

Optimisation of reverse transcription for two-step QRT-PCR:

A comparison of RT priming methods and the addition of a new enhancer for efficient removal of double-stranded DNA contamination

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QRT-PCR

- One-step
 - RT step and QPCR combined in one reaction
 - Gene-specific priming of mRNA
- Two-step
 - Separate RT step and QPCR
 - Allows different gene amplifications from one cDNA pool
 - Variety of primers for cDNA synthesis

Factors that affect QPCR efficiency

- Primer design
- Consumables
- Pipetting errors
- Cycle duration and temperature
- **RNA quality**
- **Reverse transcription step**

Variability and lack of reproducibility in QRT-PCR

“...little effort has been made to draw attention to the different priming approaches used to synthesise cDNA.”

Bustin *et al.* (2005) *Journal of Molecular Endocrinology*

Optimisation of RT step

- Priming strategies
- Blends of enzyme
 - ABsolute™ MAX QRTase blend
- Removal of contaminating DNA
 - ABsolute™ QRTase enhancer

RNA priming strategies

What choice to make?

- Are you getting equal conversion of RNA to cDNA?
 - Between two samples
 - Between gene and standard
- If using DNA copy standard for normalisation you need to be sure of 100% efficiency to be able to compare directly.
- Can use RNA but still assumes both target and standard have same efficiency.

Zhang and Byrne (1999) Biochem J.

Common priming methods

Gene-specific

- Anti-sense PCR primer
- Generates highest yield of specific product
- Only one gene can be amplified from cDNA pool



Random priming

- Typically 6-10 nucleotides in length
- Generates most diverse pool of cDNA
- Prime all RNA including rRNA



What about low gene copy number?

Gene	Primer	Slope	<i>Ex</i>	<i>r</i>	Ct (1 μ g RNA)
GAPDH	RH	-3.1	1.1	-1	23.49
	Anti-sense	-3.3	1.0	-1	11.8
ANF	RH	-2.0	2.2	-0.95	-
	Anti-sense	-3.1	1.1	-0.99	12.69

RH: Random hexamers

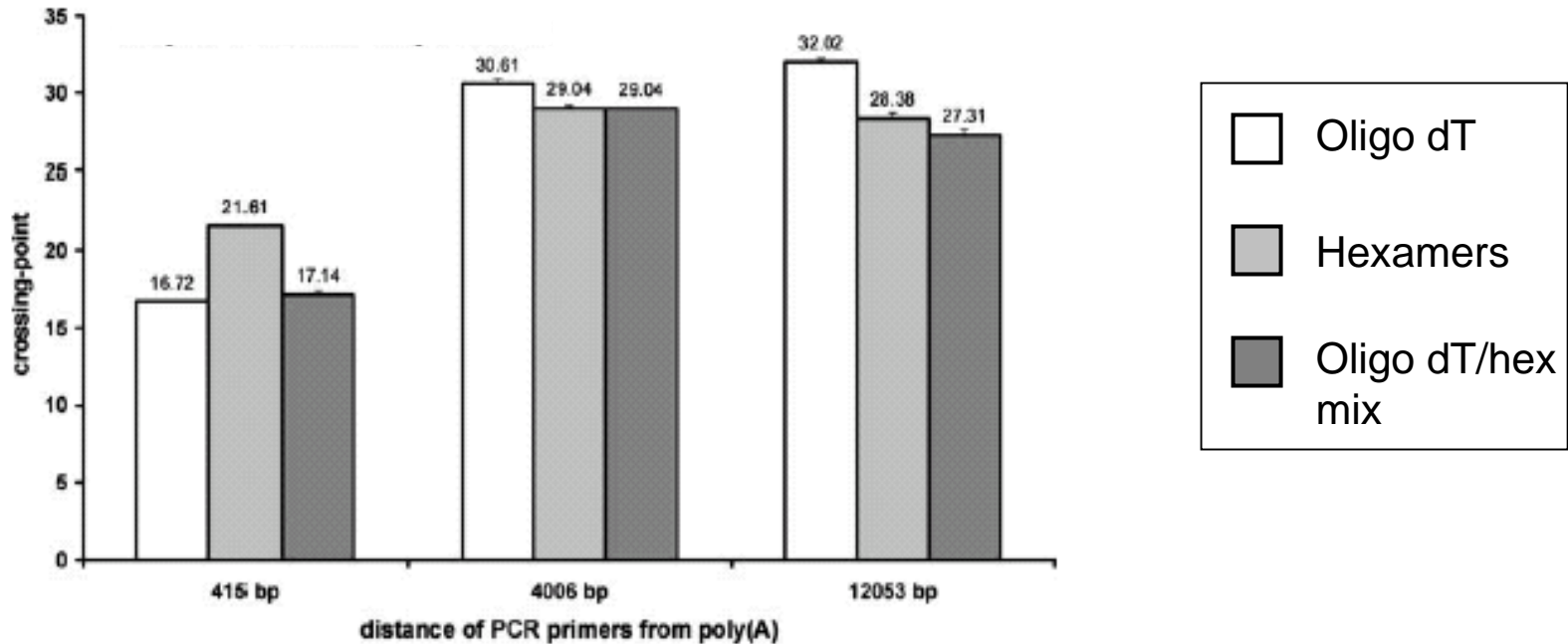
Lekanne Deprez *et al.* (2002) Analytical Biochemistry

