PCR Amplification efficiency,relative quantification, and the analysis of alterations in cellular gene expression patterns.

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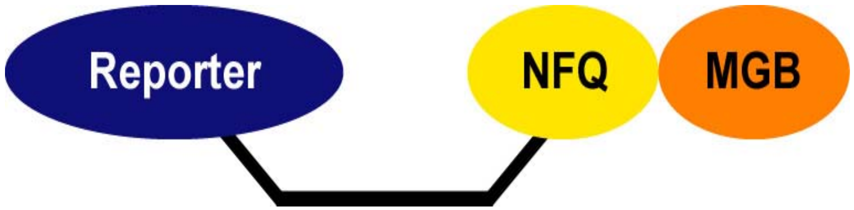
Regulation of Gene Expression

Occurs at multiple levels. Changes in transcript levels is a key mechanism for regulation.

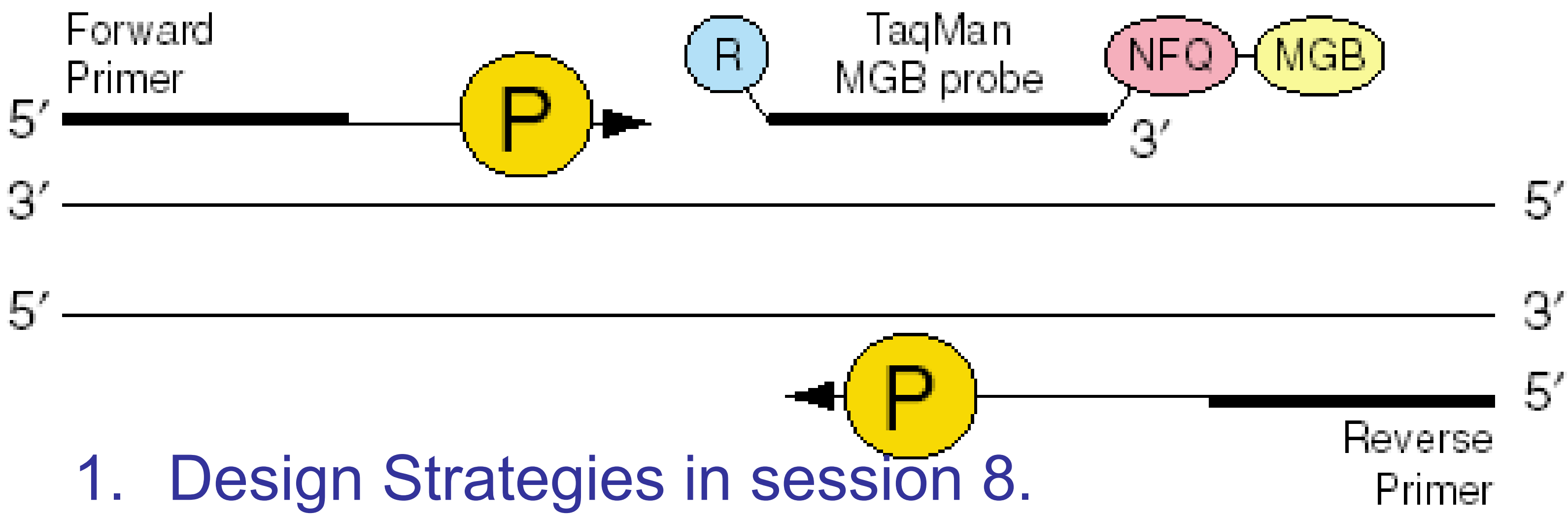
- Study of changes in transcript levels by relative quantification is useful if it can be done in HT mode without the need for internal controls and / or standard curves.
- Assays with near 100% amplification efficiency.
- Endogenous controls that do not change during the experiment.
- Large number of genes involved in cellular regulatory networks / pathways.
- Specificity: distinguish between transcripts from paralogous genes.

Gene Expression:5’ Nuclease Chemistry

- High sensitivity
- Large dynamic range: 10^6
- Closed tube chemistry - no post-PCR processing
- High efficiency (near 100%) associated with good designs. Useful for HT relative quantification.
- High degree of specificity from two primers and a probe.
- Contamination control is possible
- MGB enables shorter probes for greater discrimination:
  - increased fidelity to the intended target
  - increased flexibility of design

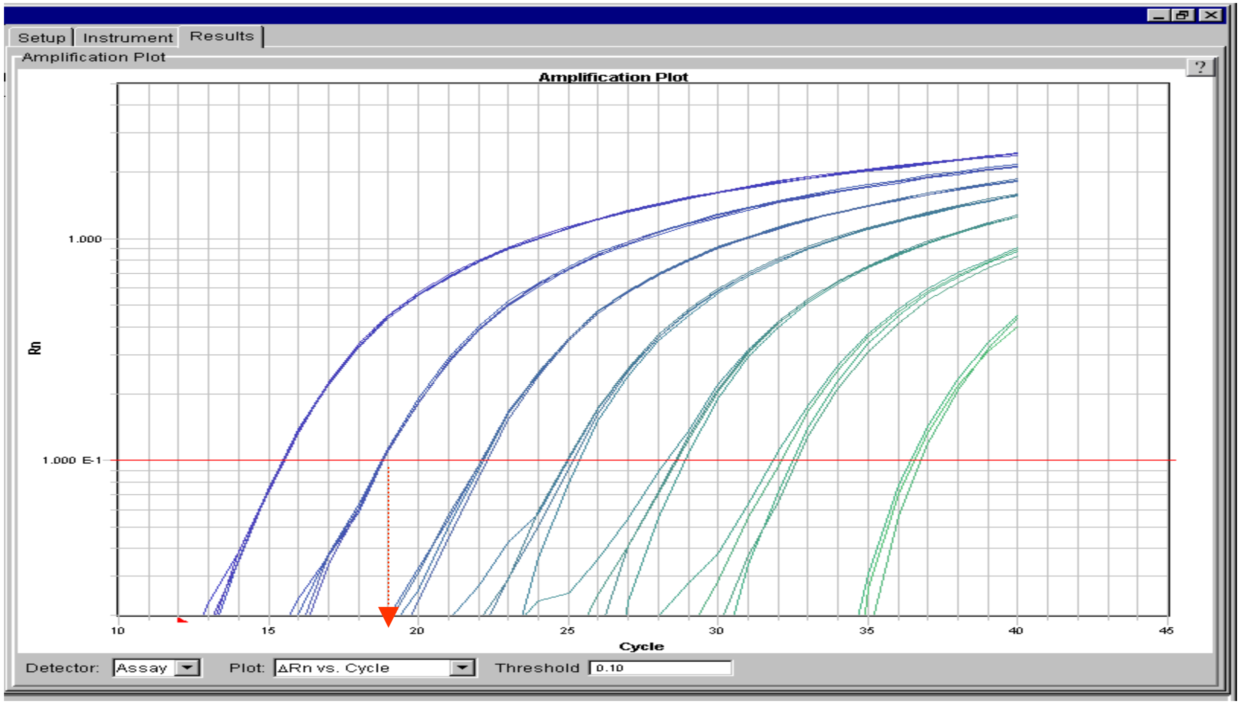


5’ Nuclease Assay



1. Design Strategies in session 8.
2. Expression Testing.
3. Standards for Quantification
4. Amplification efficiency.
5. Relative Quantification (RQ).
6. Requirements for assay specificity / selectivity.
7. Application to IFN regulation.
8. Gene Silencing:RNAi & DNA methylation

