QPCR Reagent Optimisation

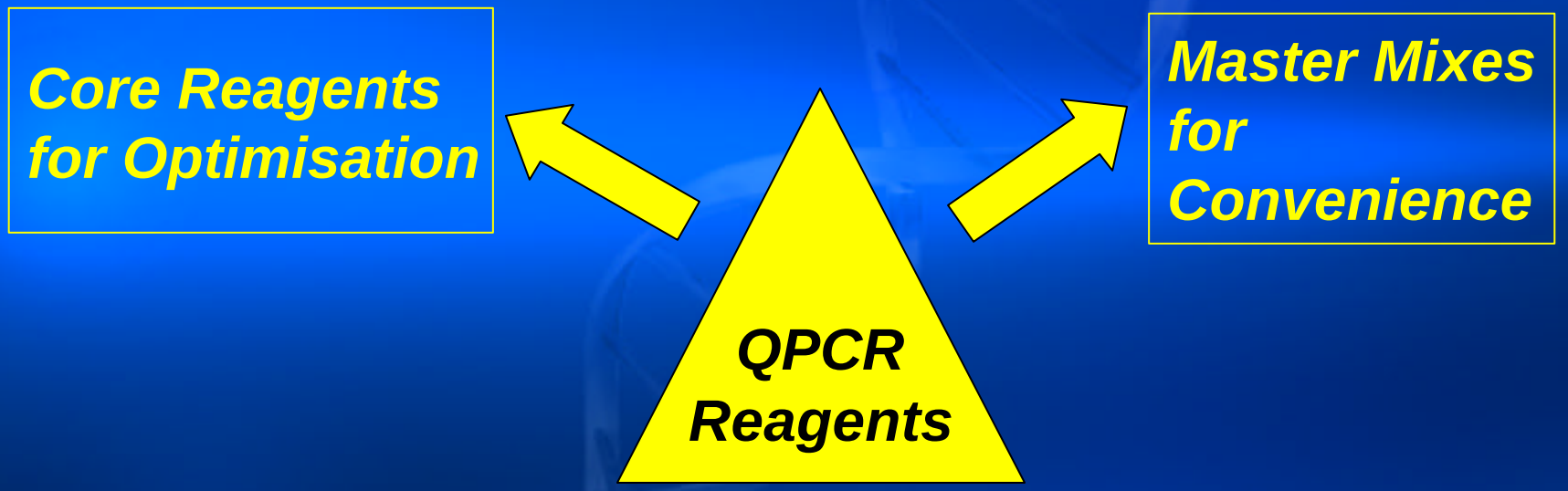
2nd Nucleic Acid Quantification Meeting

An Independent workshop for real-time PCR and RT-PCR

Morris Lecture Theatre, Robin Brook Centre,
St Bartholomew's Hospital, West Smithfield, London
29th May 2003

Reinhold Mueller
Stratagene

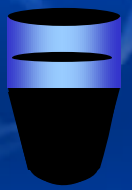
Qualified QPCR Reagents: Maximum Optimisation and Convenience



Core reagents can be optimised for best results.

DNA Polymerase *10X Buffer* *dNTPs* *$MgCl_2$* *Passive Reference Dye*

Master Mixes are easy-to-use and convenient.



All components in 1 tube!

PCR Amplification

PCR: Correlation of amount of amplified DNA to amount of initial target DNA

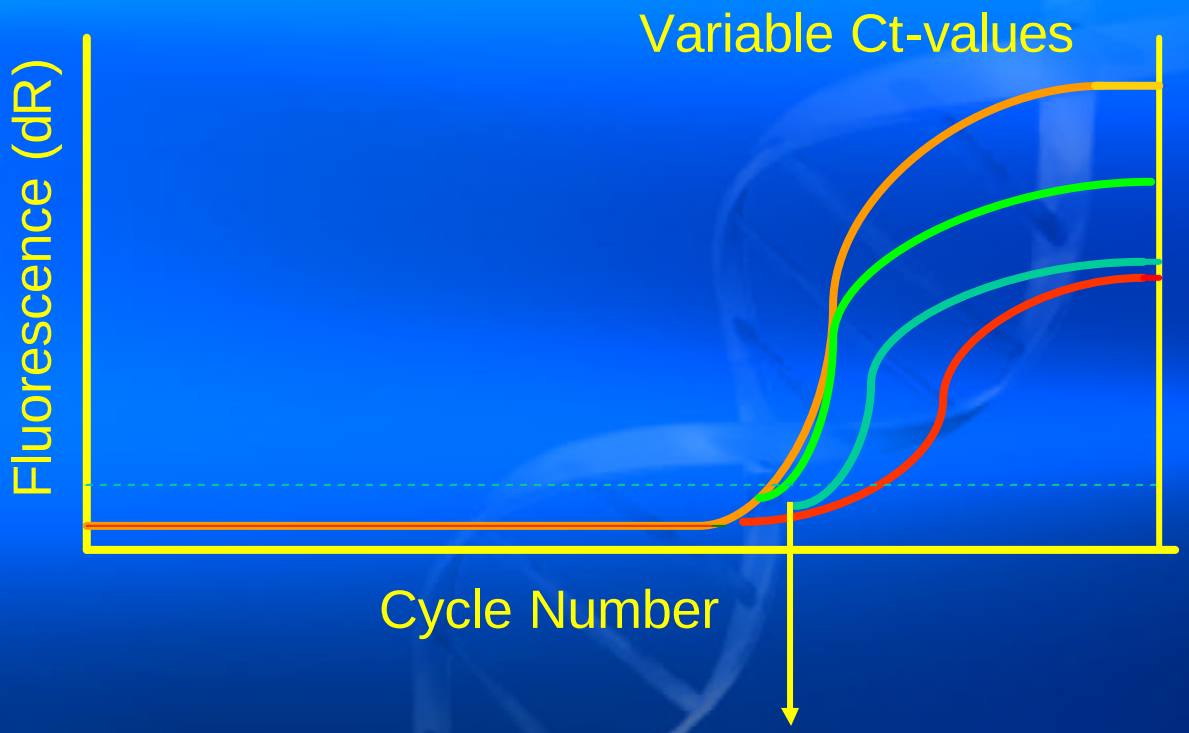
$$Y = X\,(1 + E)^n$$

Y = PCR amplified quantity
X = target DNA quantity prior to PCR
E = amplification efficiency
n = number of cycles

Reagent Optimisation: What Do You Mean?

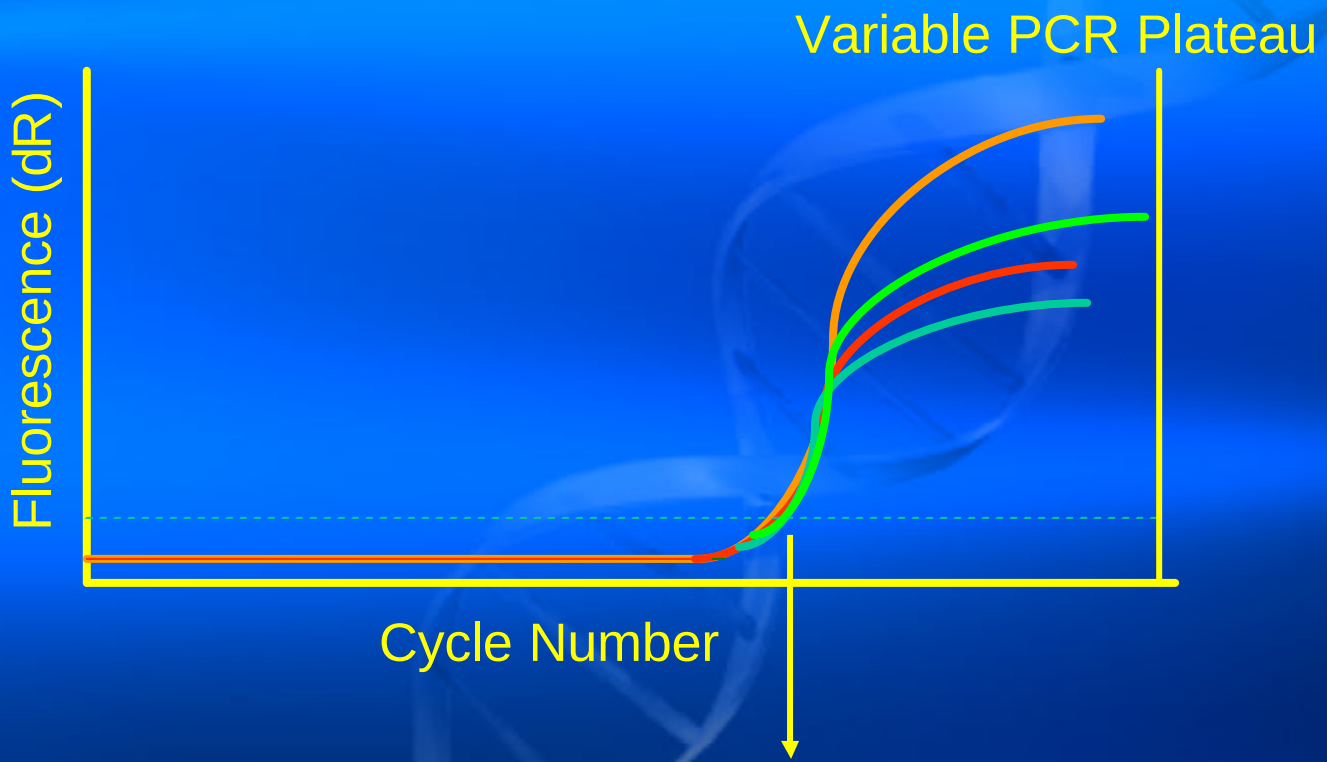
- Optimisation (of the reagents) by the manufacturer
- Optimisation (of the assay) by the customer

Replicate Samples in Reagents, Which Have Not Been Optimized



The replicate samples do not have the same Ct value. A variability in the assay data is observed. However, operator and instrumentation may also affect the results.

Replicate Samples in a Robust Assay



The replicate samples have the same Ct value. Tight data obtained when calculating the initial target concentration.

