Quantitative Real-Time PCR Application Workshop

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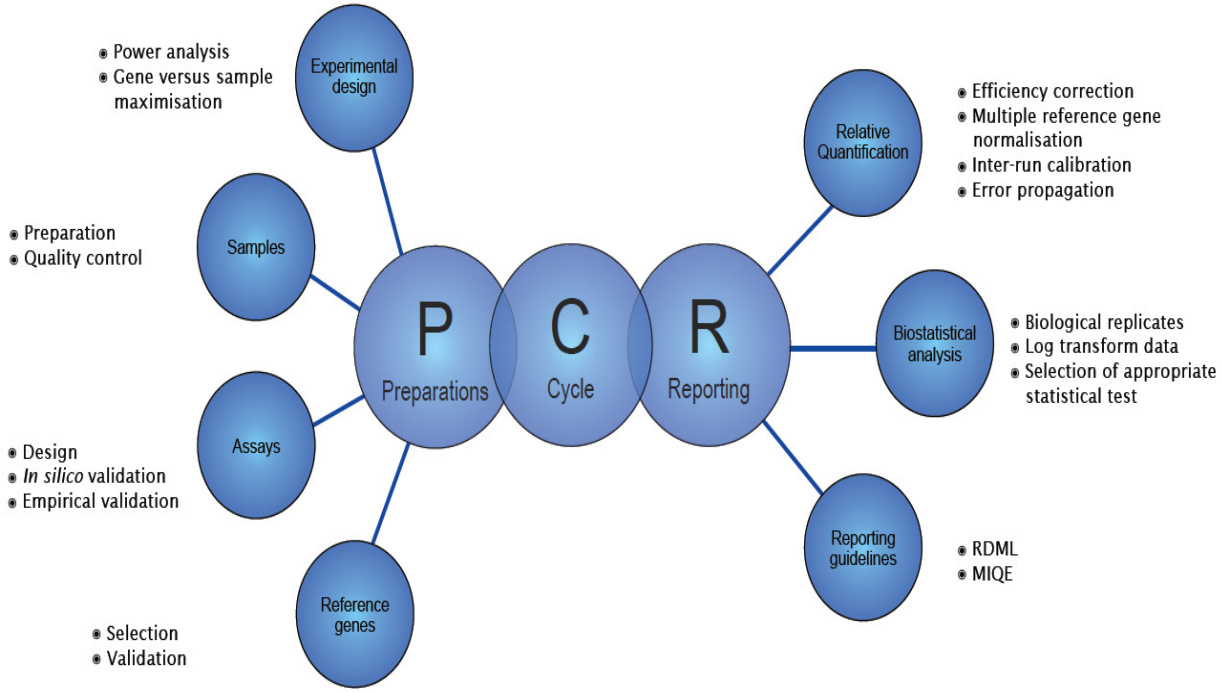
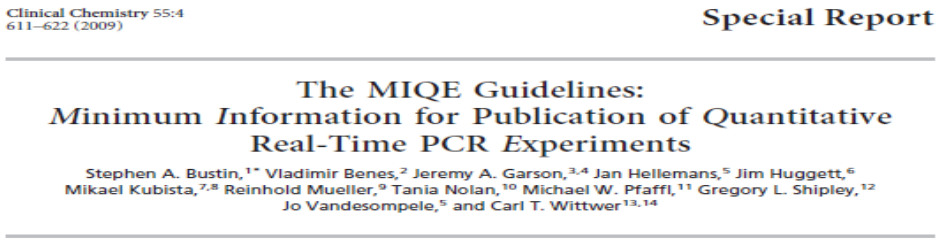
Quantitative real time pcr critical factors contributing to success

MIQE guidelines: minimum set of information that researchers should provide for their qPCR data




Clinical Chemistry 55:4
611–622 (2009)

Special Report

The MIQE Guidelines:
Minimum Information for Publication of Quantitative Real-Time PCR Experiments

Stephen A. Bustin,[1*] Vladimir Benes,[2] Jeremy A. Garson,[3,4] Jan Hellemans,[5] Jim Huggett,[6] Mikael Kubista,[7,8] Reinhold Mueller,[9] Tania Nolan,[10] Michael W. Pfaffl,[11] Gregory L. Shipley,[12] Jo Vandesompele,[5] and Carl T. Wittwer[13,14]

Author: Dr Joanna Brzeszczyńska (UWS)

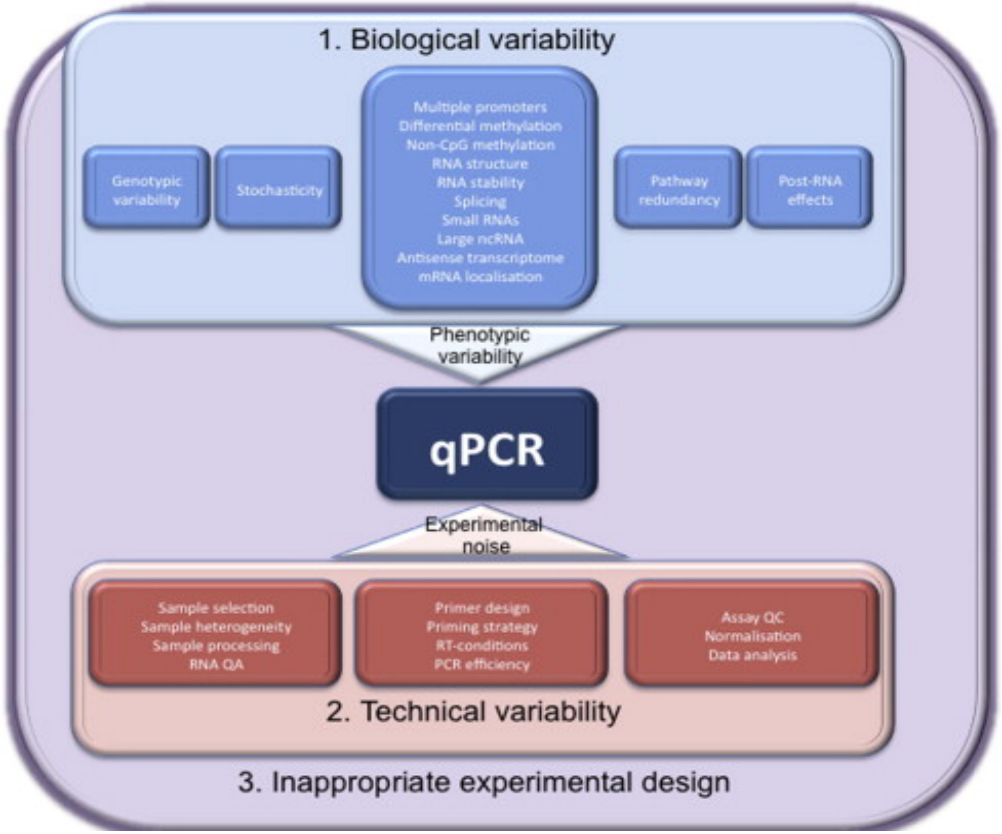


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Table 1. MIQE checklist for authors, reviewers, and editors.[a]

Item to check	Importance	Item to check	Importance
Experimental design		qPCR oligonucleotides	
Definition of experimental and control groups	E	Primer sequences	E
Number within each group	E	RTPrimerDB identification number	D
Assay carried out by the core or investigator's laboratory?	D	Probe sequences	D[d]
Acknowledgment of authors' contributions	D	Location and identity of any modifications	E
Sample		Manufacturer of oligonucleotides	D
Description	E	Purification method	D
Volume/mass of sample processed	D	qPCR protocol	
Microdissection or macrodissection	E	Complete reaction conditions	E
Processing procedure	E	Reaction volume and amount of cDNA/DNA	E
If frozen, how and how quickly?	E	Primer, (probe), Mg^{2+}, and dNTP concentrations	E
If fixed, with what and how quickly?	E	Polymerase identity and concentration	E
Sample storage conditions and duration (especially for FFPE[b] samples)	E	Buffer/kit identity and manufacturer	E
Nucleic acid extraction		Exact chemical composition of the buffer	D
Procedure and/or instrumentation	E	Additives (SYBR Green I, DMSO, and so forth)	E
Name of kit and details of any modifications	E	Manufacturer of plates/tubes and catalog number	D
Source of additional reagents used	D	Complete thermocycling parameters	E
Details of DNase or RNase treatment	E	Reaction setup (manual/robotic)	D
Contamination assessment (DNA or RNA)	E	Manufacturer of qPCR instrument	E
Nucleic acid quantification	E	qPCR validation	
Instrument and method	E	Evidence of optimization (from gradients)	D
Purity (A_{260}/A_{280})	D	Specificity (gel, sequence, melt, or digest)	E
Yield	D	For SYBR Green I, C_q of the NTC	E
RNA integrity: method/instrument	E	Calibration curves with slope and *y* intercept	E
RIN/RQI or C_q of 3′ and 5′ transcripts	E	PCR efficiency calculated from slope	E
Electrophoresis traces	D	CIs for PCR efficiency or SE	D
Inhibition testing (C_q dilutions, spike, or other)	E	r^2 of calibration curve	E
Reverse transcription		Linear dynamic range	E
Complete reaction conditions	E	C_q variation at LOD	E
Amount of RNA and reaction volume	E	CIs throughout range	D
Priming oligonucleotide (if using GSP) and concentration	E	Evidence for LOD	E
Reverse transcriptase and concentration	E	If multiplex, efficiency and LOD of each assay	E
Temperature and time	E	Data analysis	
Manufacturer of reagents and catalogue numbers	D	qPCR analysis program (source, version)	E
C_qs with and without reverse transcription	D[c]	Method of C_q determination	E
Storage conditions of cDNA	D	Outlier identification and disposition	E
qPCR target information		Results for NTCs	E
Gene symbol	E	Justification of number and choice of reference genes	E
Sequence accession number	E	Description of normalization method	E
Location of amplicon	D	Number and concordance of biological replicates	D
Amplicon length	E	Number and stage (reverse transcription or qPCR) of technical replicates	E
In silico specificity screen (BLAST, and so on)	E	Repeatability (intraassay variation)	E
Pseudogenes, retropseudogenes, or other homologs?	D	Reproducibility (interassay variation, CV)	D
Sequence alignment	D	Power analysis	D
Secondary structure analysis of amplicon	D	Statistical methods for results significance	E
Location of each primer by exon or intron (if applicable)	E	Software (source, version)	E
What splice variants are targeted?	E	C_q or raw data submission with RDML	D

[a] All essential information (E) must be submitted with the manuscript. Desirable information (D) should be submitted if available. If primers are from RTPrimerDB, information on qPCR target, oligonucleotides, protocols, and validation is available from that source.
[b] FFPE, formalin-fixed, paraffin-embedded; RIN, RNA integrity number; RQI, RNA quality indicator; GSP, gene-specific priming; dNTP, deoxynucleoside triphosphate.
[c] Assessing the absence of DNA with a no–reverse transcription assay is essential when first extracting RNA. Once the sample has been validated as DNA free, inclusion of a no–reverse transcription control is desirable but no longer essential.
[d] Disclosure of the probe sequence is highly desirable and strongly encouraged; however, because not all vendors of commercial predesigned assays provide this information, it cannot be an essential requirement. Use of such assays is discouraged.

Author: Dr Joanna Brzeszczyńska (UWS)

MIQE key issues:

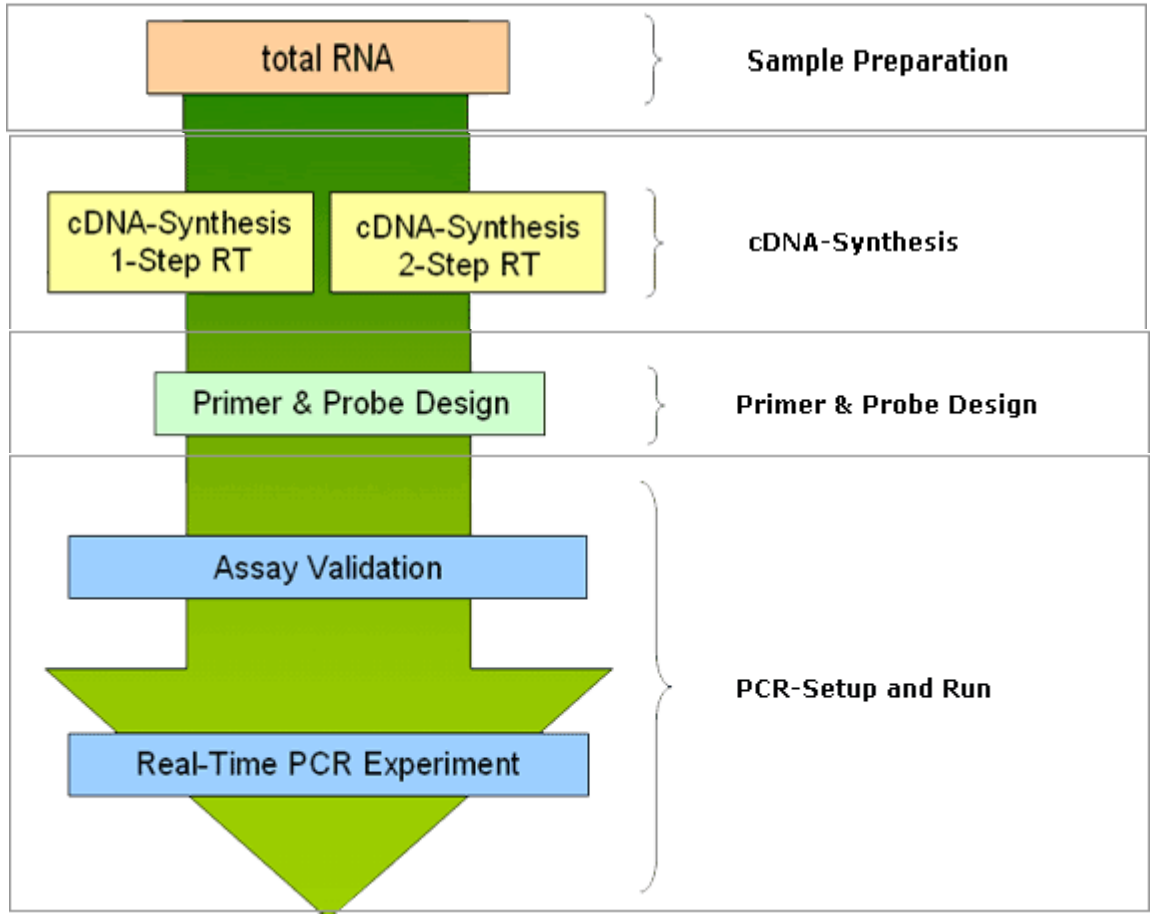
1. Detailed sample information: sample selection, acquisition, handling and storage, processing procedures.

2. (RT)-qPCR quality control metrics: purity and integrity.

3. (RT)-qPCR efficiency: amplification efficiencies of target and reference genes under treatment and control conditions.

4. Justification for normalisation procedure: use more than 1 reference gene (3 is recommended).

5. Importance of controls: NTC, (-)ve RT, (+)ve control.



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(RT)-qPCR quality control metrics

RNA validation:

Nanodrop: Purity (A260/A280 & A260/A230)

Bioanalizer: Integrity (RIN)

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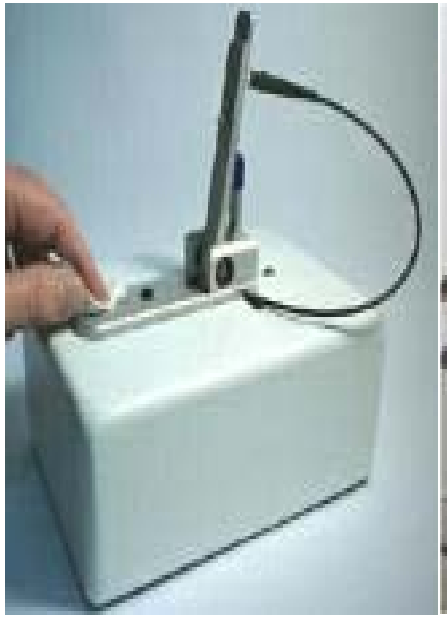
Measuring the Quantity of RNA using the Nanodrop.

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RNA has its absorption maximum at 260 nm and the ratio of the absorbance at 260 and 280 nm is used to assess the RNA purity of an RNA preparation.

Pure RNA has an A260/A280 of 2.1.

You will see in many protocols that a value of 1.8-2.0 indicates that the RNA is pure.

It is important that not only the OD A260/A280 ratio should be very close to 2.0, but that in addition, also the OD A260/A230 ratio should be very close to 2.0.

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The Good

Both the OD A260/A280 as the OD A260/A230 ratio are 2.0 or more.

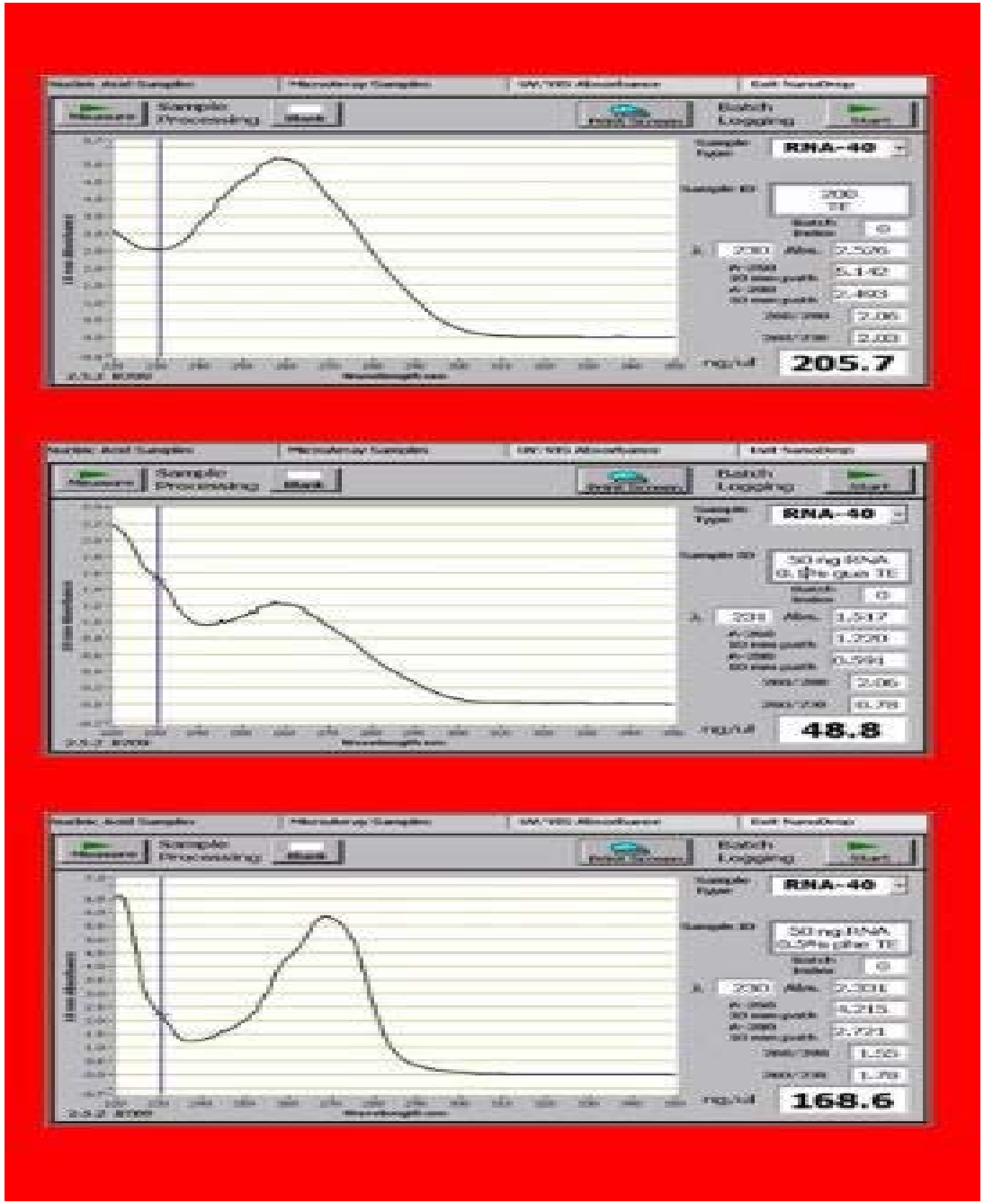
Perfect, you can do with this RNA whatever you like, everything should work.

