The Ups & Downs of Gene Expression:

Using Lipid-Based Transfection and RT-qPCR to Deliver Perfect Knockdown and Achieve Optimal Expression Results

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Hilary Srere, Ph.D., presenter

BIO-RAD

Topics

- What is RNAi?
- Methods of Delivery and Detection
- RNA Preparation
- Reverse Transcription
- qPCR Detection
- Case Study: ODC Pathway

What is RNAi?

RNA interference (RNAi) is a phenomenon where dsRNA specifically blocks the expression of its homologous gene. Also known as post-transcriptional gene silencing (PTGS) and quelling.

1990 RNAi was discovered as an endogenous property in petunias

1998 Fire & Mello at the Carnegie in Washington showed gene silencing pathway in c.elegans

2000 Tuschl and Elbashir at the Max Planck Institute showed that short interfering RNAs could be introduced into mouse cells.

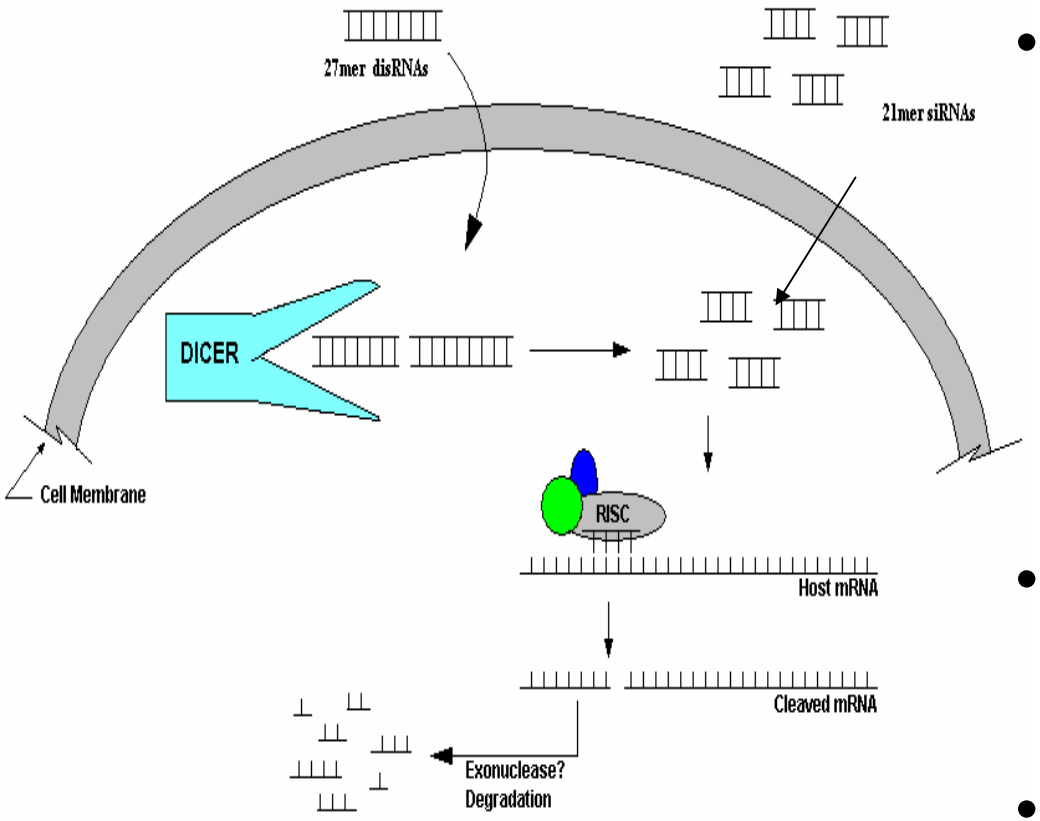
The Power of RNA Interference

Why is RNAi so powerful?

- Allows fast characterization of gene/protein function
- Enables study of pathways
- Facilitates rapid identification and validation of targets
- Therapeutic potential