Oligo dT

- Anneal to poly-adenosine tails of mRNA
- More consistent cDNA pool
- 18S, Histones
- Biased to 3' ends



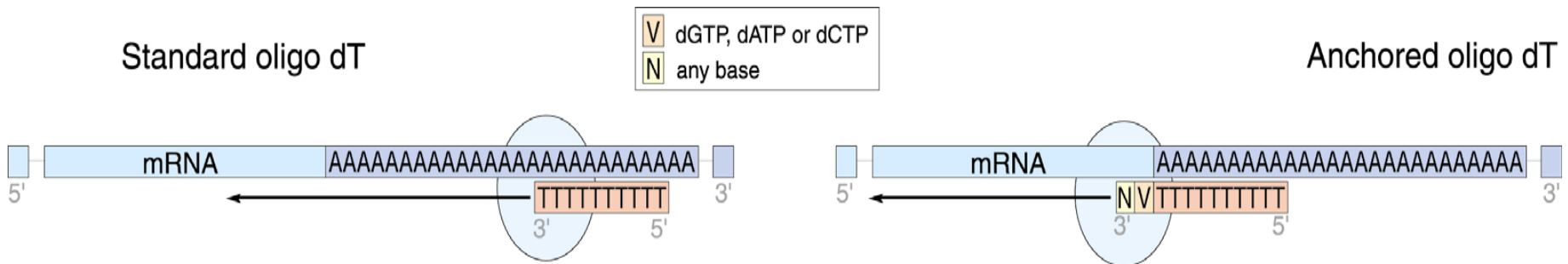
Primer choice is dependent on PCR primer location



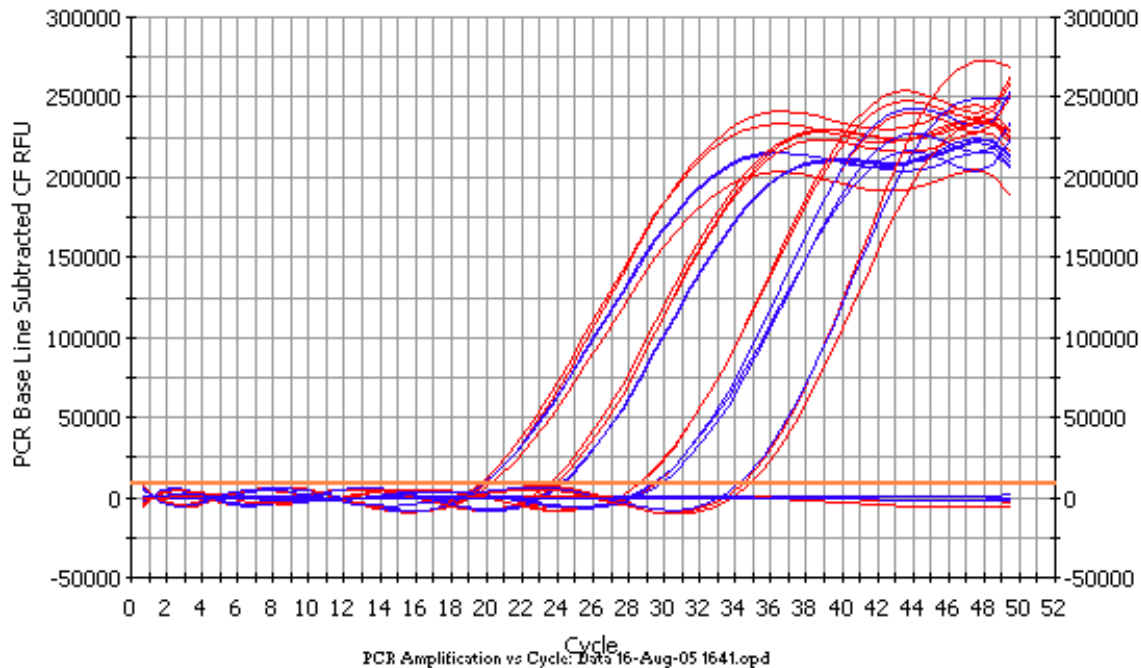
Resuehr and Spiess (2003) Analytical Biochemistry