The Bad

The OD A260/A280 ratio is over 2.0 but the OD A260/A230 ratio is below 1.0. Be careful! This indicates that the sample contains impurities. Some downstream procedures may work perfectly while others may give problems.

The Ugly

Don’t even think of using this RNA!
Just perform an extra purification step.



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Measuring the Integrity of RNA using the Bioanalyzer

RNA validation:

RNA Integrity Number (RIN) –Standardization of RNA Quality Control

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Bioanalyzer Applications:

Check RNA integrity for downstream applications

Genomic and ribosomal contamination of mRNA samples

Small RNA (6-150 nt) analysis of miRNA, siRNA, and oligonucleotides

PCR product purity and size

Protein induction in cell lysates

Protein purification

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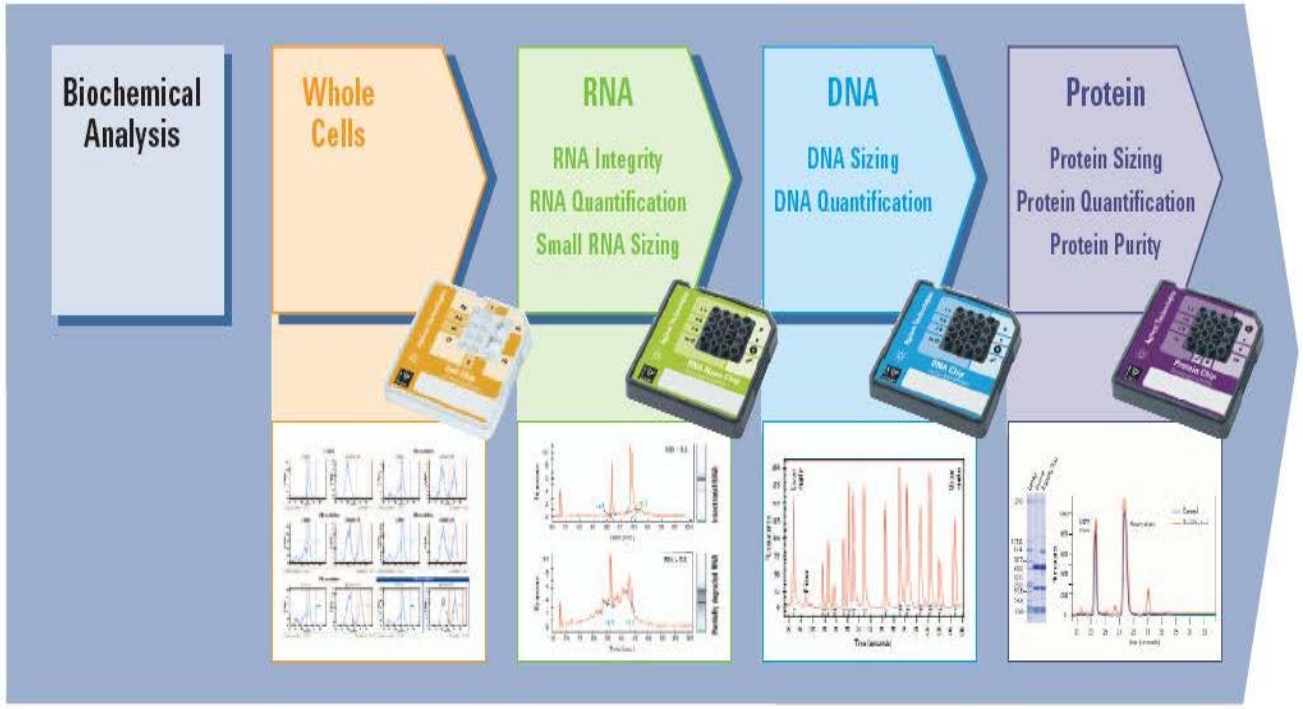

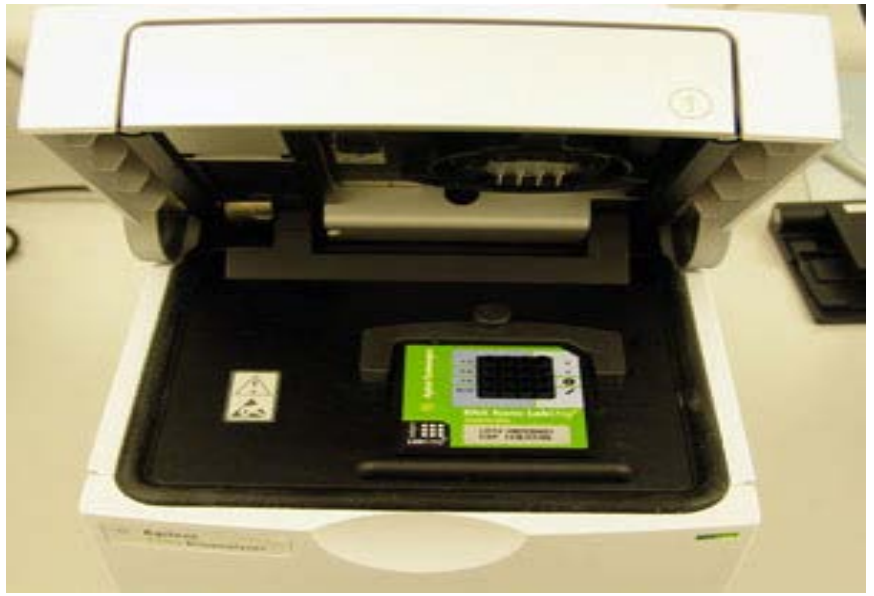

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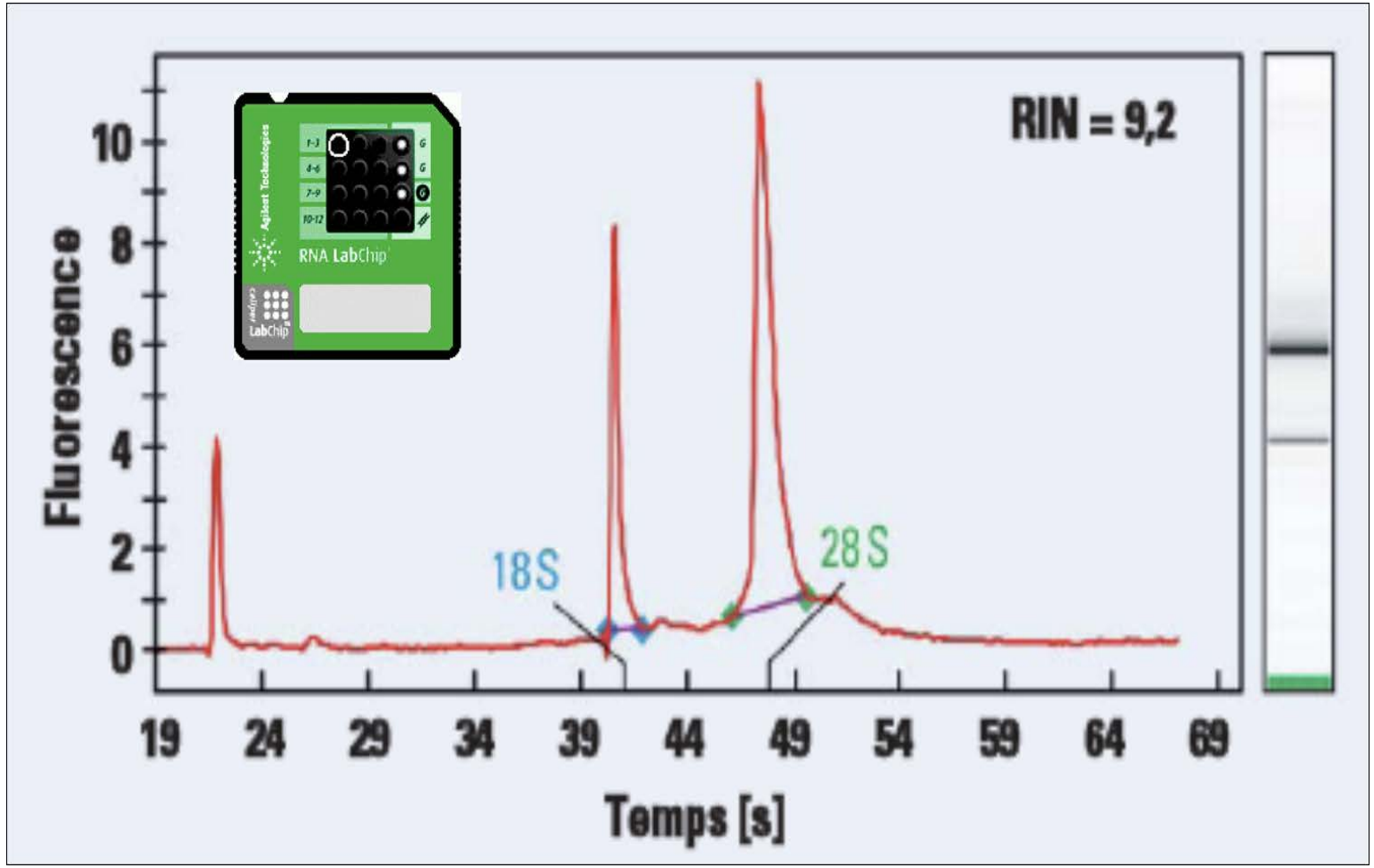
The RNA Integrity Number (RIN), was developed to remove individual interpretation in RNA quality control.



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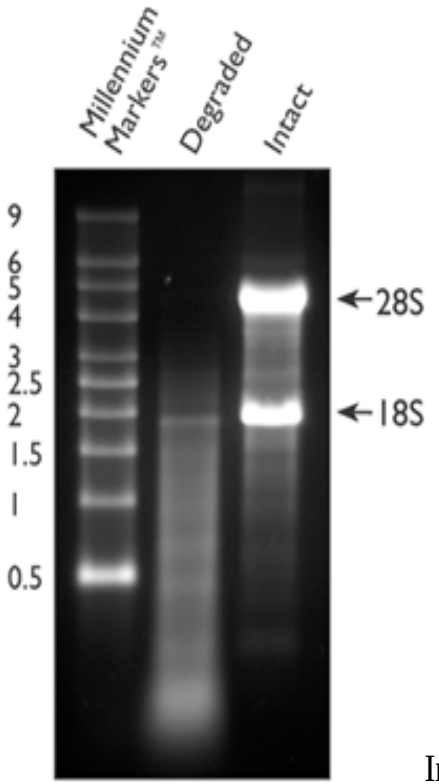

Intact vs. Degraded RNA. Two µg of degraded total RNA and intact total RNA were run beside Ambion's RNA Millennium Markers™ on a 1.5% denaturing agarose gel. The 18S and 28S ribosomal RNA bands are clearly visible in the intact RNA sample. The degraded RNA appears as a lower molecular weight smear.

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RNA Storage

- Storing the purified RNA
- Immediately after removing the 1µL aliquot from the purified RNA product, add 1u/µL Superase-IN (Ambion) RNAse inhibitor to each tube of purified RNA.
- Vortex to mix.
- Wrap the sealed tube with Parafilm and store at -80˚ C for future use.

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Conclusions:

- Should be free of protein (absorbance 260nm/280nm > 1.8)
- Should be intact (28S/18S ~2:1)
- High RIN (use Agilent Bioanalyzer)
- Should be free of DNA (treat with DNAse)
- Should be free of PCR inhibitors
  - Purification methods
  - Clean-up methods

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cDNA Synthesis

Obtain tissue

Extract RNA

Copy into cDNA
(reverse transcriptase)

Real-time PCR

Analyze results

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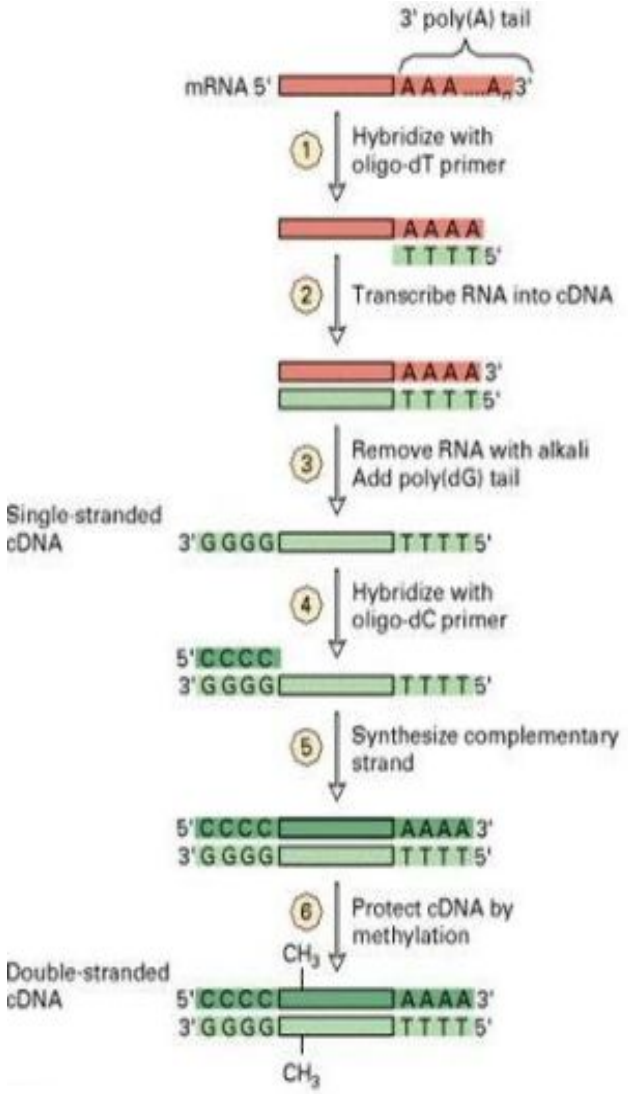
cDNA Synthesis



mRNA → (RT) → cDNA → (Taq pol) → qPCR

•efficiency usually not known

There are some aspects that have to be considered for cDNA-synthesis.

This is on one hand the <u>primer-type</u>

(random oligomer vs. oligodT)

and

<u>the PCR-type</u> (one step vs. two-step)

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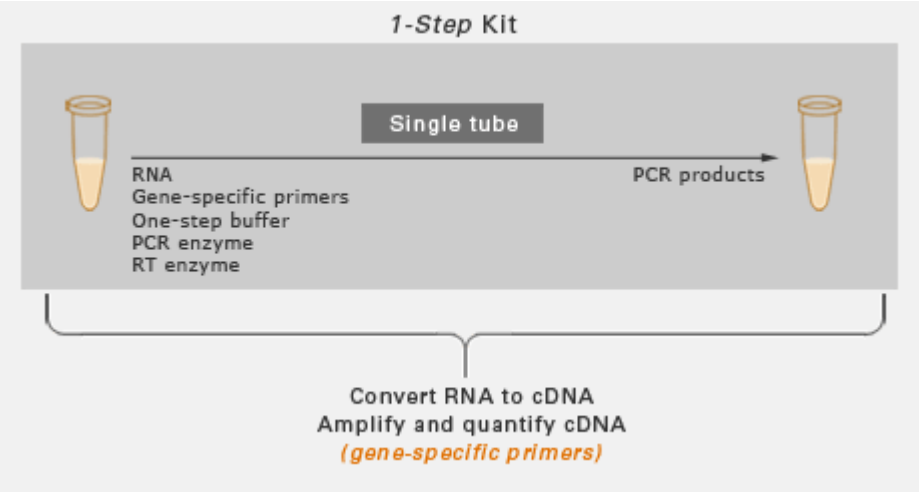
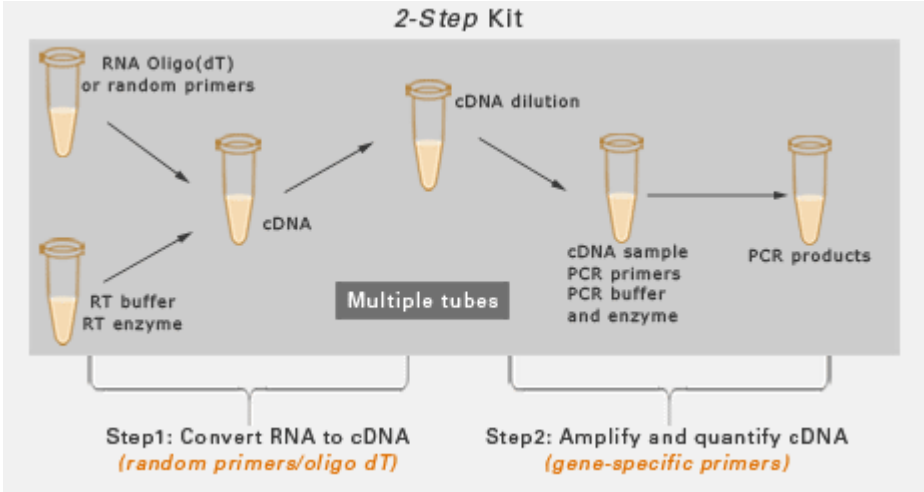
PCR-type (one step vs. two-step)).





Choose the 1-Step Kit, if you...

- Do not store cDNA
- Dispose samples after one or few uses
- Have many samples with one or few targets
- Use liquid handling robotics
- Choose to reduce chance of cross contamination during procedure
- Need to reduce time to results

Choose the 2-Step Kit, if you...

