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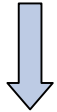
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Increase Consistency From Sample to Ct

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ABgene

Presentation Outline

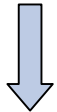
Sample



← **1 - DNA contamination**

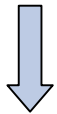
RNA

← **2 - RNA priming strategies**



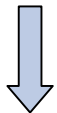
← **3 - Reverse transcription**

cDNA



← **4 - PCR plates**

Amplification



← **5 - Master mix composition**

Data



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1. Removal of DNA contamination

DNA Contamination

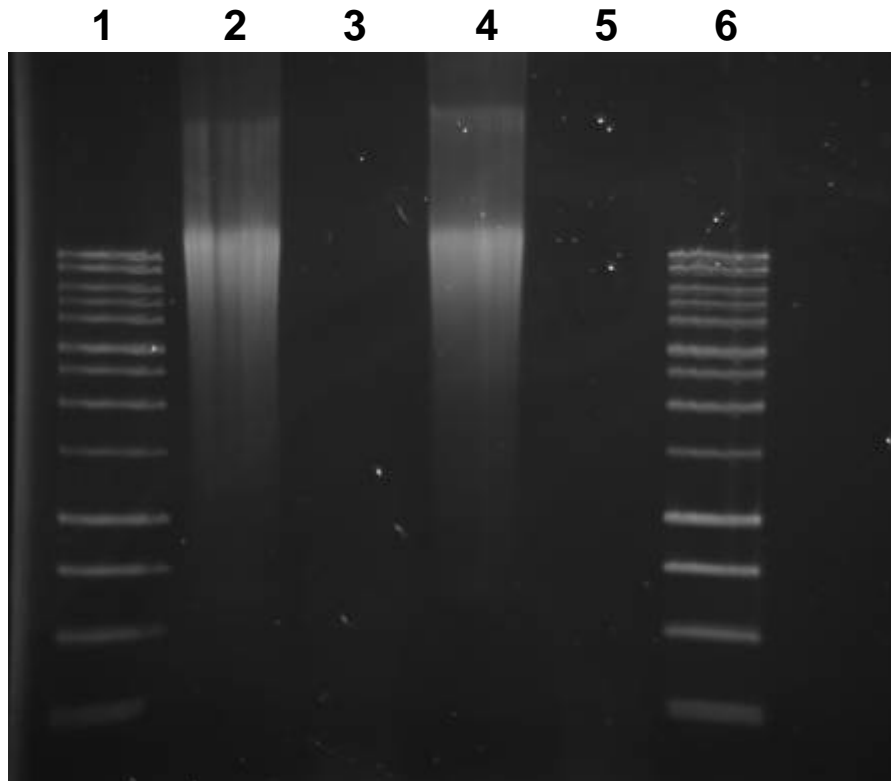
- PCR will not discriminate between cDNA and gDNA
 - DNA contamination will provide false positives
- Ways of combating contamination
 - Intron-spanning primers
 - Design problems
 - Processed pseudogenes
 - High quality RNA isolation
 - Cost
 - DNase I treatment
 - Risk of RNA degradation
 - Time and effort

RT Enhancer for specific removal of dsDNA

- Removal of contaminating DNA from sample
 - Active during the RT step
 - No degradation of RNA
- Easily inactivated at high temperatures
 - during the RT deactivation/hot start incubation step
- Eliminates need for DNase treatment
 - Same data generated as with DNase
 - Saves time, effort, money

The RT enhancer increases the sensitivity and accuracy of a QRT-PCR reaction, especially when using difficult or crudely purified samples.

RT Enhancer: dsDNA Degradation



Key (left to right)

- Lane 1: 1kb DNA size marker
- Lane 2: DNA only
- Lane 3: **DNA incubated with RT Enhancer**
- Lane 4: DNA only
- Lane 5: **DNA incubated with DNase I**
- Lane 6: 1kb DNA size marker

RT Enhancer is equal to the 'gold standard' DNase 1



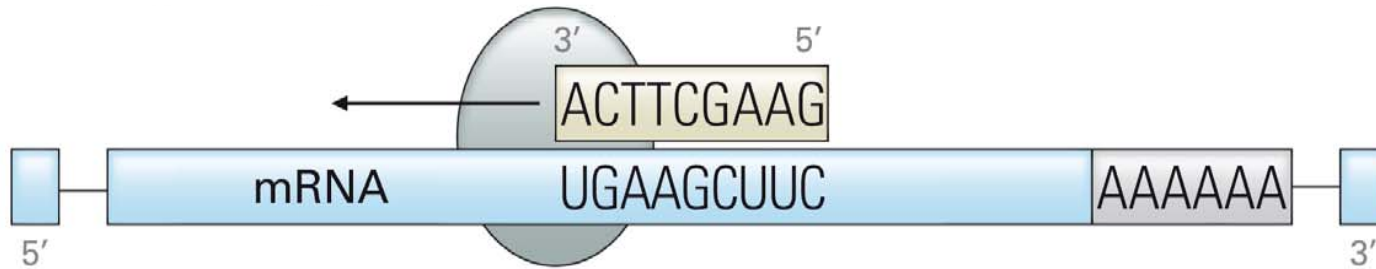
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2. RNA Priming Strategies

