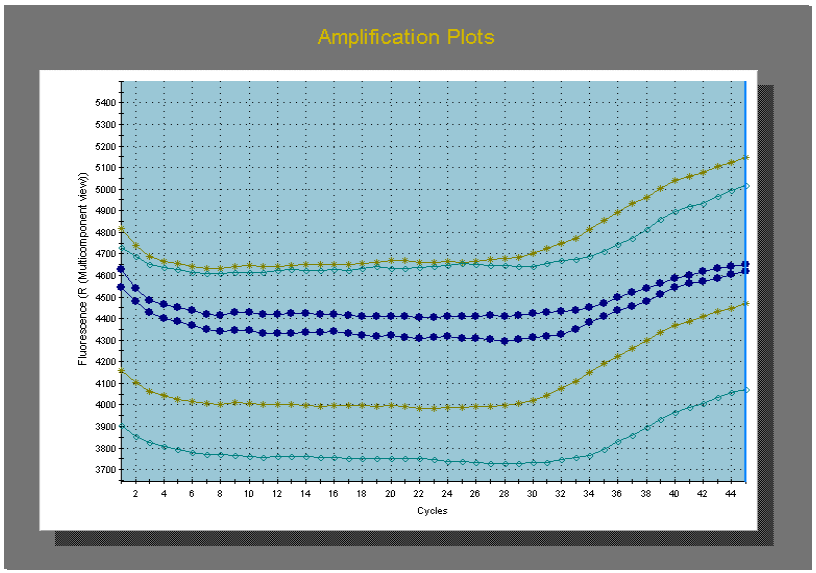
High Variability



- High replicate variability is often introduced by low signal to noise ratio

- Reasons are:

Chemistry related
- Inefficient probe binding
- Inefficient probe cleavage
- Low labeling efficiency of the probe
- **Poor optimization**

Instrument related
- Instrument is not able to measure at emission peak maximum of dye
- Fluorescence is near saturation level

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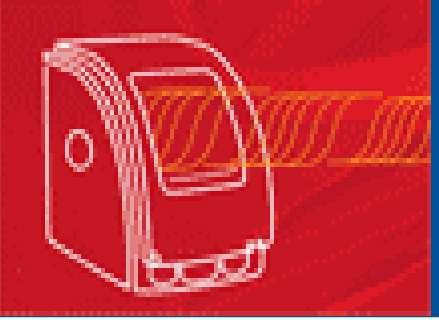

Troubleshooting Summary

- Controls and SYBR meltcurves are important tools to troubleshoot problems with your assay
- Comparing raw data and baseline corrected data helps addressing issues caused by invalid baselines
- Raw data allows identifying chemistry or instrument issues
- It can be important to quality control freshly ordered primers and probes to understand issues occuring after using new lots
- Varying template quality can lead to highly variable results

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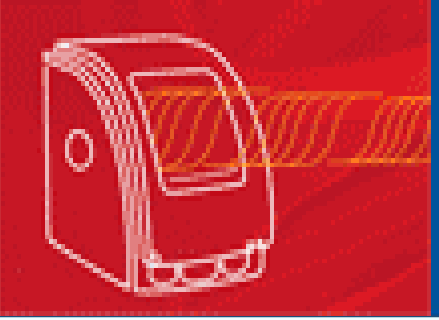

The Quiz

Let’s give it a try……..