- Need to store cDNA
- Have limited sample quantity
- Have many targets per sample
- Require maximum performance of both RT and PCR steps

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Obtain tissue

Extract RNA

Copy into cDNA
(reverse transcriptase)

Real-time PCR

Analyze results

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Importance of primers in qPCR

- Specific
- High efficiency
- No primer-dimers
- Ideally should not give a genomic DNA signal
  - cross exon/exon boundary

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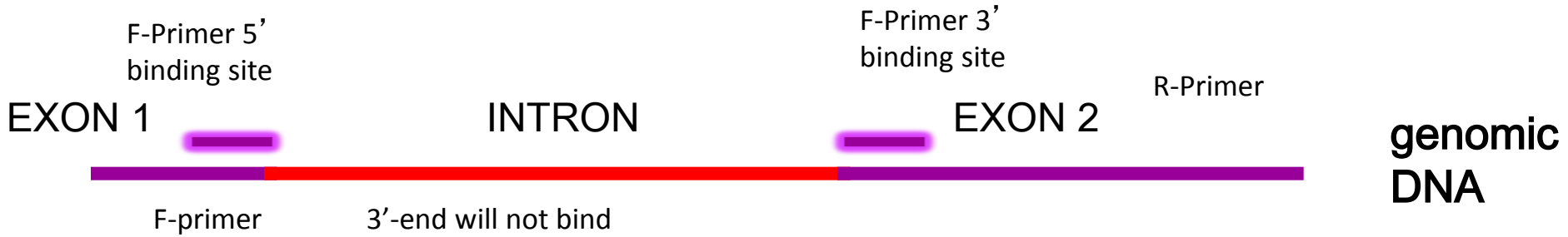
Primer will not bind to genomic DNA because the 3’ end is not complementary to the Intron



genomic DNA

cDNA

Primer will bind to the cDNA because the primer is complementary to the Exon-Exon boundary after the intron is cleaved out

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General guidelines for primer design

- 18-30 nucleotides
- G/C content: 40-60%
- Avoid complementary sequences of primers (especially at the 3’ end)
- Avoid mismatches at the 3’ end
- Avoid 3 or more G or C at the 3’ end
- Avoid a 3’ end T

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General guidelines for primer design

PerlPrimer - Open-source, downloadable PCR primer design software

Primer3 - Open-source PCR primer design software. Offers both downloadable and web versions

Primer-BLAST - Web software for designing primers that combines features of both Primer3 and BLAST.

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matches are found for GenBank Accession ()"NM_022114„

- **Gene Descriptions: GenBank Accession** NM_022114 **NCBI Protein Accession** NP_071397 **Species** Human **Coding DNA Length** 3831
- **Gene Description** PR domain containing 16; transcription factor MEL1 [Homo sapiens].
  Primer Pair 1 (Click here for cDNA and amplicon sequence)**: PrimerBank ID** 11545831a1
- **Amplicon Size** 191
- **Sequence** (5' -> 3') **Length** **Tm** **Location**
- Forward Primer AAGGCGGTCTGTTAGCTTTGG 21 62.6 3539-3559
- Reverse Primer GTCTTCGGAAAGGGACAGCA 20 61.8 3729-3710

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EFFECTS OF EFFICIENCY

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PCR

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

The amount of DNA doubles after each cycle

Assuming 100% efficient PCR reactions

After n cycles there will be 2^n times as much DNA

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CYCLE	AMOUNT OF DNA 100% EFFICIENCY	AMOUNT OF DNA 90% EFFICIENCY	AMOUNT OF DNA 80% EFFICIENCY
0	1	1	1
1	2	2	2
2	4	4	3
3	8	7	6
4	16	13	10
5	32	25	19
6	64	47	34
7	128	89	61
8	256	170	110
9	512	323	198
10	1 024	613	357
11	2 048	1 165	643
12	4 096	2 213	1 157
13	8 192	4 205	2 082
14	16 384	7 990	3 748
15	32 768	15 181	6 747
16	65 536	28 844	12 144
17	131 072	54 804	21 859
18	262 144	104 127	39 346
19	524 288	197 842	70 824
20	1 048 576	375 900	127 482
21	2 097 152	714 209	229 468
22	4 194 304	1 356 998	413 043
23	8 388 608	2 578 296	743 477

AFTER 1 CYCLE

100%= 2.00x

90% = 1.90x

80% = 1.80x

70% = 1.70x

Much different values depending on the efficiency

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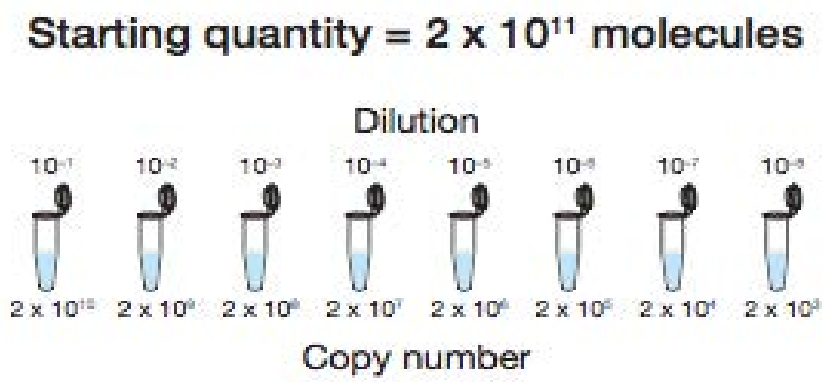
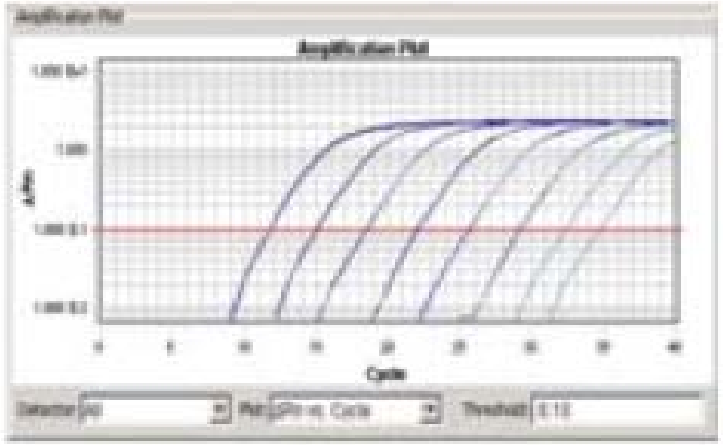
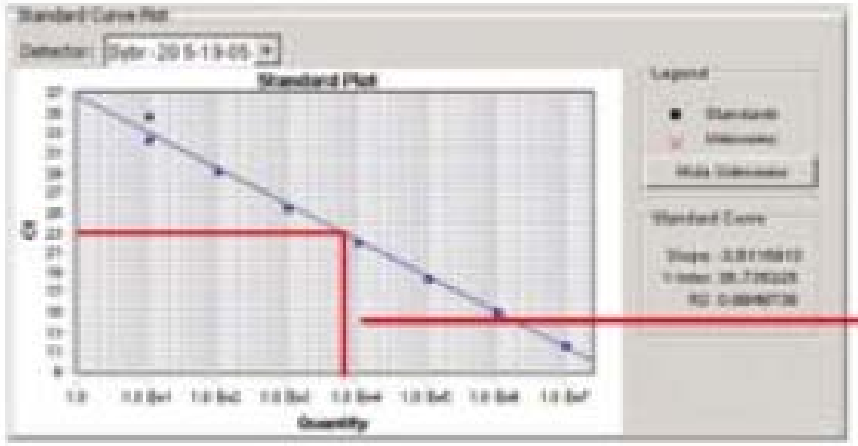
Absolute quantification

A standard curve is generated using a single template species that is diluted over several orders of magnitude.

C_t (C_p) vs concentration is plotted.







$y = mx + b$

$y = C_t$

m = slope

b = y-intercept

x = copy number

Plot the Ct values for the dilutions vs concentration, the slope of the line can be used to calculate the PCR efficiency

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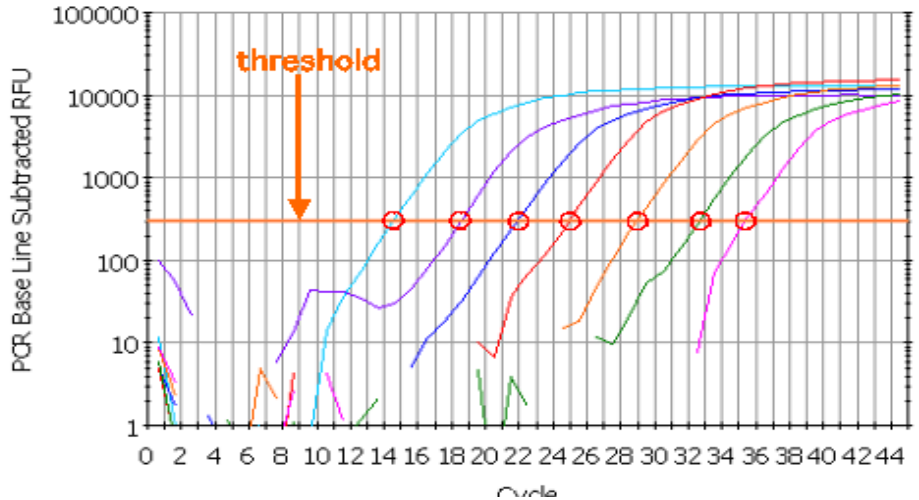
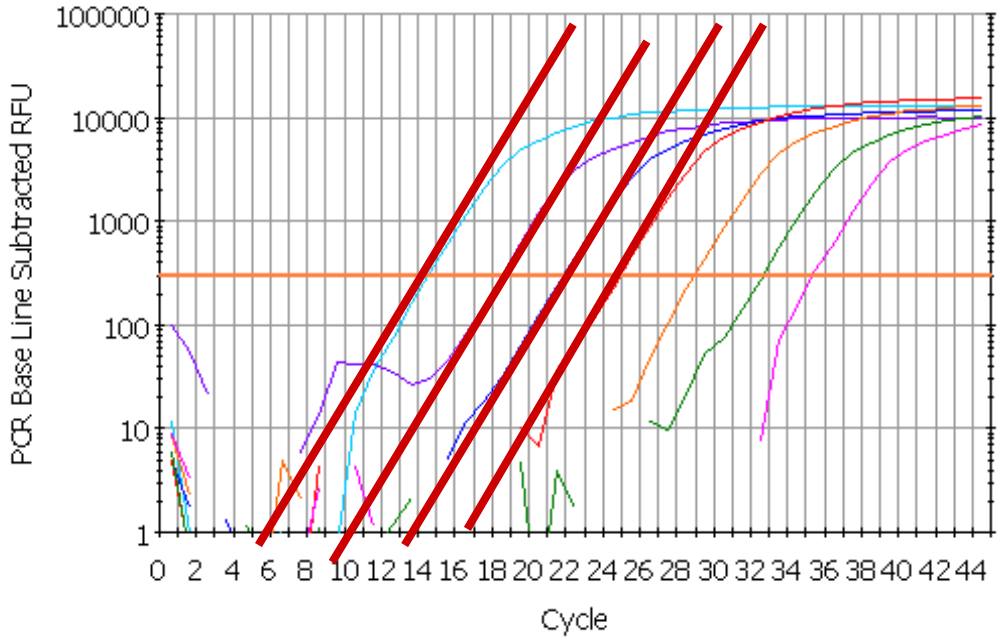
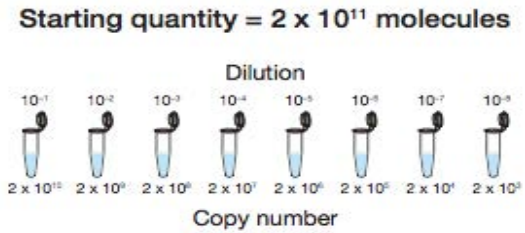
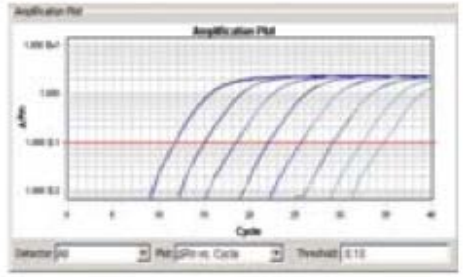
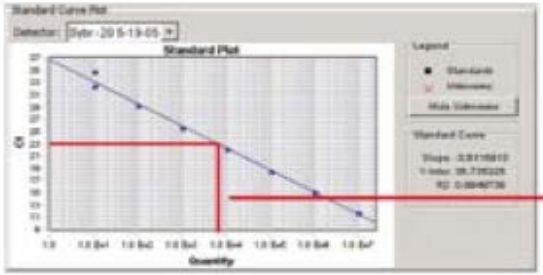




SERIES OF 10-FOLD DILUTIONS







$y = mx + b$

$y = C_t$

m = slope

b = y-intercept

x = copy number

Plot the Ct values for the dilutions vs concentration, the slope of the line can be used to calculate the PCR efficiency

Same slope = Same efficiency

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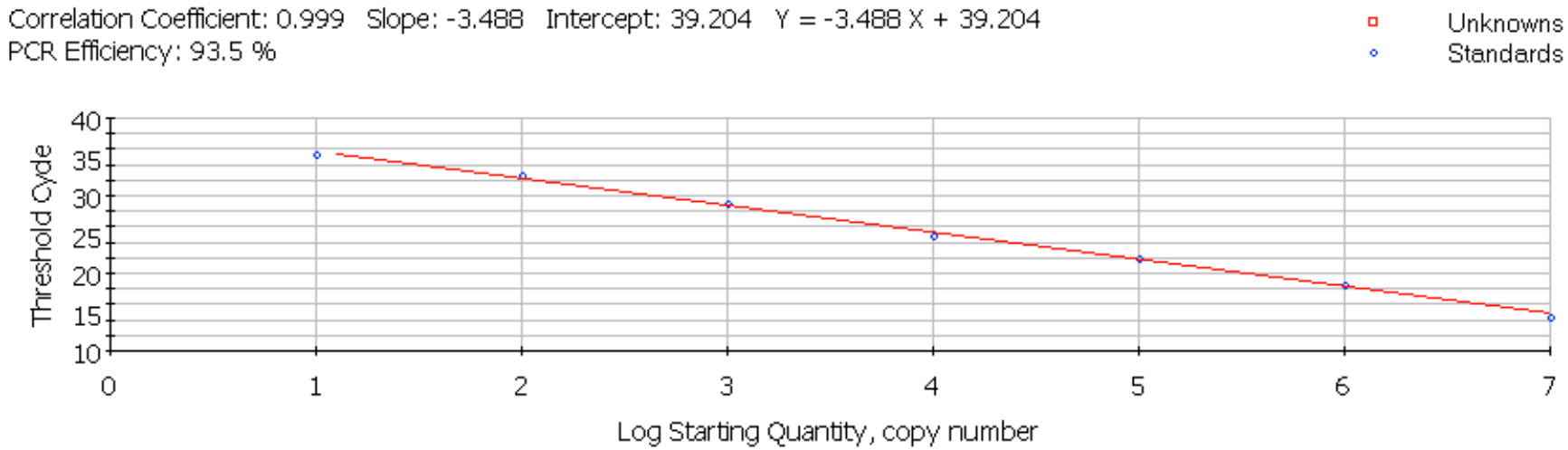
The ideal slope is -3.32, which correlates to an amplification efficiency of 100% correlation coefficient R2 =0.999.

Slopes in the range of -3.60 to -3.10 are generally considered acceptable for real-time PCR. These slope values correlate to amplification efficiencies between 90% and 110%



Note: A 100% efficient reaction will yield a 10-fold increase in PCR amplicon every 3.32 cycles during the exponential phase of amplification (log2 10= 3.3219).

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PCR Amplification Efficiency

GAPDH	RNA (ng/rxn)	log RNA	1	2	3	AVG	SD	CV
std curve	50	1.699	16.587	16.789	16.712	16.696	0.102	0.61%
std curve	10	1.000	19.344	19.208	18.977	19.176	0.185	0.97%
std curve	2	0.301	21.672	21.564	21.125	21.454	0.290	1.35%
std curve	0.4	-0.398	24.524	24.093	23.472	24.030	0.529	2.20%
std curve	0.08	-1.097	26.746	27.020	26.453	26.740	0.284	1.06%
std curve	0.016	-1.796	29.693	29.916	29.829	29.813	0.112	0.38%
std curve	-RT	-	36.540	36.367	37.249	36.719	0.467	1.27%
HT 29 parent	5	0.699	20.406	20.498	20.126	20.343	0.194	0.95%
HT 29 parent	0.5	-0.301	23.745	23.729	23.363	23.612	0.216	0.92%
HT 29 parent	-RT	-	36.422	35.035	Undetermined	35.728	0.980	2.74%
HT 29 x8	5	0.699	20.523	20.389	20.026	20.313	0.258	1.27%
HT 29 x8	0.5	-0.301	23.667	23.509	23.176	23.451	0.251	1.07%
HT 29 x8	-RT	-	Undetermined	37.032	37.136	37.084	0.074	0.20%
no temp	0	-	34.101	34.199	36.068	34.790	1.108	3.19%
		slope	-3.714	22.805	intercept			
		std error m	0.094	0.112	std error b			
		R-squared	0.997	0.274	std error y-est			
		F-stat	1574.115	4.000	deg F			
		reg sum sq	117.908	0.300	ris sum sq			
		Efficiency	85.90%					

Gene of Interest	RNA (ng/rxn)	log RNA	1	2	3	AVG	SD	CV
std curve	50	1.699	21.052	21.843	20.798	21.231	0.545	2.57%
std curve	10	1.000	24.184	24.233	24.062	24.160	0.088	0.36%
std curve	2	0.301	27.002	27.179	26.820	27.000	0.180	0.67%
std curve	0.4	-0.398	29.888	29.983	29.859	29.910	0.064	0.22%
std curve	0.08	-1.097	32.720	32.810	33.044	32.858	0.168	0.51%
std curve	0.016	-1.796	35.617	36.395	36.138	36.050	0.396	1.10%
std curve	-RT	-	Undetermined	Undetermined	Undetermined	-	-	-
HT 29 parent	5	0.699	29.769	29.553	29.124	29.482	0.328	1.11%
HT 29 parent	0.5	-0.301	36.648	36.134	34.556	35.779	1.090	3.05%
HT 29 parent	-RT	-	Undetermined	Undetermined	Undetermined	-	-	-
HT 29 x8	5	0.699	25.079	25.198	24.418	24.899	0.420	1.69%
HT 29 x8	0.5	-0.301	28.781	29.234	28.293	28.770	0.471	1.64%
HT 29 x8	-RT	-	Undetermined	Undetermined	Undetermined	-	-	-
no temp	0	-	Undetermined	Undetermined	Undetermined	-	-	-
		slope	-4.214	28.331	intercept			
		std error m	0.039	0.046	std error b			
		R-squared	1.000	0.113	std error y-est			
		F-stat	11976.157	4.000	deg F			
		reg sum sq	151.849	0.051	ris sum sq			
		Efficiency	72.70%					

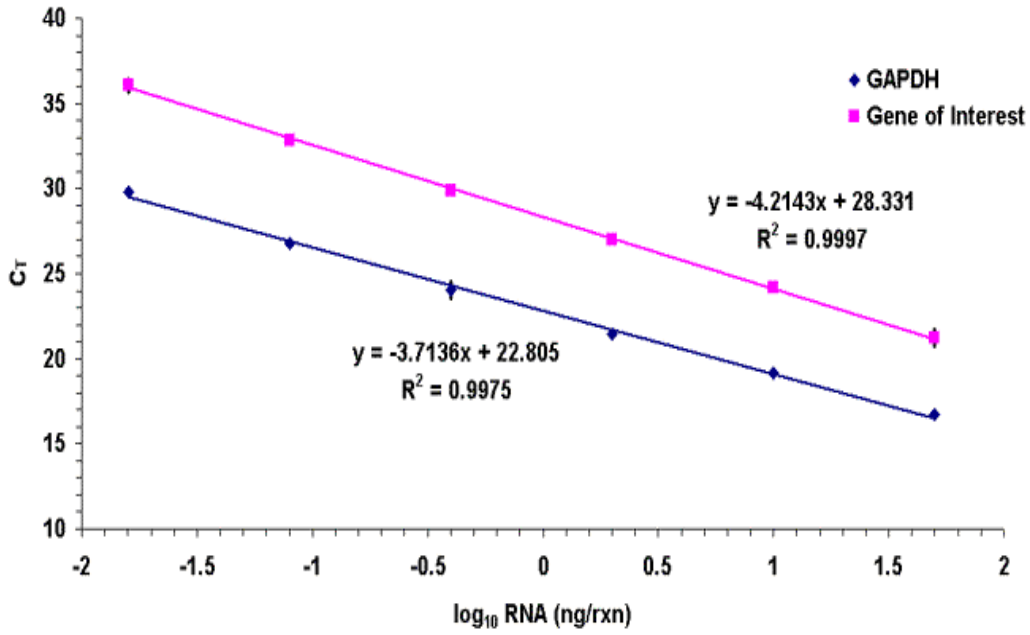


Figure 2: Standard Curves for Primers in Real Time Assay

Author: Dr Joanna Brzeszczyńska (UWS)

PCR Amplification Efficiency

A standard curve slope of –3.32 indicates a PCR reaction with 100% efficiency.