RNAi Mechanism

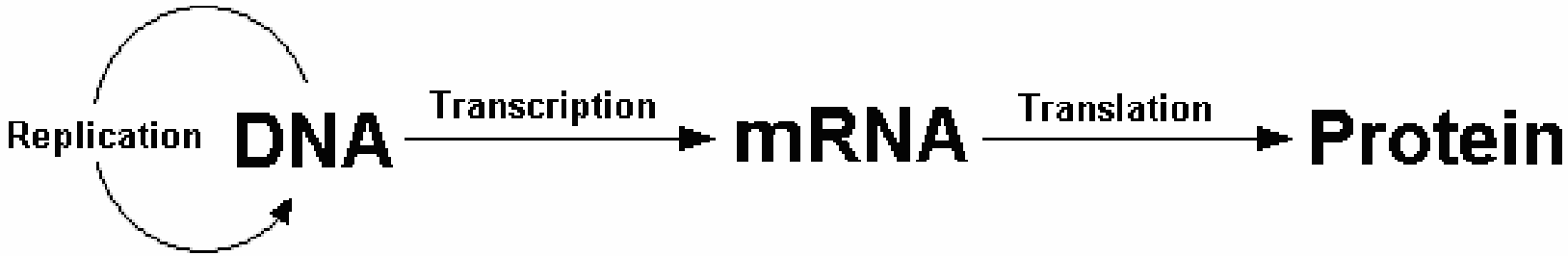
Sequence-specific message degradation



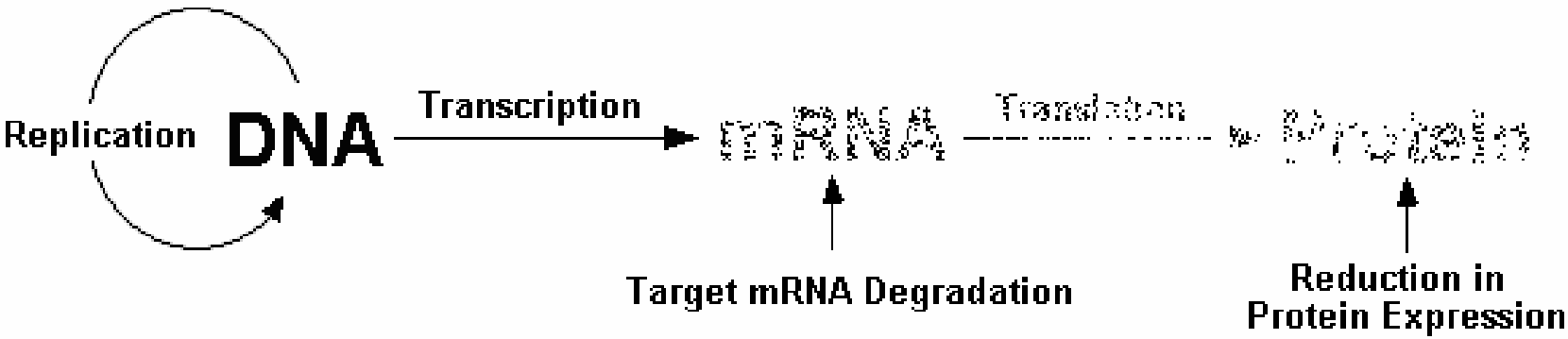
- Conserved Cellular Mechanism (two steps):
  - -Initiation - DICER
  - -Effector – RISC (RNA-Induced Silencing Complex)
- Natural defense against viral infection
- Transfect with siRNA (21mer and 27mer)

Molecular Biology and RNAi

Central Dogma of Molecular Biology:



Basic RNA interference Mechanism:



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RNAi: Challenge of Delivery

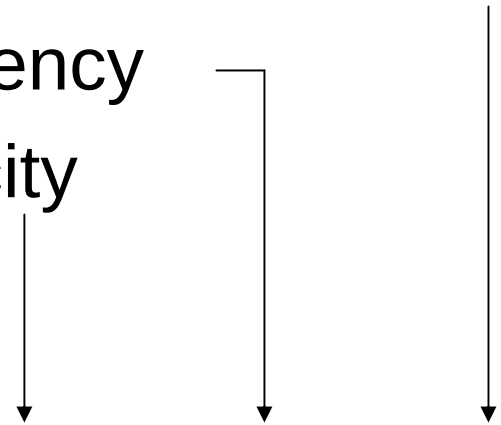
What delivery method is best?

- Electroporation – good for suspension & difficult cells
- Biolistics – good for neural & primary cells
- MicroInjection – offers greatest specificity
- Viral – very high efficiency
- ➔ Lipid Mediated – low cost, simple protocol, consistent results, good for high throughput applications

Lipid Mediated Delivery

Three Major Lipid Characteristics to Consider:

- Design / Development
- Efficiency
- Toxicity



Silencing (siRNA Activity)

Lipid Mediated Delivery

Important Transfection Conditions

- Cell line maintenance
- Cell line confluence / density
- siRNA quality (design / purity)
- siRNA concentration*
- Lipid concentration*