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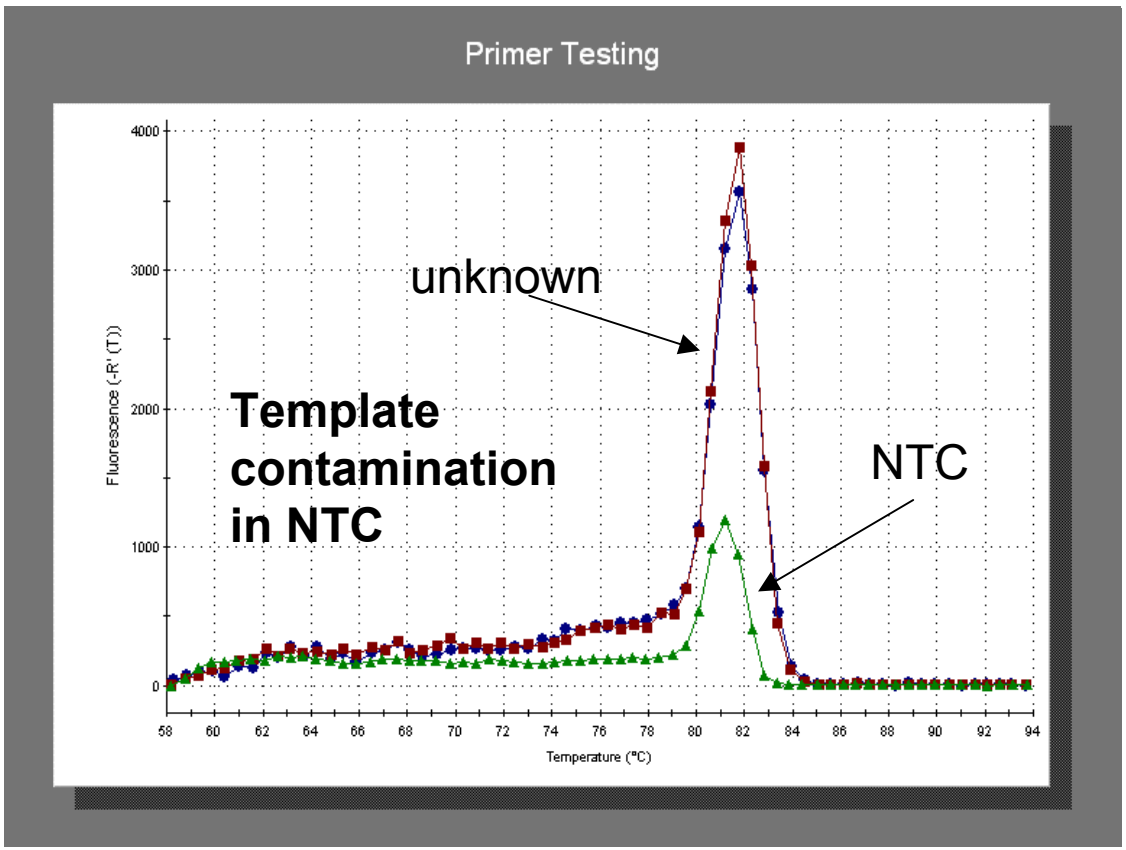
The Quiz