Slopes more negative than –3.32 (ex. –3.9) indicate reactions that are less than 100% efficient.

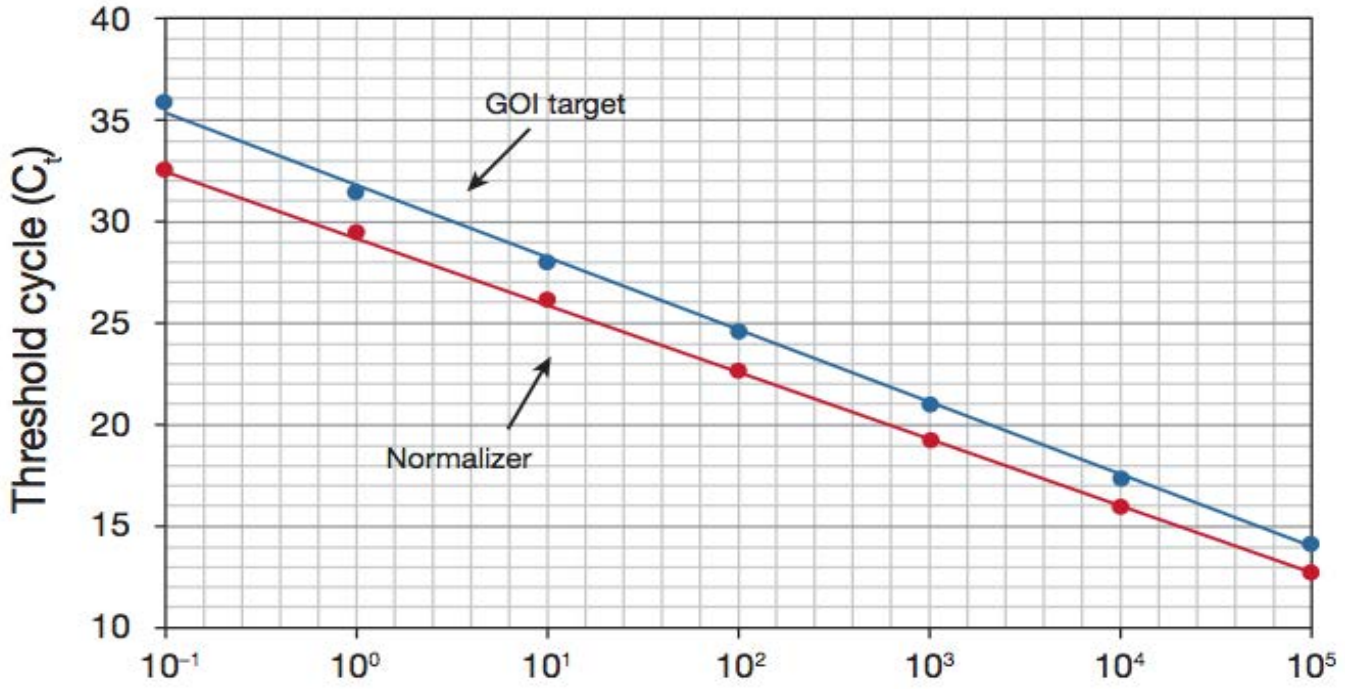
Slopes more positive than –3.32 (ex. –2.5) may indicate sample quality or pipetting problems.

Author: Dr Joanna Brzeszczyńska (UWS)

E = efficiency from standard curve

A calculation for estimating the efficiency (E) of a real-time PCR assay is:

$$E = (10^{-1/\text{slope}} - 1) \times 100$$



To ensure comparability: determine the PCR efficiency of each individual assay - PCR efficiency must be similar for GOI and Ref.Gene. An efficiency between 96% and 100%.

Author: Dr Joanna Brzeszczyńska (UWS)

Importance of controls

- Negative control (no cDNA)
  - checks reagents for contamination

- No reverse transcriptase control (involves carrying out the RT step in the absence of reverse transcriptase)
  - detects if signal from contaminating DNA

- Positive control
  - checks that reagents and primers work
  - especially importance if trying to show absence of expression of a gene

Author: Dr Joanna Brzeszczyńska (UWS)

Importance of controls

RNA from control cells → cDNA from control cells

RNA from treated cells → cDNA from treated cells

Is there any change in your gene expression?

Author: Dr Joanna Brzeszczyńska (UWS)

Importance of controls

RNA from control cells

→ cDNA from control

→ No RT for control
(to see if any genomic DNA signal)

RNA from treated cells

→ cDNA from treated cells

→ No RT for treated cells
(to see if any genomic DNA signal)

Is there any change in your gene expression?

*RT - Reverse Transcriptase

Author: Dr Joanna Brzeszczyńska (UWS)

Importance of Normalization Standards - corrects for loading errors

Definition?

- same copy number in all cells
- expressed in all cells
- medium copy number advantageous

Suzuki et al., 2000: In 1999 over 90% of the RNA transcription analyses published in high impact journals, used only one reference gene. Prominent genes were: GAPDH, Beta-actin, 18S and 28S rRNAs.

Several publications agree with the finding: 'GAPDH, Beta-actin vary considerably and are consequently unsuitable references for RNA transcription analysis'

Unreasonable STATEMENT: the transcription of any gene in a living cell is absolutely resistant to cell cycle fluctuations or nutrient status, etc.

Author: Dr Joanna Brzeszczyńska (UWS)

Normalization Standards - corrects for loading errors

- Commonly used standards
  - Glyceraldehyde-3-phosphate dehydrogenase mRNA (GAPDH)
  - Beta-actin mRNA
  - MHC I (major histocompatability complex I) mRNA
  - mRNAs for certain ribosomal proteins
    - **E.g. RPLP0 (ribosomal protein, large, P0; also known as 36B4, P0, L10E, RPPO, PRLP0, 60S acidic ribosomal protein P0, ribosomal protein L10, Arbp or acidic ribosomal phosphoprotein P0)**
  - 28S or 18S rRNA

Author: Dr Joanna Brzeszczyńska (UWS)

Importance of Normalization Standards

- The perfect standard does not exist
- You have to determine which is best for your experiment / organism / tissue / cells

Author: Dr Joanna Brzeszczyńska (UWS)

Importance of Normalization Standards

- There are no true “House keeping” genes
- Uses more than 1 reference gene (3 is recommended) and takes the geometric mean to normalize fold expression
- Using a single reference gene leads to incorrect normalization up to 3.0-fold and 6.4-fold in 25% and 10% of the cases, respectively, with sporadic values above 20-fold
- geNorm site: http://medgen.ugen.be/~jvdesomp/genorm/
  - geNorm is a popular algorithm to determine the most stable reference (housekeeping) genes from a set of tested candidate reference genes in a given sample panel

Author: Dr Joanna Brzeszczyńska (UWS)

Normalization Standards - corrects for loading errors

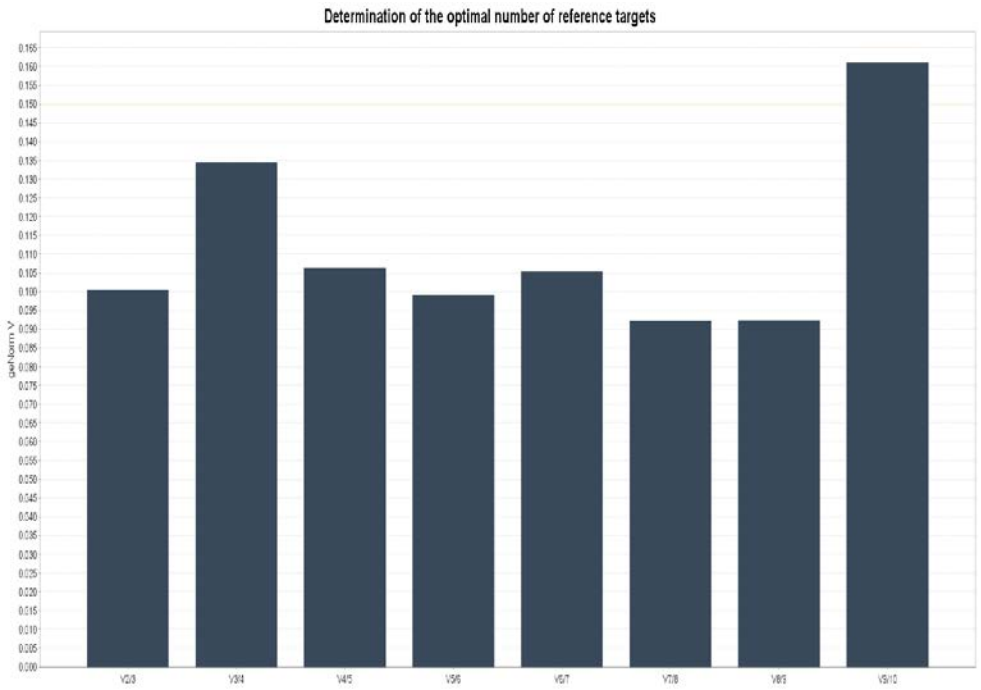
Normalisation of differences in the amount of cDNA in individual samples generated by:

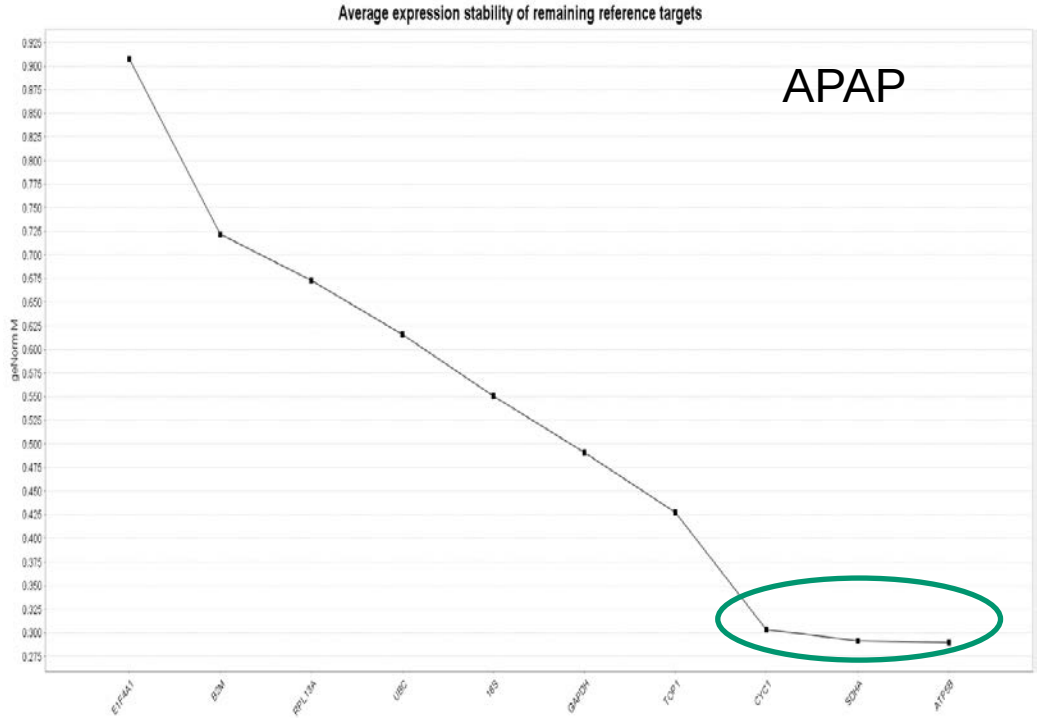
(i) different amounts of starting material,
(ii) the quality of the starting material,
(iii)differences in RNA preparation and cDNA synthesis

same copy number in all cells

expressed in all cells

Author: Dr Joanna Brzeszczyńska (UWS)

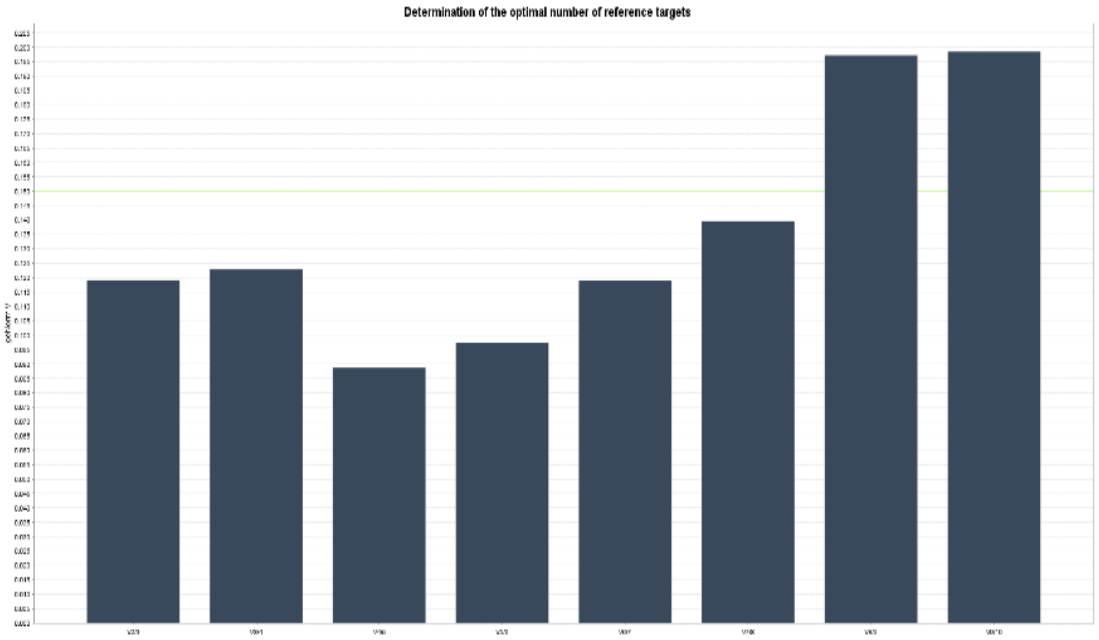


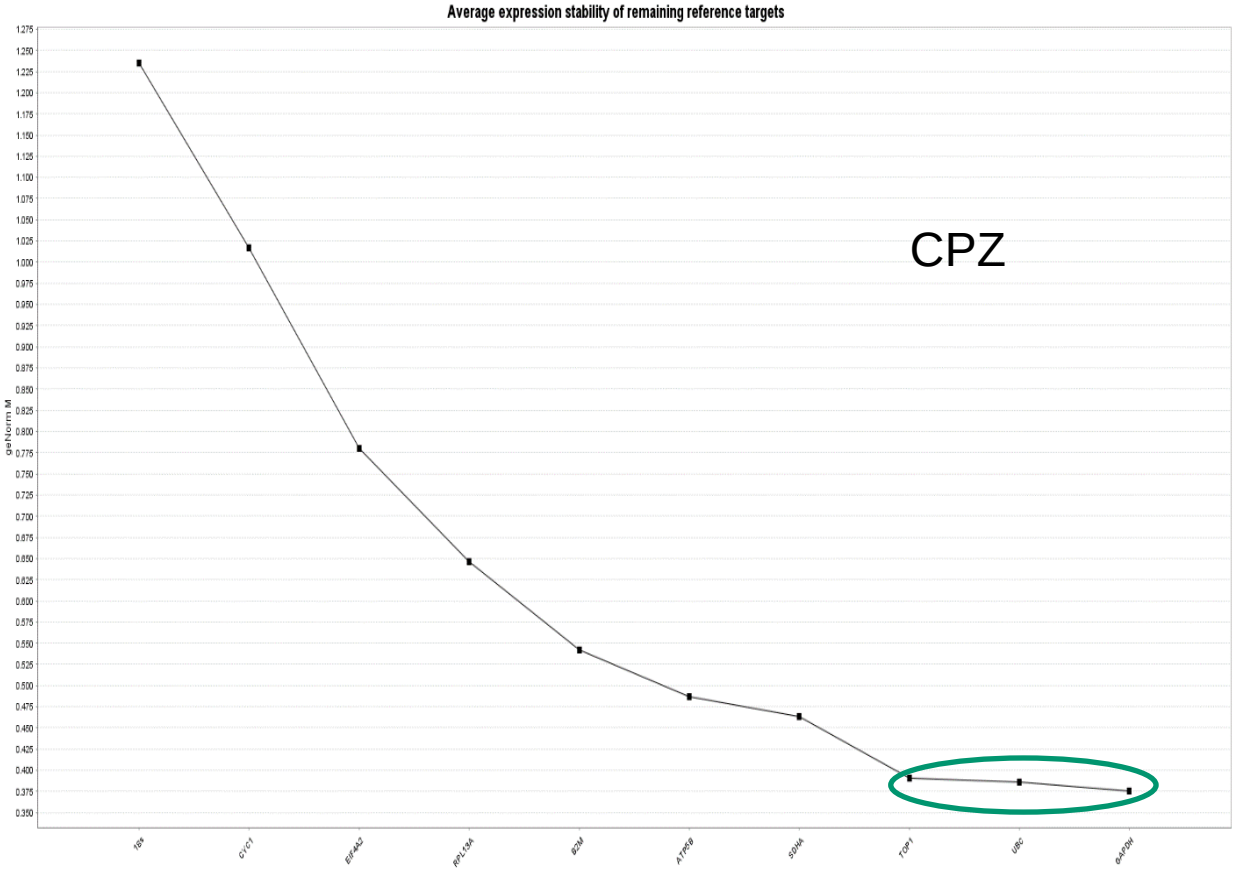


APAP

APAP: Optimal reference target selection: The optimal number of reference targets in this experimental situation is 2 (geNorm V < 0.15 when comparing a normalization factor based on the 2 or 3 most stable targets). As such, the optimal normalization factor can be calculated as the geometric mean of reference targets CYC1, SDHA and ATP5B.