Common Priming Methods

- Gene-specific
- Random priming
- Oligo dT
- Combination priming

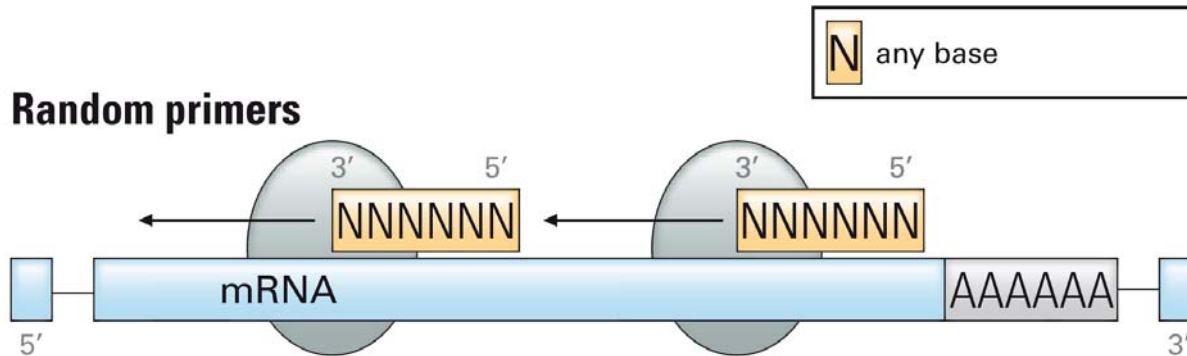
Gene-Specific Priming



Advantages	Disadvantages
<ul style="list-style-type: none">▪ Specific cDNA pool▪ Increased sensitivity▪ Use anti-sense QPCR primer	<ul style="list-style-type: none">▪ Amplification is limited to one gene of interest

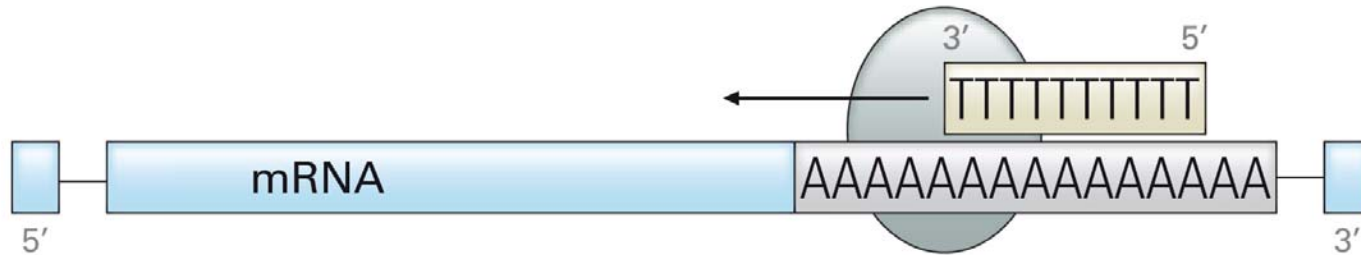
Random Priming

- Typically 6-10 nucleotides in length



Advantages	Disadvantages
<ul style="list-style-type: none">▪ Primes RNA sequences▪ Amplification of rRNA	<ul style="list-style-type: none">▪ Truncated cDNA▪ Transcribing all RNA could dilute mRNA signal

Oligo dT

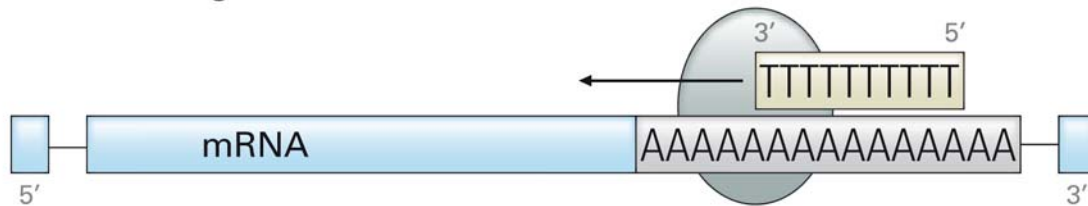


Advantages	Disadvantages
<ul style="list-style-type: none">▪ Priming of mRNA only resulting in a more representative cDNA pool	<ul style="list-style-type: none">▪ Only amplify genes with a poly (A) tail▪ Truncated cDNA from priming internal poly (A) sites▪ Bias towards 3' end