Anchored oligo dT_(VN)

- Binds to mRNA/poly A tail junction
- Increased consistency of cDNA pool



Oligo dT vs. anchored oligo



Anchored oligo-dT
efficiency = 82%

Oligo-dT
efficiency = 76%

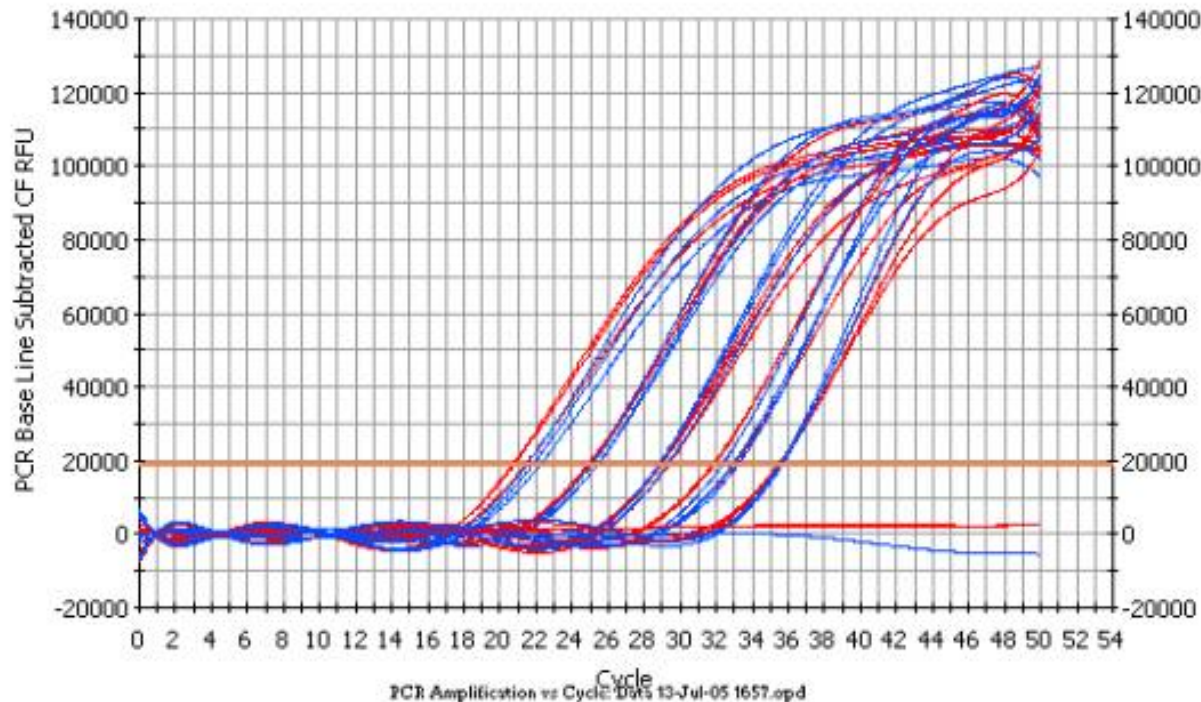
Primer combinations



- Mixture of random hexamers (RH) and anchored-oligo dT (AO)
- Versatility over a wider range of applications
- Improved sensitivity and efficiency with a 3:1 ratio of RH:AO