Author: Dr Joanna Brzeszczyńska (UWS)



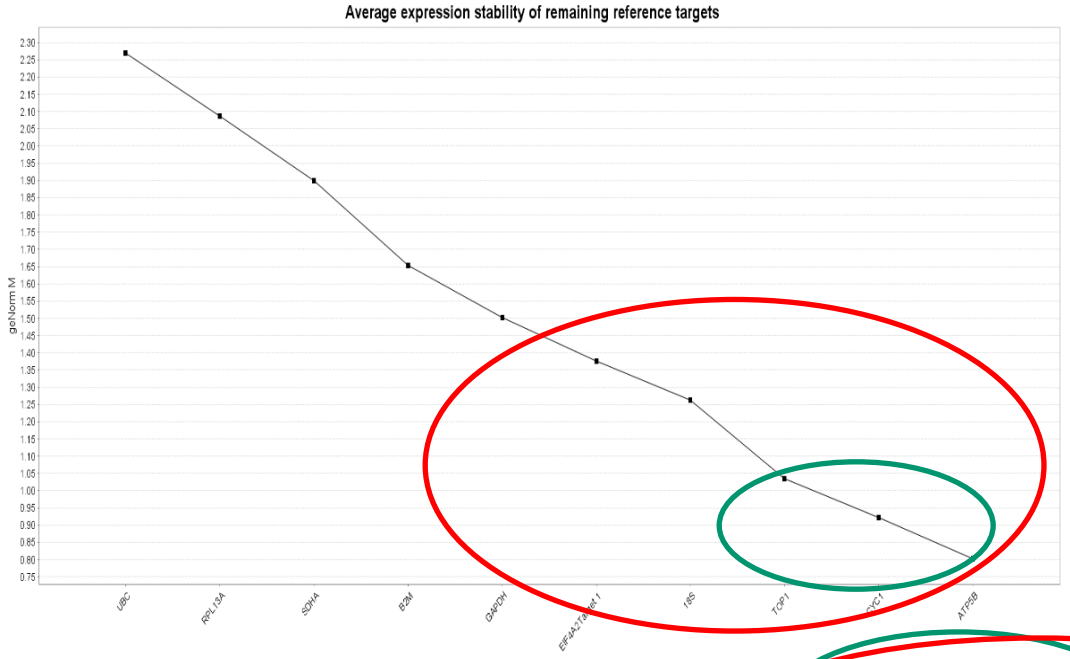


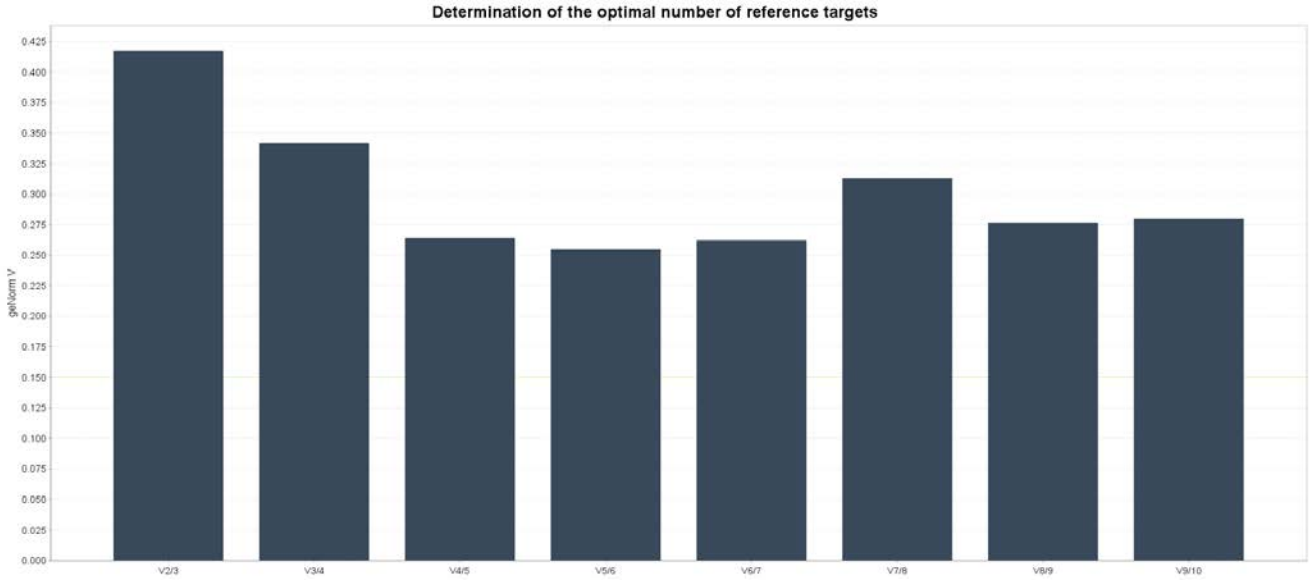
CPZ

CPZ: Optimal reference target selection: The optimal number of reference targets in this experimental situation is 2 (geNorm V < 0.15 when comparing a normalization factor based on the 2 or 3 most stable targets). As such, the optimal normalization factor can be calculated as the geometric mean of reference targets TOP1, UBC and GAPDH.

Author: Dr Joanna Brzeszczyńska (UWS)

Comparative analyses : APAP vs CPZ – as additional option





TOP, CYC1, ATP5B - APAPandCPZ

18S, eif4

APAP and CPZ: Optimal number of reference targets: The optimal number of reference targets in this experimental situation is 5. Genorm recommends to use 5 reference targets with lowest M value, as the use of multiple (non-optimal in this case) reference targets results in more accurate normalization compared to the use of a single non-validated reference target.

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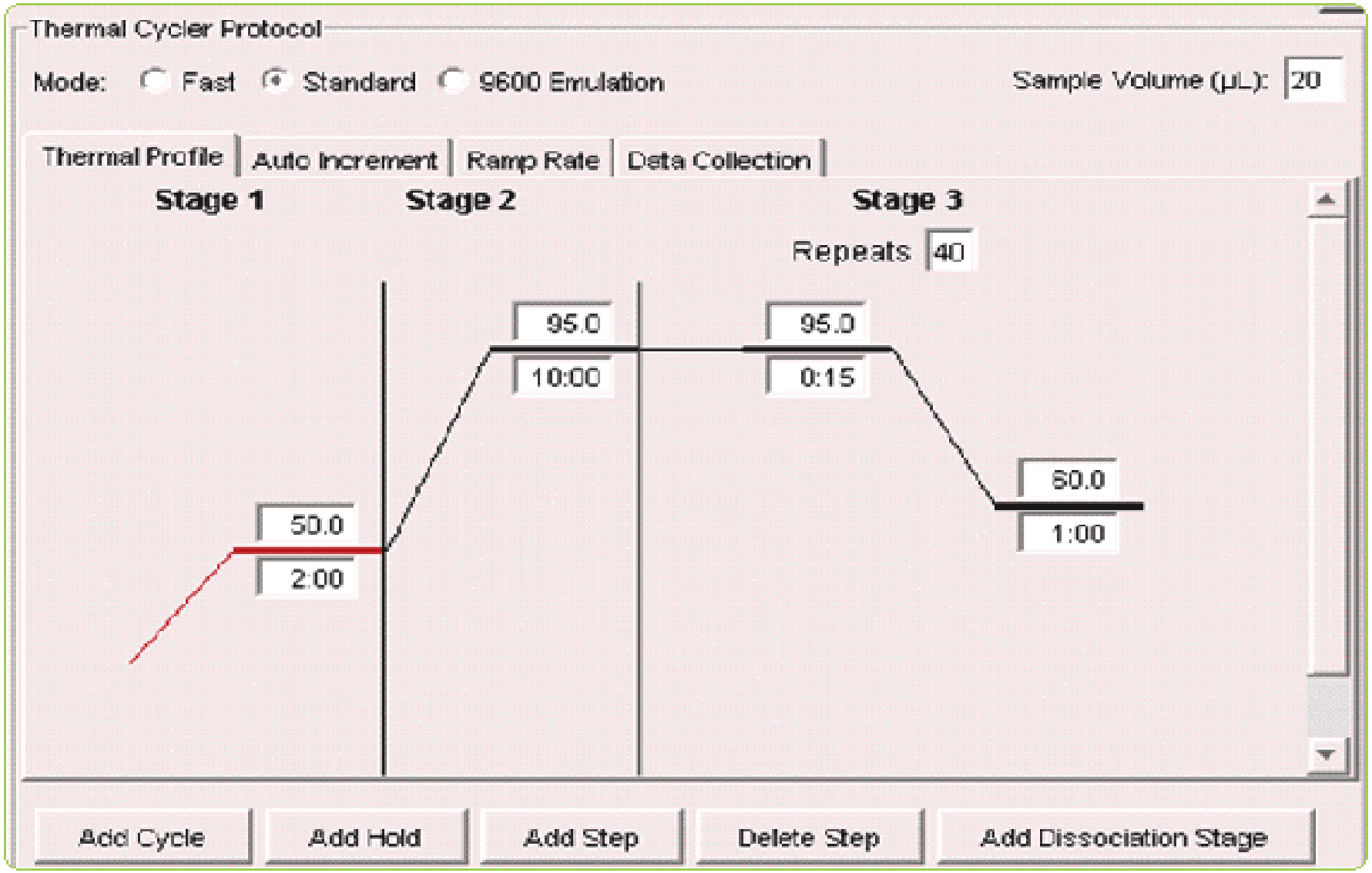
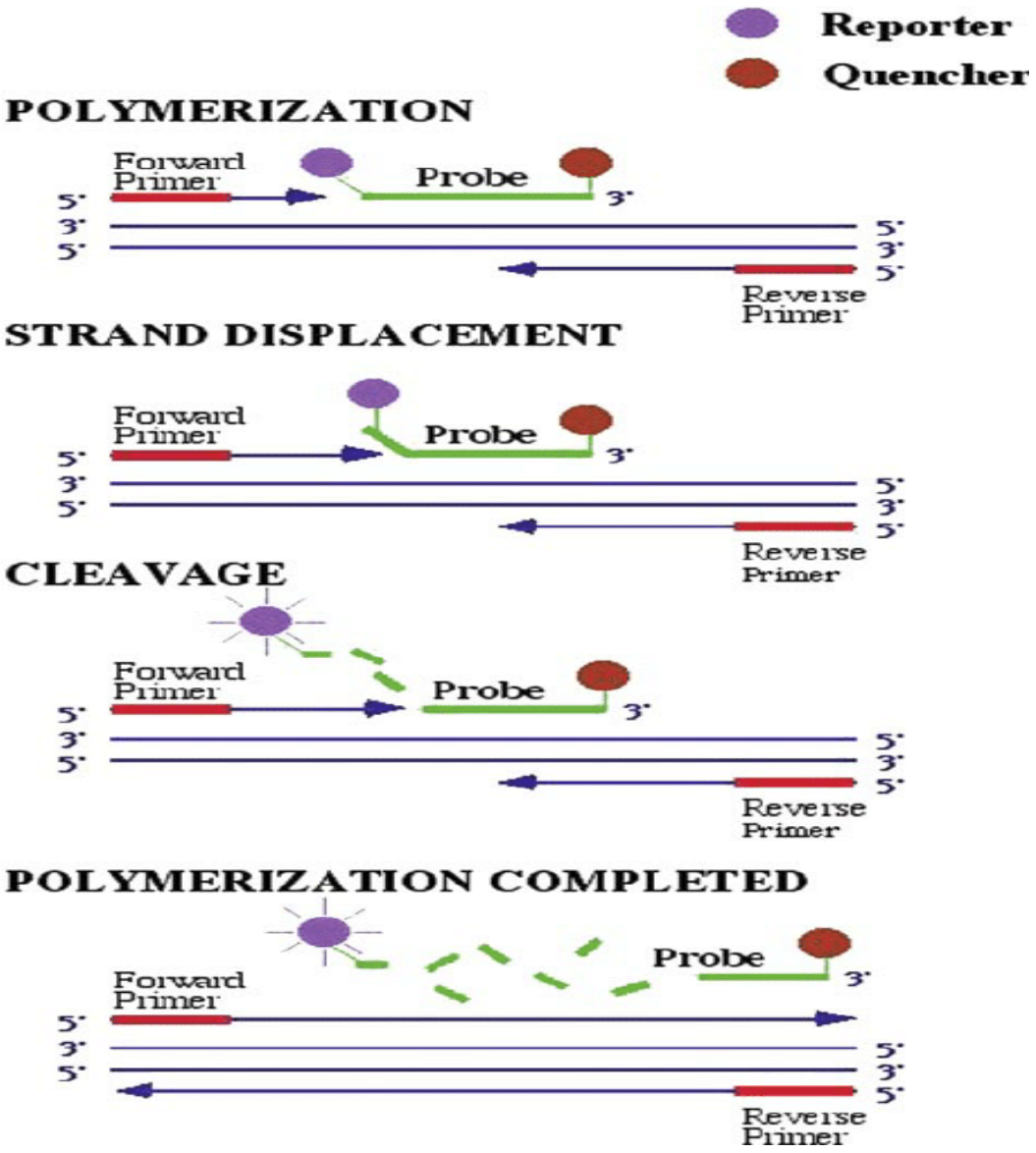
TaqMan



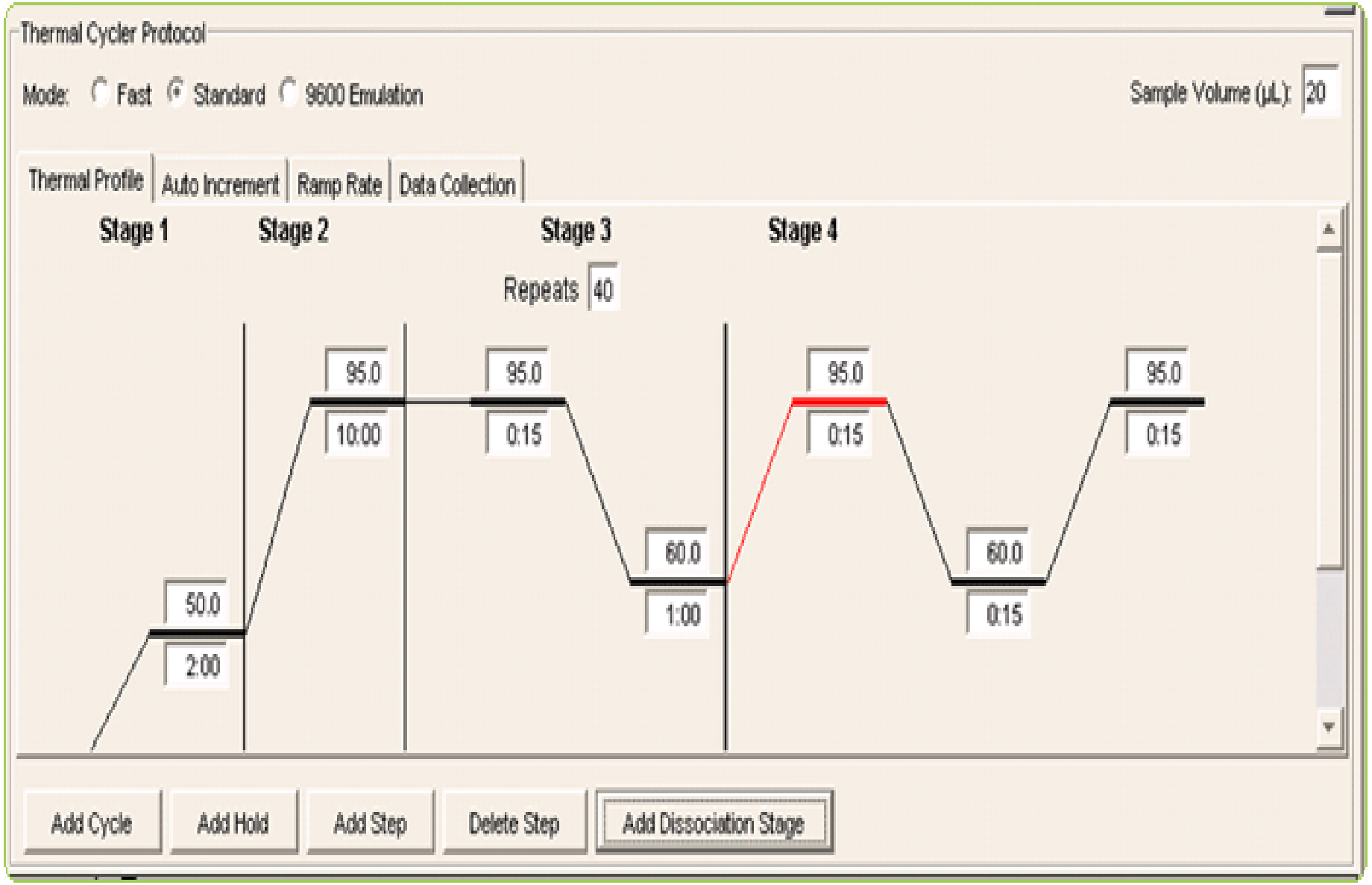
Author: Dr Joanna Brzeszczyńska (UWS)

TaqMan



Author: Dr Joanna Brzeszczyńska (UWS)

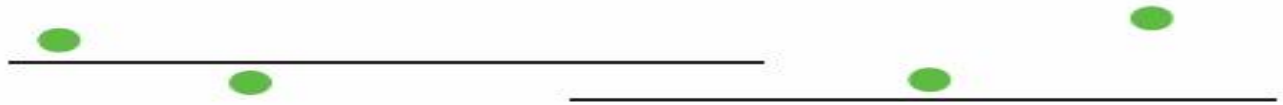
SybreGreen



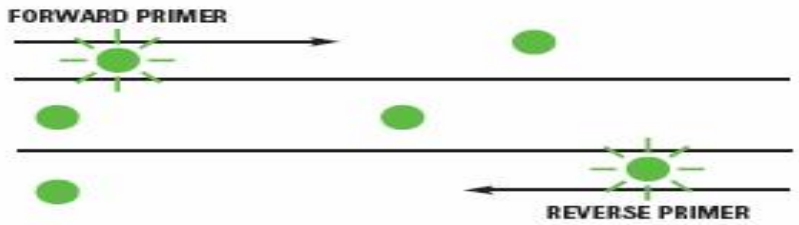
Author: Dr Joanna Brzeszczyńska (UWS)

1. **Reaction setup:** The SYBR® Green I Dye fluoresces when bound to double-stranded DNA.

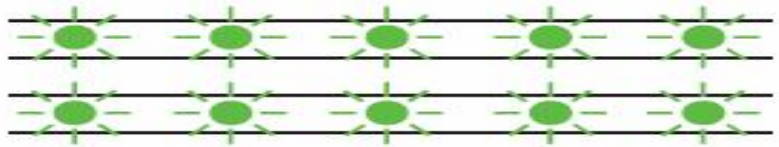
2. **Denaturation:** When the DNA is denatured, the SYBR® Green I Dye is released and the fluorescence is drastically reduced.