2-Tube QRT-PCR Core Reagent Kit

RNA

cDNA Synthesis

cDNA

PCR

Cycler/Detector

Single-Tube QRT-PCR Core Reagent Kit

RNA

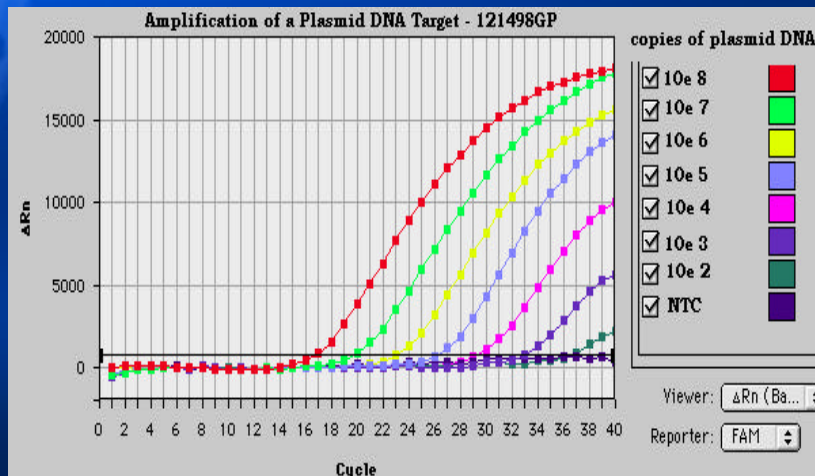
RT-PCR

QPCR Core Reagent Kit

DNA

PCR

Amplification Plot



STRATAGENE

Amplification of RNA Template: Which Route to Take?

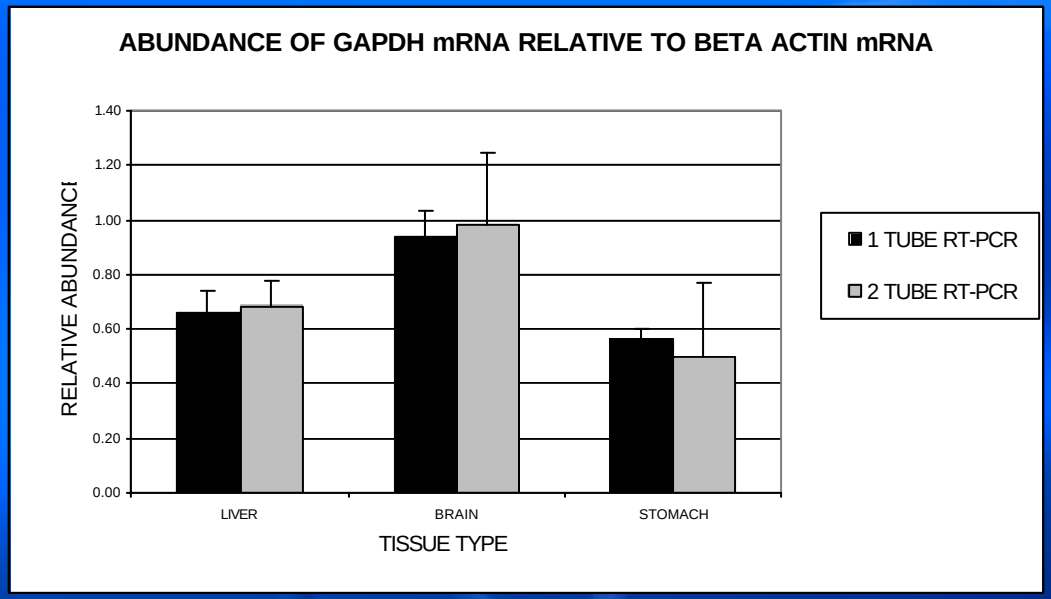
- Single-tube QRT-PCR

  Fast, homogenous assay with possibly lower sensitivity.

- Two-tube QRT-PCR

  Retain (archived) material; must open tube and maybe obtain higher sensitivity.

Performance of Optimized Single-Tube and 2-Tube QRT-PCR Reagents



Template: 10 ng of human total RNA (liver, brain and stomach)
Target: β-actin (FAM-MB) and GAPDH (JOE-MB) in multiplex

Using relative quantification, the difference in performance of a single-tube and a two-tube QRT-PCR assay might be small.

Configuration of Qualified QPCR Core Reagents

	GATC + ROX	GATC - ROX	GAUC + ROX	GAUC - ROX	GAUC/UNG + ROX
PCR	Eurogentec/Stratagene		Eurogentec	Eurogentec	ABI/Stratagene
-Tube RT	Stratagene/Takara Mirus		Stratagene		ABI
-Tube RT	Stratagene		Eurogentec	Eurogentec	Stratagene
YBR Green I QPCR	Eurogentec/Stratagene		Eurogentec	Eurogentec	ABI
YBR 1-Tube RT	Sigma	Ambion			
YBR 2-Tube RT			Eurogentec	Eurogentec	

Buffer Components

QPCR

- (SYBR® Green I)
- 10X Core PCR Buffer
- Taq DNA Polymerase
- $MgCl_2$
- dGATC Mix (dGAUC Mix)
- UNG
- Glycerol
- DMSO
- Other Enhancing Agents
- Reference Dye

Two-tube QRT-PCR

- (SYBR® Green I)
- 10X Core RT Buffer
- 10X Core PCR Buffer
- Reverse Transcriptase
- RNase Inhibitor
- Taq DNA Polymerase
- $MgCl_2$
- dGATC Mix and dGAUC Mix
- UNG
- Glycerol
- DMSO
- Other Enhancing Agents
- Random Primers
- Oligo(dT) Primer
- Reference Dye

Single-tube QRT-PCR

- (SYBR® Green I)
- 10X Core RT Buffer
- Reverse Transcriptase
- RNase Inhibitor
- Taq DNA Polymerase
- $MgCl_2$
- dGATC Mix (dGAUC M
- Glycerol
- DMSO
- Other enhancing agent
- Reference Dye

List of Core Reagent Components That Have a Known Effect on Ct and/or Fluorescence Intensity

- 10X Core RT-PCR Buffer
- 10X Core PCR Buffer
- SYBR Green I
- Reverse Transcriptase
- RNase Inhibitor
- Taq DNA Polymerase
- $MgCl_2$
- dGATC mix (dGAUC mix)
- UNG
- Glycerol
- DMSO
- Other enhancing agents
- Random Primers
- Oligo(dT) Primer
- Reference Dye

Different Strokes for Different Blokes?

- TaqMan® probes, Molecular Beacons, Hybridization probes, Scorpions® and Amplifluor® primers can be detected in the same Core Reagents.

- SYBR® Green I Core Reagents have their own set of ingredients.

- Core Reagents for samples to be run in plastic tubes have a different make-up than Core Reagents to be run in glass capillaries.