Dynamic Range and Sensitivity



C_t

Sensitivity 50 –100 copies

Dynamic Range: 6-logs

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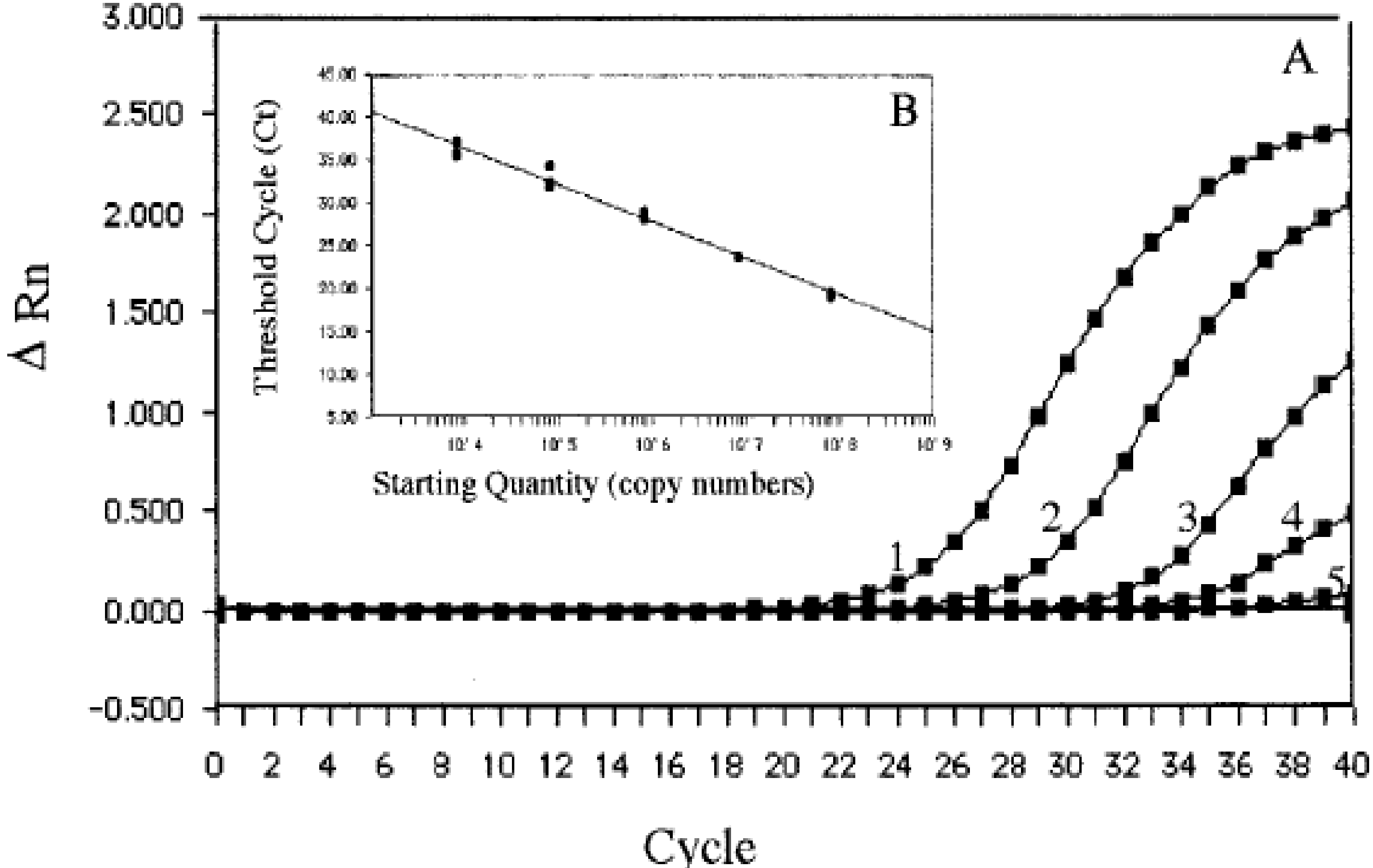
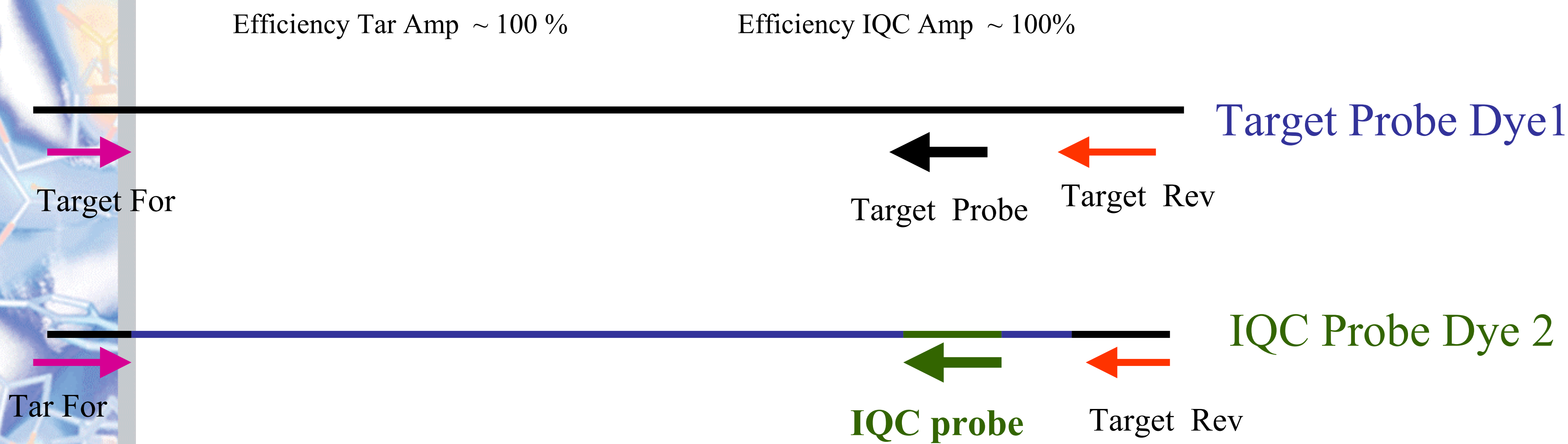
Absolute Quantification:Use of Standard Curves



Wang and Morris Anal. Biochem. 269:198-201 (1999)

Difficult to generate standard curves for large gene sets.

Co-amplification of a Competing IQC Target Identical in Size to the Target



Introduction of 200- 500 copies/ml of IQC does not inhibit target amplification in the 50 to 5 x 10^6 copies/mL range. Needs extensive optimization

Co amplifying targets the same size as the Target amplicon.

1. Furtado M R;Murphy R; Wolinsky S M. Quantification HIV-1 tat mRNA as a marker for assessing the efficacy of antiretroviral therapy. Journal of Infectious Diseases. 167(1): 213-6, **1992.**

2. Mulder J, McKinney N, Christopherson C, Sninsky J, Greenfield L, Kwok S. Rapid and simple PCR assay for quantitation of human immunodeficiency virus type 1 RNA in plasma: application to acute retroviral infection. *J Clin Microbiol*. 1994;32:292-300. BASIS FOR THE ROCHE AMPLICOR ASSAY

Co amplifying targets smaller in size compared to the Target amplicon as IQCs

3. Furtado, MR, DS Callaway, JP Phair, KJ Kunstman, JL Stanton, CA Macken, AS Perelson and SM Wolinsky. Persistence of HIV-1 Transcription in Peripheral Blood Mononuclear Cells of Patients Receiving Potent Antiretroviral Therapy [see editorial review p1672]. New England Journal of Medicine. 340(21): 1614-1622, **1999.**

4. Wang AM, Doyle MV, Mark DF. Quantitation of mRNA by the polymerase chain reaction. *Proc Natl Acad Sci U S A*. 1989;86:9717-21 . CYTOKINES

Need for Quantification Standards

Non synthetic standards

Bacteria or virus grown in culture and titered
Cellular RNA (reference RNA pools)
DNA from well characterized cell lines.
(Quantified by culture, EM , biochemical methods).

Synthetic standards

Synthetic RNA and DNA templates.
DNA cloned in plasmids.
RNA generated by *in vitro* transcription.
Recombinant retrovirus with target sequence.
Recombinant phage with target sequence.

The phage and viral based standards can be spiked into the sample at the sample preparation stage and carried through the entire process.

NIST web site

Quantification Relative to an Endogenous Control

Cellular transcripts whose levels do not change during the course of the experiment can serve as endogenous controls / reference.

18S RNA	ADA	β-actin
GAPDH	Rb	
EF-1α	UbcH5B	MLN51

Warrington et al., *Physiol. Genomics*, **2**:143-147 (2000)
Hamalainen et al., *Anal Biochem*. **299**:63-70 (2001)

Is there an endogenous control ?

User defined from experience with the system.

Needs validation.

Multiple controls.

Spike in a constant amount of an exogenous standard RNA to serve as an QS into all samples.

Normalize data across all samples to identify an endogenous control that changes minimally across the study.

Relative Quantification

1. Using efficiency values in the PCR equation

$$\frac{(1+Etar)^{\{Ct\ tarcal\ -\ Ct\ tarsam\}}}{(1+\ Eref)^{\{Ct\ refcal\ -\ Ct\ refsam\}}} = \text{Fold Change Ratio}$$

Liu and Saint, Anal. Biochem.**302**:52-59; 2002; Pfaffl et al., NAR 29:2002-2007

Efficiency values derived for target gene and reference can be used with the Ct values obtained to calculate fold change.

Accurate determination of assay efficiency is difficult. Requires the use of a broad dynamic range (5 to 6-logs) of target dilution and multiple determinations.