Anchored Oligo dT(VN)

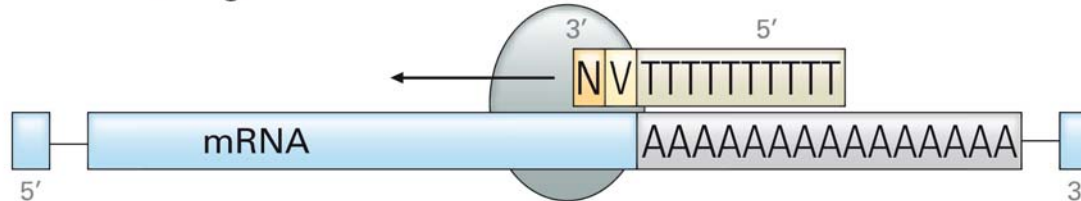
- Binds to mRNA/poly A tail junction
- Prevent priming from internal sites of the poly (A) tail and poly (A) rich regions

Standard oligo dT



V dGTP, dATP or dCTP

Anchored oligo dT



Primer Combinations

- Combines advantages of different primers
- Oligo dT with gene specific for 18S (Zhu L. 2005)
- Mixture of random hexamers (RH) and oligo dT
- 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

▪ Efficiency is the calculated efficiency of QPCR based on a 10-fold dilution standard curve ranging between 100ng and 10pg of Human total RNA

Priming Strategy Conclusions

- Gene specific
 - greatest sensitivity
 - least versatility
 - Researchers that know their GOI
- Random hexamers
 - Interest in non-mRNA RNA
- Anchored Oligo dT
 - Working with mRNA only
 - Distance from poly-A tail, rRNA, viral RNA
- Blend of primers
 - Good versatility
 - Increased specificity of PCR products
 - Increased cDNA yield

Utilising the combination of random hexamers and anchored oligo dT is the recommended strategy for general use.

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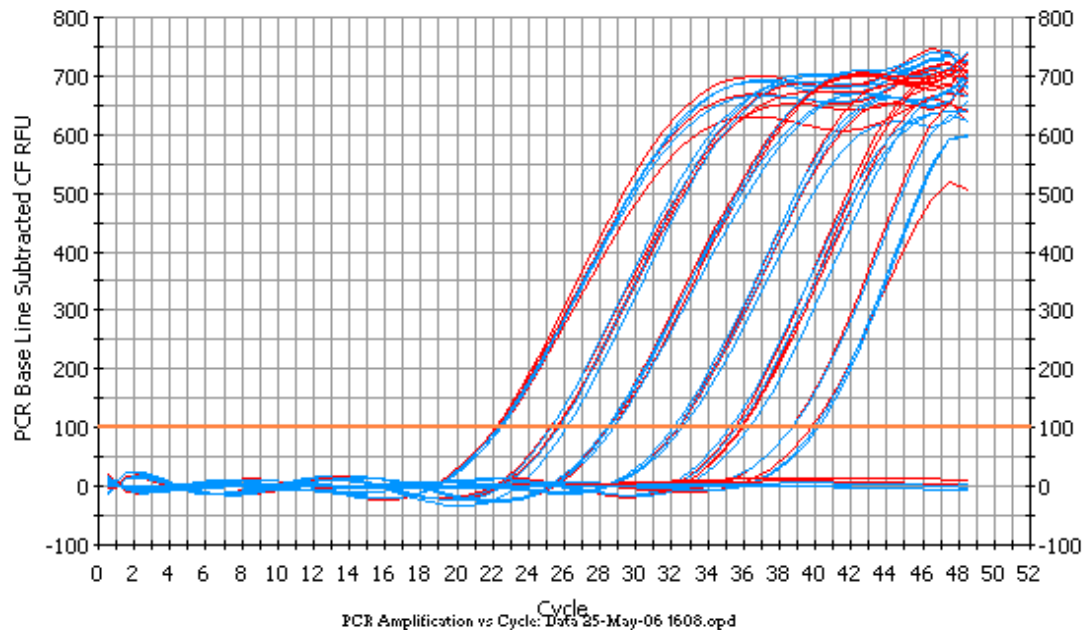
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3. Reverse Transcriptase

Requirements for Reverse Transcription

- Sensitivity
 - Ability to transcribe low copy number mRNA molecules
- Dynamic range
 - demonstrates equal efficiency at high and low mRNA levels
- Specificity
 - Reduction of non-specific cDNA
 - Works well in combination with Taq during 1-step QRT-PCR
- Length of cDNA transcript
 - Long mRNA are fully transcribed
 - Less important for QRT-PCR