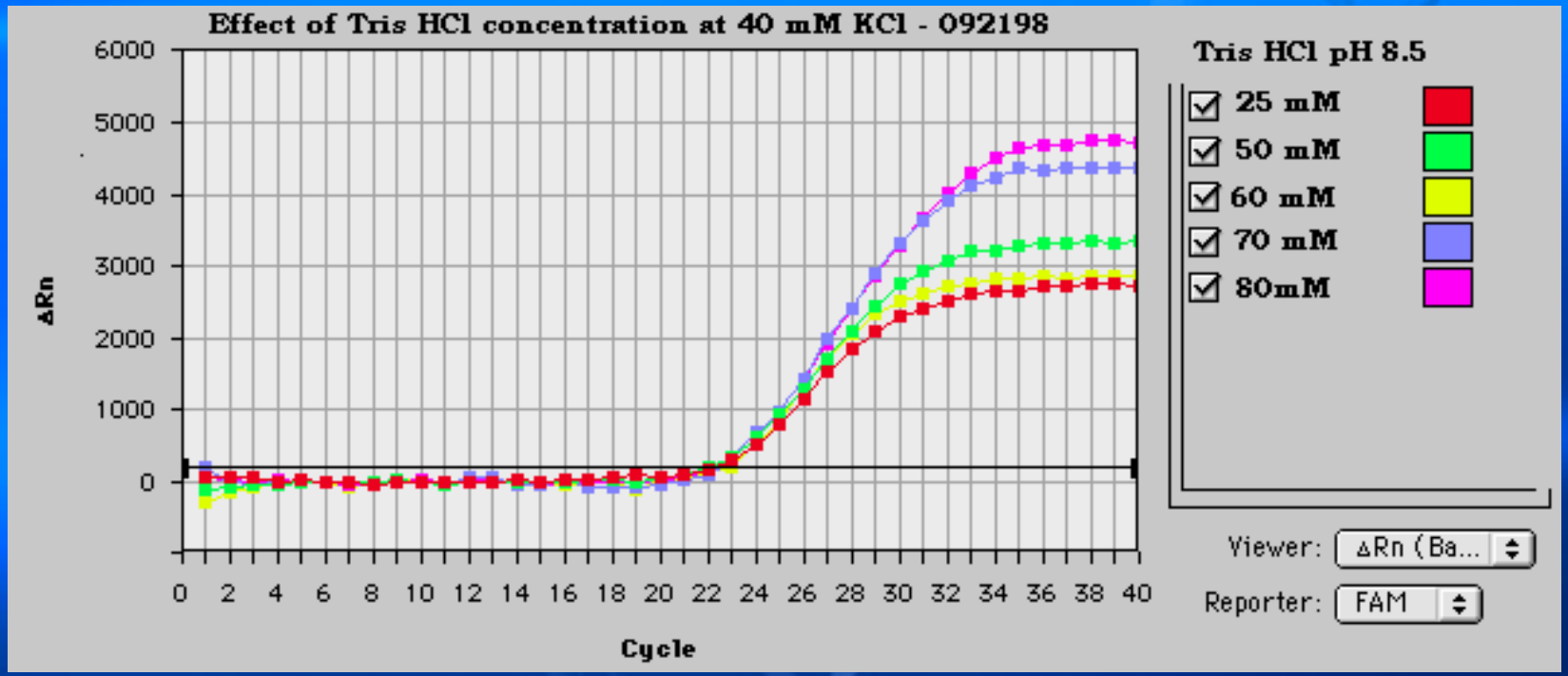
Requirements for Core Reagents

- Sensitivity
- Reproducibility
- Accuracy
- Versatility

Part I: Optimising the Reagents

Probe Detection

Optimisation of [Tris-HCl] for Single-Tube QRT-PCR Core Reagents



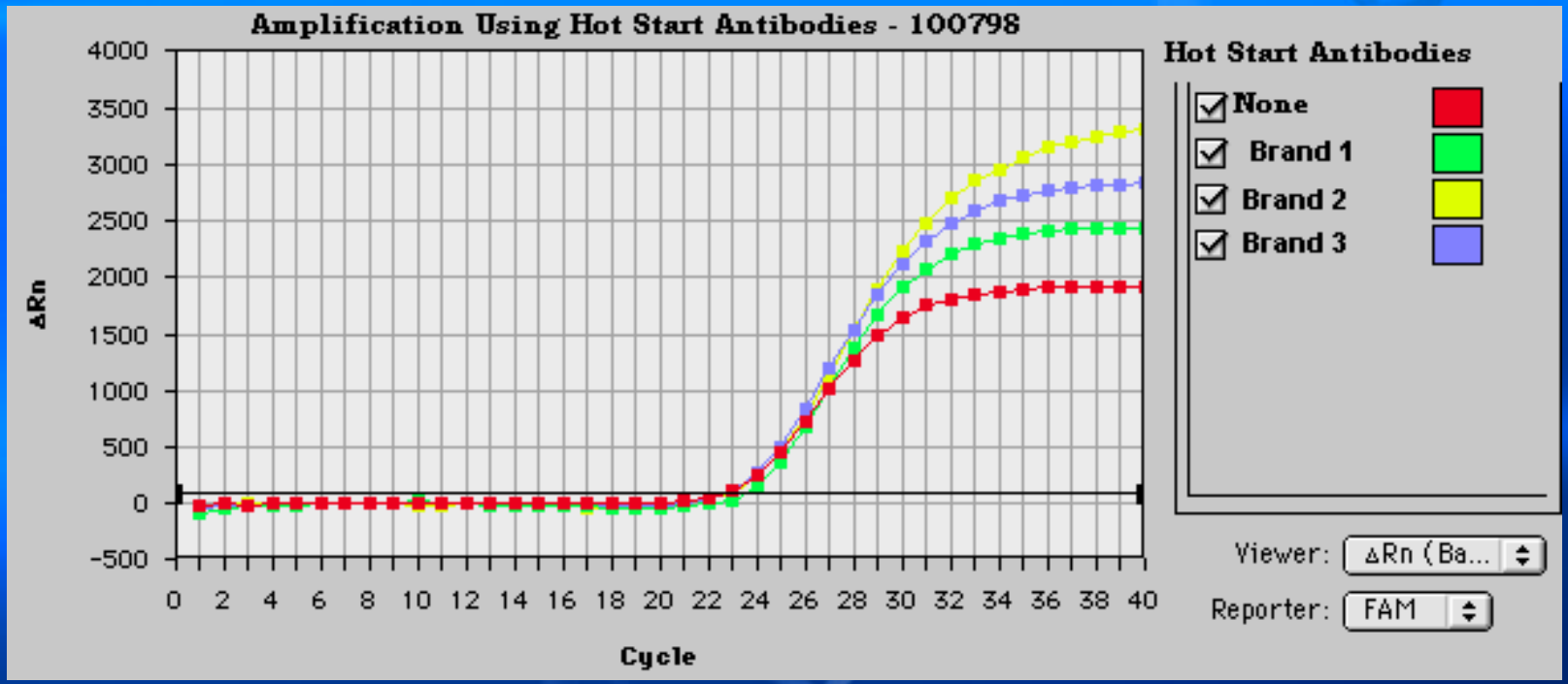
Template: 10 ng of mouse total RNA

Target: β-actin (FAM-MB)

A matrix for [Tris] and [KCl] was set up. This plot represents one part of the matrix. Low Ct and high final fluorescence is desired.

DNA Polymerase (Taq)

Hot Start vs. Non-Hot Start



The average of replicate QPCR reactions is shown. A FAM-labeled Molecular Beacon is used for detection.

Chemical and antibody hot start Taq DNA polymerase yields in higher final fluorescence, compared to reactions run with a non-hot start enzyme.

Polymerase (Taq) Concentration

(Chemically Modified Taq)

Target Amount	1.25U	1.5U	1.75U	2.0U	2.5U
10ng	21.1	20.5	20.8	20.5	20.3
0.1ng	27.8	27.2	27.4	27.4	27.2
0.01ng	31.1	30.4	30.7	30.7	30.7
NTC	No Ct	No Ct	No Ct	No Ct	No Ct

Average Ct of duplicate 50 µl QPCR reactions using GAPDH TaqMan probe (PDAR) and cDNA from PMA-treated Raji cells.

The higher the [Taq], the lower the Ct.

Do We Need Contamination Control?

Security, but loss of sensitivity.

Reverse Transcriptase Concentration

TaqMan Probe
Detection of GAPDH

Units of RT	Target conc (ng)	Avg Ct
1.25	10	17.9
	0.1	24.2
	0.001	30.9
2.5	10	17.5
	0.1	24.1
	0.001	31.1
5.0	10	17.6
	0.1	24.3
	0.001	31.2

Molecular Beacon
Detection of GAPDH

Units of RT	Target conc (ng)	Avg Ct
1.25	100	18.7
	1	24.9
	0.1	28.5
2.5	100	16.9
	1	22.8
	0.1	26.1
5.0	100	15.5
	1	22
	0.1	25.3

The GAPDH targets used are very likely different (the TaqMan PDAR site is not identified). The data indicate that [RT] may have a different effect on different targets.

RNase H (+) vs. RNase H (-) Reverse Transcriptases

TaqMan Detection of Different Target genes

Target	Target Amt.	RNAse H (+)	RNAse H (-)
b-2 micro	1ng	27.4	27.3
	0.01ng	34.3	34.5
	NTC	no ct	no ct
Cyclophilin	1ng	26.7	25.7
	0.01ng	33.7	33.4
	NTC	no ct	no ct
GUS	10ng	26.1	25.7
	0.1ng	32.7	32.9
	NTC	no ct	no ct
IL-6	10ng	30.6	30.6
	1ng	34.0	34.4
	NTC	no ct	no ct

The results indicate that in this single-tube QRT-PCR there is no significant difference in using RTs with or without RNase H activity.

Effect of RNase Block on QRT-PCR

Amt RNAse Block	Target conc (ng)	Avg Ct
None	100	25.9
	0.1	35
10U	100	25.7
	0.1	34.8
20U	100	26
	0.1	36.2
30U	100	26.7
	0.1	36
40U	100	26.5
	0.1	36.1
50U	100	26.3
	0.1	36.6

Target: TATA Box Binding Protein/TaqMan Probe

Template: QPCR Human Reference RNA

Only at low template concentration is the effect of RNase inhibitor really apparent.

Optimising dNTP (GATC) Concentration

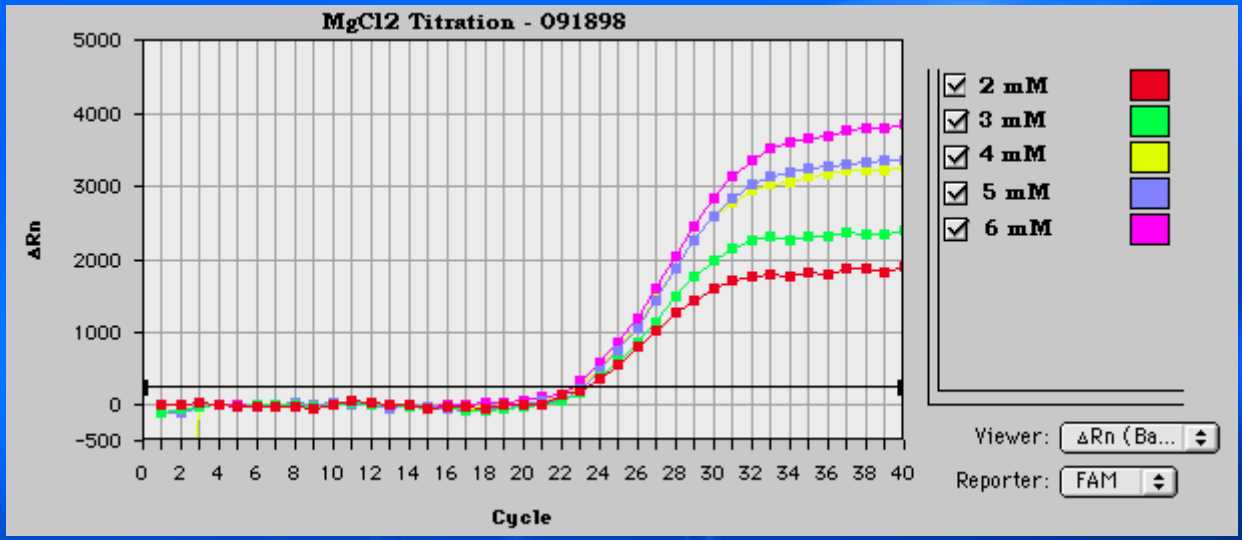
Conc. dNTP each	Ct	FI
50uM	23.6	2500
100uM	22.8	3800
200uM	21.9	4700
300uM	21.7	4700

Template: 10 ng mouse total RNA

Target: b-actin using single-tube QRT-PCR with Molecular Beacon detection (FAM)

By increasing the [dNTP] a decrease in Ct and an increase in final fluorescence is observed.