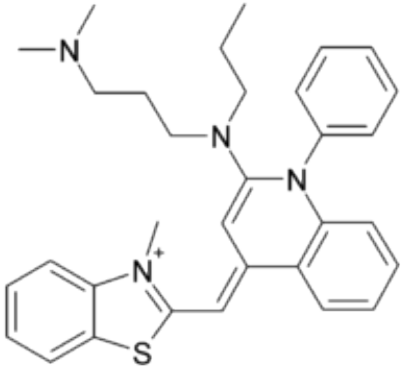


3. **Polymerization:** During extension, primers anneal and PCR product is generated.

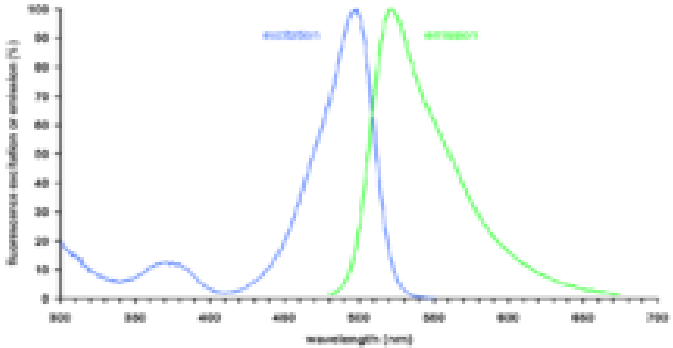


4. **Polymerization completed:** When polymerization is complete, SYBR® Green I Dye binds to the double-stranded product, resulting in a net increase in fluorescence detected by the 7900HT system.





- SYBR Green is a cyanine dye that binds to double stranded DNA.
- When it is bound to D.S. DNA it has greater fluorescence than when bound to single stranded DNA.
- This can be used to follow the production of new PCR products



Author: Dr Joanna Brzeszczyńska (UWS)

Reaction Optimization

- Melt Curve –MIQE Guidelines
- Data Analysis

Author: Dr Joanna Brzeszczyńska (UWS)

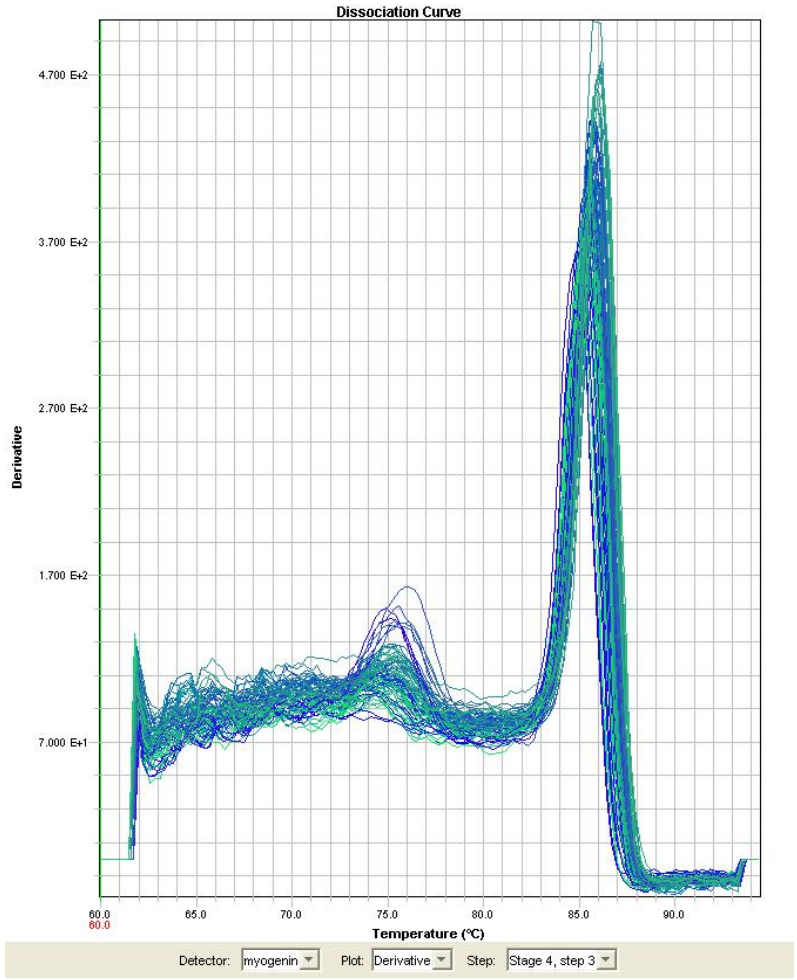
Dissociation Curve

Why Using Dissociation Curves?

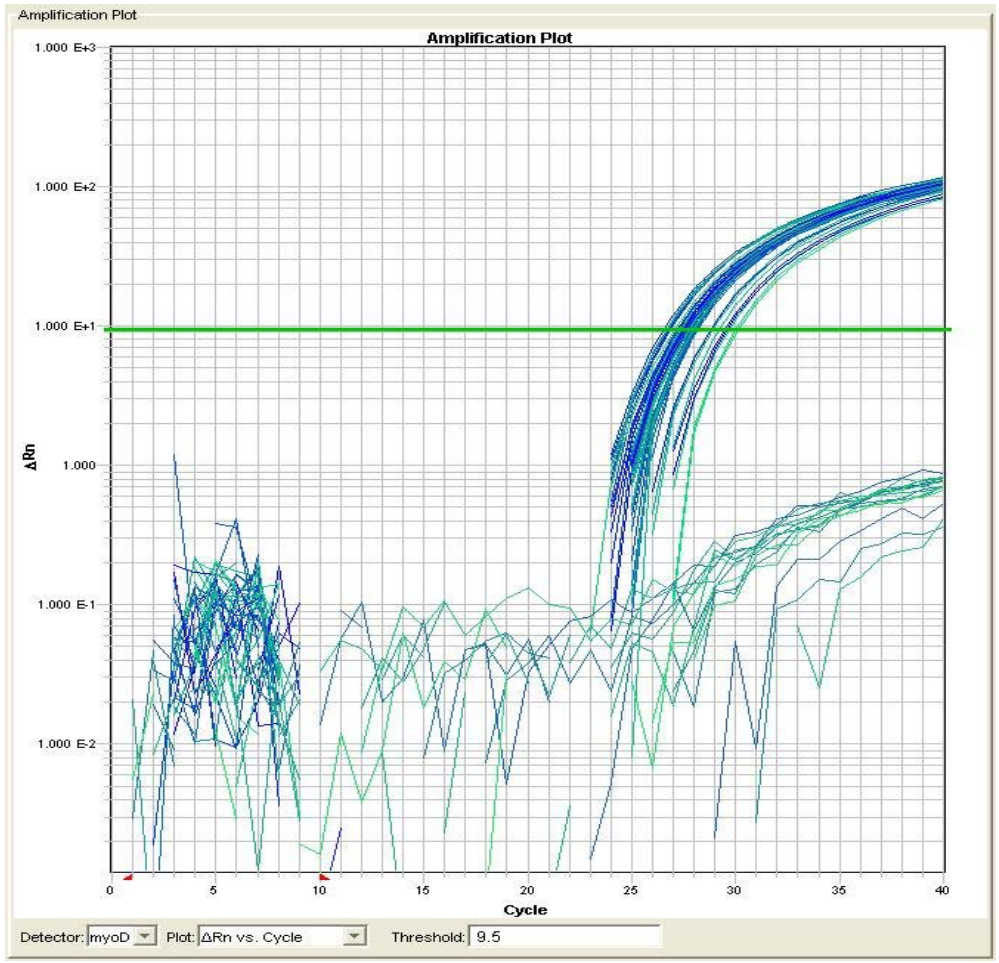
Nonspecific amplification, including primer-dimers, may affect the quality of amplification data.

Author: Dr Joanna Brzeszczyńska (UWS)

Dissociation Curve

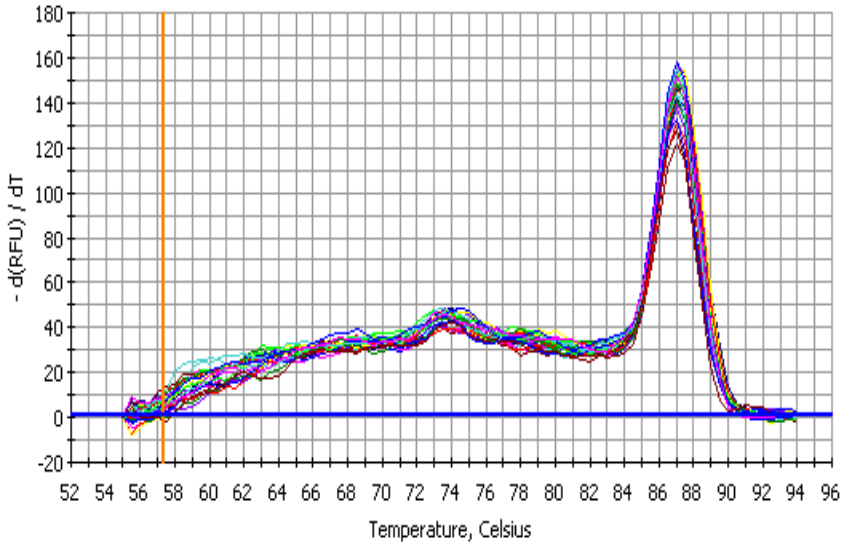


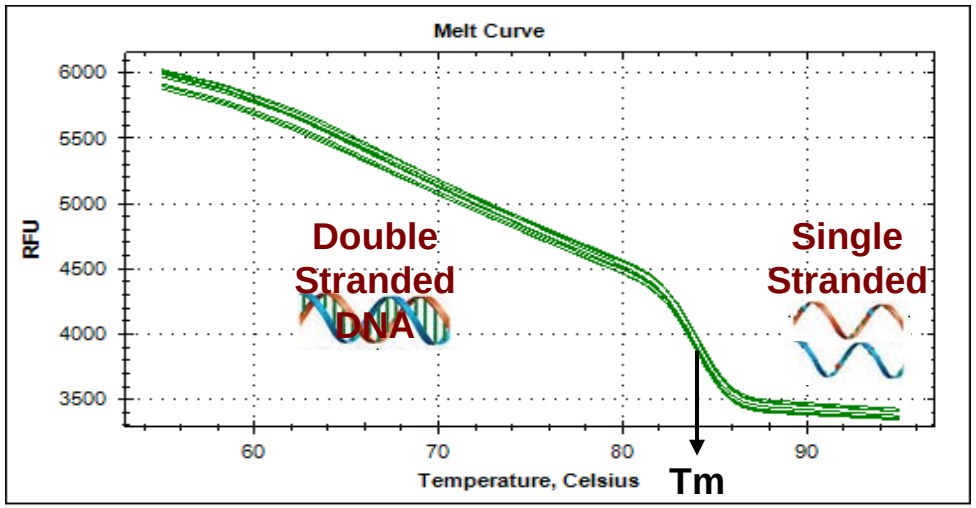
Amplification Curve



Author: Dr Joanna Brzeszczyńska (UWS)

Endpoint analysis to determine the melting temperature (Tm) of PCR products.

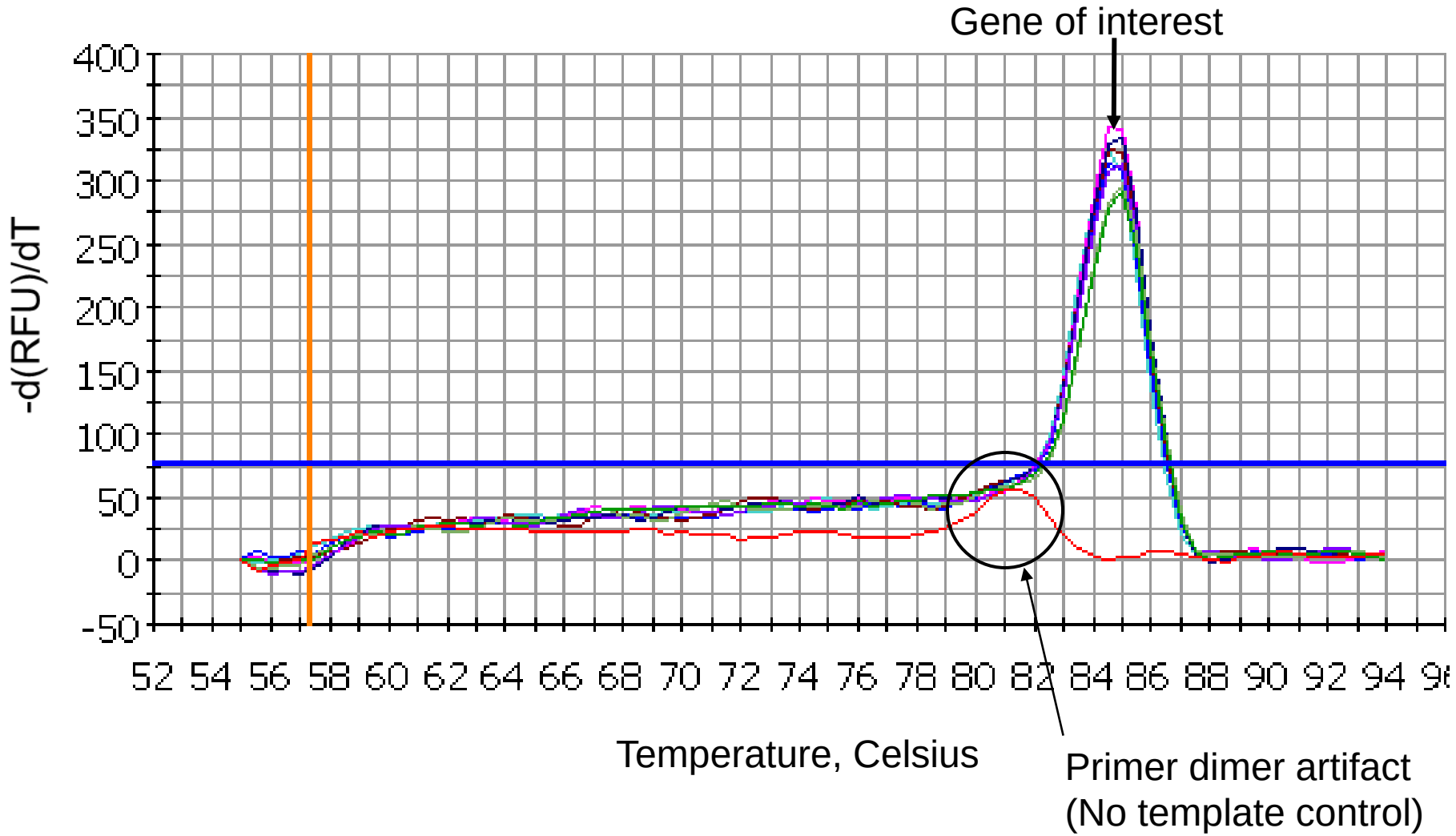




- Melting temperature (Tm) of dsDNA
  - Temperature at which half the DNA is double stranded and half is single stranded
  - Depends on nucleotide content and length

Author: Dr Joanna Brzeszczyńska (UWS)

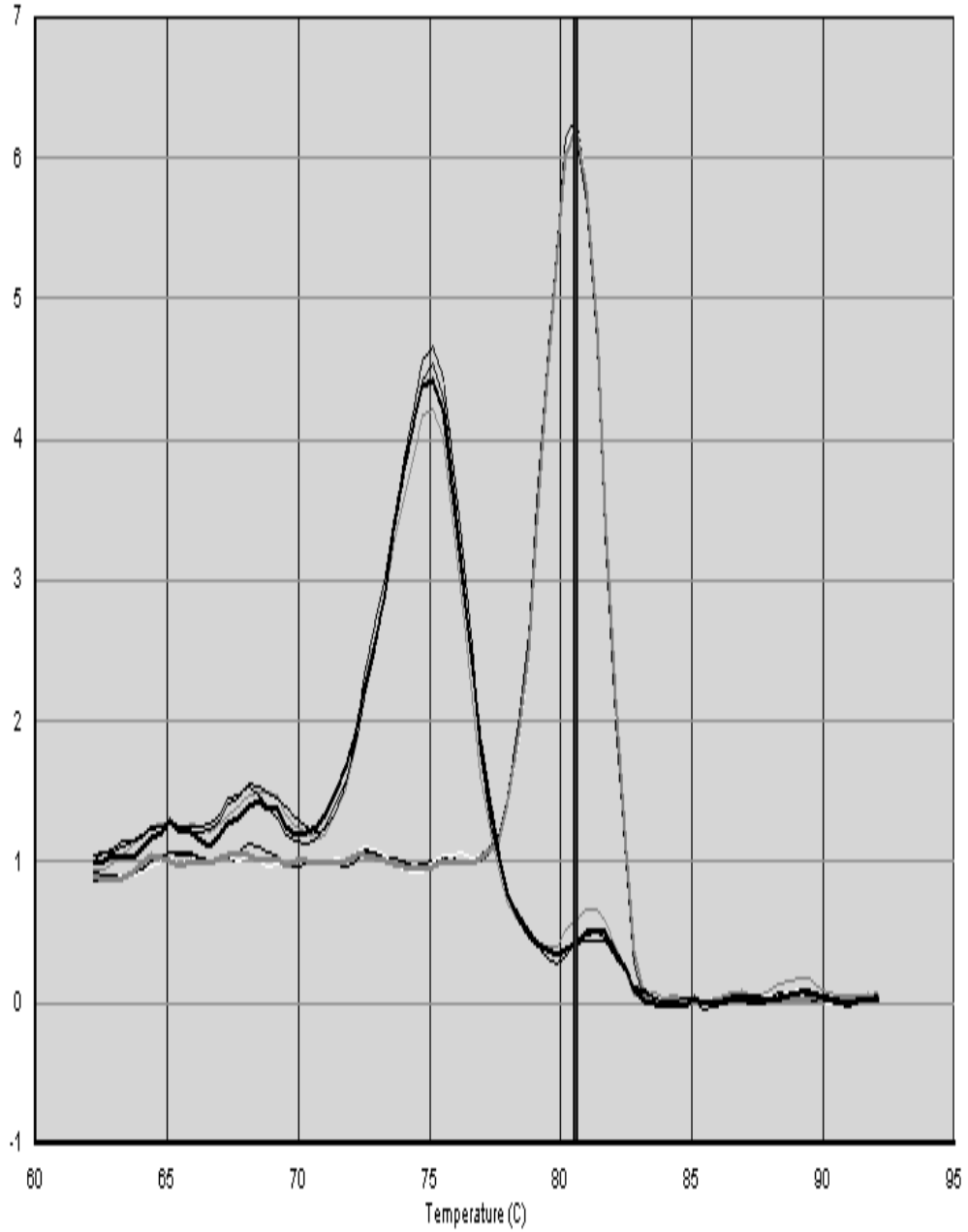
The Melt-Curve shows the different types of DNA present