Comparison of cDNA priming strategies on efficiency and sensitivity

PCR primers	cDNA primer	Efficiency (%)	Correlation	Ct @ 100ng
GAPDH (SYBR green)	AO	83.9	0.992	23.6
	RH	82.5	0.995	20.6
	RH:AO (3:1)	88.7	0.997	20.9
	Anti-sense	95.6	0.995	21.8
B-actin (Taqman)	AO	60.6	0.977	24.0
	RH	68.4	0.966	22.1
	RH:AO (3:1)	74.5	0.995	22.1
	Anti-sense	72.4	0.987	21.7

Comparison of RH:AO (3:1) vs. GAPDH anti-sense



Blend (3:1): 
Anti-sense: 

Human total
RNA :
100ng-10pg

RNA priming strategies: Conclusions

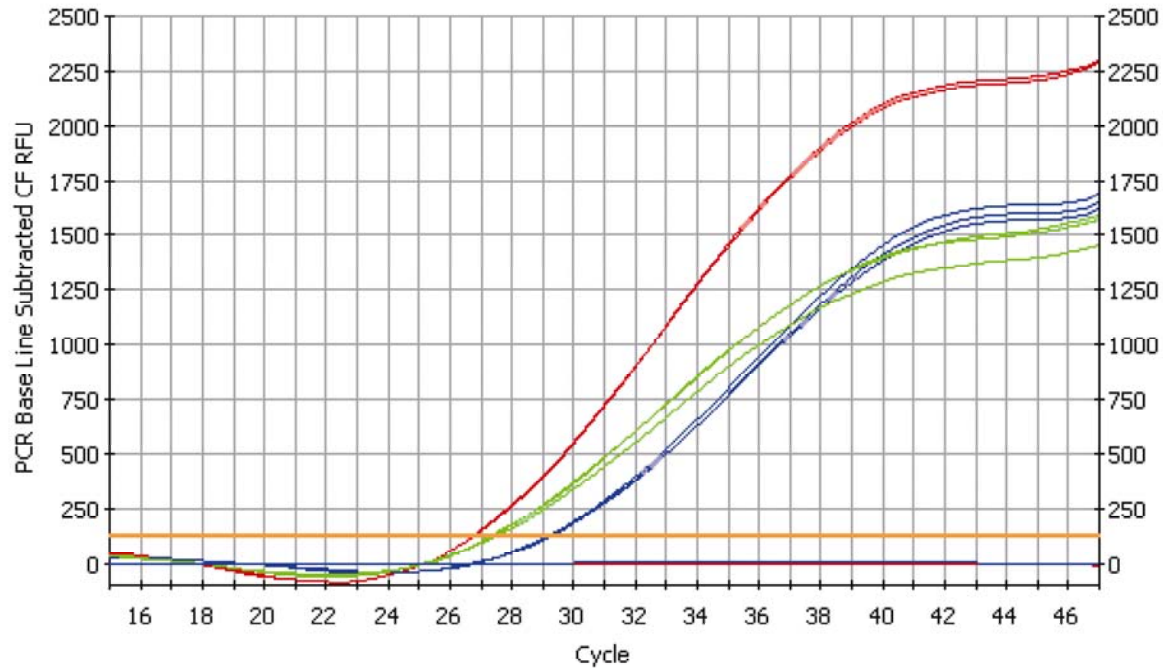
- Gene specific
 - Greatest sensitivity
 - Least versatility
- RH or AO
 - Distance from poly-A tail, rRNA, viral RNA
- Combination (RH:AO)
 - Good versatility
 - Good sensitivity