The Effect of Magnesium on QRT-PCR

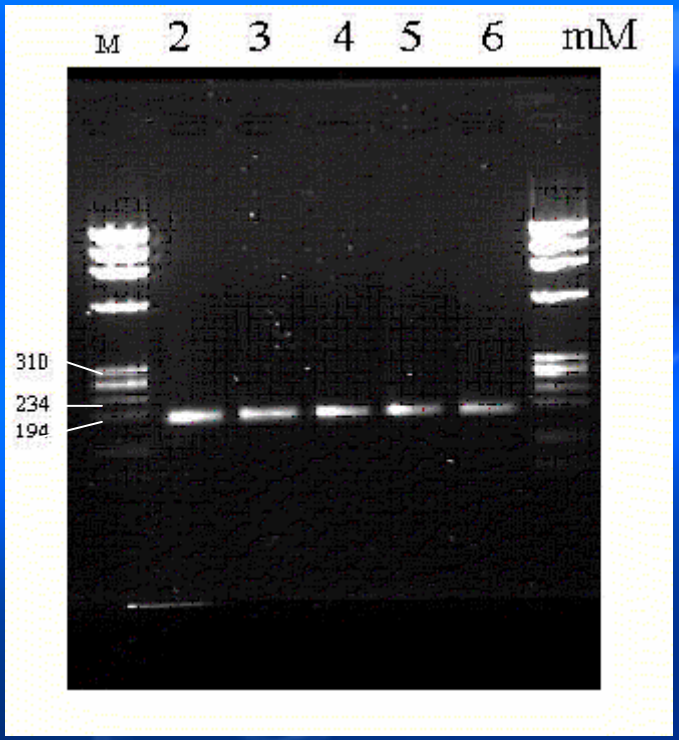


Template: 10 ng of mouse total RNA

Target: b-actin (FAM-MB) in single-tube QRT-PCR

The Effect of Magnesium on QRT-PCR

Continued



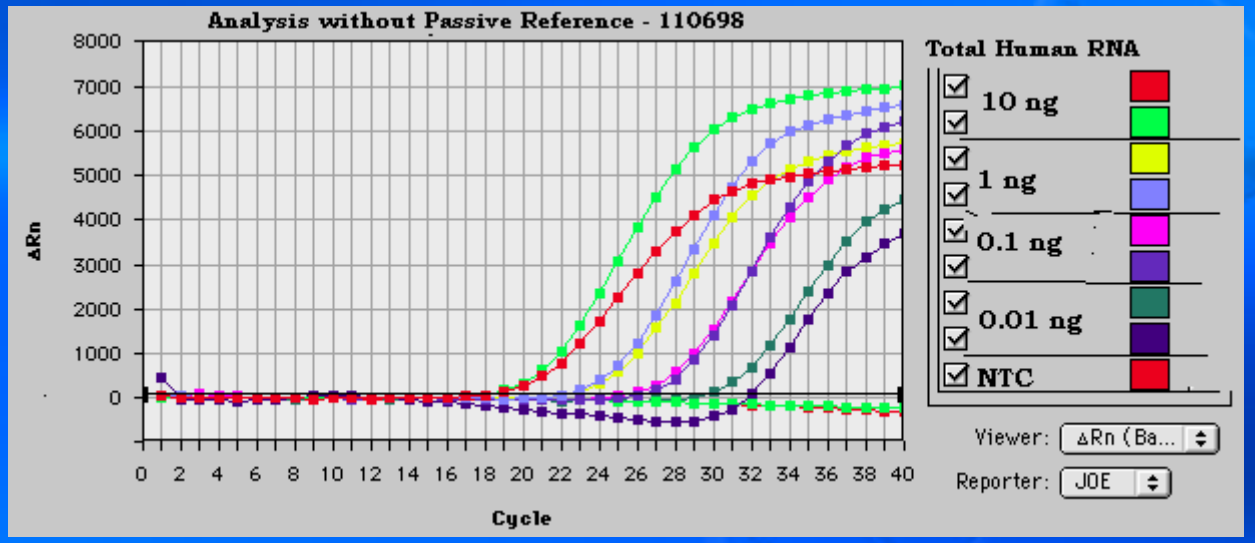
4% agarose gel, stained with ethidium bromide.

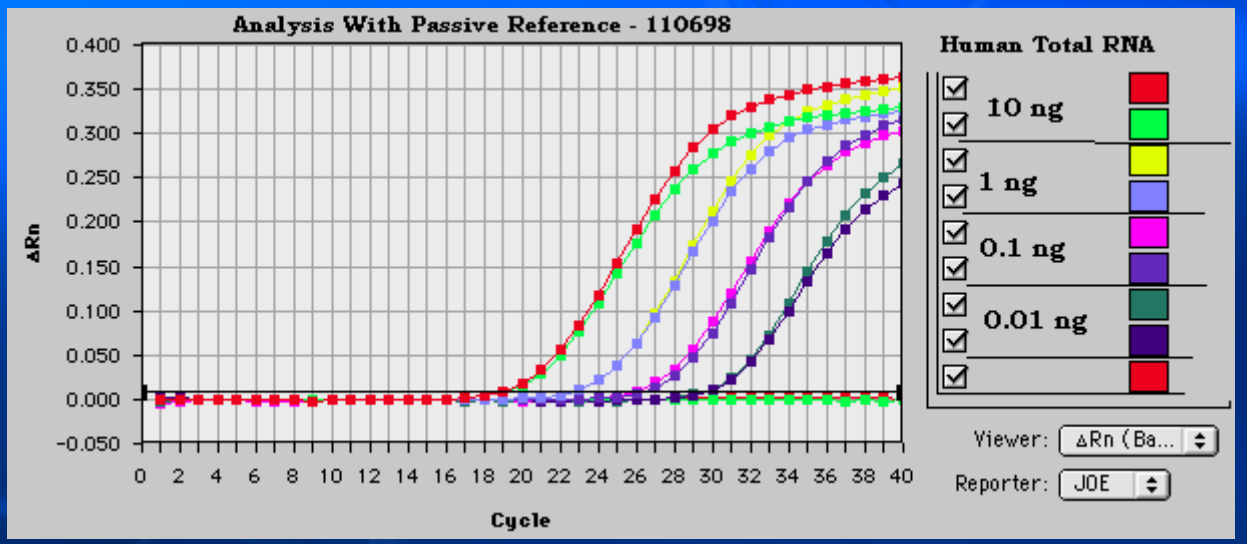
Effect of DMSO on Ct

	Average Ct	
DMSO Conc (%)	GAPDH	β-2 micro
0	27.5	36.3
0.5	26.4	32.8
1	25.9	32.7
1.5	26.2	32.4
2	25.6	32.6
2.5	25.8	32.9
3	25.8	32.7
5	26.7	33.1
10	32.4	37.7

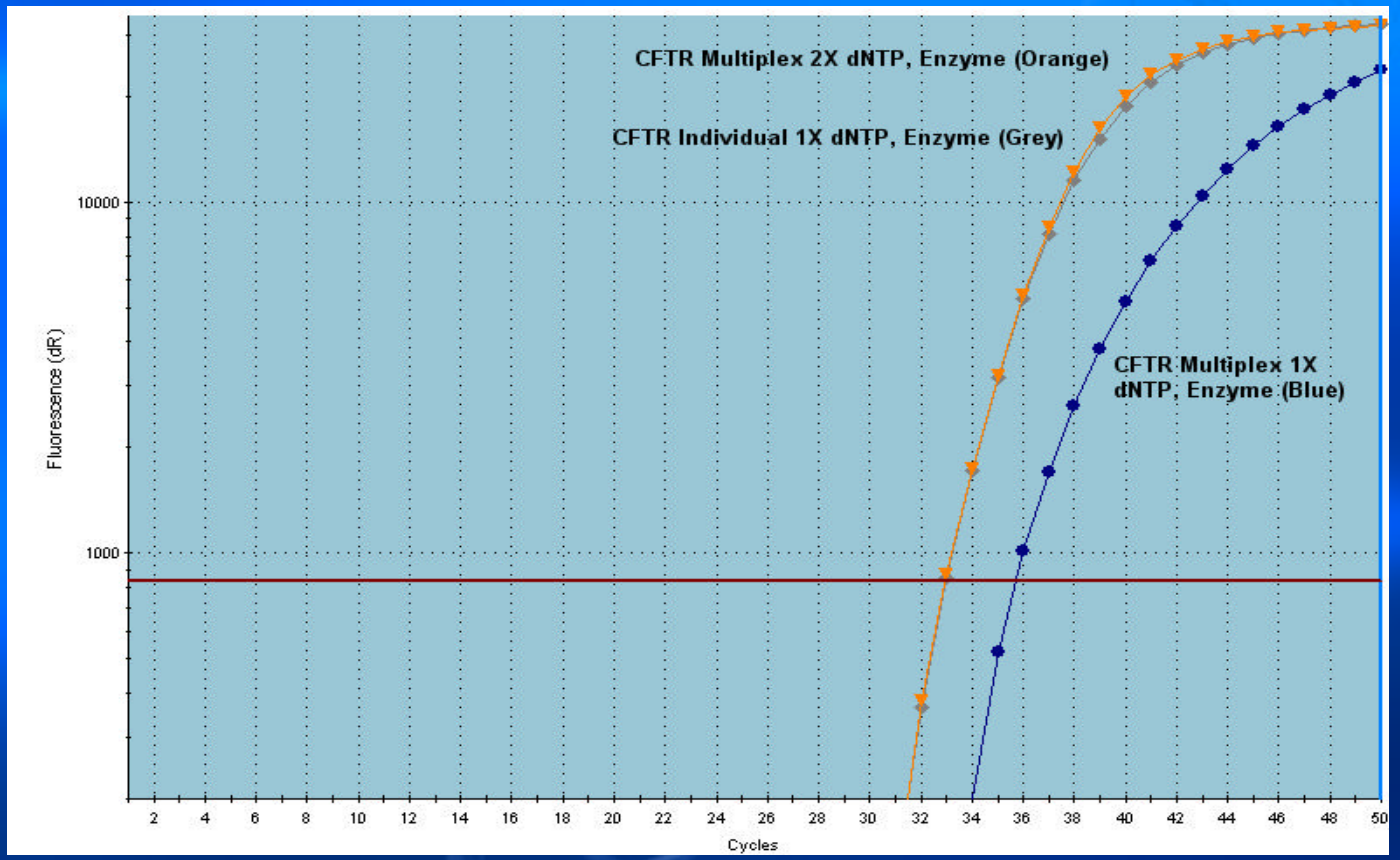
Single Tube QRT-PCR Core Reagents with TaqMan probes for GAPDH and β-2 microglobulin using 0.01ng of QPCR reference RNA.