Author: Dr Joanna Brzeszczyńska (UWS)

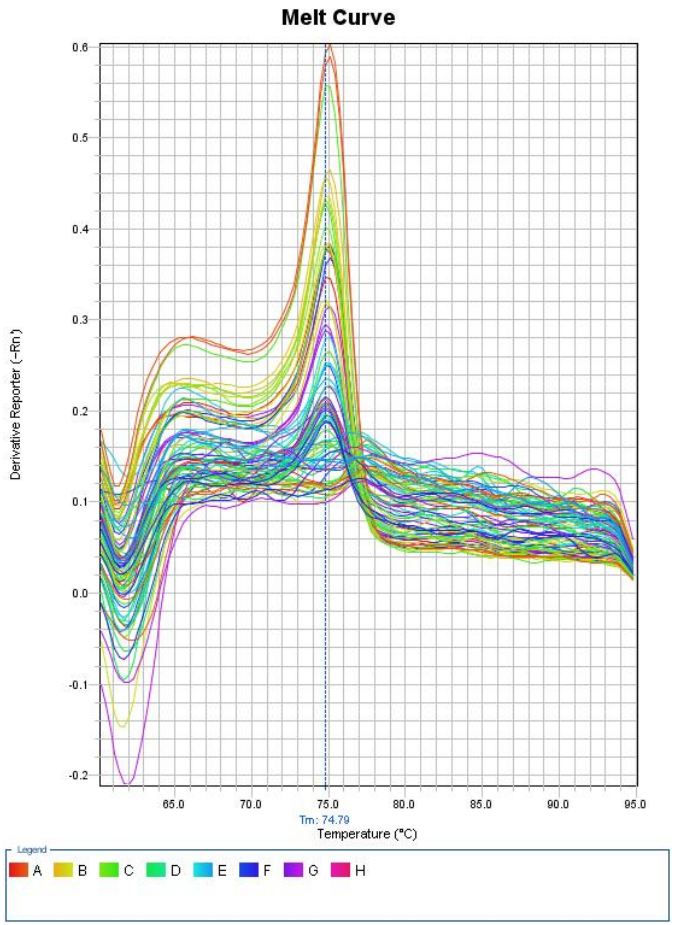
Melt Curve Analysis

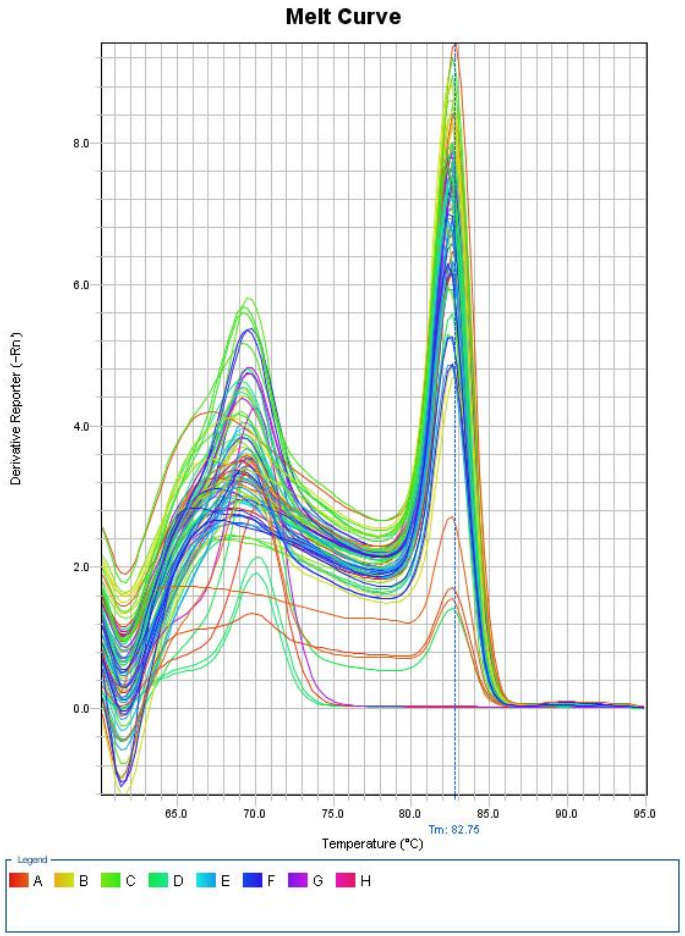
- Dissociation curves show typical primer-dimer formation.
- The specific product is shown with a melting temperature (Tm) of 80.5 ° C
- The primer-dimer has a characteristically lower Tm of 75 ° C.
- Primer-dimer will be most prevalent in No Template Control (NTC) wells or in sample wells containing low concentrations of template.

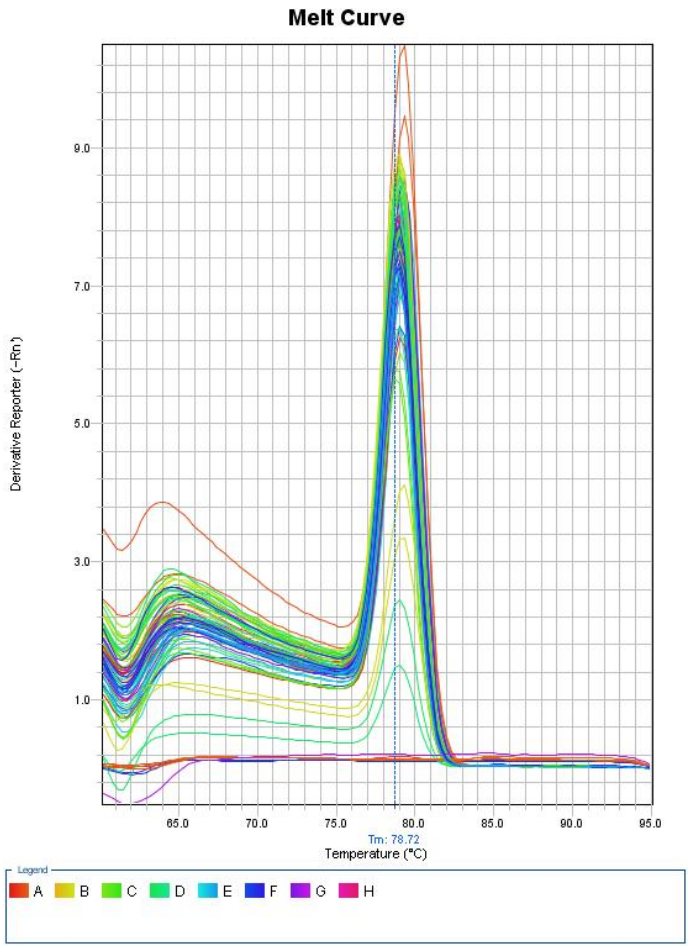


Author: Dr Joanna Brzeszczyńska (UWS)

Melt Curve Analysis







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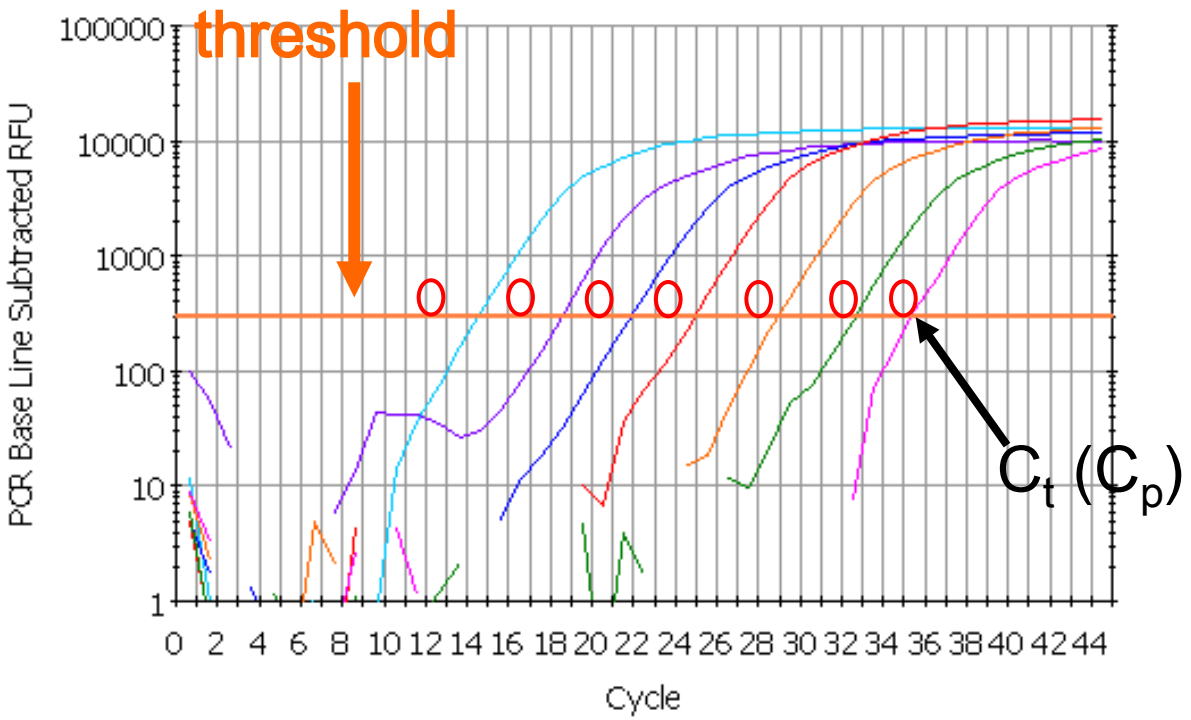
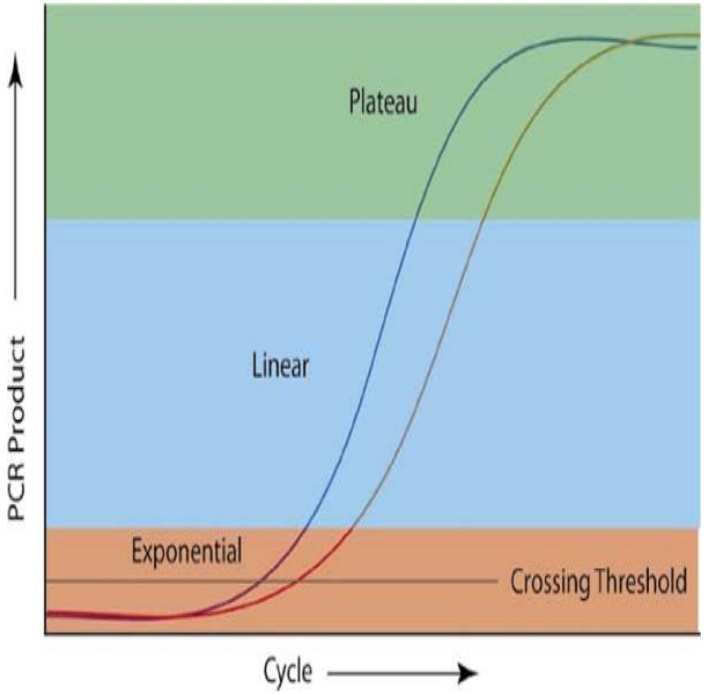
Logarithmic scale

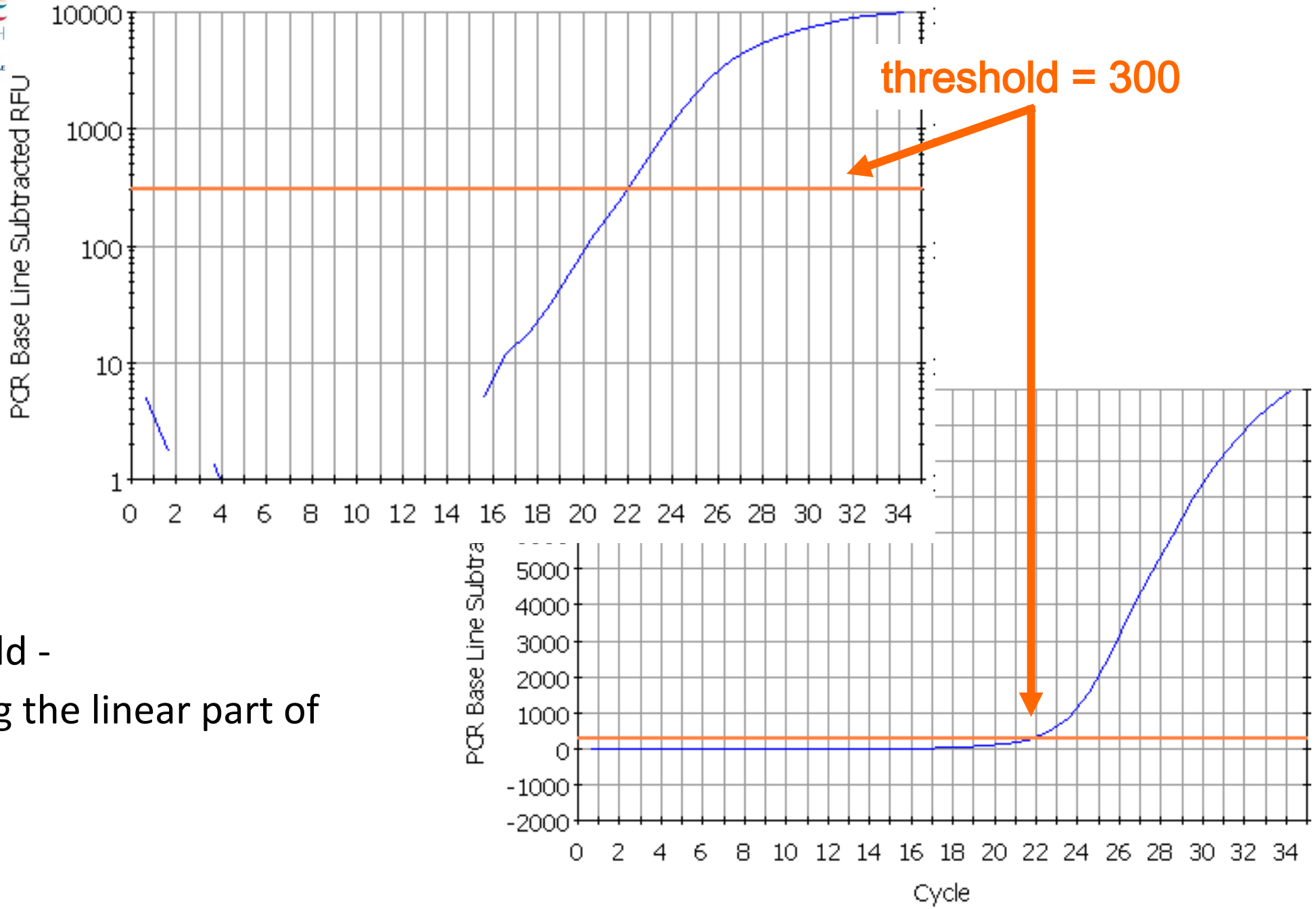




Cycle Threshold - Ct is set during the linear part of the reaction

The Ct (cycle threshold) is defined as the number of cycles required for the fluorescent signal to cross the threshold (ie exceeds background level).

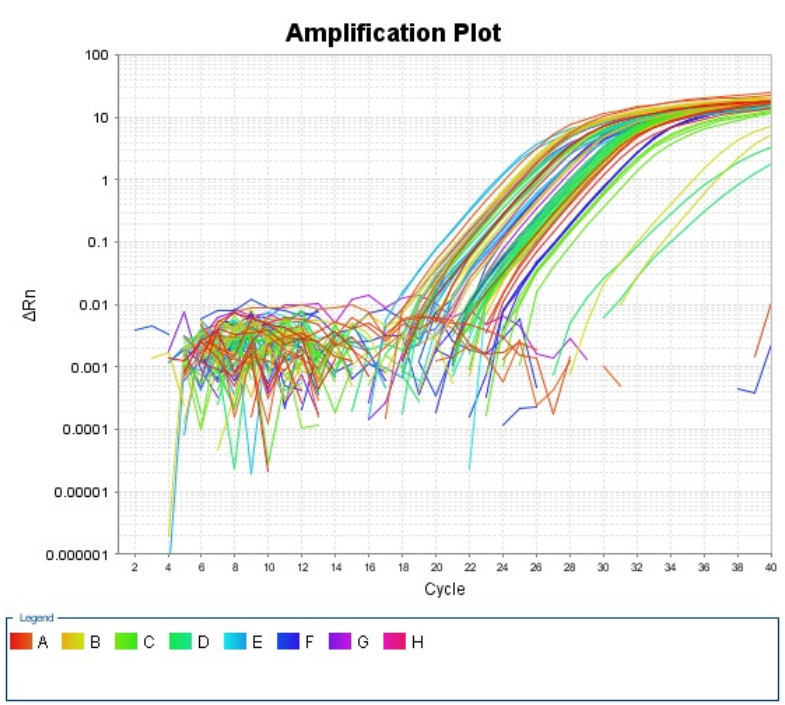
Author: Dr Joanna Brzeszczyńska (UWS)

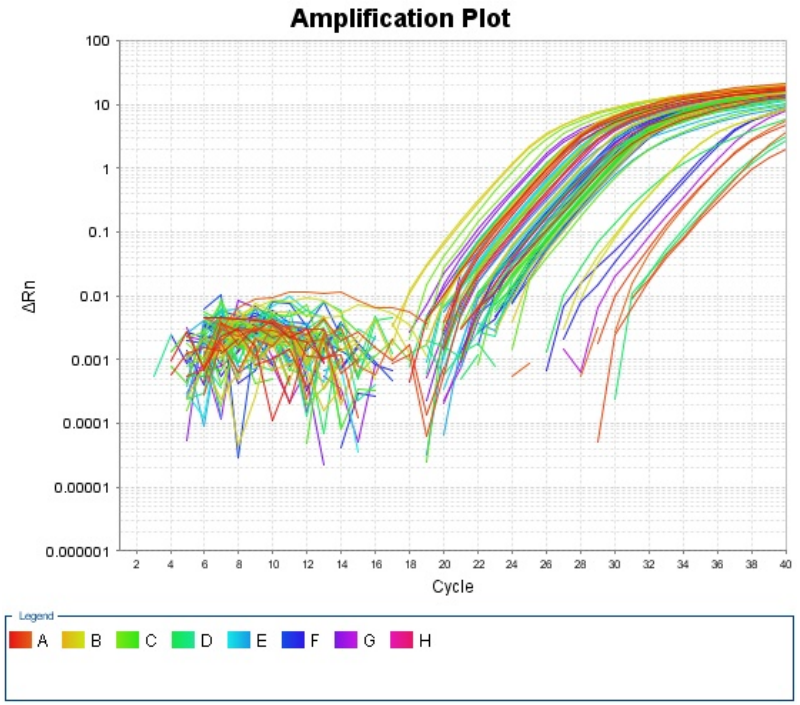


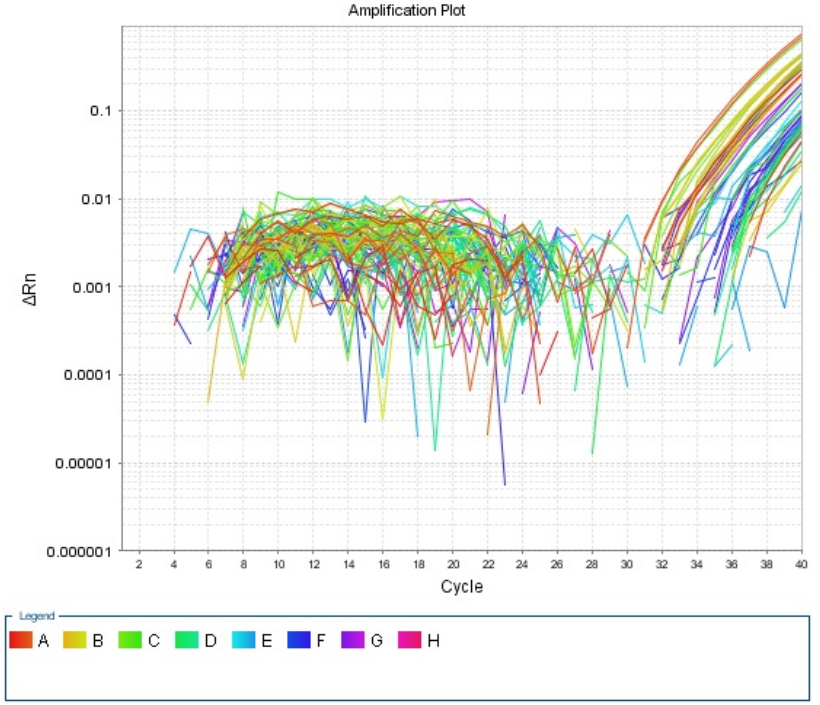
Cycle Threshold -

Ct is set during the linear part of the reaction

Author: Dr Joanna Brzeszczyńska (UWS)