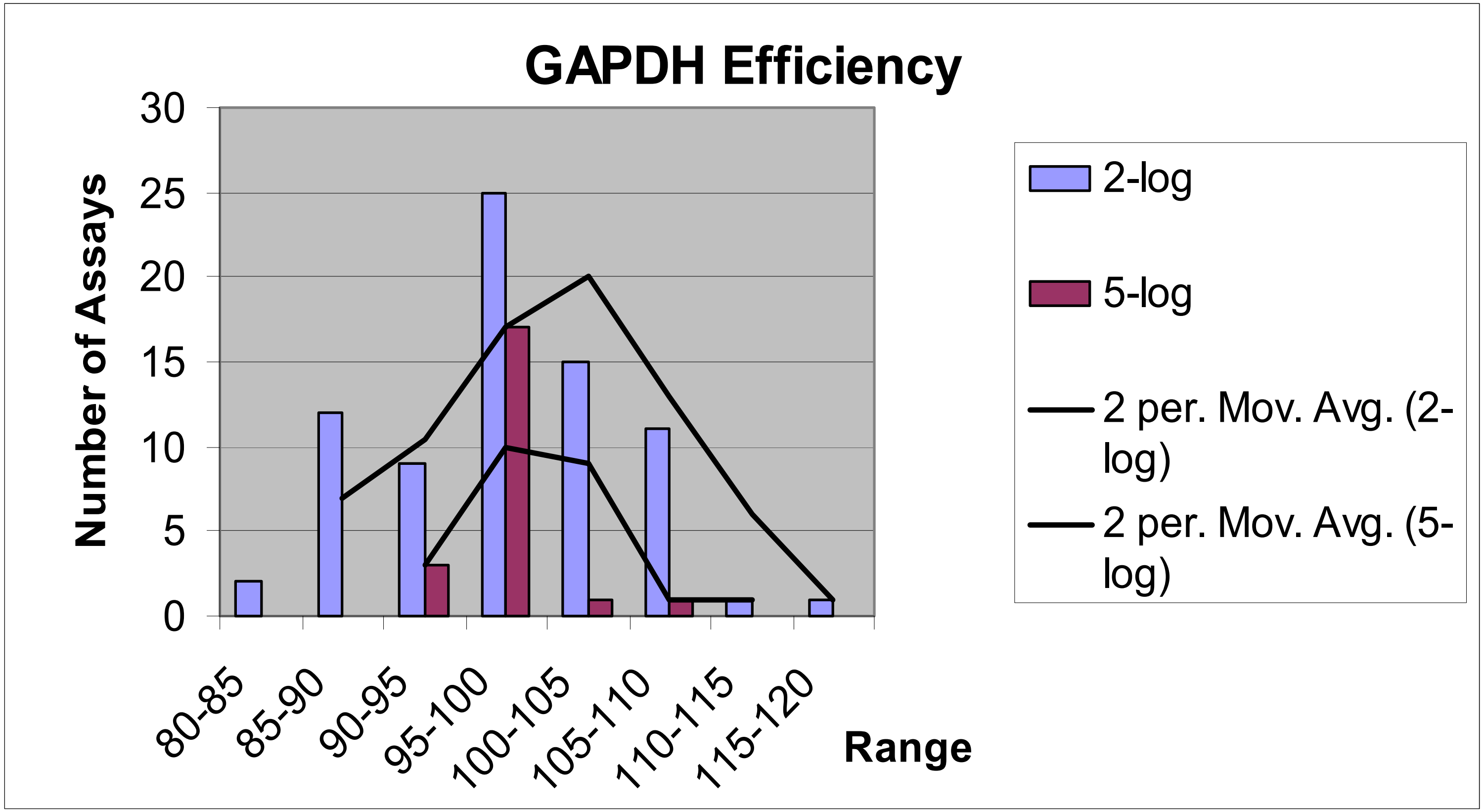
2. Using the ΔΔCt method Livak & Schmittgen; 2001 Methods 25:402 -8

If E ref = E tar =1 the above equation will change to $2^{-\Delta\Delta Ct}$

(Livak 1997; ABI Prism 7700 SDS System User Bulletin # 2)

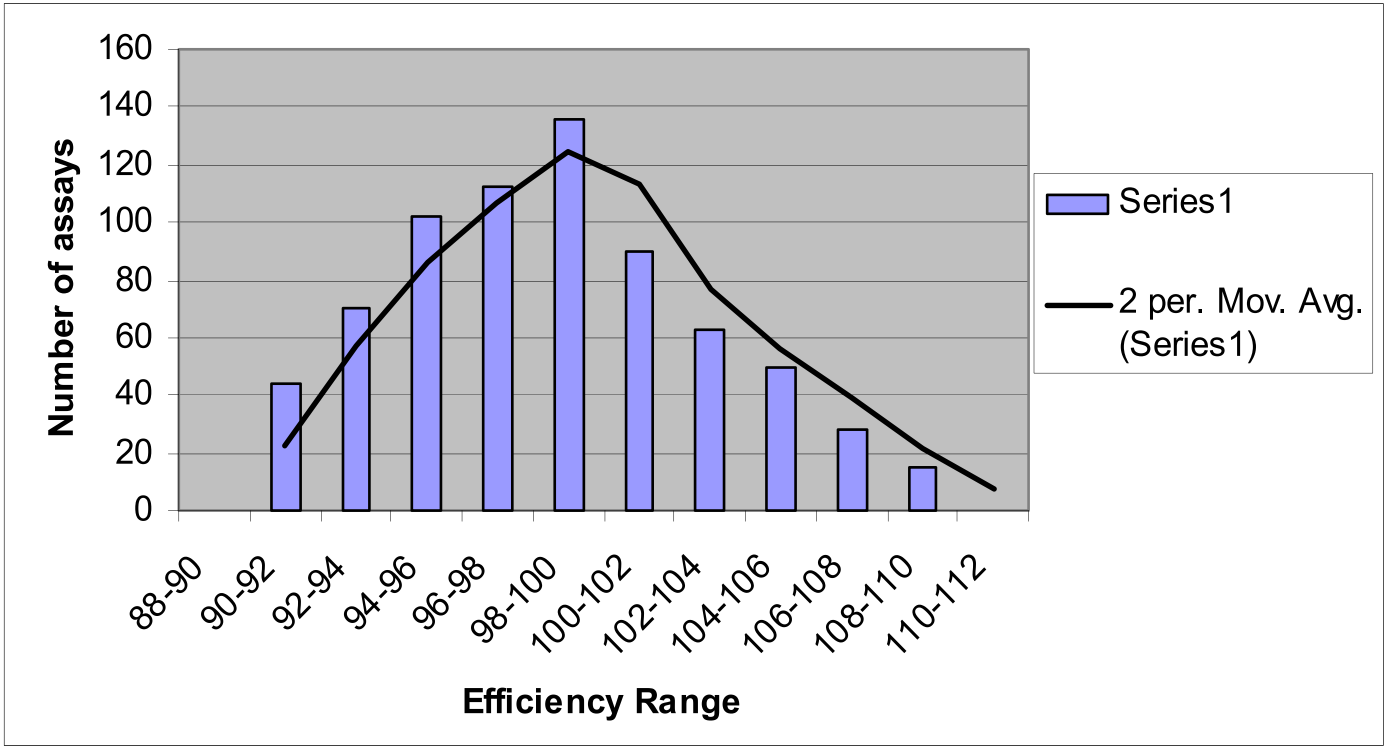
Most useful for higher throughput. No need for efficiency values.

Variability in Measuring Efficiency Values:
Dependence on dilution range.



Range 2- log	82-112	97.6 Avg	N= 76
Range 5- log	92-105	98.4 Avg	N= 22

Efficiency Determination > 1000 assays



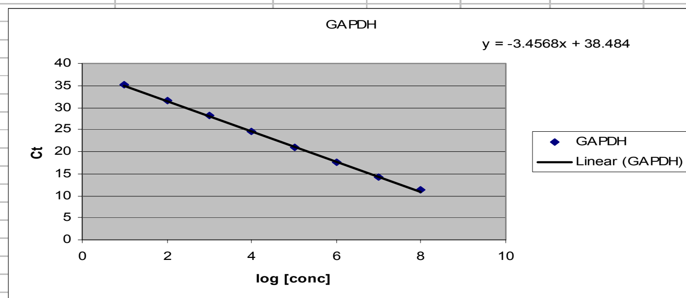
Amp Size	60-200 bp
GC content	25% to 79%
Free energy	zero to –17 kcal/mole

Designed by TaqPipe; run with UMM / universal conditions

Process validation to ensure that 99.75% of the assays would have near 100% efficiency

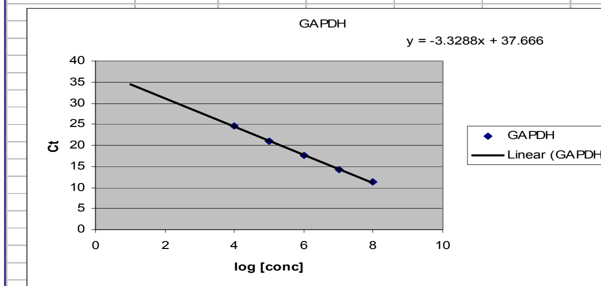
EFF.= 94.7%

GAPDH						
DILUTION	GENE NAME	Ct	Ctdev			EFF %
0.0001	GAPDH	11.235	8		-3.4568	94.66366
0.00001	GAPDH	14.22611	7			
0.000001	GAPDH	17.51864	6			
0.0000001	GAPDH	20.98957	5			
0.00000001	GAPDH	24.4975	4			
0.000000001	GAPDH	28.09658	3			
1E-10	GAPDH	31.57578	2			
1E-11	GAPDH	35.28958	1			



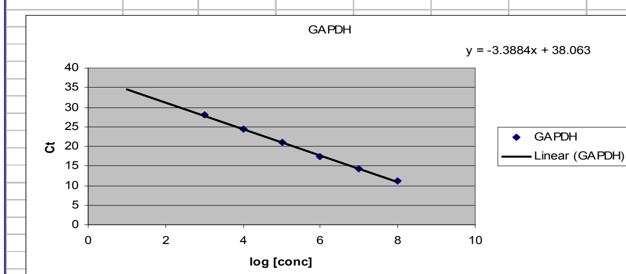
EFF.= 99.7%

GAPDH						
DILUTION	GENE NAM	Ct	Ctdev			EFF %
0.0001	GAPDH	11.234996	8		-3.3288	99.714021
0.00001	GAPDH	14.226113	7			
0.000001	GAPDH	17.518643	6			
0.0000001	GAPDH	20.989574	5			
0.00000001	GAPDH	24.497505	4			
0.000000001	GAPDH		3	28.096584		
1E-10	GAPDH		2	31.575781		
1E-11	GAPDH		1	35.28958		