Passive Reference Dye





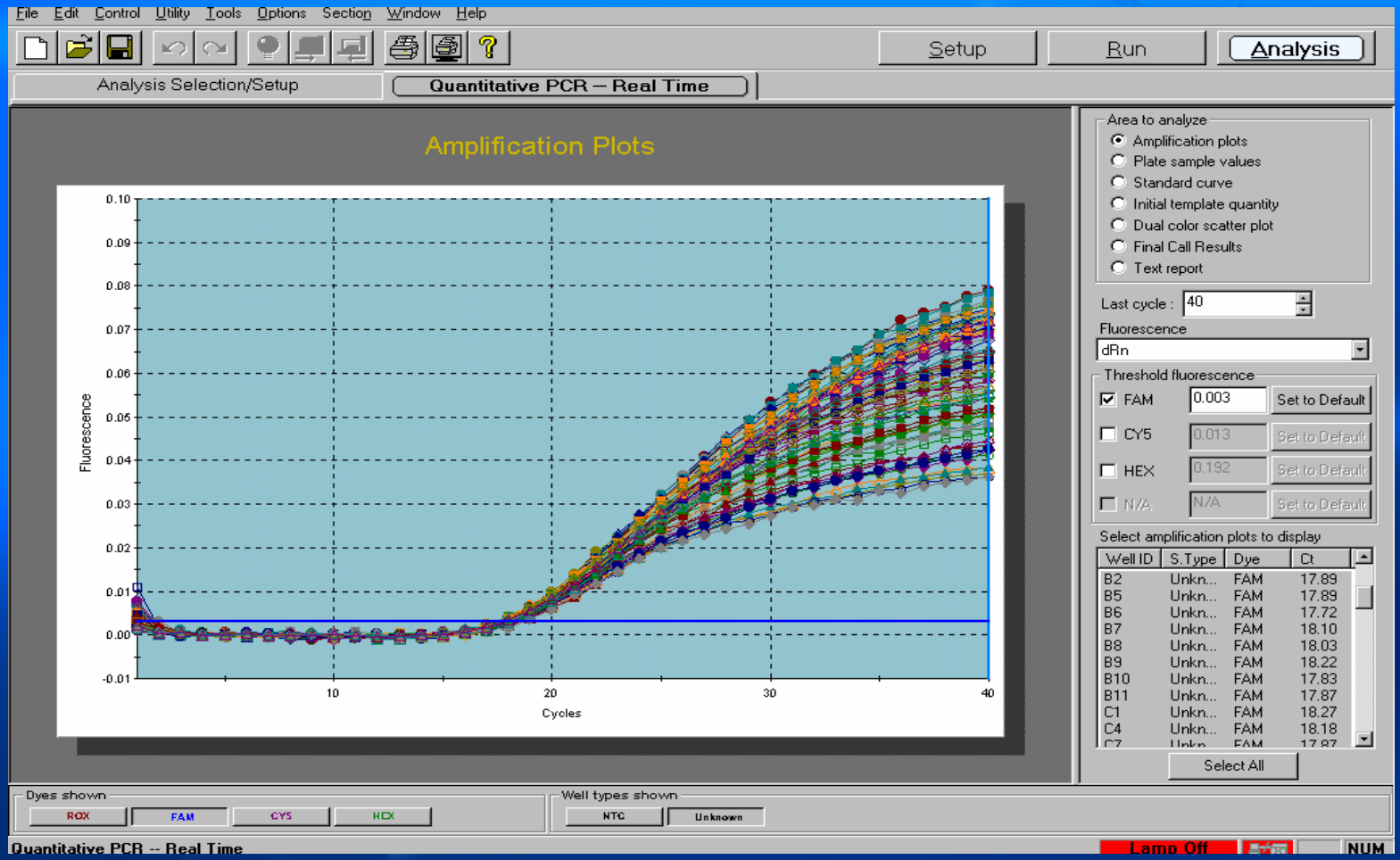
dNTP Concentration in Multiplex Reactions



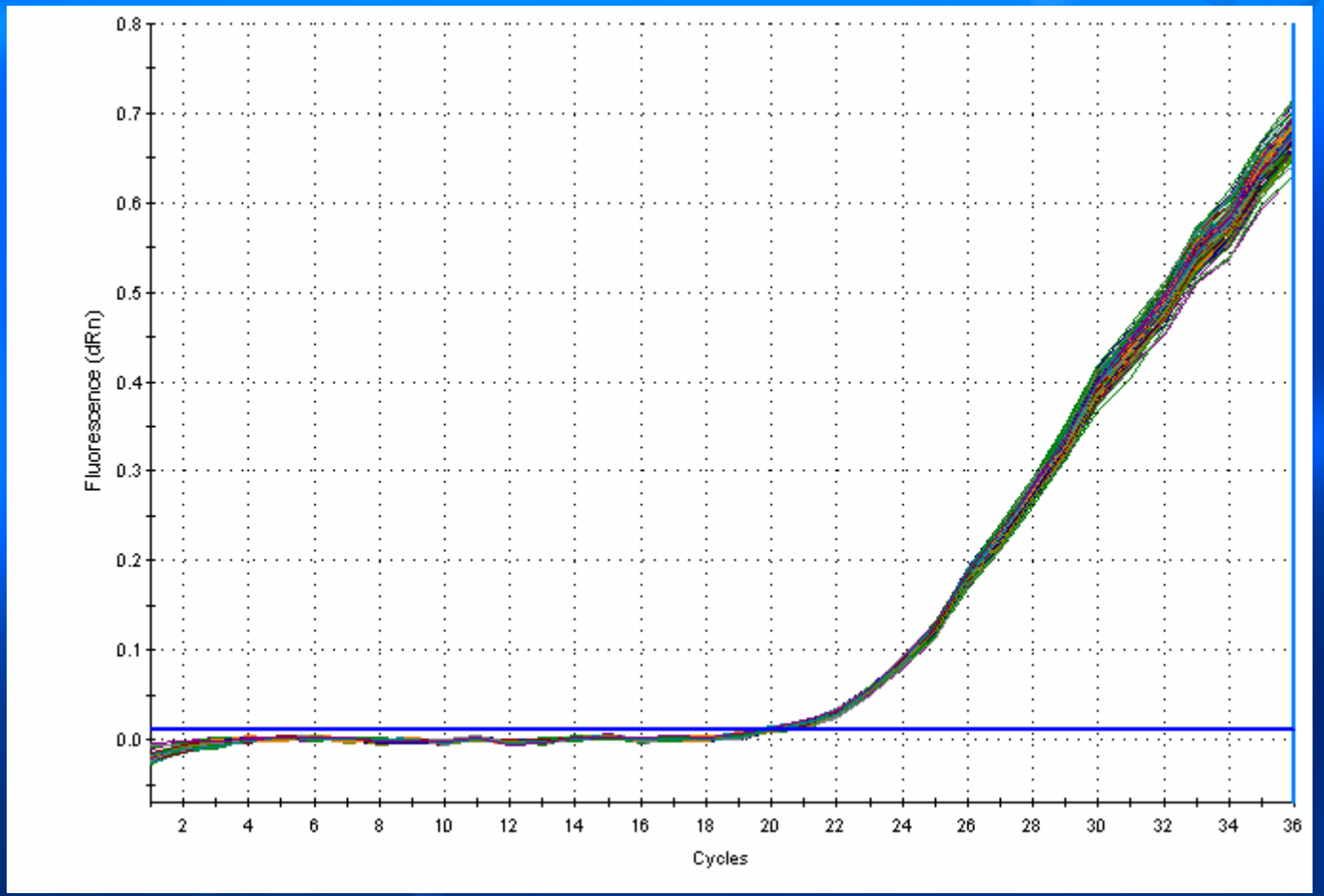
Template: QPCR human reference total RNA

Target: Cystic fibrosis transmembrane conductance regulator (CFTR); (Detection: TaqMan ROX-BHQ2)

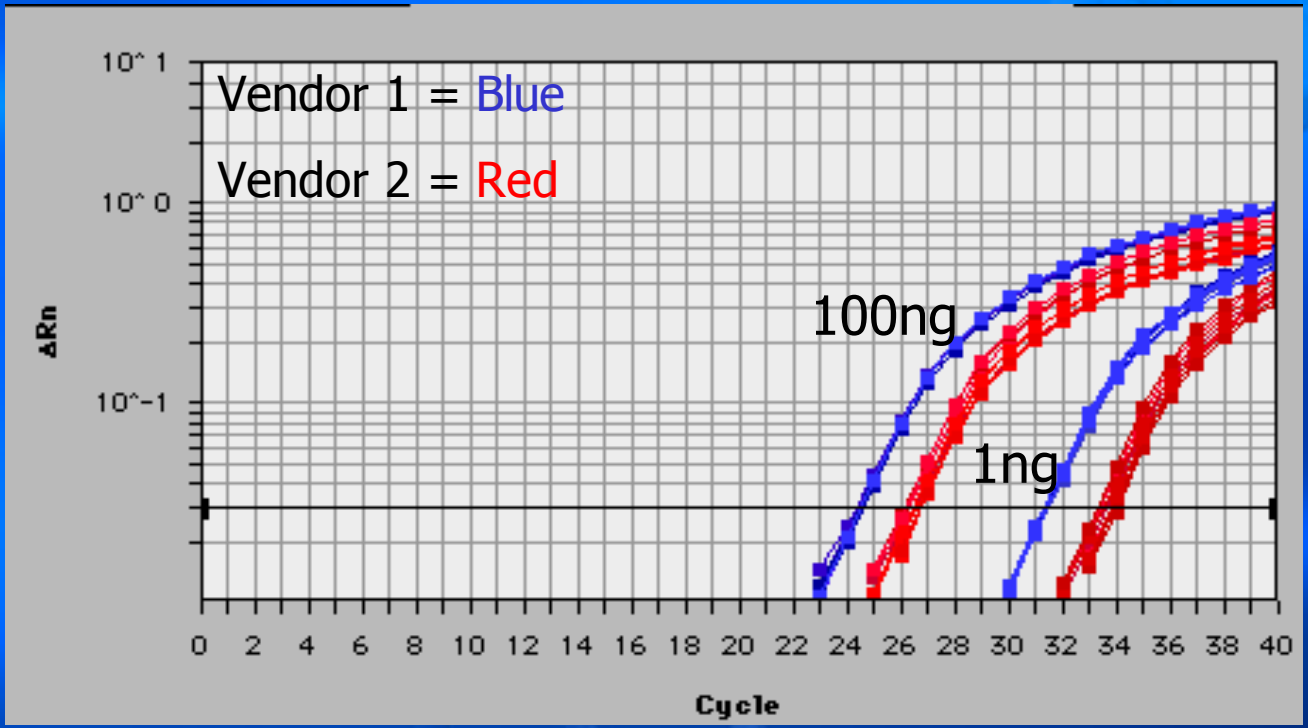
Assay Reproducibility in 96 Wells



Assay Reproducibility in 96 Wells, Very Tight Run.