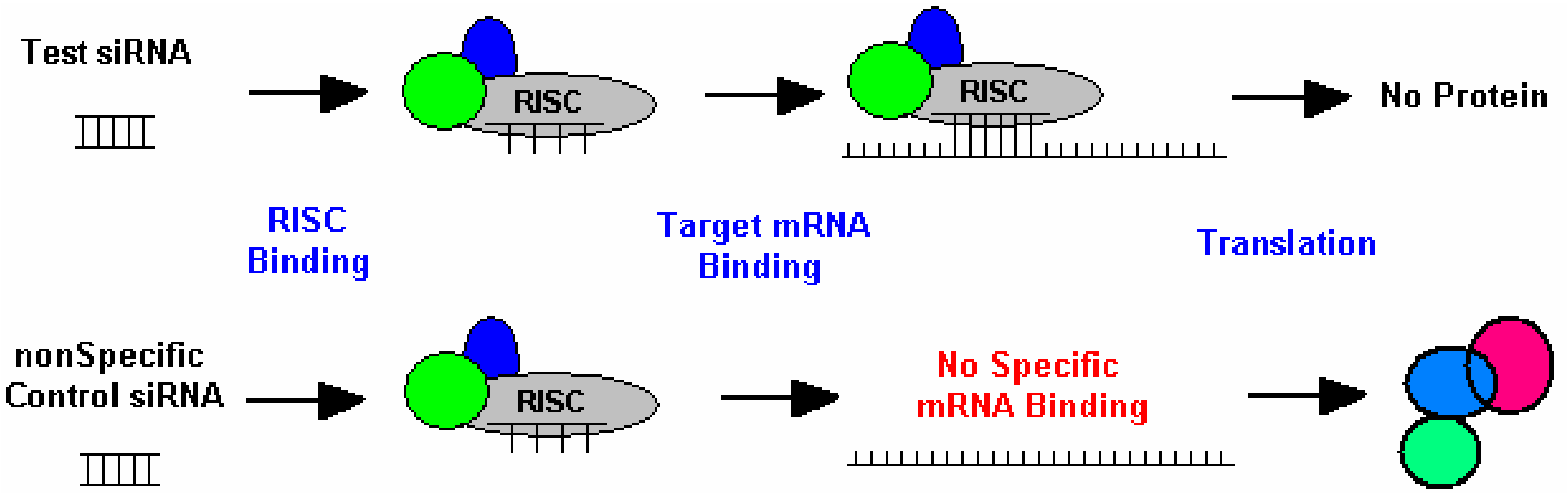
***influenced by choice of lipid reagent and cell line**

Cell Lines Tested

- LNCaP – Human Prostate Carcinoma
- HUVEC – Human Umbilical Vein Endothelial
- FS – Human fibroblast
- NIH-3T3 – Mouse embryonic fibroblast