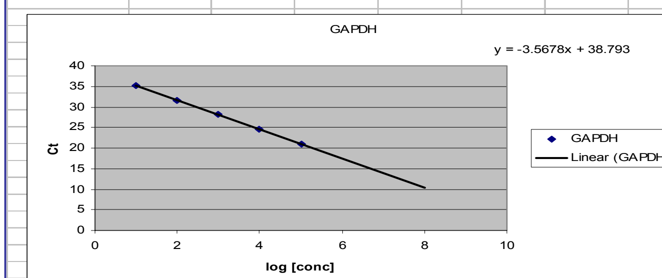
EFF.= 97.3%

GAPDH						
DILUTION	GENE NAM	Ct	Ctdev			EFF %
0.0001	GAPDH	11.235	8		-3.3884	97.29884
0.00001	GAPDH	14.22611	7			
0.000001	GAPDH	17.51864	6			
0.0000001	GAPDH	20.98957	5			
0.00000001	GAPDH	24.4975	4			
0.000000001	GAPDH	28.09658	3			
1E-10	GAPDH		2	31.57578		
1E-11	GAPDH		1	35.28958		

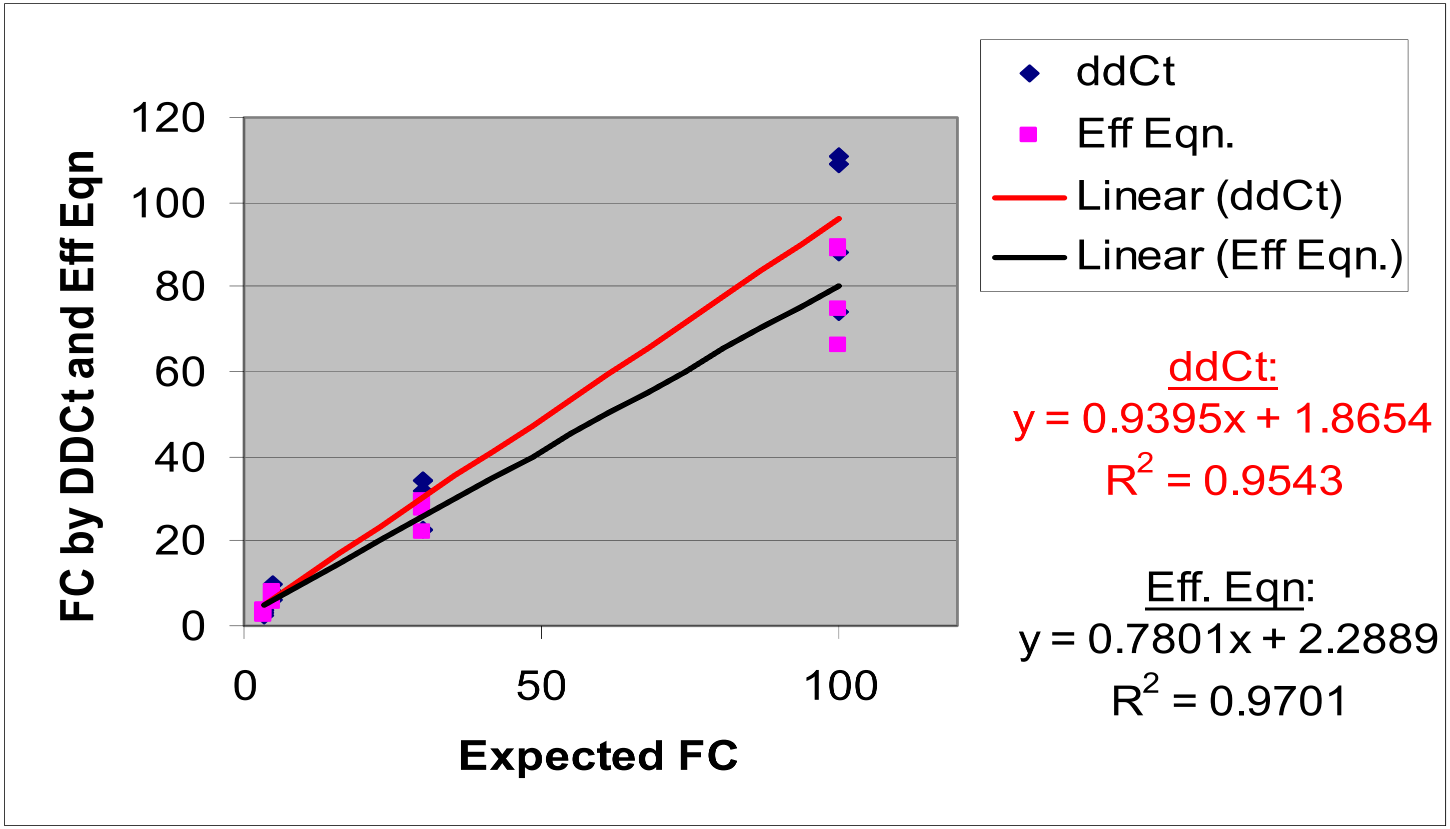


EFF.= 90.6%

GAPDH						
DILUTION	GENE NAM	Ct	Ctdev			EFF %
0.0001	GAPDH		8	11.235	-3.5678	90.67105
0.00001	GAPDH		7	14.22611		
0.000001	GAPDH		6	17.51864		
0.0000001	GAPDH	20.98957	5			
0.00000001	GAPDH	24.4975	4			
0.000000001	GAPDH	28.09658	3			
1E-10	GAPDH	31.57578	2			
1E-11	GAPDH	35.28958	1			



Relative Quantification



Dilution range of human cellular RNA 100 /50/30/10/1ng
Spike in a constant amount of an external transcript into all tubes in pool dilution series. Non-human transcript. Determine FC & compare with expected FC.

RQ Experimental Design; Reproducibility

1.	Number of Assays Selected			15
•	Low Expressors	26-30 Ct	10,000 to 2000	5
•	Moderate Expressors	22-26 Ct	100,000 to 10,000	5
•	High Expressors	18-22 Ct	1,000,000 to 100,000	5

2. Dilutions/ Amounts: 100; 50; 30; 10 and 1 ng /well

Spike in a constant amount of external transcript

3. Number of Operators 3

4. Number of Runs 3

5. Number of sites / instrument 1

6. Number of Replicates 3

RQ: Results Summary

Operator to Operator Variation: 2-Fold Change

	N	Range	AVG	SD	Median	95% CI	< 1.5	Accuracy (1.33)	Accuracy (1.50)
Exp_FC 2 and All OPER	387	0.97 to 6.03	2.23	0.5	2.18	1.23 to 3.23	6 or 1.5%	97.40%	96.80%
Exp_FC 2 and OPER 1	120	1.59 to 4.71	2.29	0.53	2.15	1.23 to 3.35	0	100%	100%
Exp_FC 2 and OPER 2	133	0.97 to 6.03	2.14	0.62	2.03	0.90 to 3.38	6 or 4.5%	92.50%	90.90%
Exp_FC 2 and OPER3	134	1.76 to 3.14	2.29	0.29	2.23	1.71 to 2.87	0	100%	100%

Robust RQ results for FC in the 1.66 to 100 range.
RQ with the ΔΔCt method is more accurate than methods using efficiency values in the PCR equation.