Reproducibility of "Optimized" QPCR Core Reagents From Two Vendors

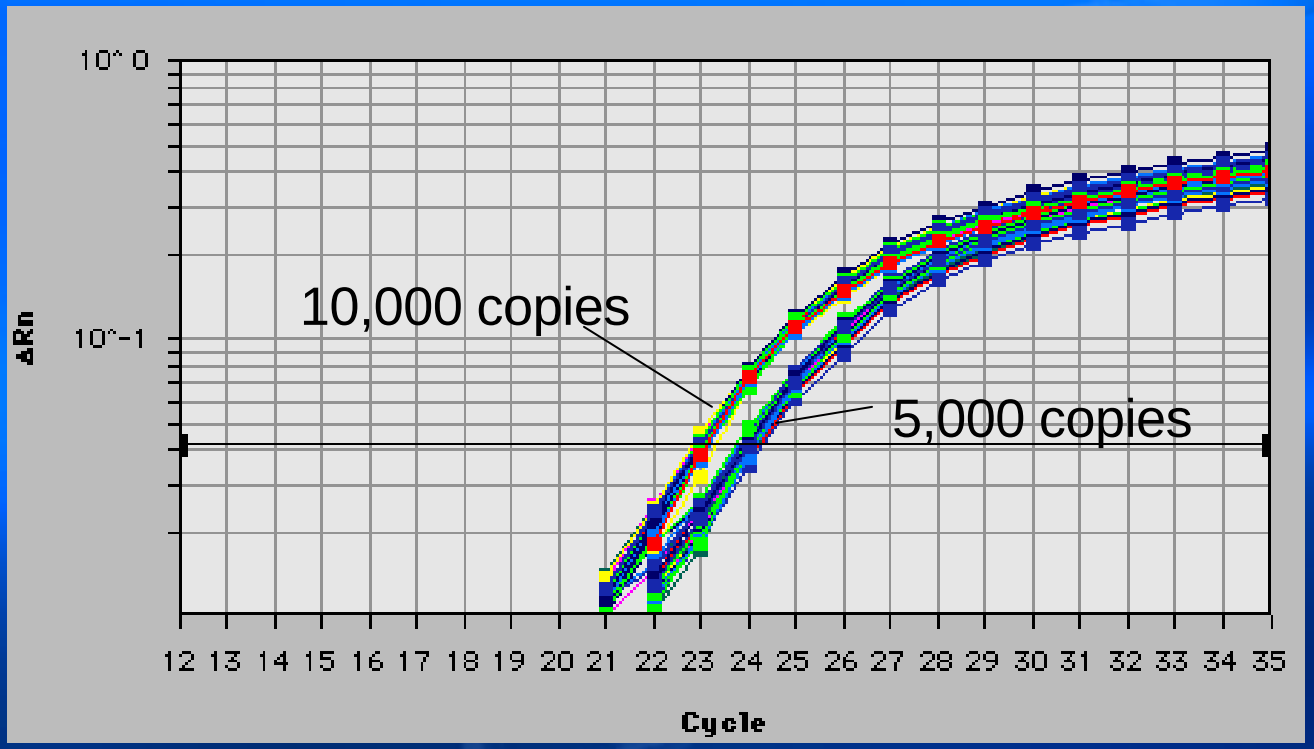


Target: b-actin detection, linear probe, human genomic DNA as template.

Platform: ABI 7700

The plot shows the reproducibility and sensitivity when 8 replicates at two target concentrations (100ng, 1ng) were run (0.3Ct spread for Vendor 1 vs 0.8 Ct spread for Vendor 2).

2-Fold Discrimination



b-actin Molecular Beacon assay

Intra-Assay Variability Using the Single-Tube RT-PCR Core Reagent Kit

pg Total RNA	Ct for Exp 1	Ct for Exp 2	Ct for Exp 3	Ct for Exp 4	Avg. Ct	Std. Dev	% CV
1	35.1	35.9	35.6	32.5	34.8	1.3	3.9
10	32.1	31.8	31.9	31.1	31.7	0.4	1.2
100	28.2	28.7	28.7	29.0	28.7	0.3	1.0
1000	24.7	25.5	25.8	25.2	25.3	0.4	1.6
10000	20.8	21.3	22.1	21.6	21.5	0.5	2.2

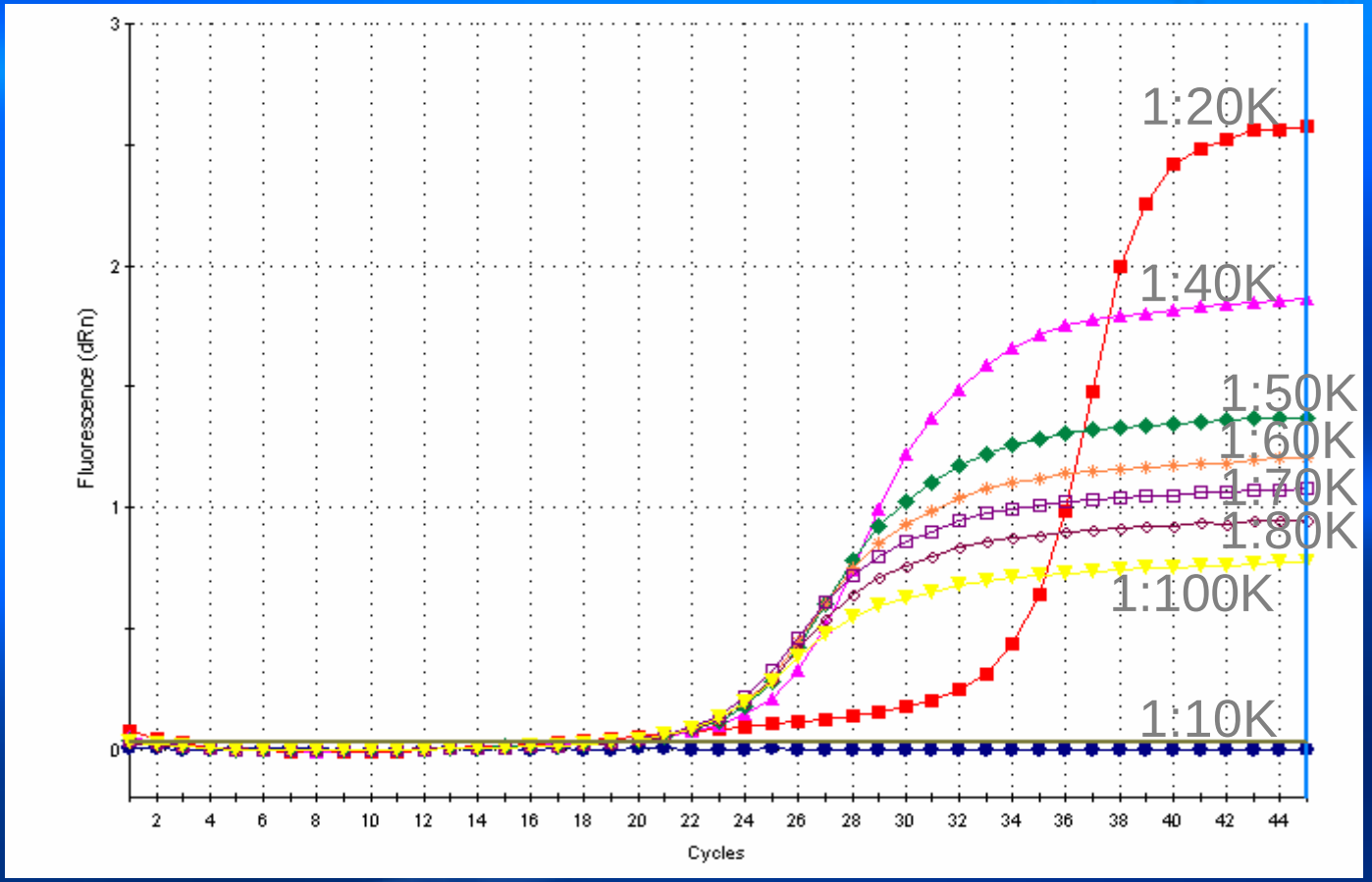
Optimized QRT-PCR Core Reagents show good reproducibility.