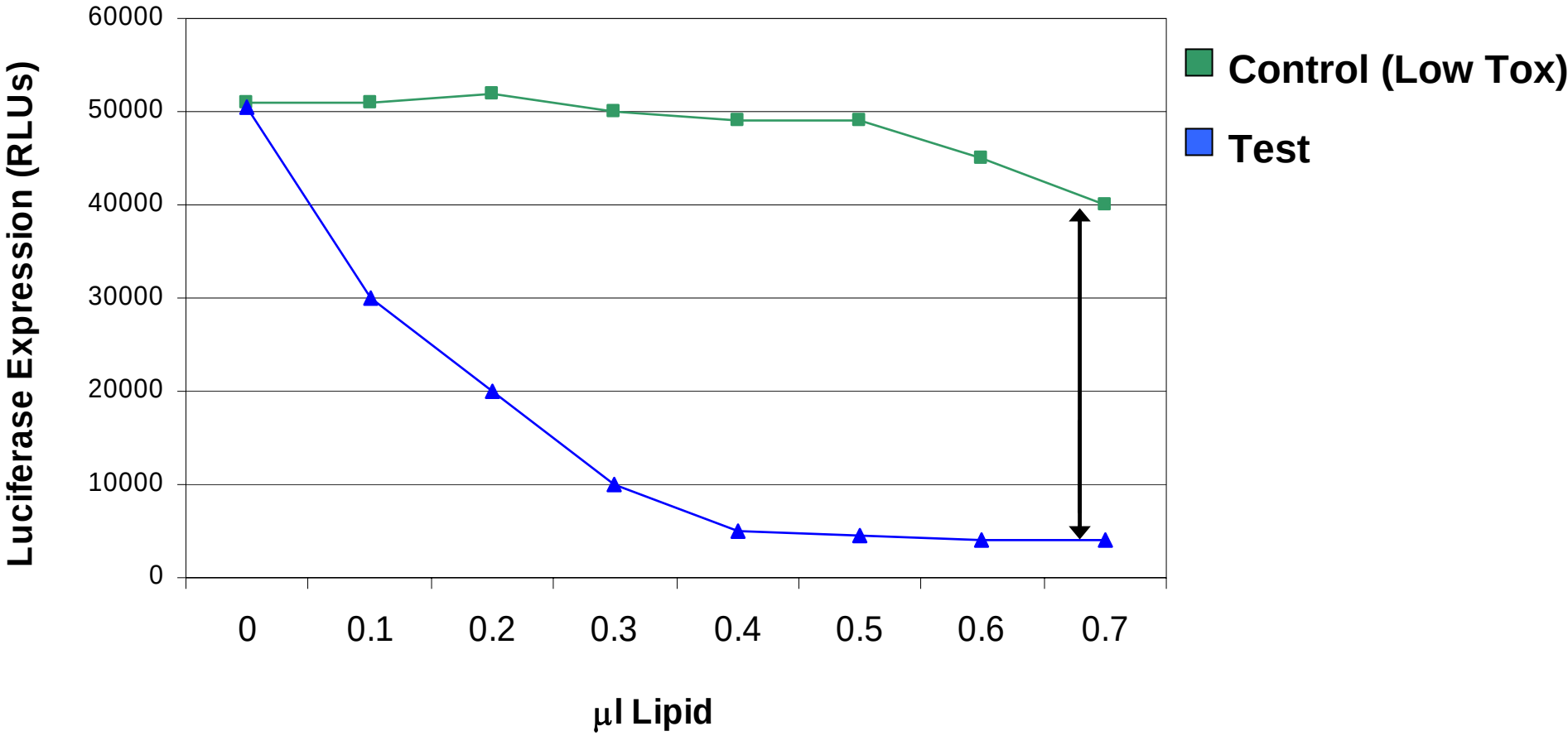
Experimental Design: Controls

Test siRNA vs. nonSpecific Control siRNA



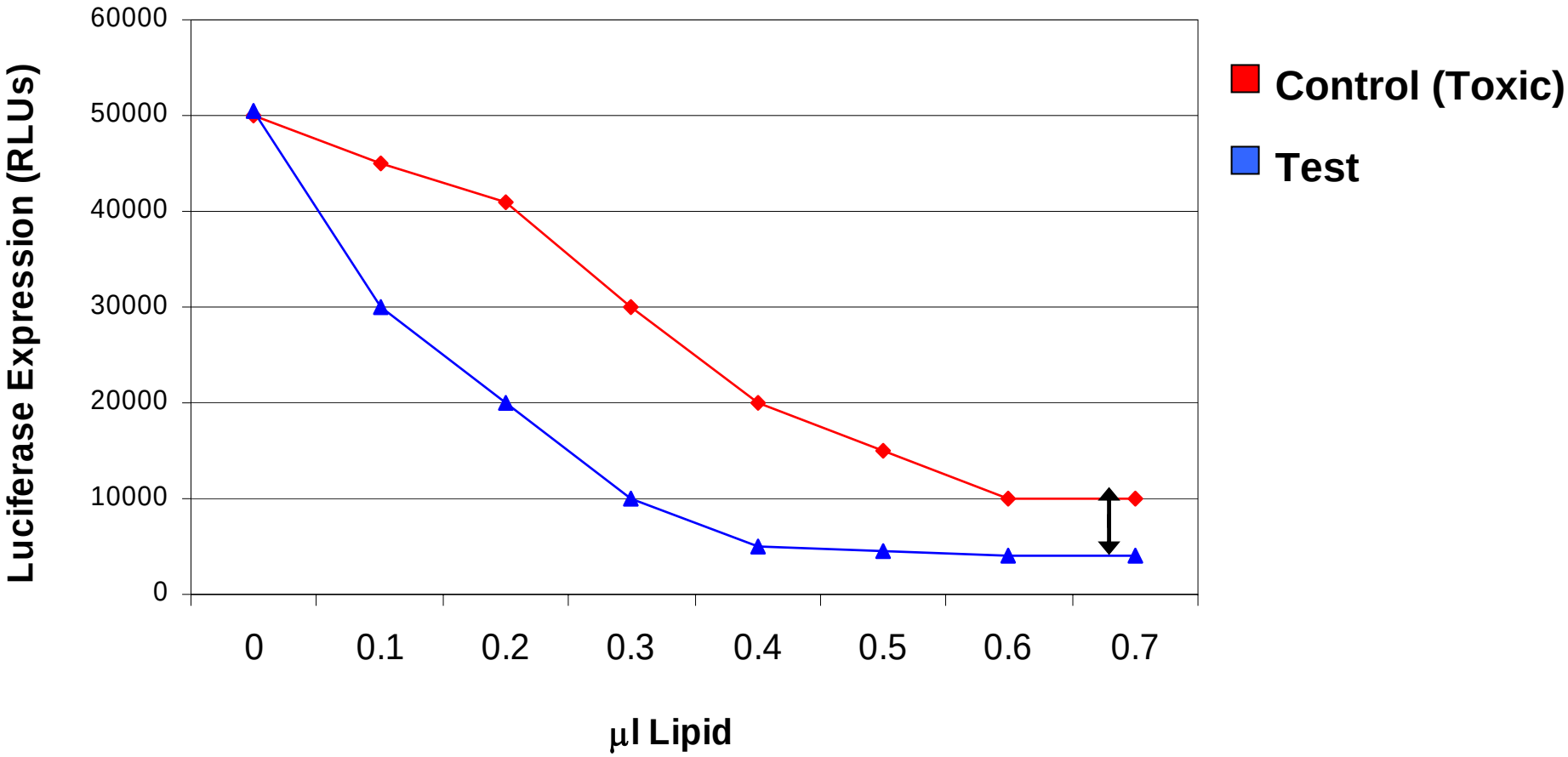
Experimental Design: Controls

How this will look as Data…….