Sensitivity and Dynamic range of Verso™

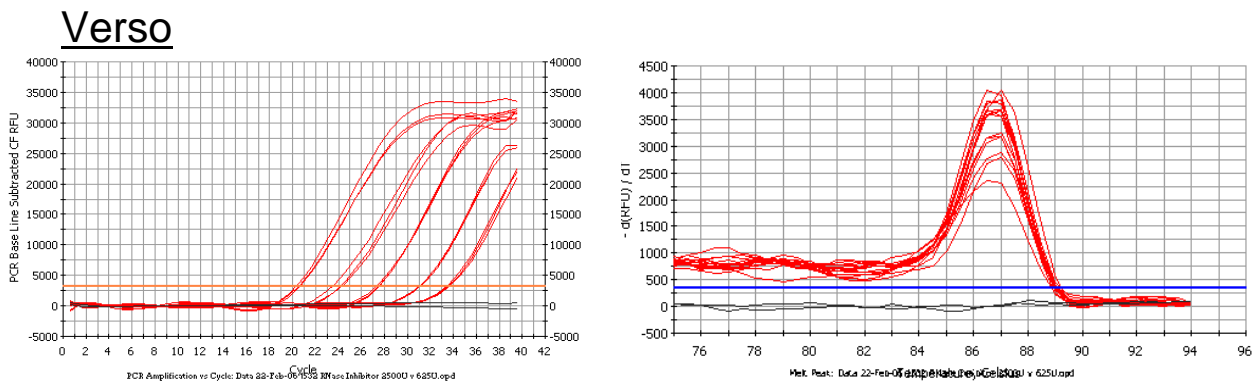
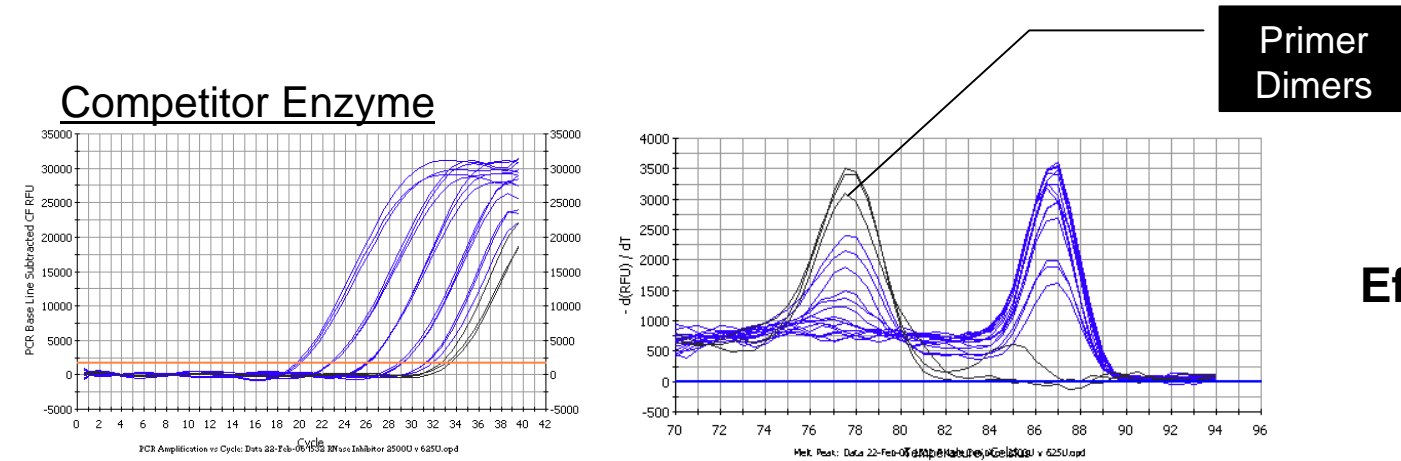


■
ABsolute Verso
(correlation 0.996, efficiency 99.1%)

■
Leading competitor enzyme
(correlation 0.997, efficiency 95.5%)