Blending RTases for improved efficiency

Reverse transcriptase

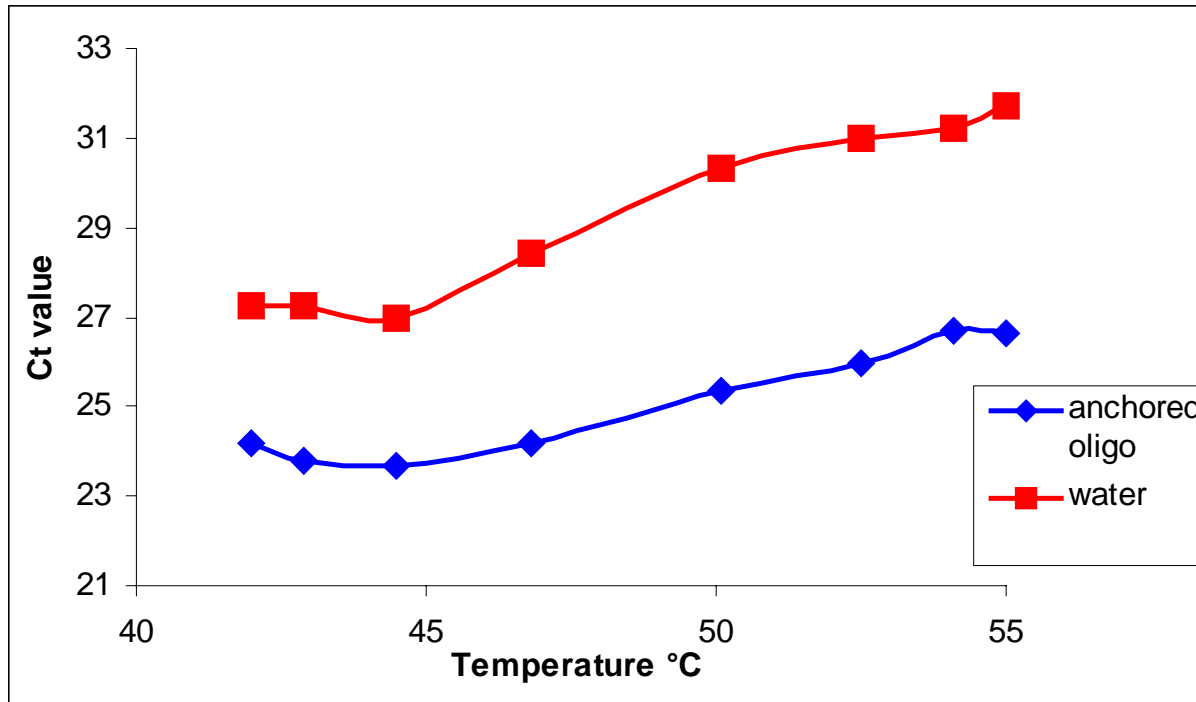
- RNA-dependent DNA polymerases
- Avian Myeloblastosis Virus (AMV)
 - Effective at elevated temperatures (57°C)
 - Recommend denature RNA 70°C before
 - Long PCR not done in real time (150bp ideal)
- Moloney Murine Leukeamia Virus (MMuLV)
 - Good dynamic range
 - Lower RNase H activity

ABsolute™ MAX QRTase blend



Amplification of β -actin using 1ng of total human RNA. Red lines represent the results obtained with ABsolute™ MAX QRTase blend, blue lines the MMuLV results, and green lines the AMV results