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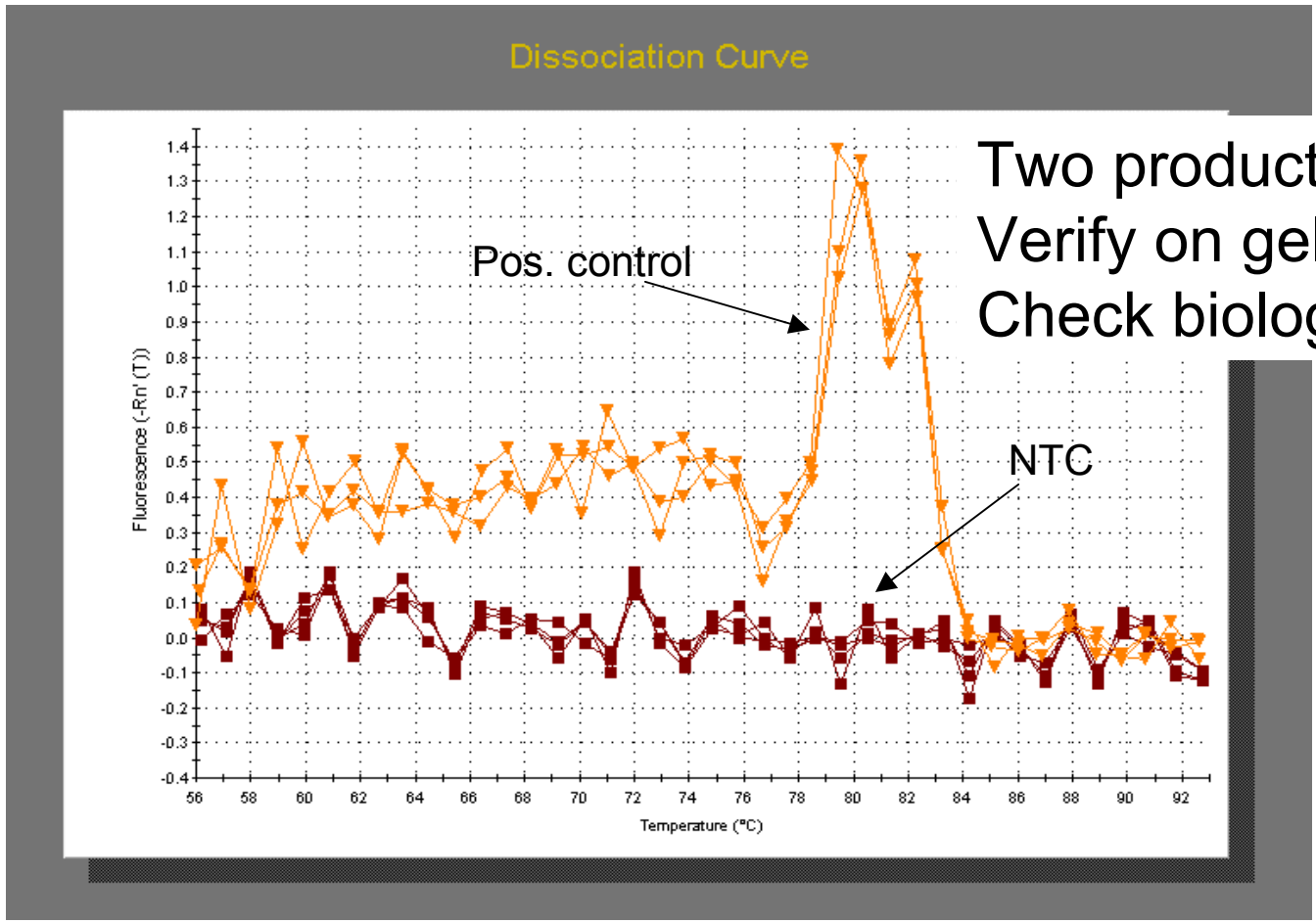
The Quiz



Assay validation

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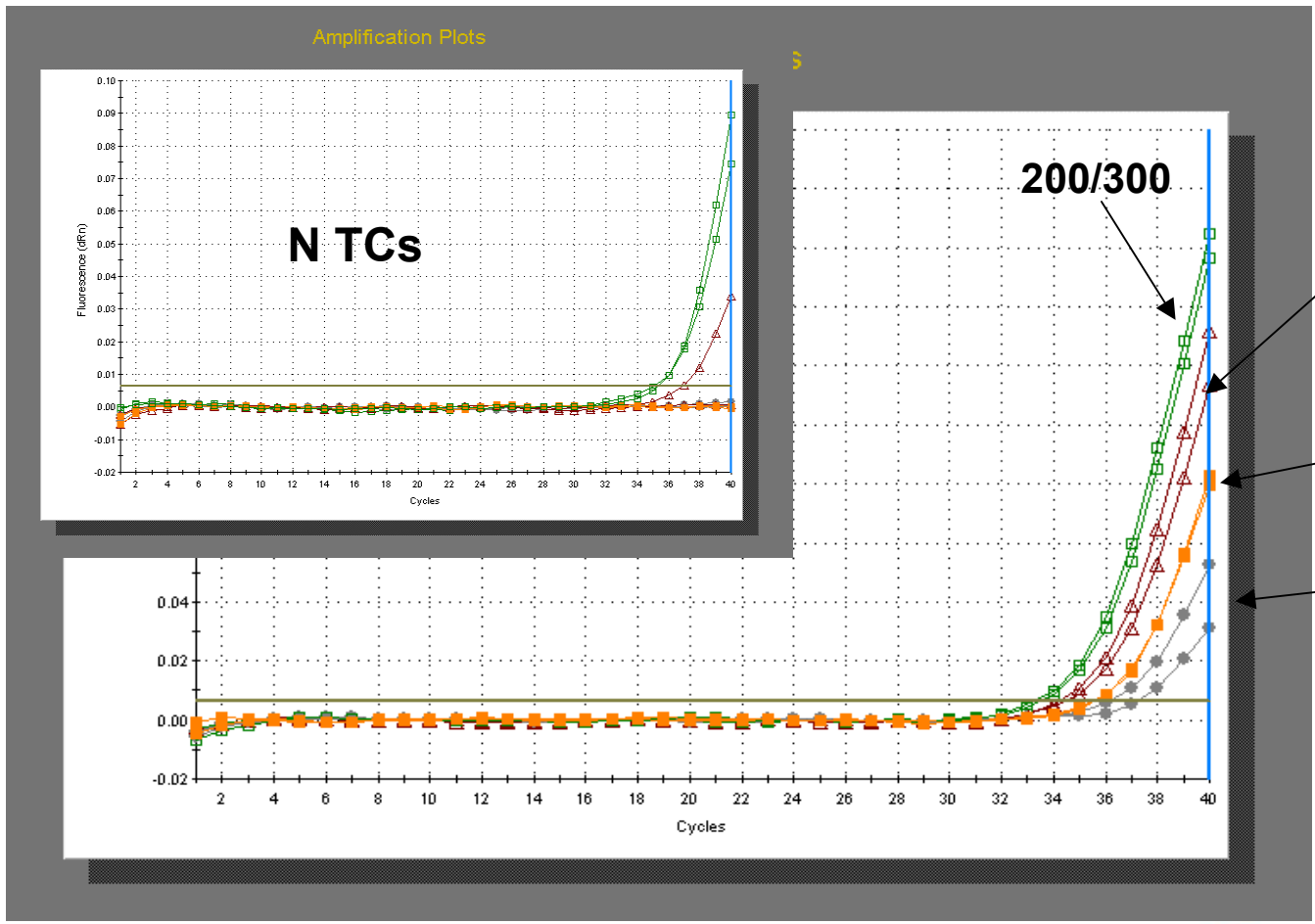
The Quiz