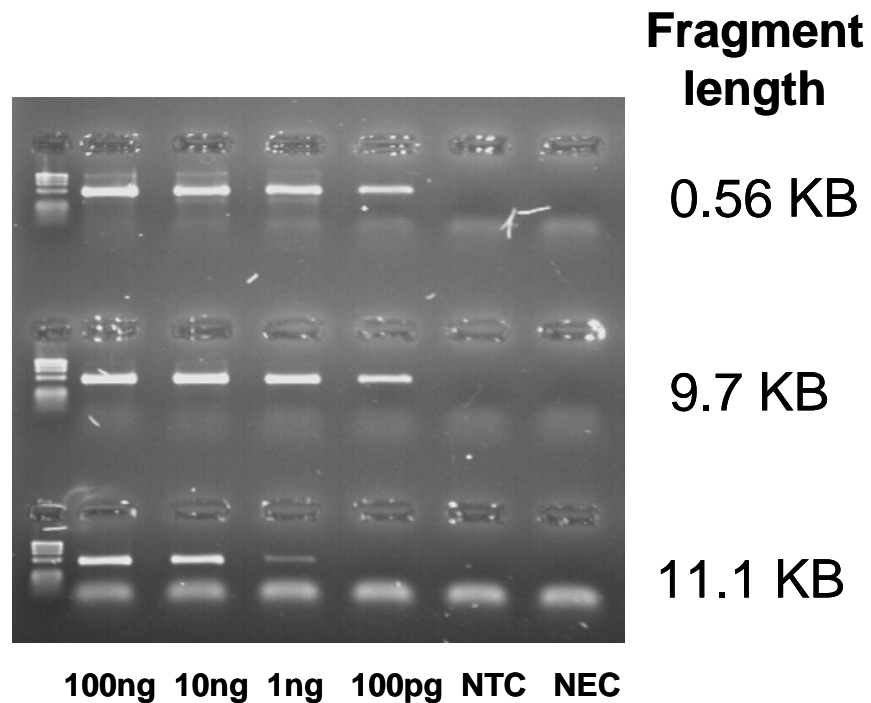
cDNA was reverse transcribed from 100ng-1pg of human RNA, using either new ABgene enzyme (red) or competitor enzyme (blue). A sample of this cDNA from both enzymes was amplified using AB-1132, thus directly comparing the reverse transcriptase's.

Specificity of Verso™: Elimination of primer dimers (1-step)



Amplification of GAPDH gene with SYBR Green master mix, using a difficult primer set. cDNA was reverse transcribed from 100ng-10pg of human RNA, using either Reverse-iT (blue) or new ABgene enzyme (red). After QPCR amplification the Reverse-iT enzyme produced primer dimers in the low RNA dilutions and the NTC, which increased the efficiency. The new ABgene enzyme did not produce primer dimers and therefore demonstrated a better PCR efficiency.

Long Transcription Length of Verso™



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4. Plastic Consumables

Plastic Consumables

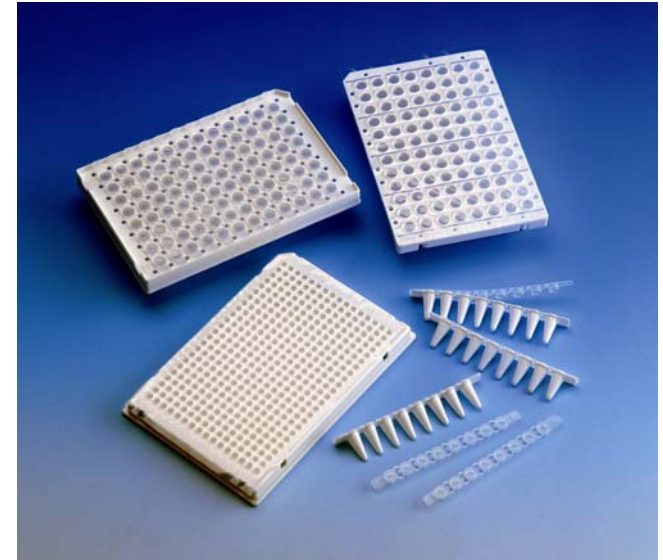
- Contamination “dust”
 - Human and Bacterial DNA contamination
 - Inhibitors of PCR
 - Cleanroom production

- Sealing
 - Avoid evaporation
 - Reduction in sensitivity and reproducibility of reaction
 - Provide a window into your reaction
 - Low transmission
 - High optical clarity

Plastic Consumables

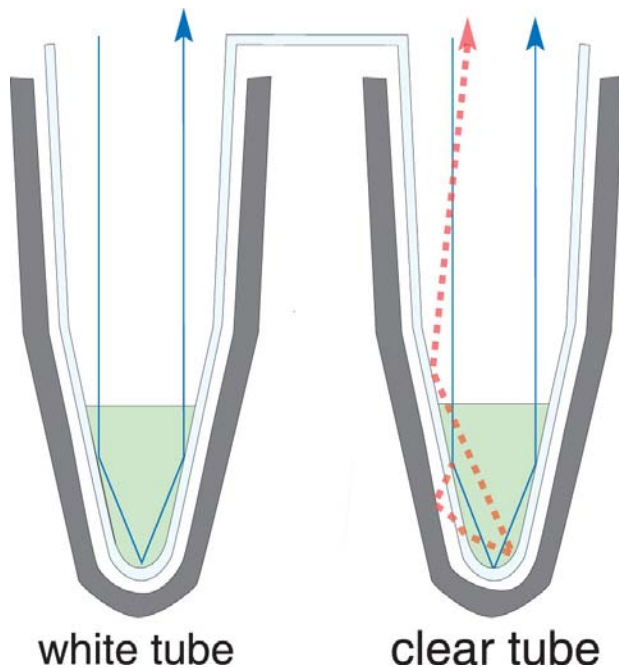
Comparison of plate types for QPCR:

- Natural
- Opaque (white)



Natural vs. Opaque Plates

Clear material allows light to be transmitted through to the block where it is either absorbed (black block) or reflected (silver block)

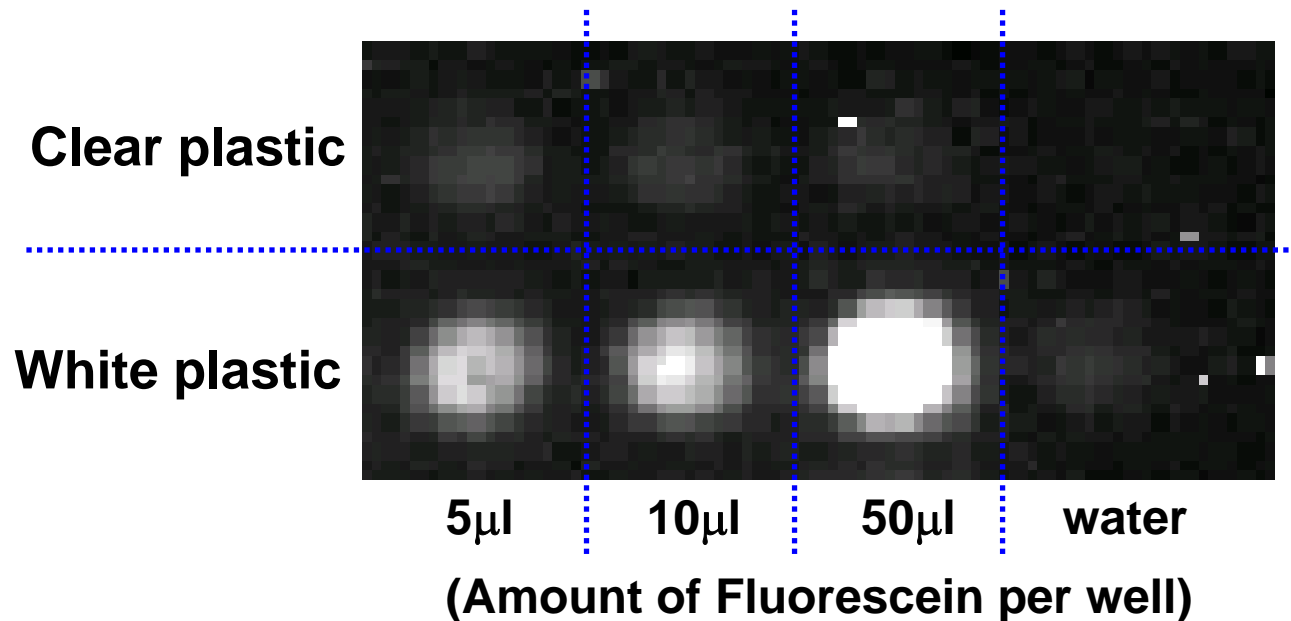


- Well-to-well cross talk can occur
- Light also reflects off outside of the tube