Effect of temperature on Ct in ABsolute™ MAX QRTase blend

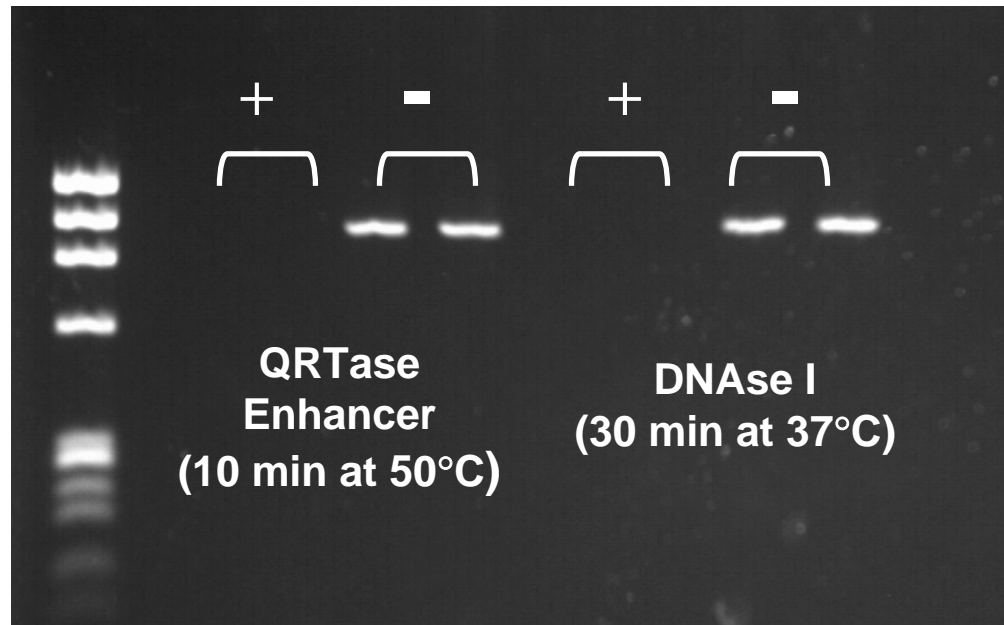


Removal of DNA contamination

DNA contamination

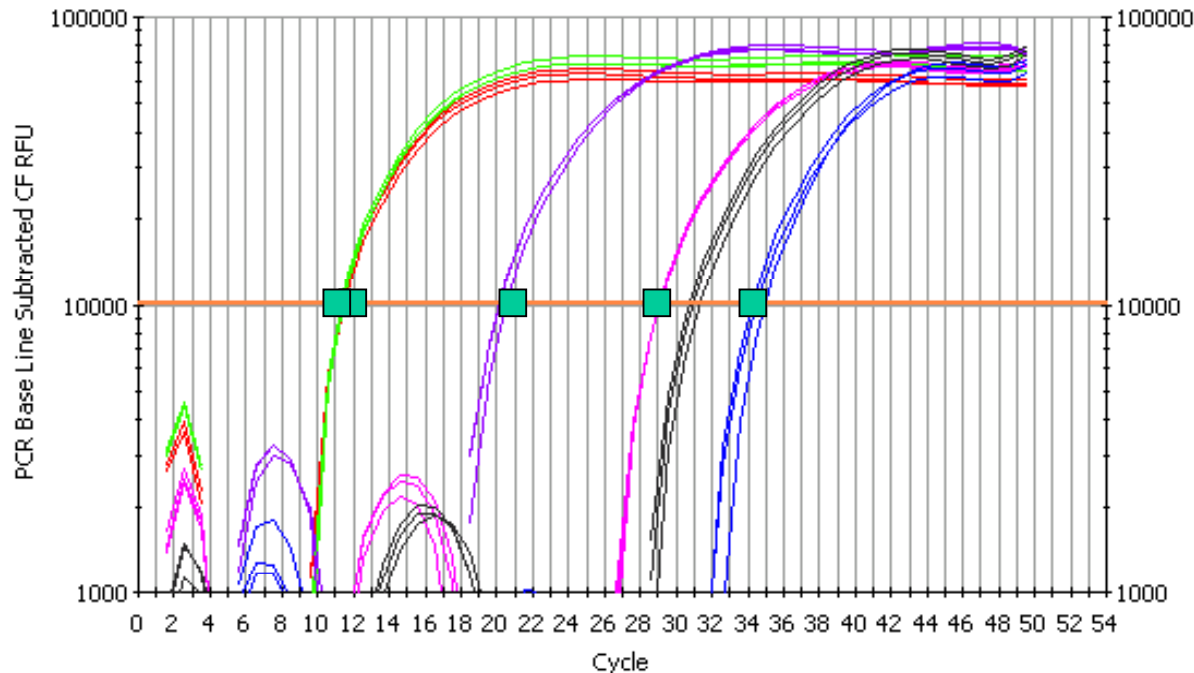
- Reduces PCR efficiency, reproducibility and sensitivity
- DNase I treatment
 - Time consuming pre-incubation
 - Harsh inactivation methods
 - Increased chance of RNase activity
- QRTase enhancer
 - Used for difficult or crudely purified samples
 - No additional incubation/inactivation needed