Primer titration

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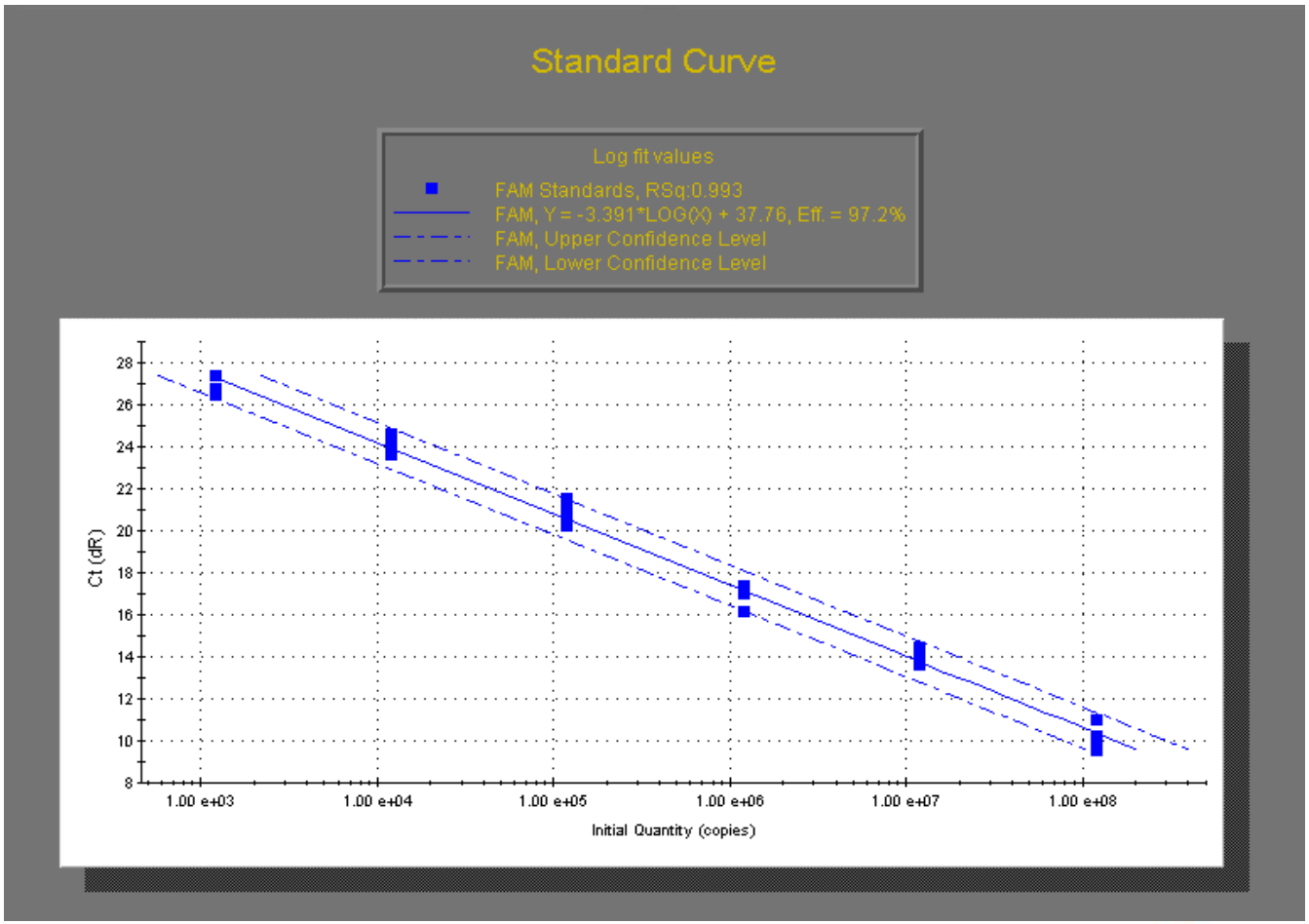
The Quiz