Part I: Optimising the Reagents:

SYBR Green I

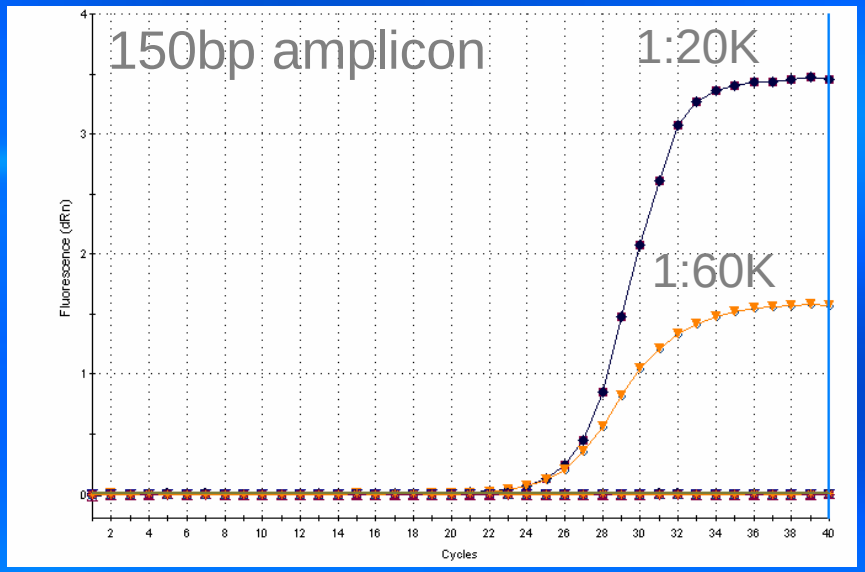
SYBR Green I Titration With 900bp

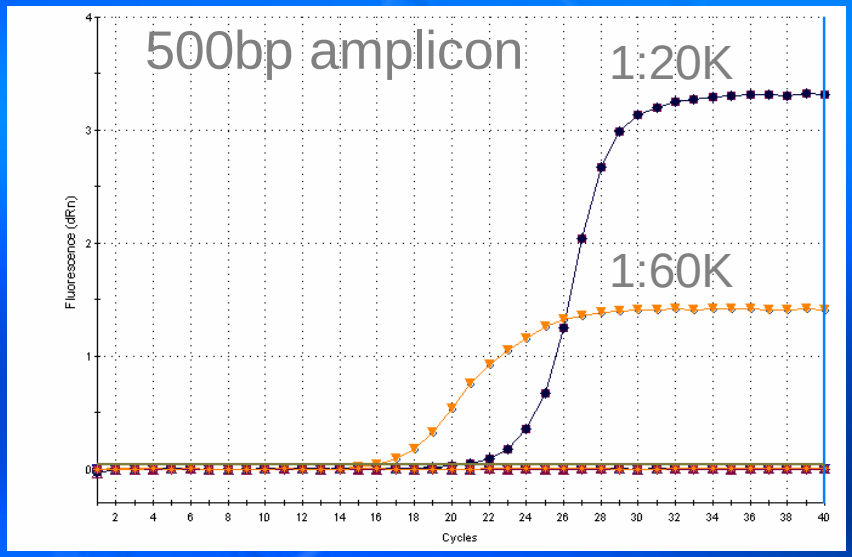
a-1 Anti Trypsin Target

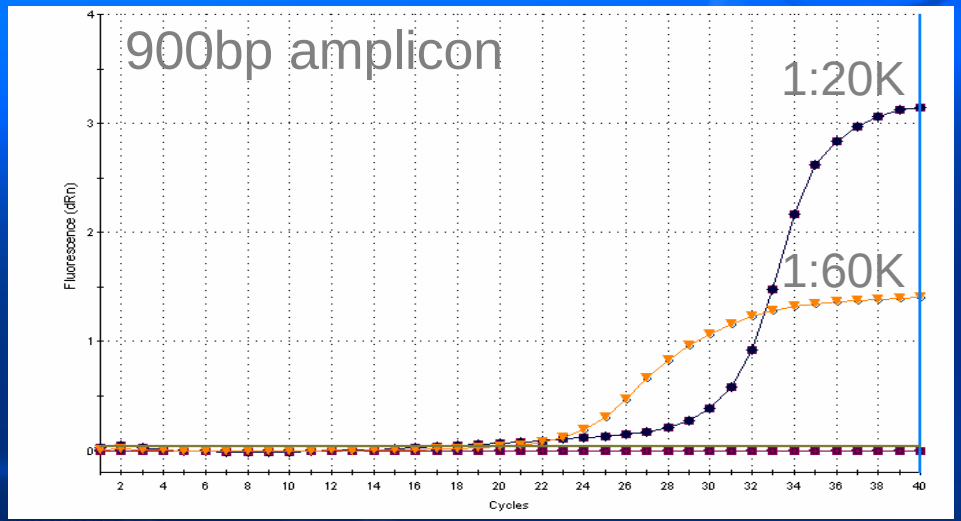


SYBR Green I has an inhibitory effect on amplification/detection of target.

Effects of SYBR Green I Concentration On Amplicon Length

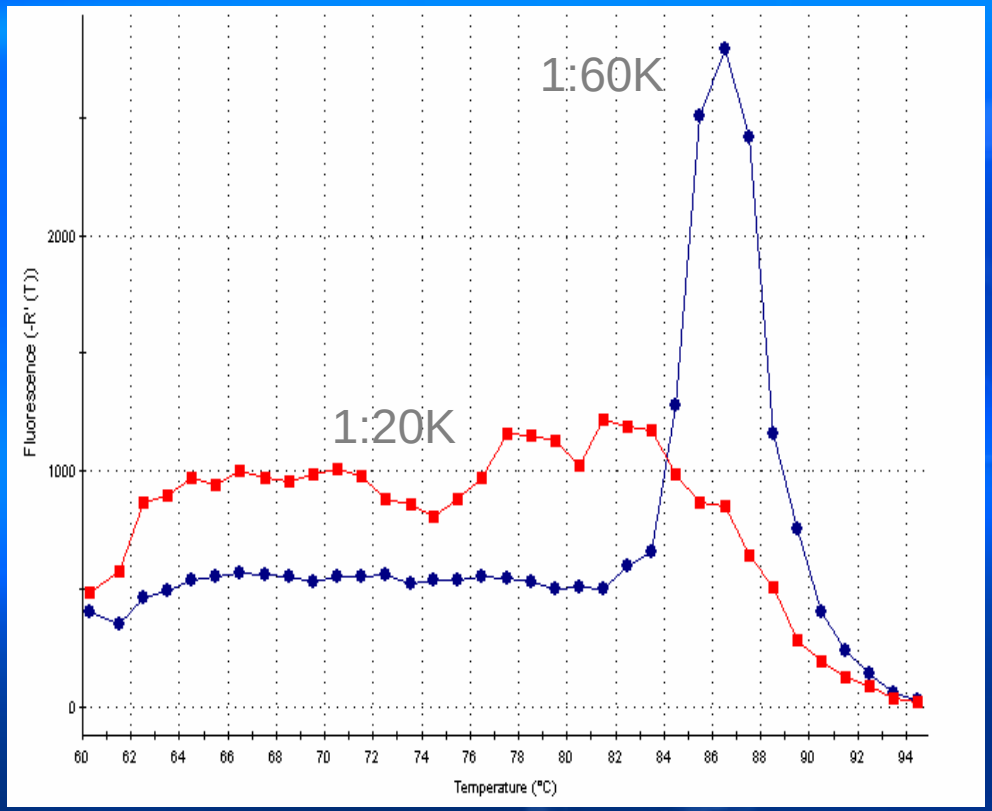




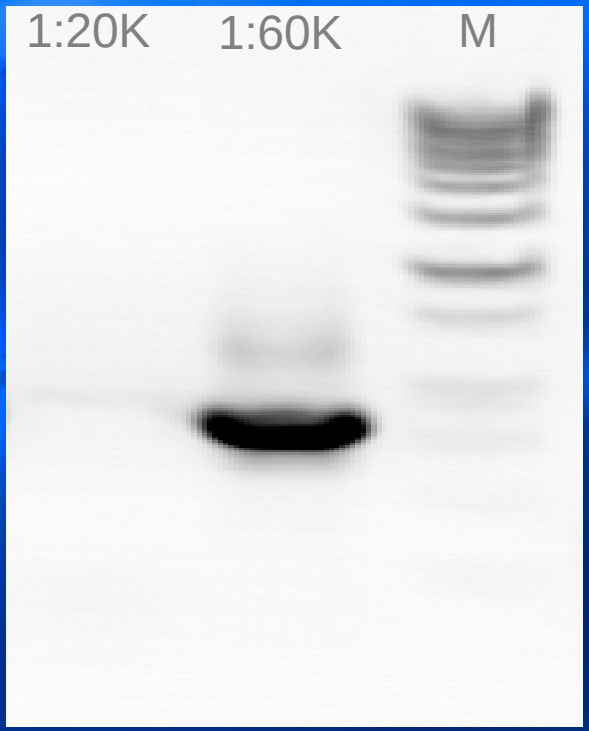


The SYBR Green I inhibitory effect is related to amplicon length.