Removal of dsDNA by QRTase enhancer compares with DNase I



1 μ g of 1000 base pair DNA standard

Effect of contamination on consistency

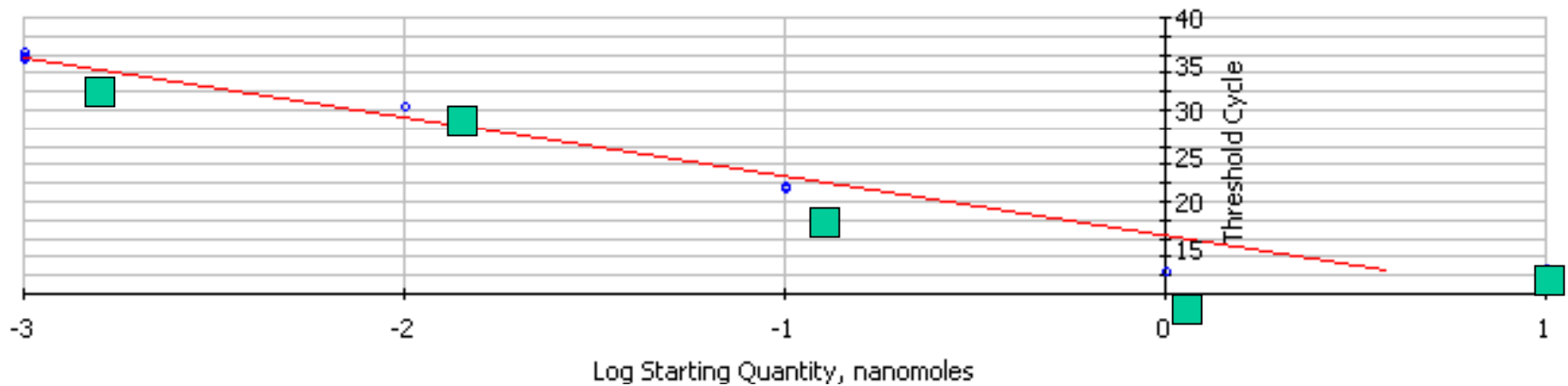


The template is contaminated by inhibitors and DNA that cause false positives and inaccurate quantification. Black - no RTase control