Experimental Design: Controls

How this will look as Data…….

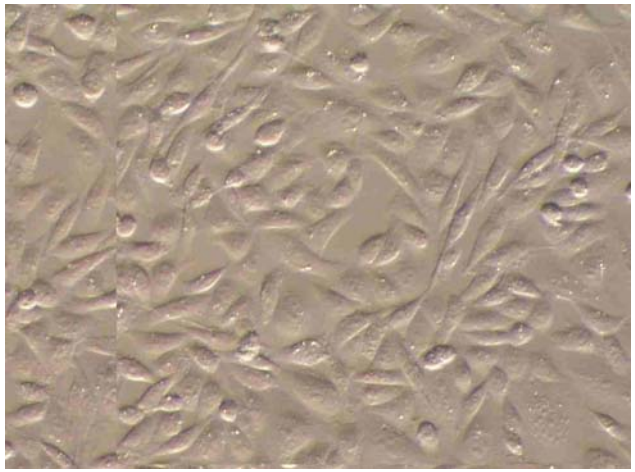


Toxicity Evaluations

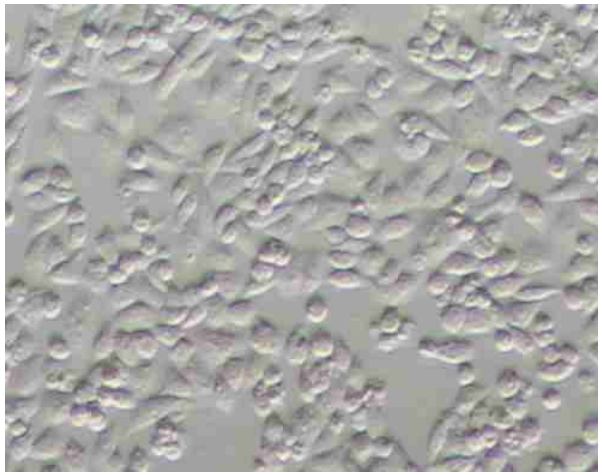
Visual Analysis

- Morphology changes
- Detachment
- Lysis