Melt curves

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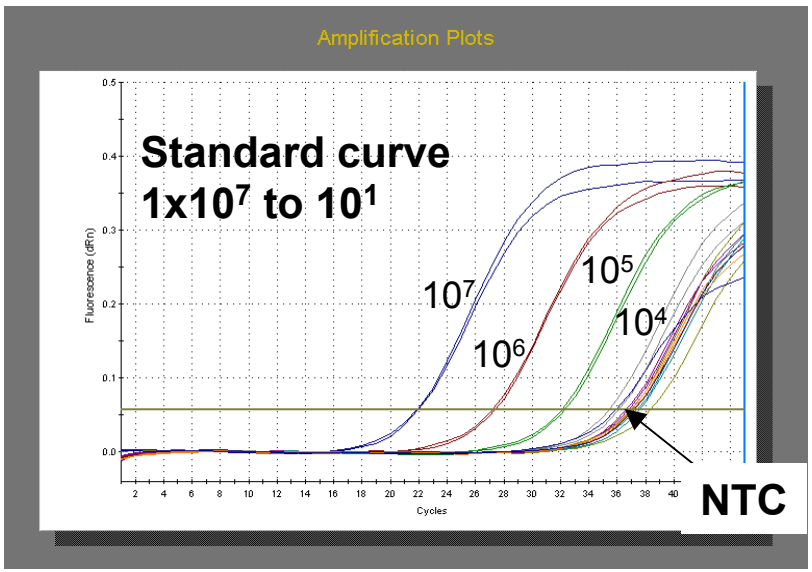
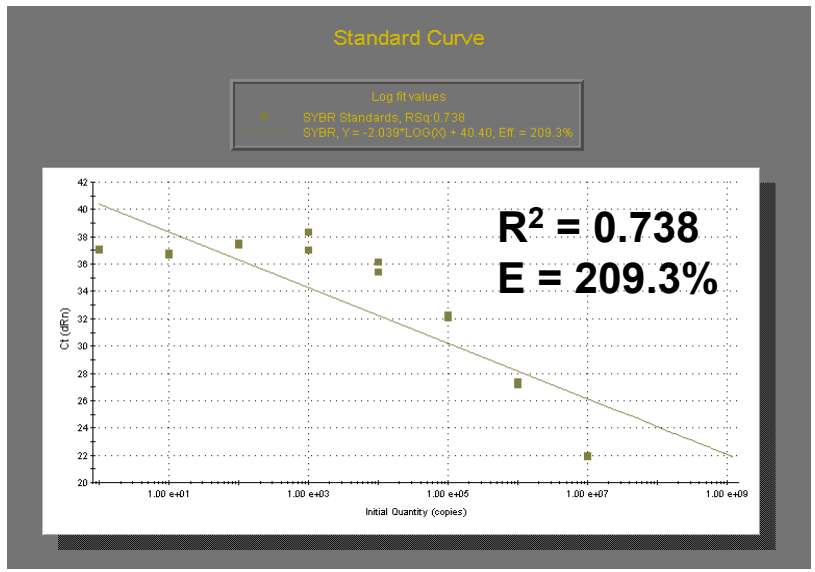
The Quiz