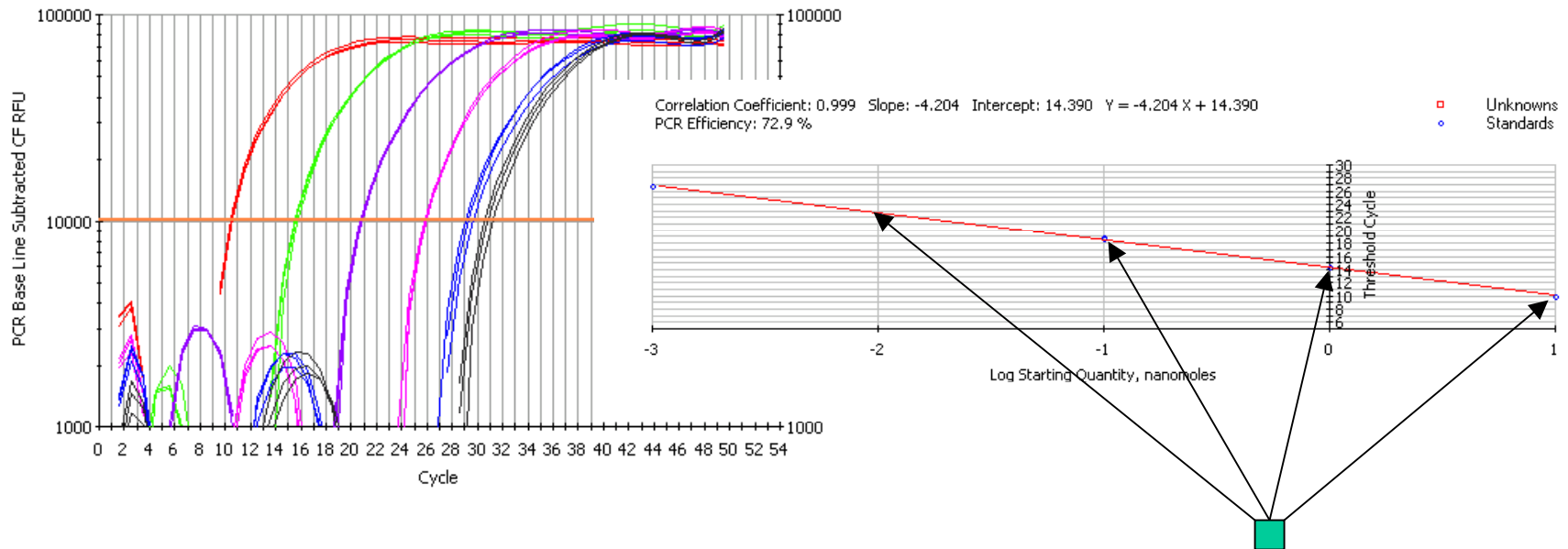
Standard curve

Correlation Coefficient: 0.971 Slope: -6.464 Intercept: 16.216 $Y = -6.464 X + 16.216$
PCR Efficiency: 42.8 %

□ Unknowns
○ Standards

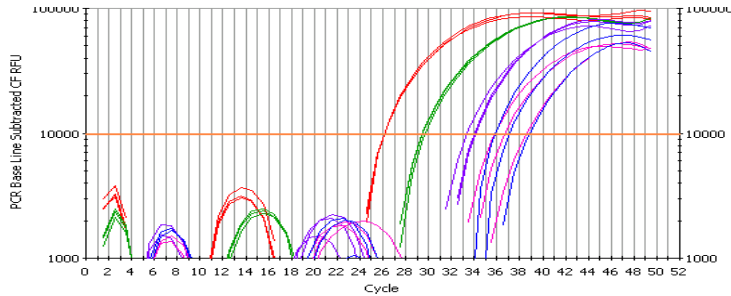


ABsolute™ QRTase enhancer

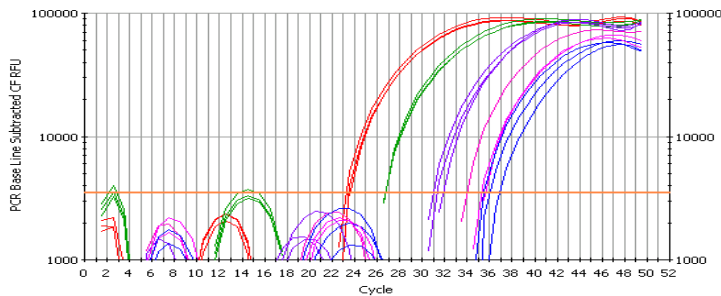


QRT-PCR using a crudely purified 'difficult' template and QRTase enhancer. The black lines show the 'no RTase' control. The coloured lines represent a serial dilution (10ng-1pg) of yeast RNA.

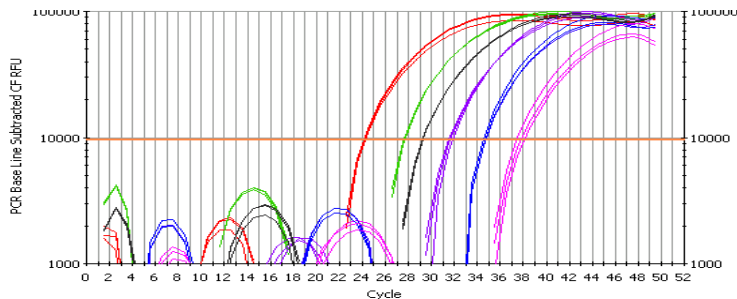
Compared against DNase I



Without pre-treatment,
efficiency 159.2%, CC 0.957
Ct values delayed.



With DNase I pre-treatment,
efficiency 92.7%, CC 0.957
Ct values improved, and sensitivity increased.



With QRT PCR enhancer
efficiency 93.3%, CC 0.998, Ct values improved, and
sensitivity and reproducibility increased even further.

Advantages of QRTase enhancer over DNase

- Thermolabile
 - Killed by Taq activation step
 - No need for prolonged heating
- Easy to use
- Can remove significant quantities of contaminating DNA
 - Not suitable for use with probes relying on hairpin formation only in a one-step reaction

Conclusions

- Efficiency and sensitivity of QPCR can be improved by optimising RT step:
 - Priming choice (experiment specific, 3:1 blend)
 - ABsolute™ MAX QRTase blend
 - ABsolute™ QRTase enhancer
- Potential benefits
 - Decrease of Ct (2-3)
 - Increase in efficiency (5-10%)

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