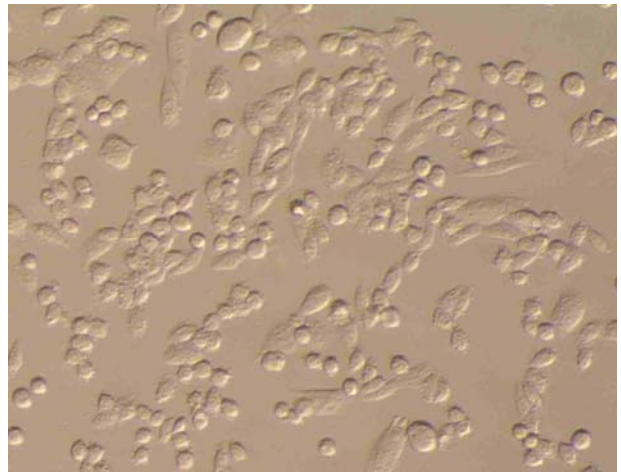
Low



Moderate

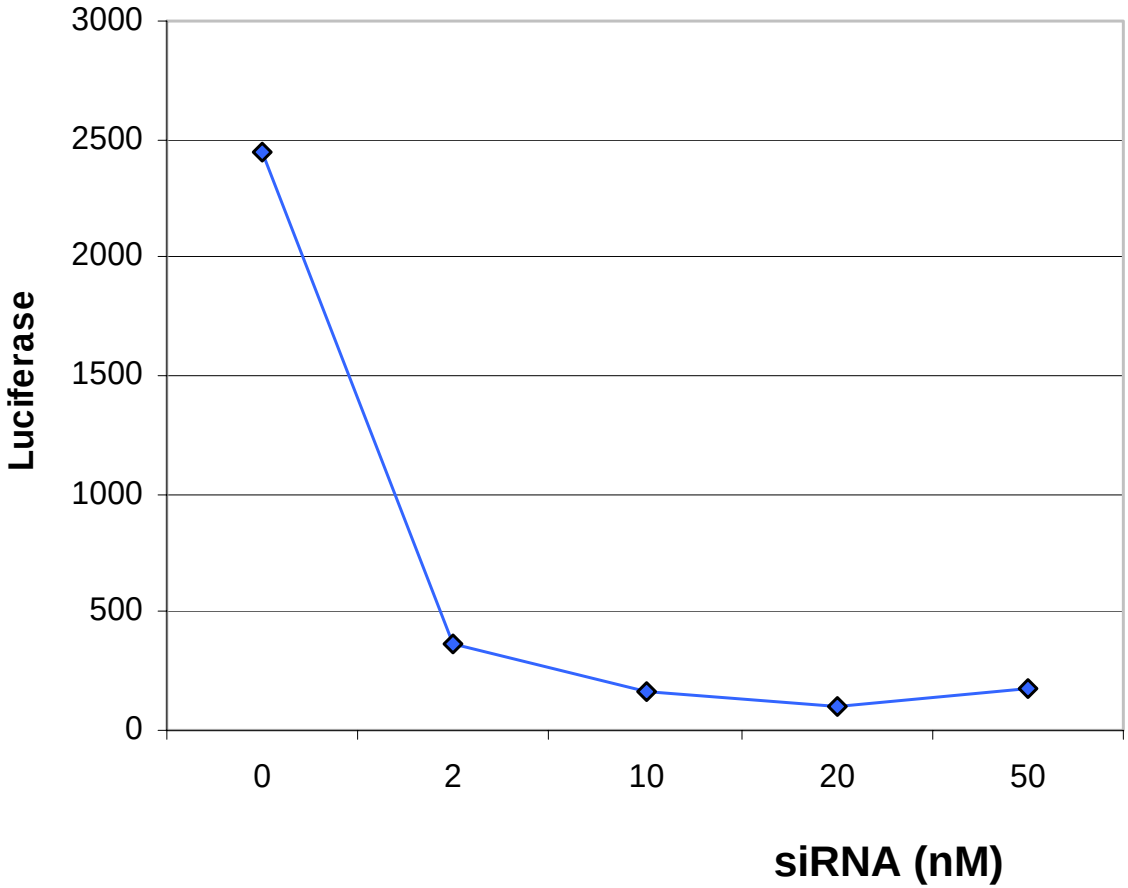


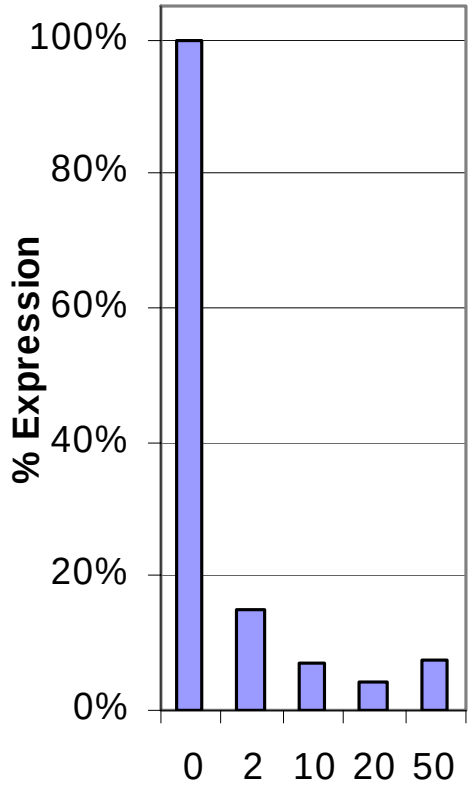
High



Efficiency: siRNA Amount

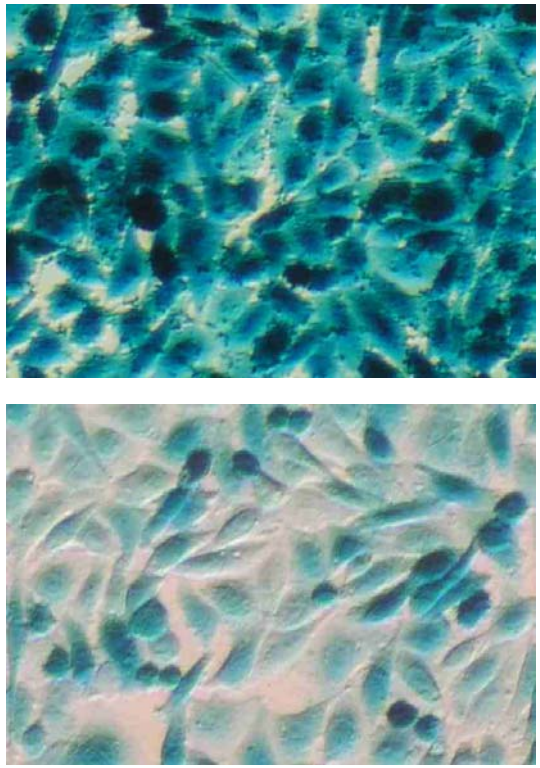
CHO-Luc / siLentFect – 0.3 µl (96-well)