ΔCt: 10^7 → 10^6 5.4; 10^6 → 10^5 5.0; 10^5 → 10^4 3.6

Primer dimers, in combination with inefficient priming/inhibition

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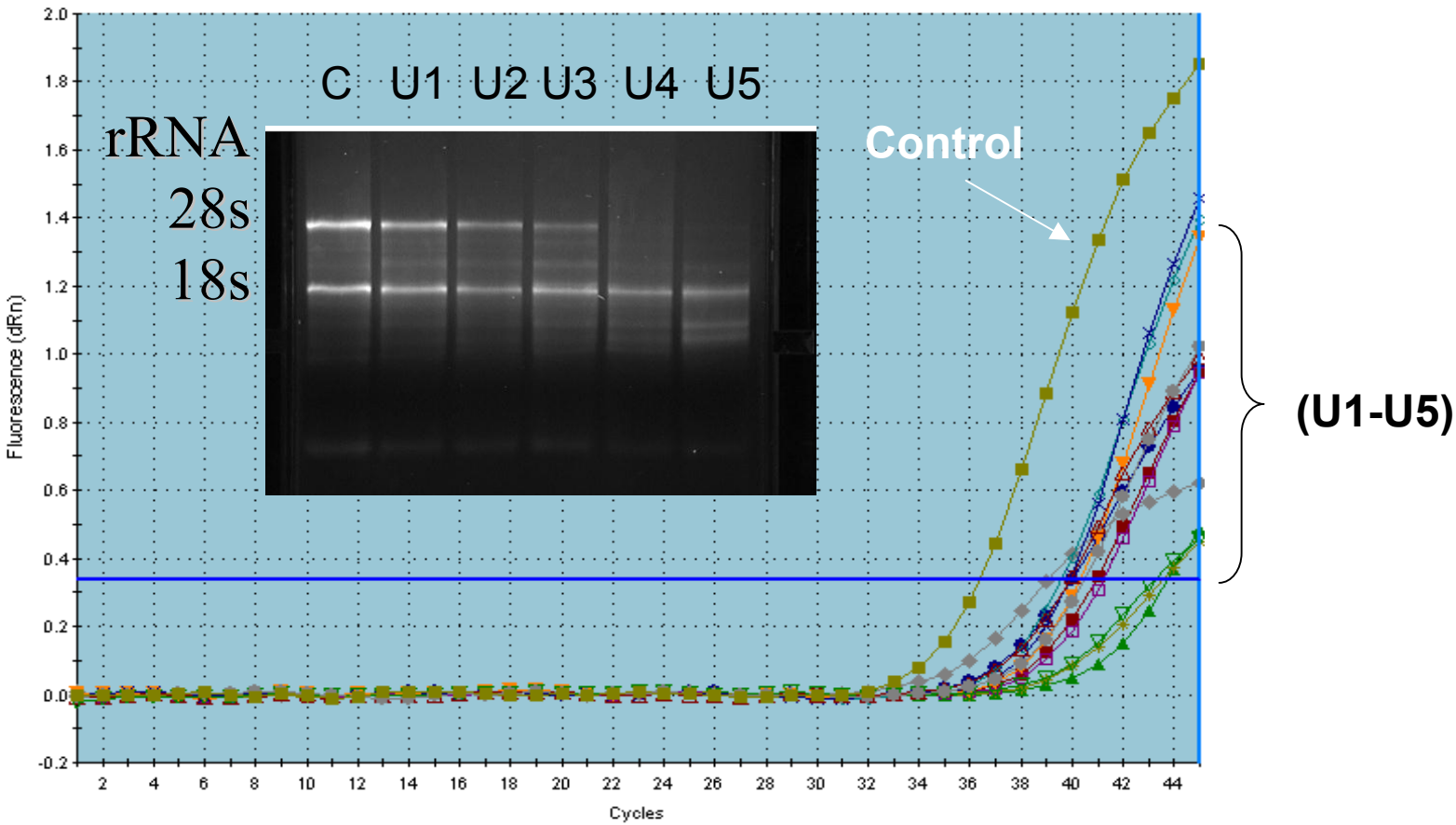
The Quiz

qRT-PCR with equal amounts of RNA for control and samples (U1-U5)