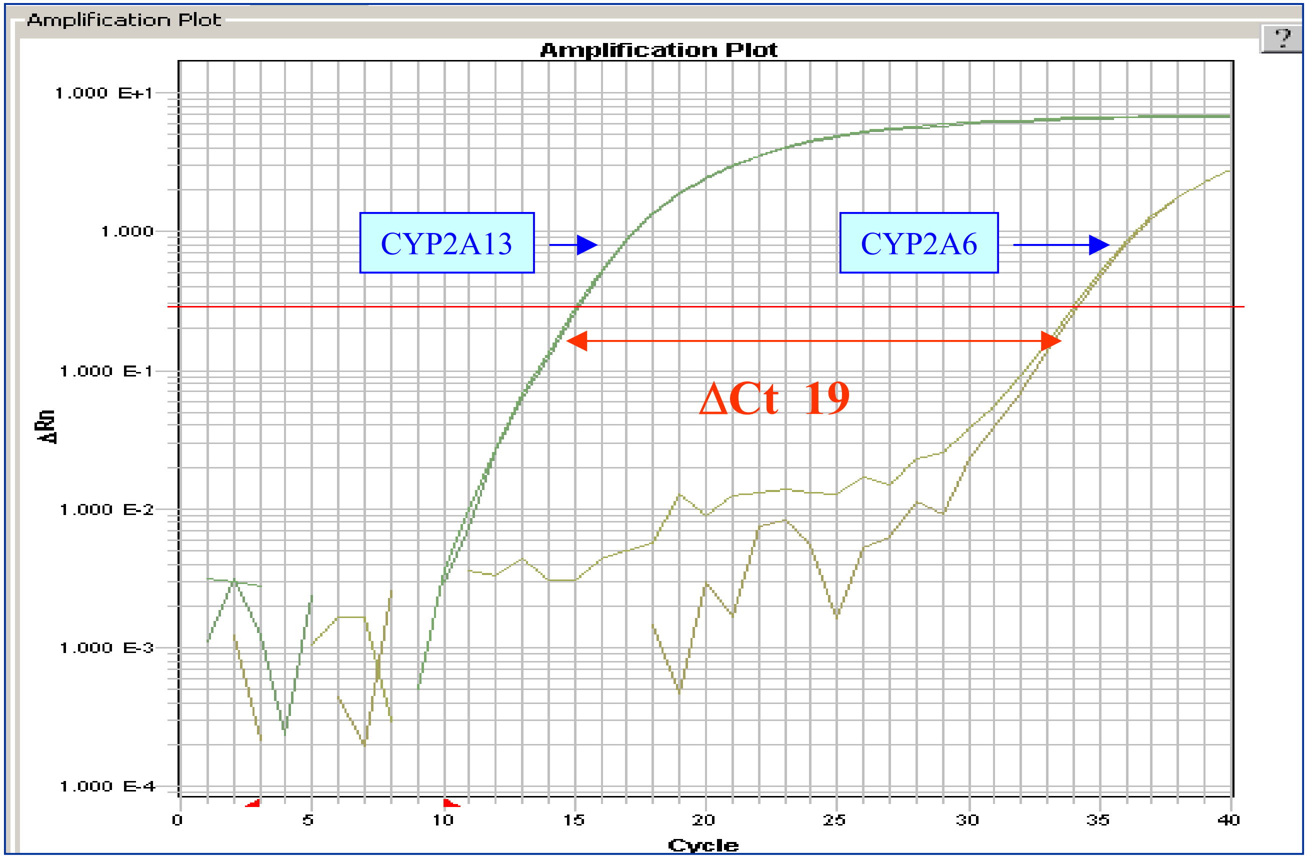
Summary : Quantification

- Internal calibrators or standard curves are necessary for accurate absolute quantification.
- For relative quantification it is useful to have an endogenous control that is minimally altered during the study / experiment.
- Quantification standards that can be added into the sample (or during NA extraction) and carried through the entire process are useful for normalization across large studies and identification of appropriate endogenous controls.

Conclusions .. continued

- Efficiency measurements have a high degree of variability. Using these values in RQ measurements gives less robust results.
- The ΔΔCt method produced a better correlation to expected results as compared to measurements using efficiency values in PCR equations.
- Good assay design strategies can ensure near 100% efficiency for real time PCR assays.
- Relative quantification using the ΔΔCt method generated reliable results and is amenable to HT processing.

Assay Specificity CYP2A13 vs CYP 2A6



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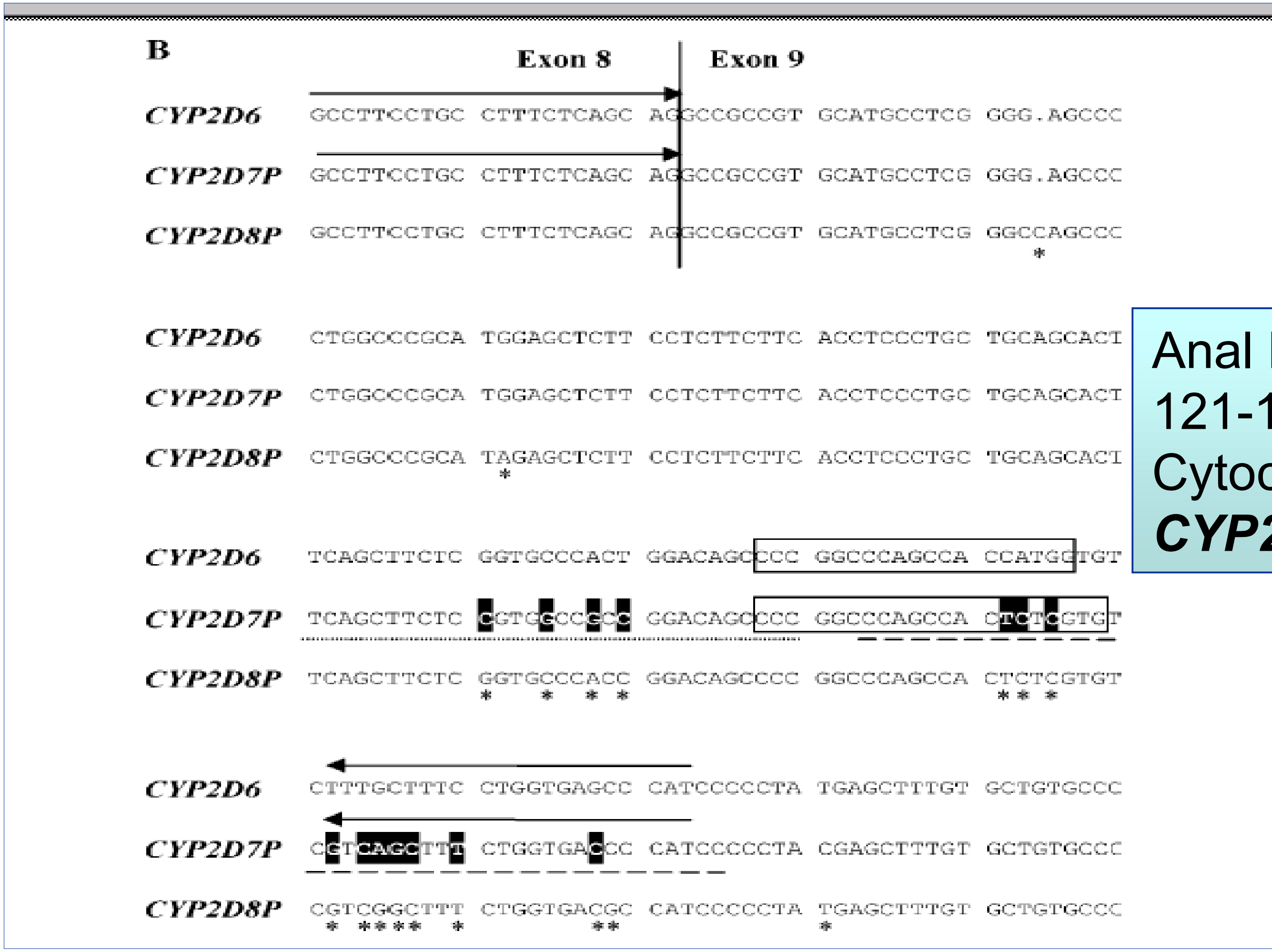
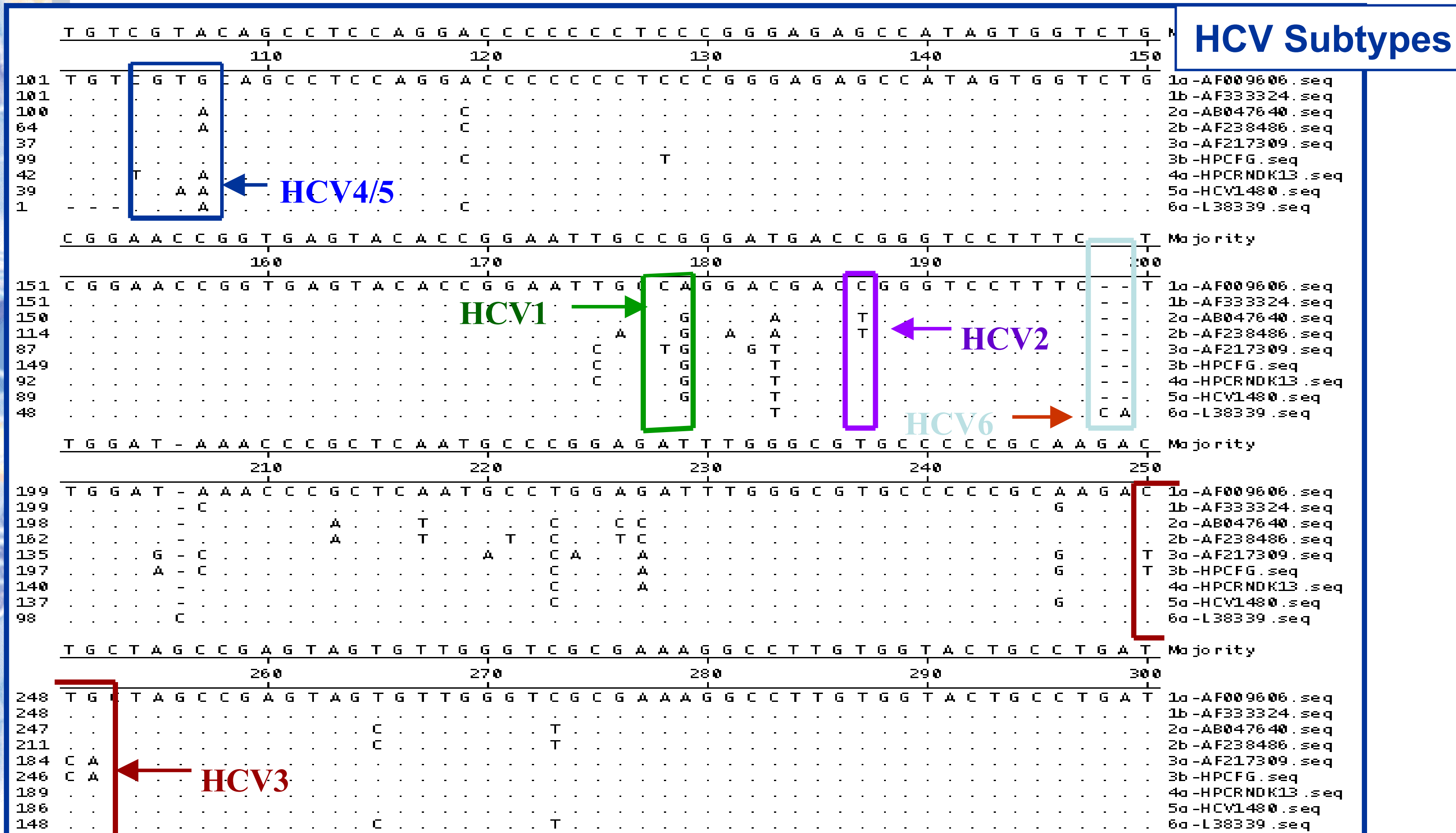
Selectivity by Primer Mismatches