RNAi Detection Strategies

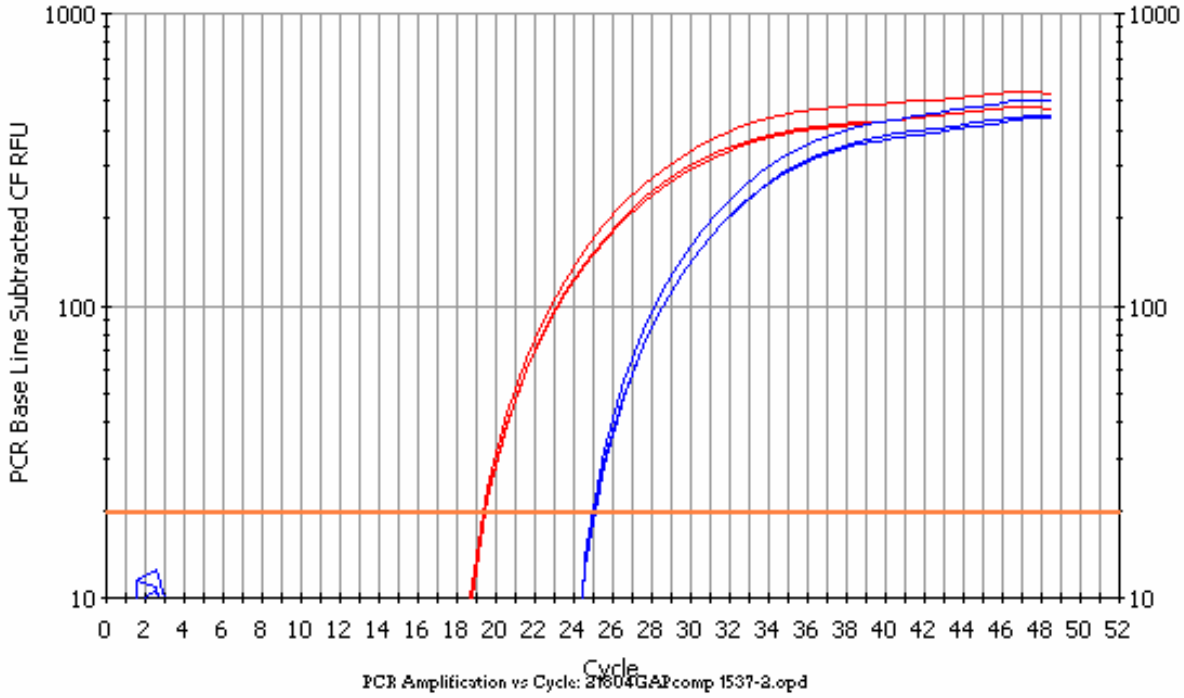
- Western Blots
- Northern Blots
- Microarrays
- qPCR
  - **1.0 Cycle Threshold = 50% silencing**
  - **3.3 Cycle Threshold = 90% silencing**
  - **6.6 Cycle Threshold = 99% silencing**

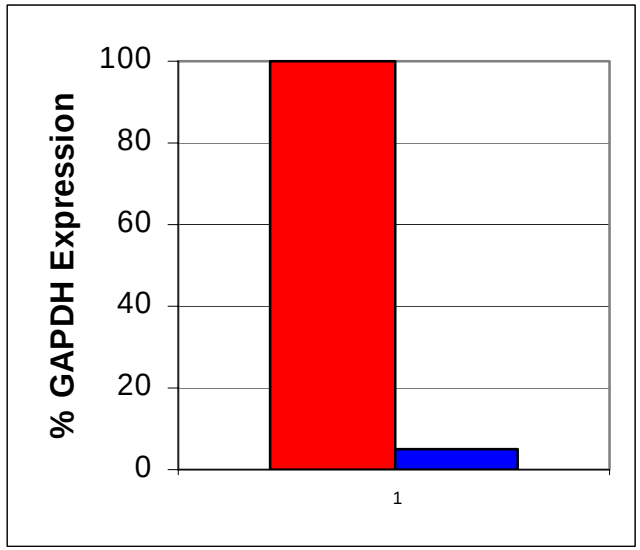


CHO-lacZ cells transfected with scrambled siRNA control (top) and beta-gal siRNA (bottom)

Detection: qPCR Analysis

GAPDH, HeLa Cells, 48 hr, 6-well

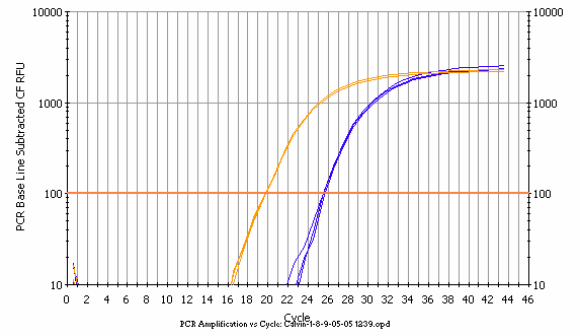




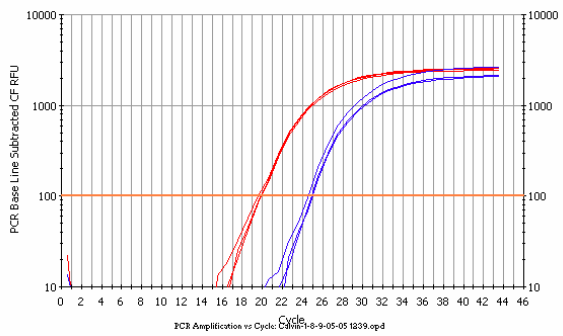
- **5.6 C_t Difference**
- **Over 95% knockdown**
- **1.25 µl siLentFect**
- **10nM siRNA**