Results in refraction and high variance

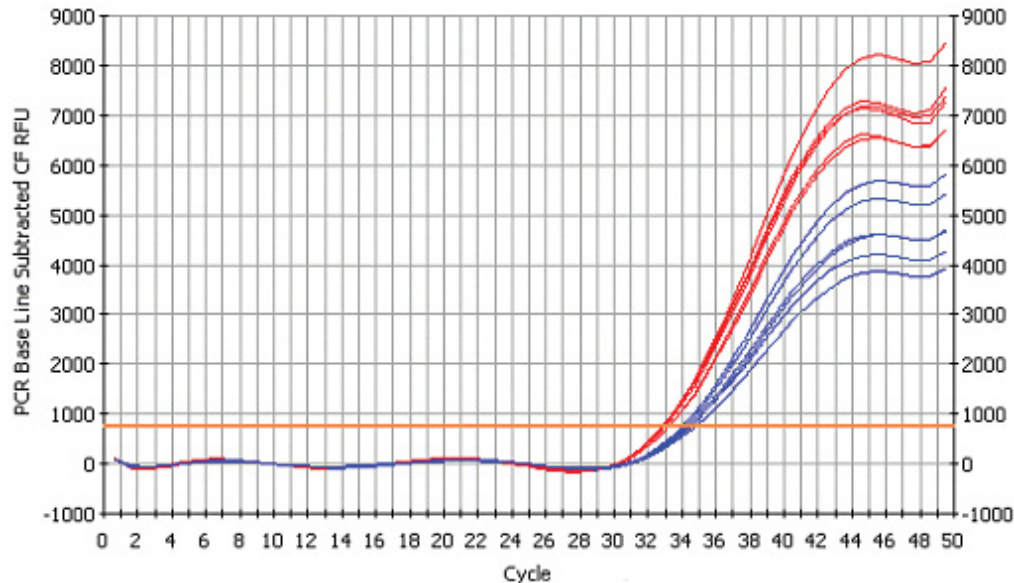
White Plates - Greater Sensitivity

- Greater reflection of light back to detector



White Plates

- More consistent and even reflection (no refraction)
- Higher signal-to-background ratio
- Eliminates possibility of well-to-well cross-talk



GAPDH amplification using 2ng Human genomic DNA SYBR® Green Mix in Natural plates (Blue) and in white plates (Red)

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5. ABsolute™ Blue QPCR Master Mixes

Pipetting Errors

- Sources of errors
 - Clear liquids going into clear/white plastics
 - Missed wells
 - Doubled up wells
- Problems!
 - Lack of confidence in data
 - Repeat assays (increased cost and time)
 - Incorrect results may go unknown

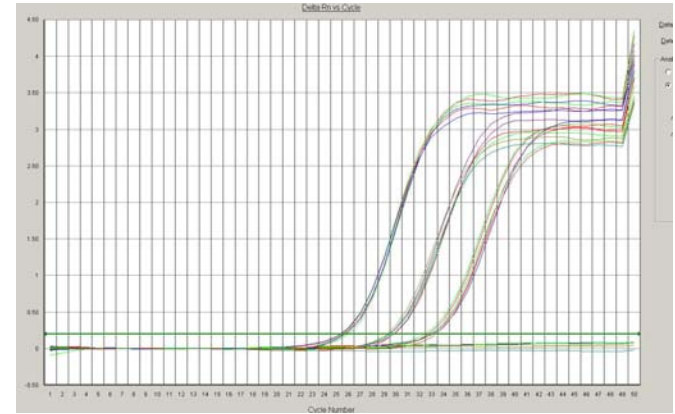
- 96-well, 384-well, 1536-well.....

Visualization of Master Mix

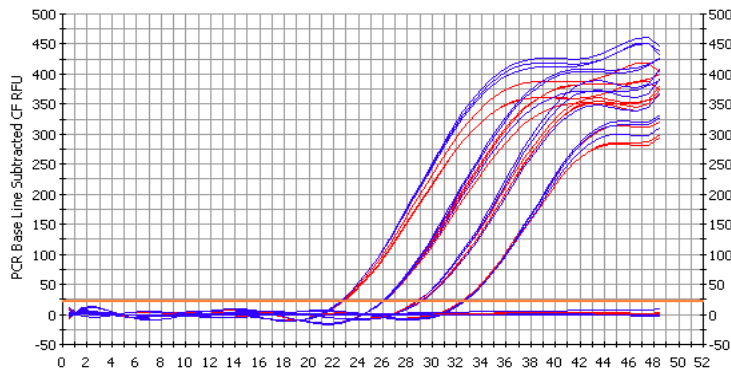
- Inert blue dye
- Easily view mix in all plates and tubes
- Minimize aliquoting errors
- Maintain ABsolute™ sensitivity, reproducibility & ease of use
- compatible with all QPCR platforms



NO QPCR inhibition



Amplification of GAODH from 10ng-100pg of human genomic DNA using Absolute™ QPCR SYBR® Green ROX Mix with and without coloured dye.



Amplification of beta-actin from 100ng-100pg of human genomic DNA using Absolute™ QPCR Mix with (red) and without (blue) coloured dye.