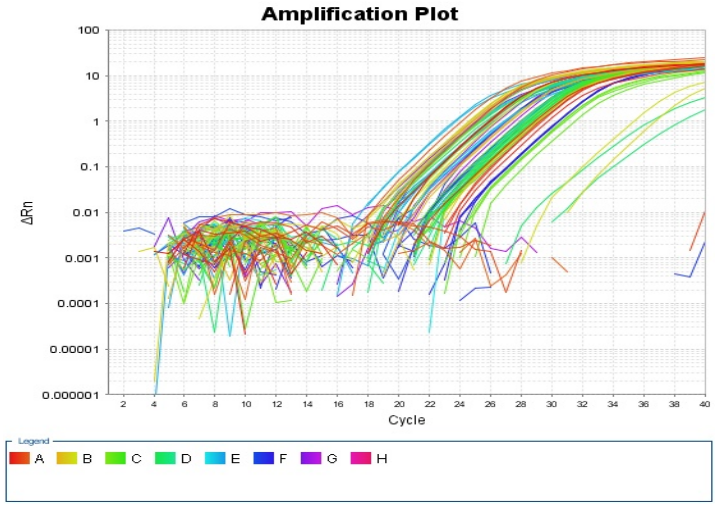






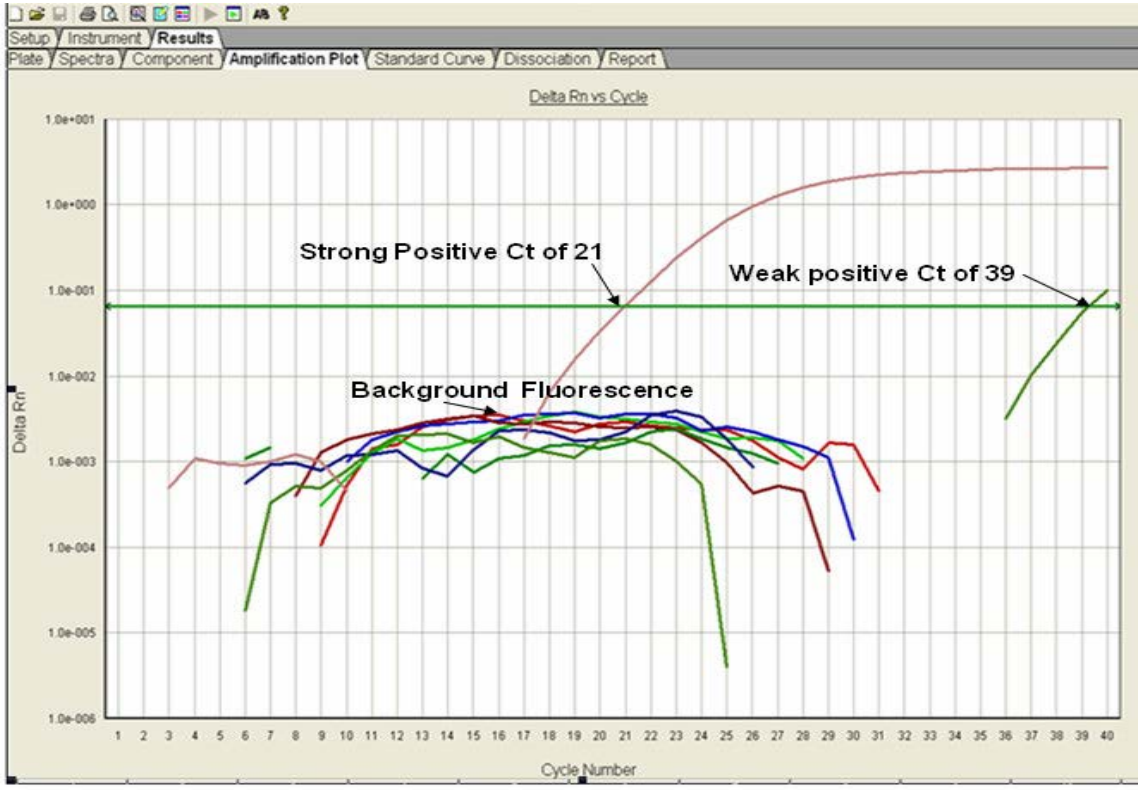
In a real time PCR assay a positive reaction is detected by accumulation of a fluorescent signal

Author: Dr Joanna Brzeszczyńska (UWS)



Ct levels are inversely proportional to the amount of target nucleic acid in the sample.

(ie. the lower the Ct level the greater the amount of target nucleic acid in the sample).



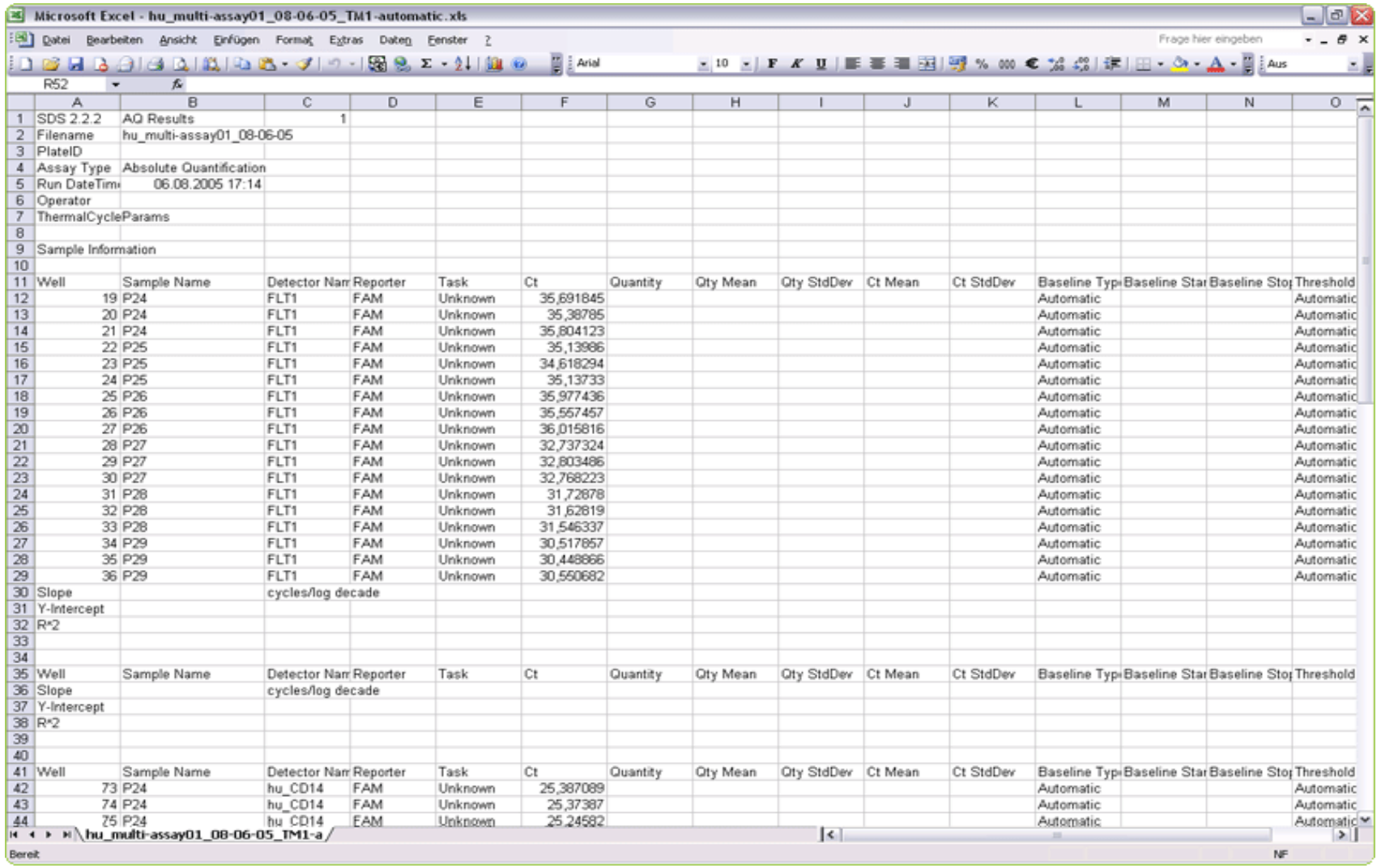
RT-QPCR assays undergo 40 cycles of amplification

Cts < 29 are strong positive reactions indicative of abundant target nucleic acid in the sample

Cts of 30-37 are positive reactions indicative of moderate amounts of target nucleic acid

Cts of 38-40 are weak reactions indicative of minimal amounts of target nucleic acid which could represent an environmental contamination

Author: Dr Joanna Brzeszczyńska (UWS)



control	MyoD1	26.0
control	MyoD1	26.6
control	MyoD1	26.5
TNF10ng/ml	MyoD1	28.8
TNF10ng/ml	MyoD1	28.4
TNF10ng/ml	MyoD1	28.4
TNF20ng/ml	MyoD1	28.1
TNF20ng/ml	MyoD1	28.1
TNF20ng/ml	MyoD1	28.1
IFN10ng/ml	MyoD1	28.8
IFN10ng/ml	MyoD1	31.0
IFN10ng/ml	MyoD1	31.0
IFN20ng/ml	MyoD1	28.9
IFN20ng/ml	MyoD1	28.5
IFN20ng/ml	MyoD1	28.5
TNF10ng/ml+IFN10ng/ml	MyoD1	28.5
TNF10ng/ml+IFN10ng/ml	MyoD1	28.4
TNF10ng/ml+IFN10ng/ml	MyoD1	28.1
TNF10ng/ml+IFN20ng/ml	MyoD1	29.3
TNF10ng/ml+IFN20ng/ml	MyoD1	28.0
TNF10ng/ml+IFN20ng/ml	MyoD1	27.7

Author: Dr Joanna Brzeszczyńska (UWS)

qPCR Data Analysis

Comparative quantification determines relative abundance rather than exact copy.

Considerations:

- Gene Expression Assays have amplification efficiencies of 100%, and because of this, when using Gene Expression Assays, calculated fold change values correlate better to expected fold change values when the comparative CT method is used.

- If you are using custom primers and probes, an initial validation relative standard curve is recommended to validate the PCR efficiencies of the target and endogenous control(s), particularly when you are looking for low-expression-level fold changes.

- The comparative CT method is useful when a high number of targets and/or number of samples are tested.

Author: Dr Joanna Brzeszczyńska (UWS)

qPCR Data Analysis

Absolute quantification allows actual copy numbers to be determined but is labor intensive.

Considerations:

- This method requires that each reaction plate contain standard curves, and requires more reagents and more space on a reaction plate.
- This approach gives highly accurate quantitative results because unknown sample quantitative values are interpolated from the standard curve(s).
- Consider this method when testing low numbers of targets and small numbers of samples and if you are looking for very discrete expression changes.

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Comparative quantification – ΔC_t method

This basic method does not incorporate a normalizer or corrects for efficiency.
It assumes that the same amount of template was present and the amplification efficiency is the same

Most basic form is to obtain a C_t value for the gene of interest and a calibrator sample (such as time zero sample). The difference is the ΔC_t

Fold difference = $2^{\Delta C}t$

ΔΔ Ct-Method:

$$\Delta \mathbf{CT} = CT_{target} - CT_{ref}$$

$$\Delta\Delta \mathbf{Ct} = \Delta CT_{treated} - \Delta CT_{control}$$

$$Ratio = 2^{-\Delta\Delta CT}$$

target = target gene
ref = reference / housekeeping gene
treated = treated tissue or timepoint
control = reference tissue or timepoint

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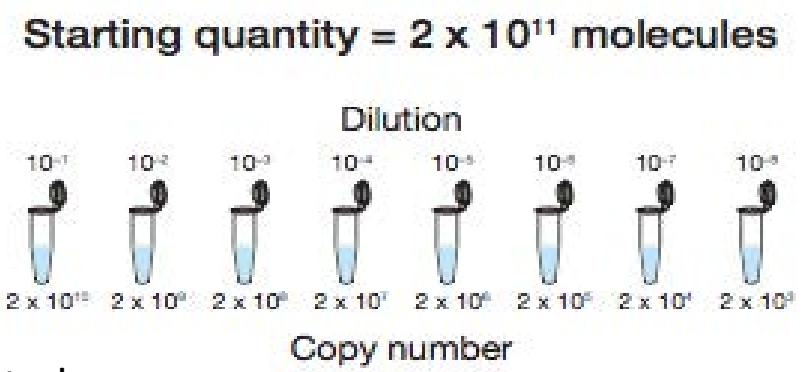
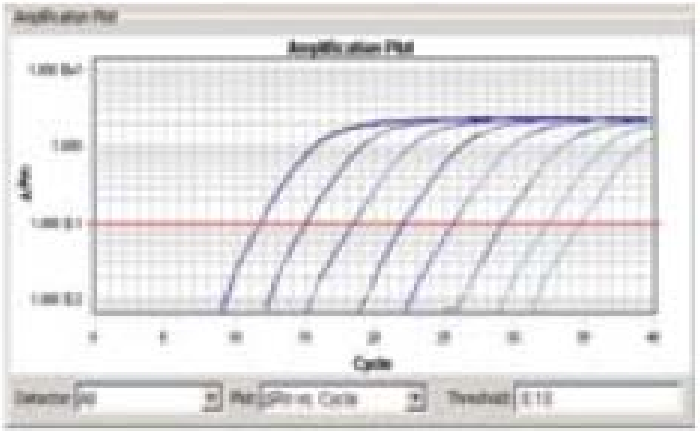
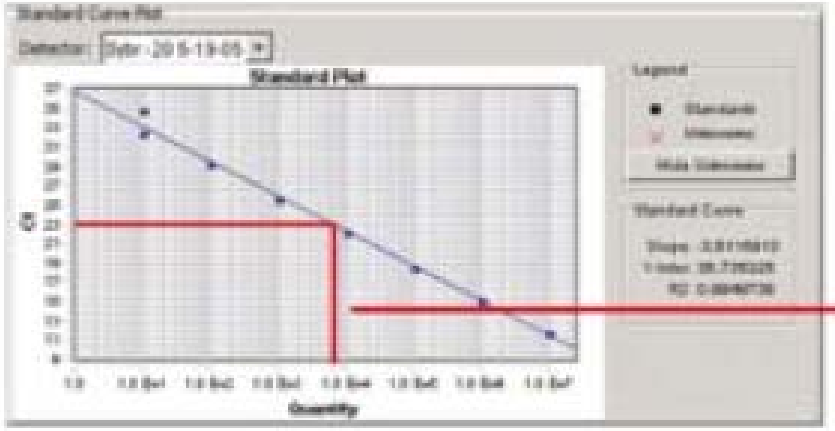
Absolute quantification

A standard curve is generated using a single template species that is diluted over several orders of magnitude.





C_t (C_p) vs concentration is plotted.



$$y = mx + b$$

$y = C_t$

m = slope

b = y-intercept

x = copy number

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Absolute quantification – standard curve method

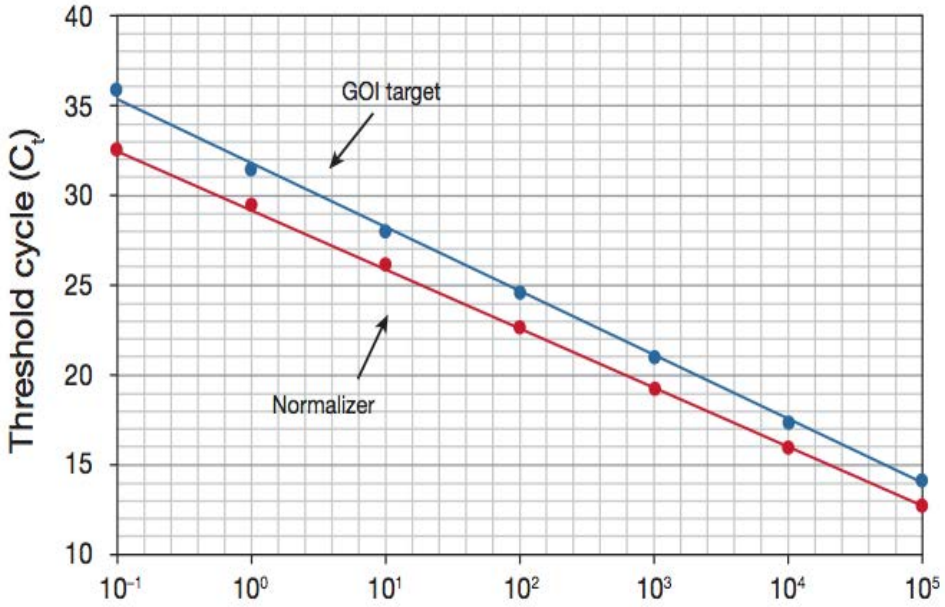
E = efficiency from standard curve $E = 10^{[-1/slope]}$

Fold difference = $(E_{target})^{\Delta Ct\ target} / (E_{normalizer})^{\Delta Ct\ normalizer}$

$\Delta Ct\ target = C_{t\ GOI}c - C_{t\ GOI}s$

$\Delta Ct\ normalizer = C_{t\ norm}c - C_{t\ norm}s$

Fold difference equation derived from
M.W. Pfaffl in A-Z of Quantitative PCR



Starting quantity (pg total RNA)

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References:

Several pdfs for this talk are available at:
http://botany.okstate.edu/resources/pcr_core.html

Another good website with loads of information:
http://www.gene-quantification.de/