Samples: paraffin embedded tissue
same tissue, varying storage times

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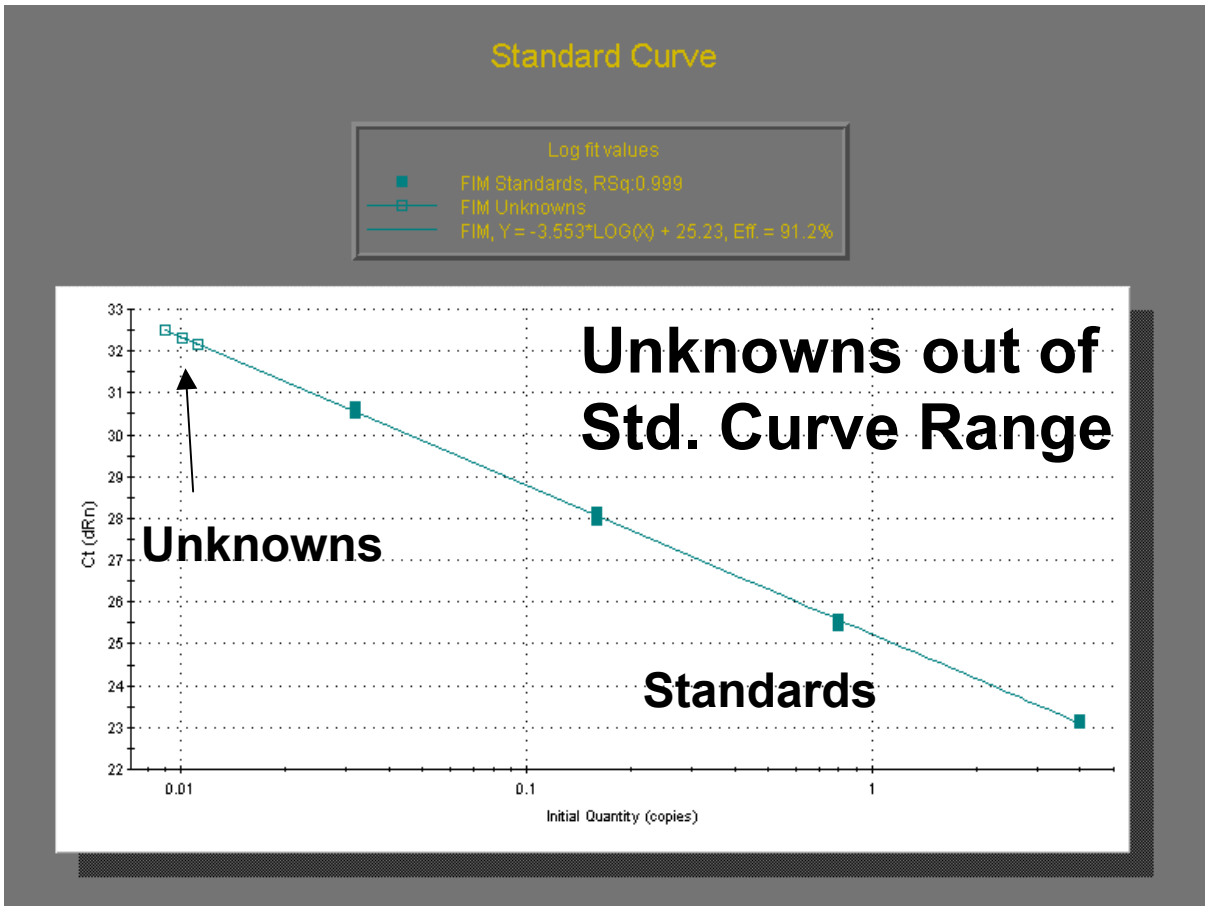
The Quiz



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Thanks for your attention!

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