Effects of SYBR Green I Concentration Continued



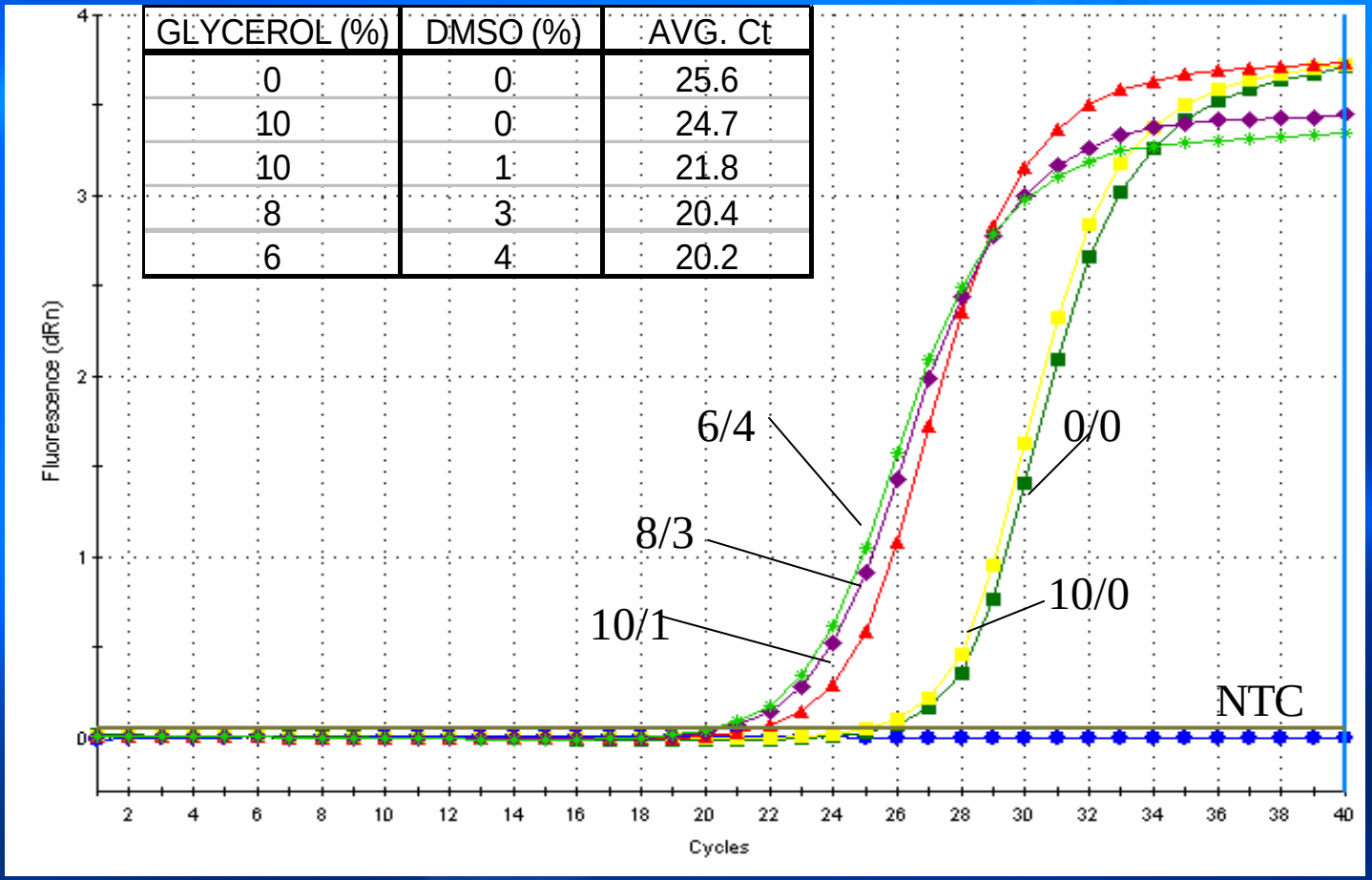
Dissociation profile



Agarose gel stained with EtBr

SYBR Green I: Effects of DMSO and Glycerol (Amplification Plot)

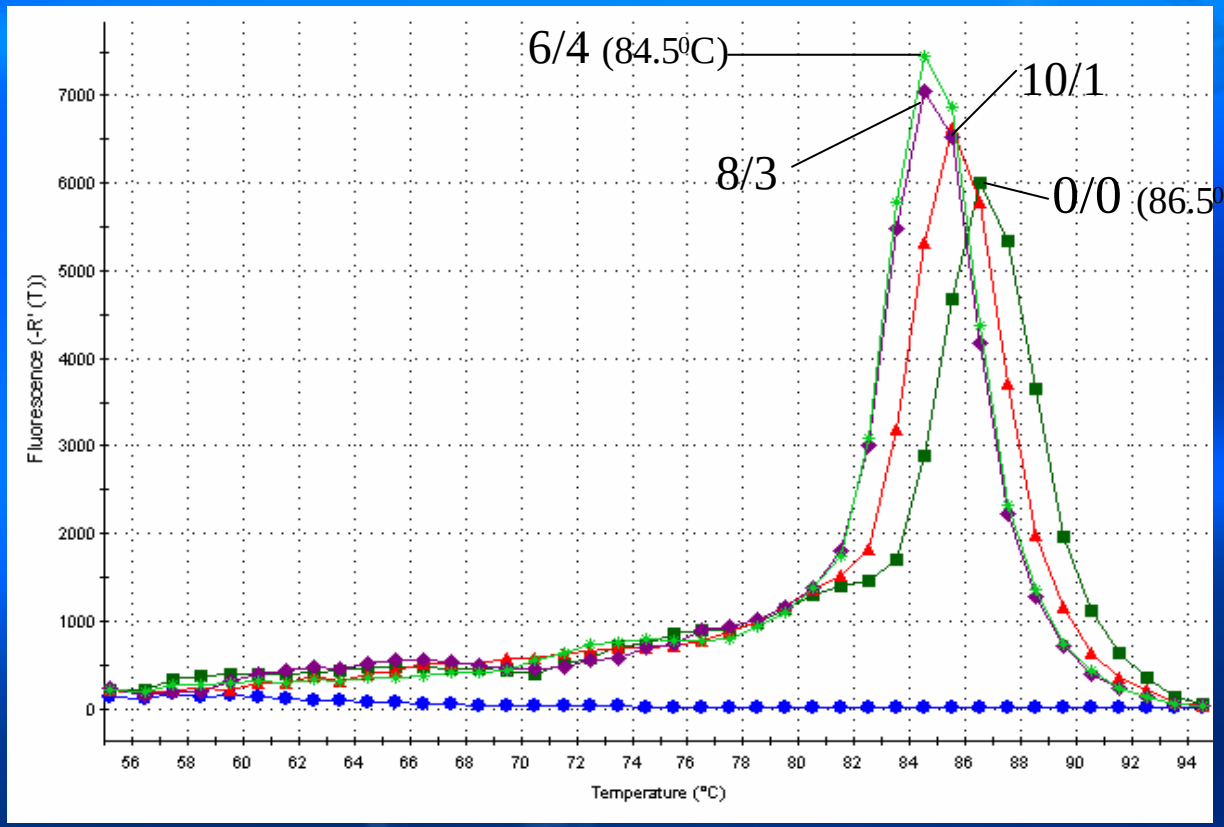
GLYCEROL (%)	DMSO (%)	AVG. Ct
0	0	25.6
10	0	24.7
10	1	21.8
8	3	20.4
6	4	20.2



Template: Plasmid DNA

Target: Mouse muscle nicotinic acetylcholin receptor g (FAM-MB)

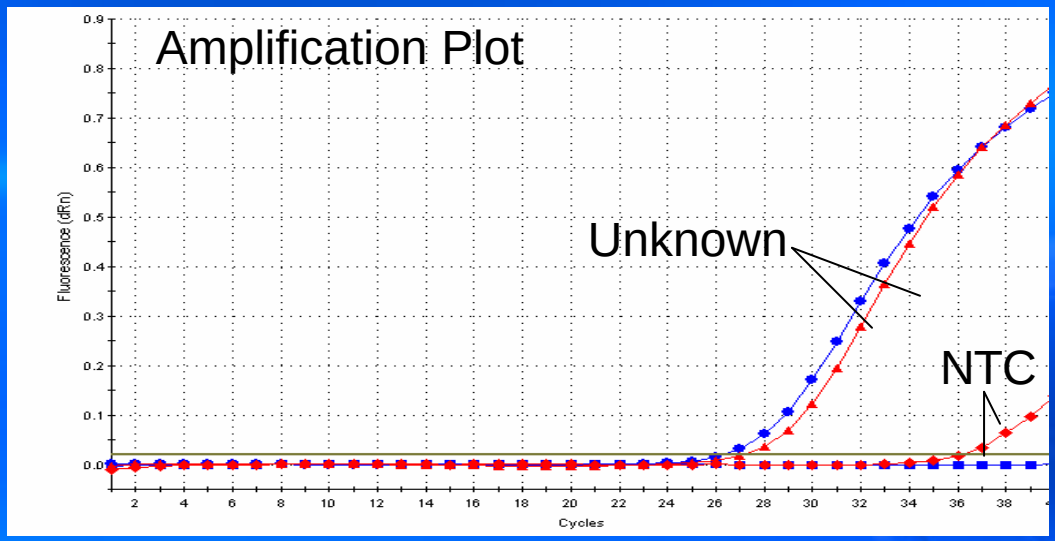
SYBR Green I: Effects of DMSO and Glycerol (Dissociation Run)



As expected, glycerol and DMSO change the melting of specific amplicon, in this case by 2°C.

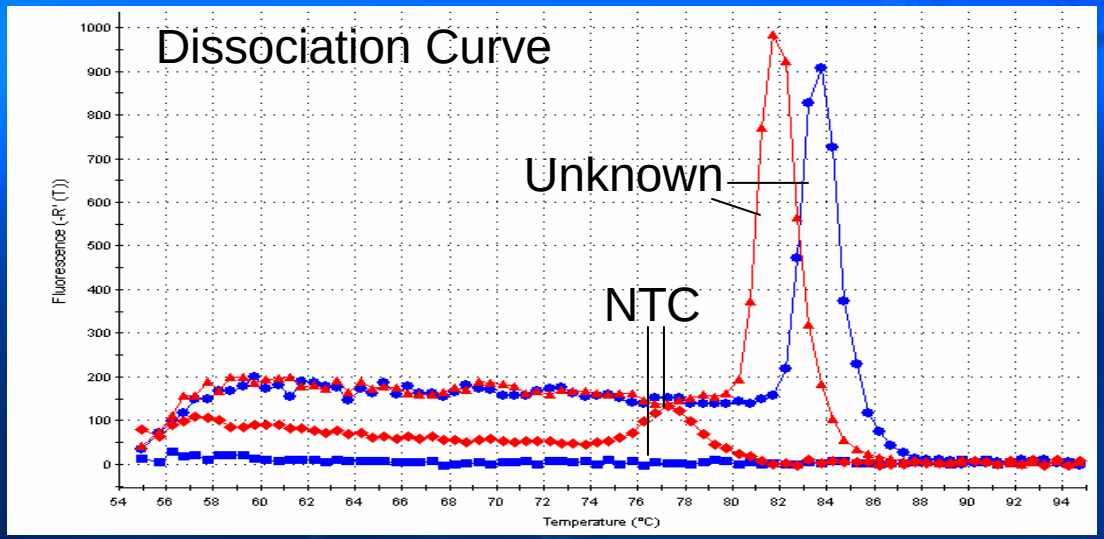
Part II: Optimising the Reactions

Optimizing Primer Sets for SYBR Green



Blue = Primer set 1 (247bp)

Red = Primer set 2 (151bp)



Target = SERCA1(ATP2A1

Template = cDNA from Human QPCR Reference Total RNA

Acknowledgements

Gothami Padmabandu

Jeanette Quinn

Andrew Firmin

For Optimisation of Reactions Using Core Reagents One Should Consider Varying First:

- Primers
- Template
- $MgCl_2$
- Enzymes
- dNTPs
- DMSO,Glycerol
- (SYBR Green I)