Anal Biochem. 300: 121-131. 2002
Cytochrome P450
CYP2D6

Selectivity by Probe Mismatch: HCV Alignment



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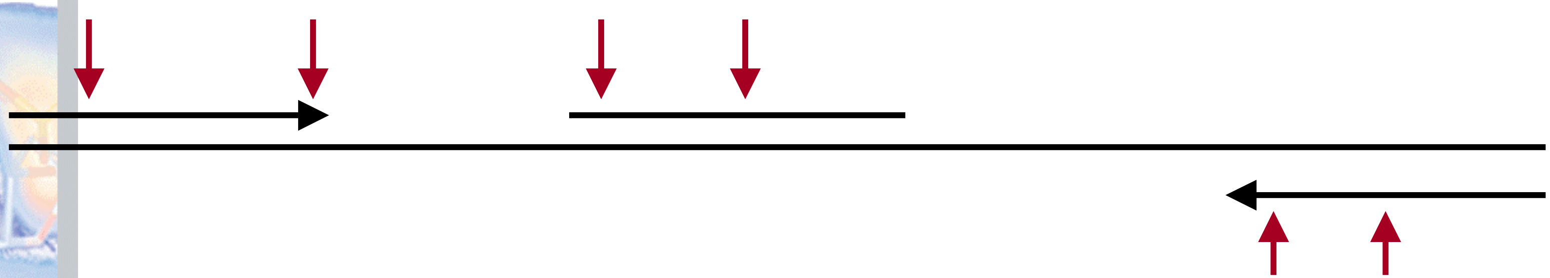
Effect of a Mismatch on Ct



The Ct value is dependent on both PCR amplification & probe cleavage.

Effect of mismatches in primers & probe on Ct.

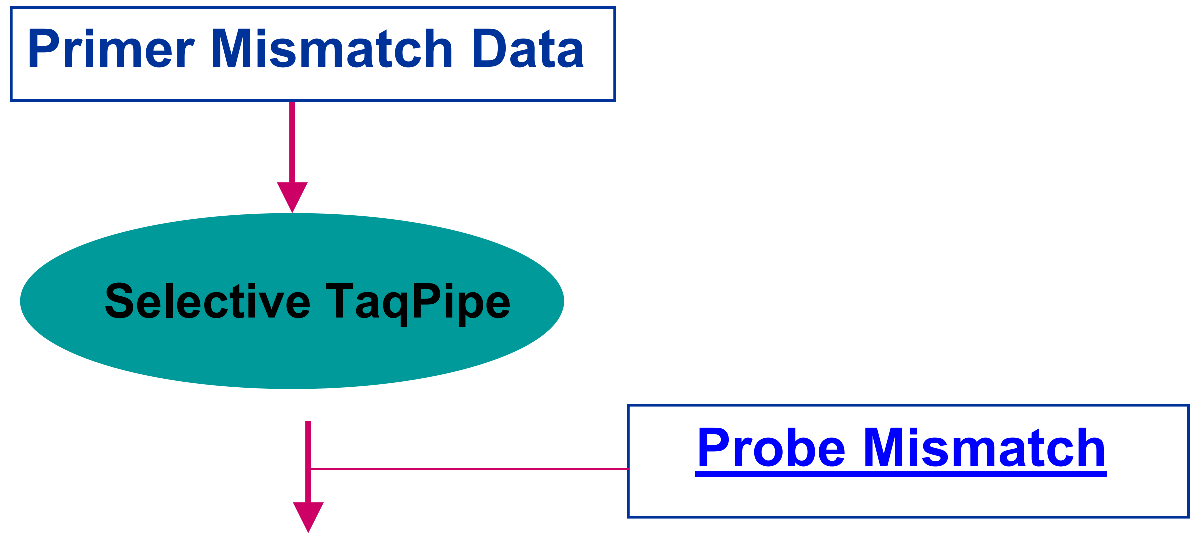
- Position specific effects.
- Mismatch specific effects.
- Combinatorial effects of multiple mismatches

Relative contributions from primers & probes.

Over 3,000 designs tested to generate data.

In silico QC against the known transcriptome.

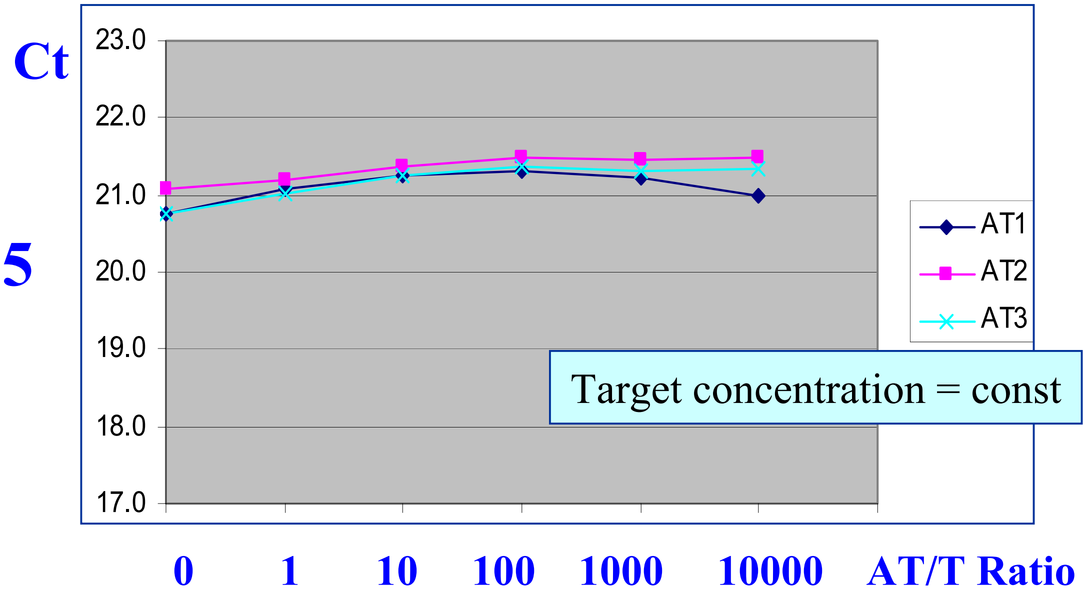
Selective TaqPipe: Bioinformatics Tool

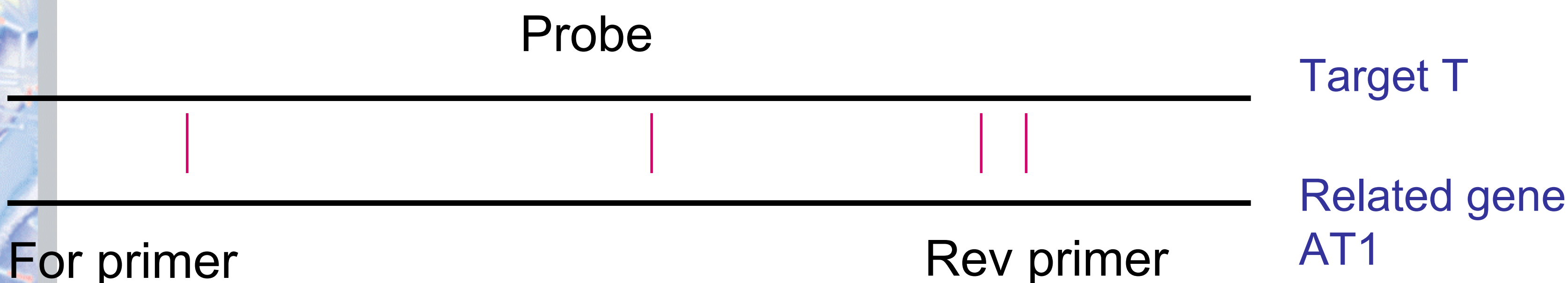


Testing a large number of designs to define contributions to selectivity by **mismatch on a primer and a probe: type and location**