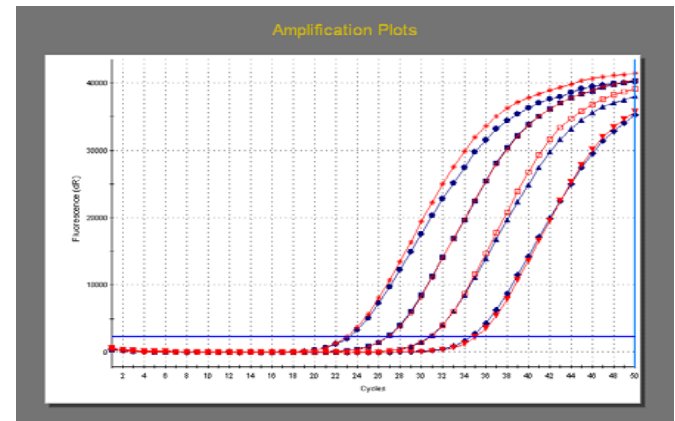
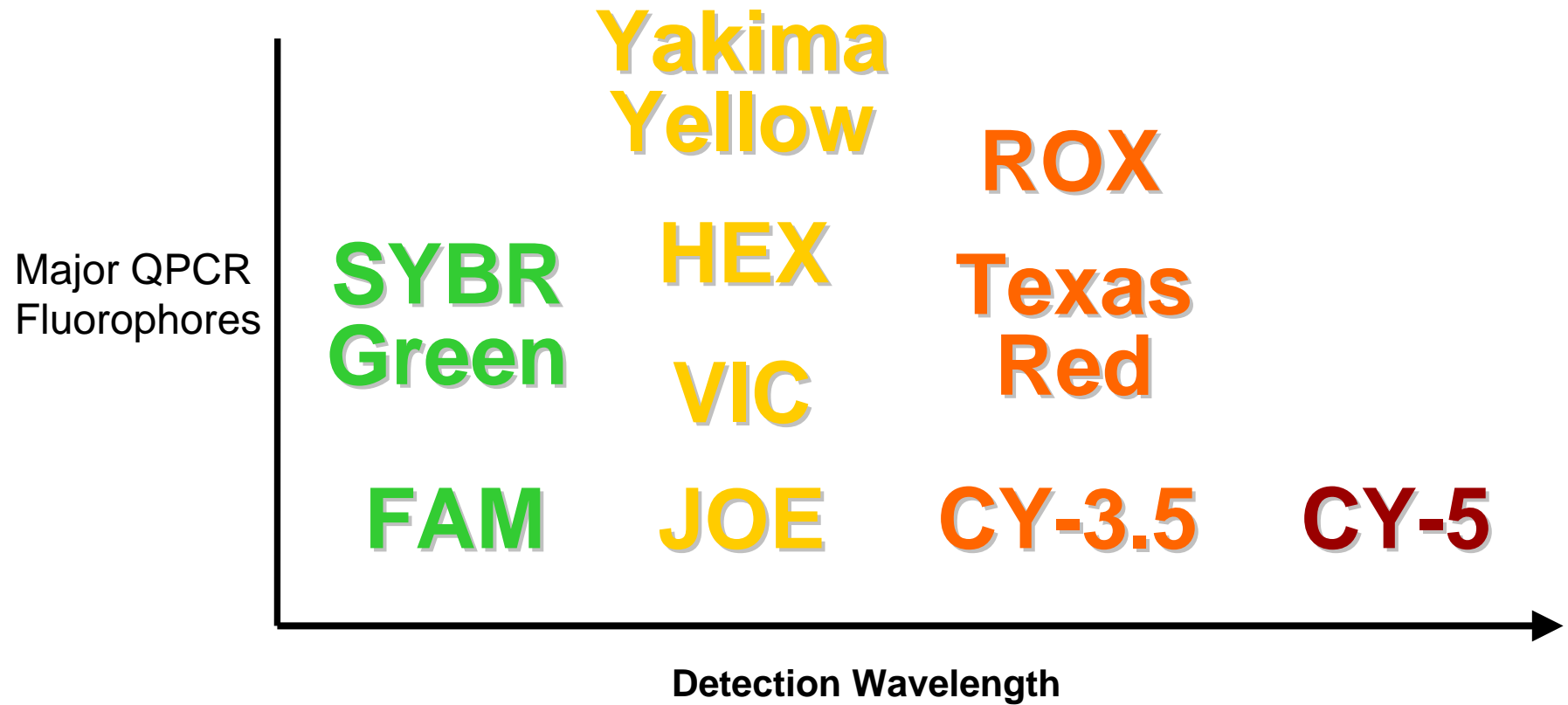


Figure shows a Stratagene Mx3005P™ amplification/cycle graph of a 295bp target of the β -Actin gene via TaqMan® probe chemistry labelled with FAM using human genomic DNA (200ng-200pg) in triplicate reactions comparing Absolute™ Blue QPCR Master Mix (Blue) and Absolute™ QPCR Master Mix (Red).

Dyes validated with ABsolute™ Blue



Conclusions

- DNA contamination removal
 - RT enhancer
- RNA priming
 - Blend of RH and AO provides versatility and sensitivity
- Reverse transcriptase
 - Sensitivity and dynamic range for 1-step and 2-step reactions
- Plastic consumables
 - Contamination free plastic
 - Good seal to avoid evaporation
 - White plastics for maximum sensitivity
- ABsolute Blue™ Master Mixes
 - Blue dye for a clear advantage!