Determining Level of Assay Specificity

Ct diff AT1 vs T= 15

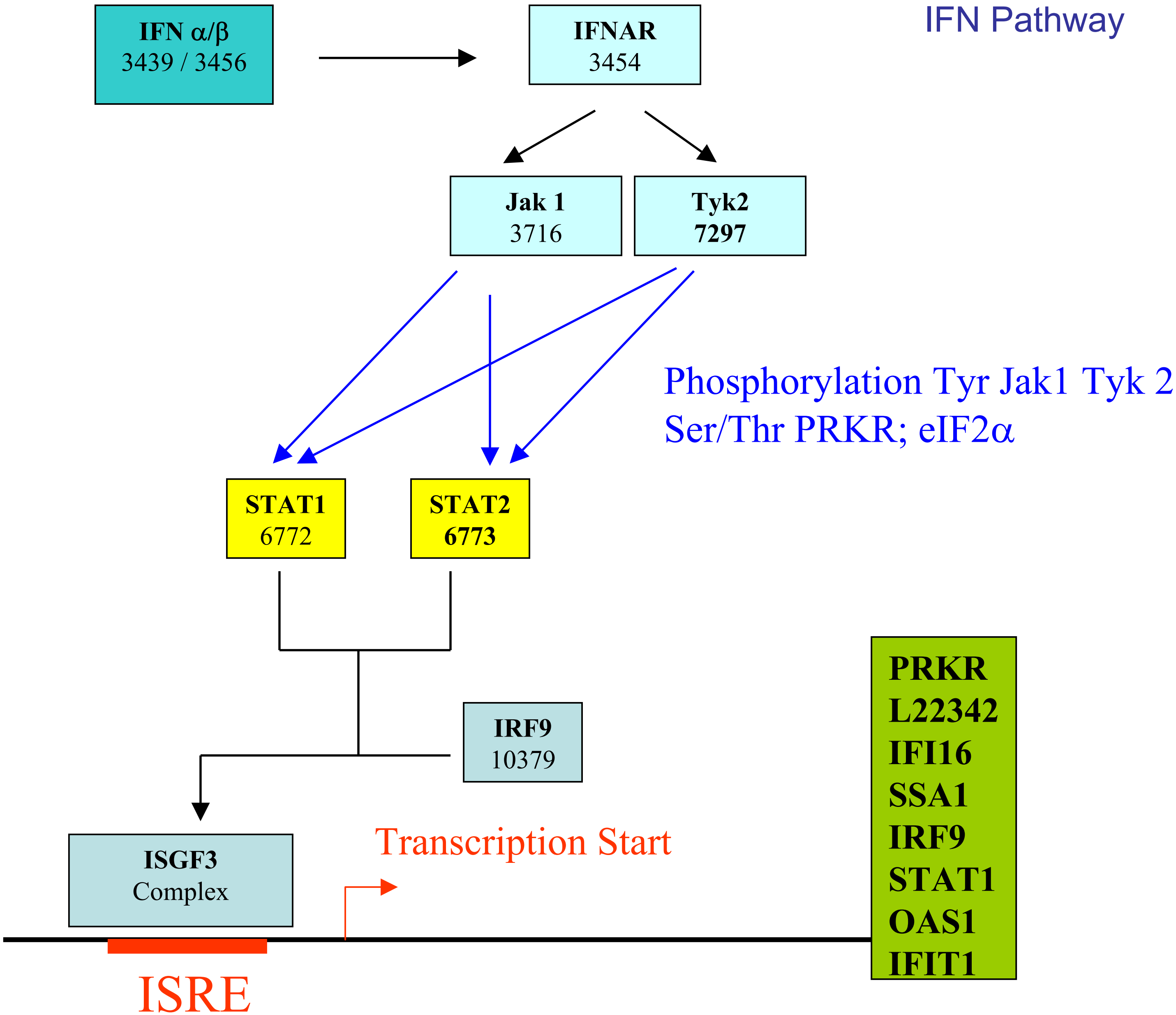


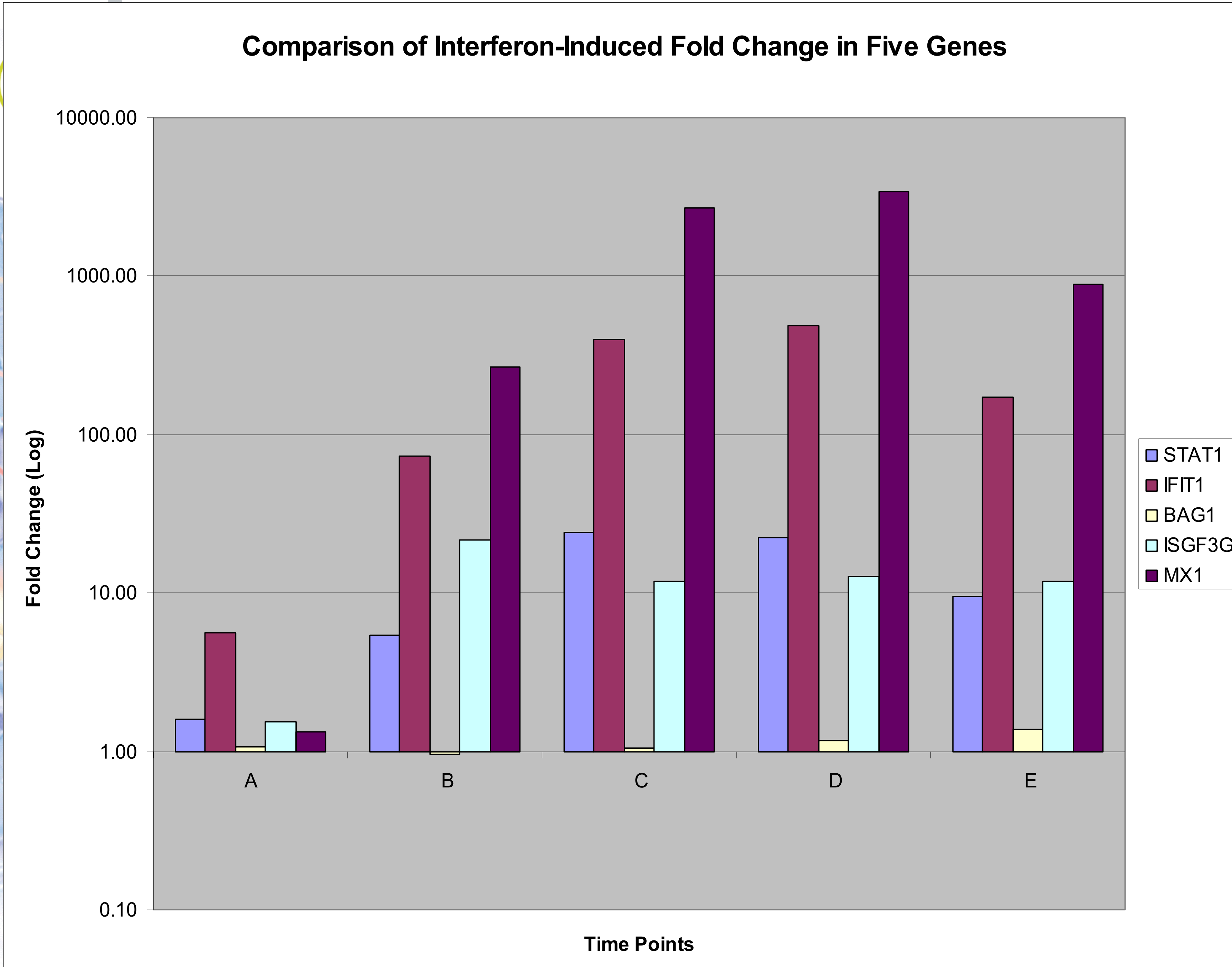


Conclusions

- Real time PCR assays that target transcripts emanating from closely related genes should have high selectivity.
- Suppression of co-amplification can be achieved by placing primers at positions where there are mismatches.
- Mismatches within probe binding regions will also enhance selectivity.
- The level of selectivity should be defined by the user based on the study / experiment and is related to transcript abundance and levels of induction / suppression of the related genes.

IFN Pathway


IFN α/β
3439 / 3456
IFNAR
3454
Jak 1
3716
Tyk2
7297
Phosphorylation Tyr Jak1 Tyk 2
Ser/Thr PRKR; eIF2α
STAT1
6772
STAT2
6773
IRF9
10379
ISGF3
Complex
Transcription Start
ISRE
PRKR
L22342
IFI16
SSA1
IRF9
STAT1
OAS1
IFIT1



Comparison of Interferon-Induced Fold Change in Five Genes
10000.00
1000.00
100.00
10.00
1.00
0.10
Fold Change (Log)
A
B
C
D
E
Time Points
STAT1
IFIT1
BAG1
ISGF3G
MX1

ms

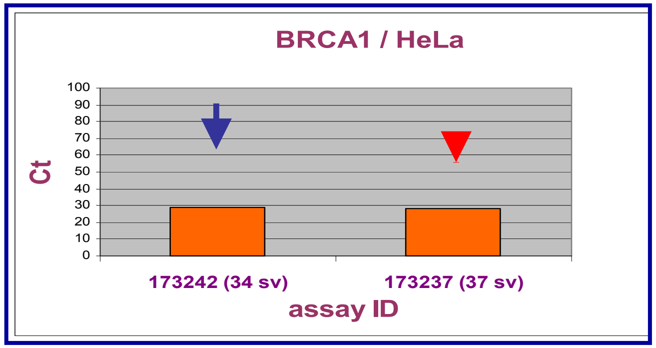
Gene Name	Fold Change at 24 hours (Literature)	Fold Change at 24 hours in study
PRKR	7	7.03
L22342	10	12.87
IFI16	12.7	15.1
SSA1	6.6	18.8
ISGF3/IRF9	9.6	12.7
STAT1	11.2	22.3
OAS1	25.2	29.6
IFIT1	124.6	490.12

Yuen et al., NAR 30 No 10 e48 ; 2002 Arrays PCR

Tested over a 100 genes with good correlation to literature.
Microarray analysis, mass spec analysis for protein profile and PTMs in progress

BRCA1 (3 siRNA vendors):

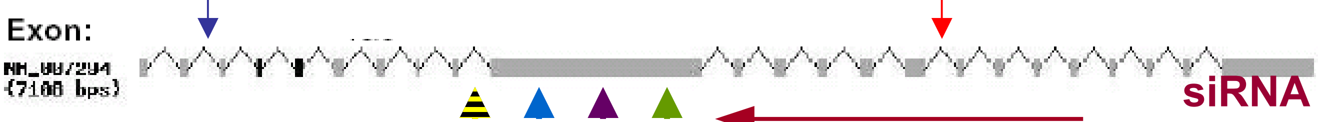
AoD (3') and AbD (5') assays detect 30-85% RNA knock-down by different siRNAs



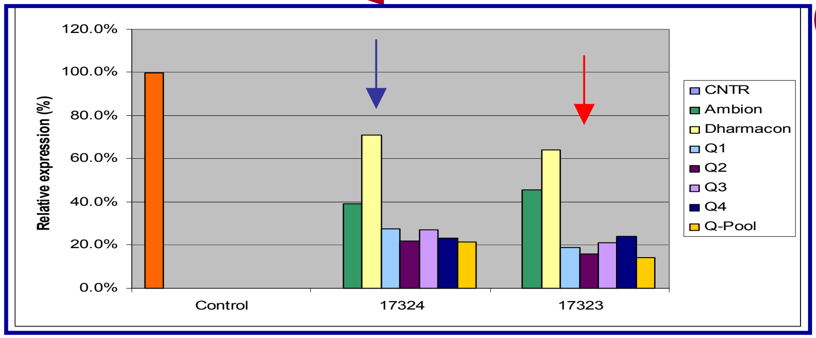
HeLa cells Control

AbD (exon 2-3)

AoD (exon 15-16)

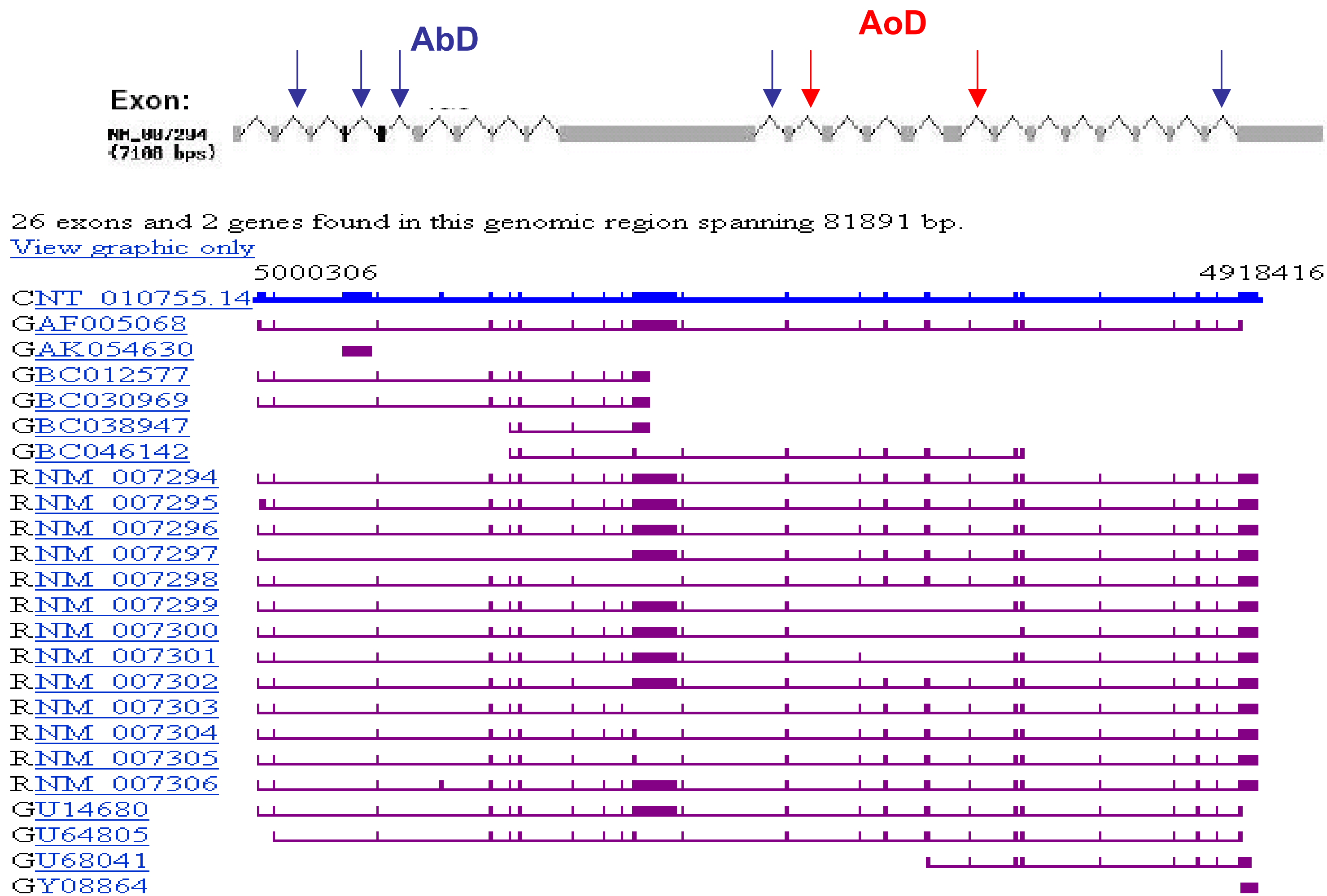


siRNA (target exon 10)



HeLa cells siRNA treated 24 Hours

BRCA1 gene: 23 splice isoforms



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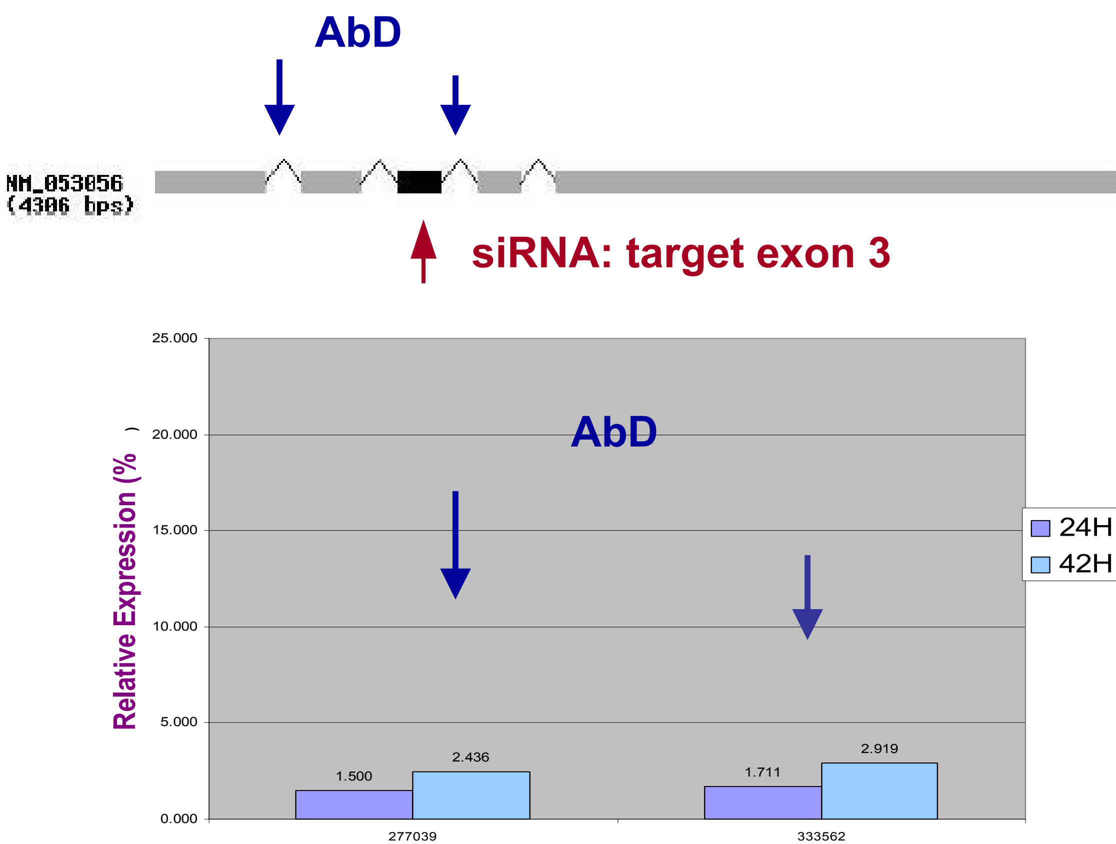
CyclinD1: siRNA



More than 90% knock-down detected

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Karen Poulter
Rixun Fang
Michael Malicdem
Ada Wong
Sonny Young
Eileen Westphale
Kathleen Shelton
Jennifer MacLean
Jeff Peralta
Ben Curzon

Labeling

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