Silencing Endogenous Genes in Multiple Cell lines

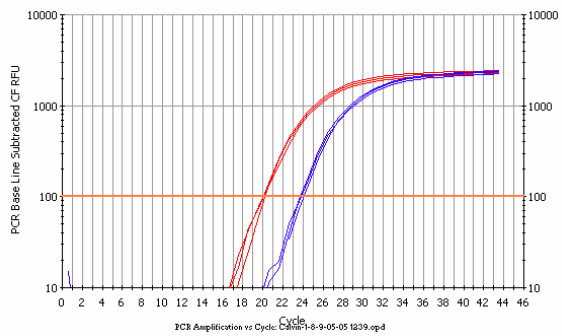
FS GAP

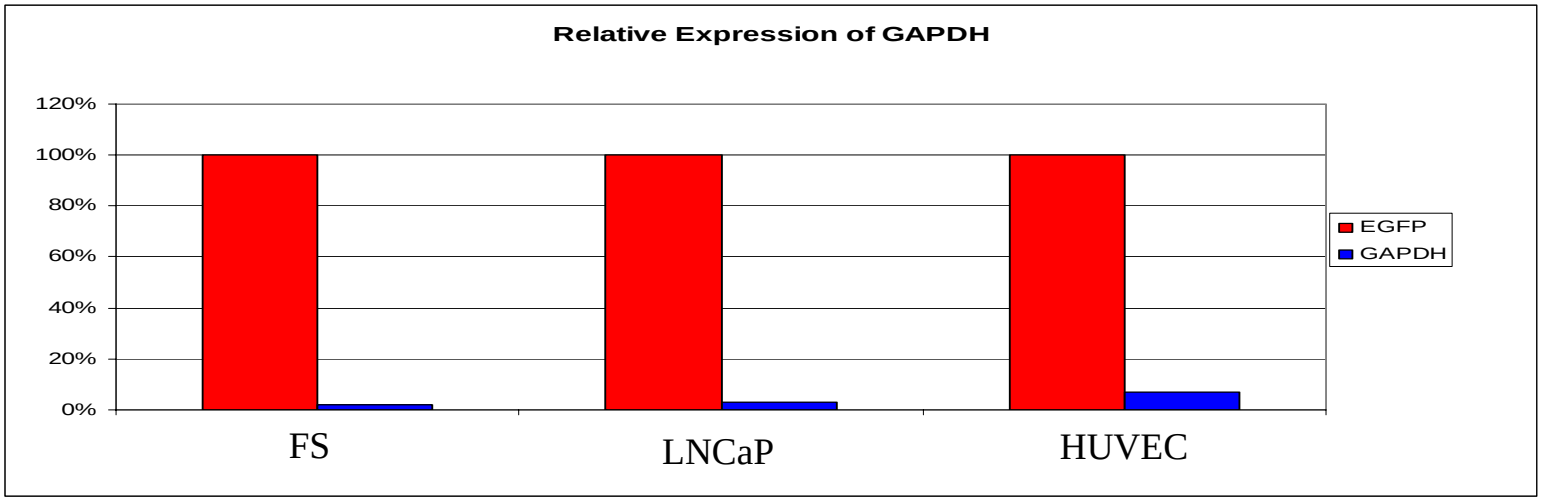


LNCaP GAP



HUVEC GAP

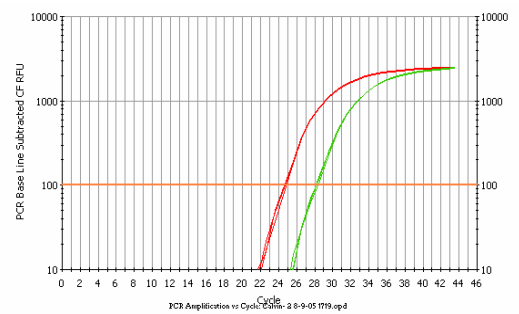




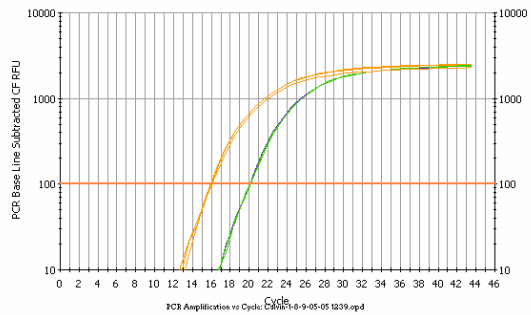
Several siRNAs in single transfection

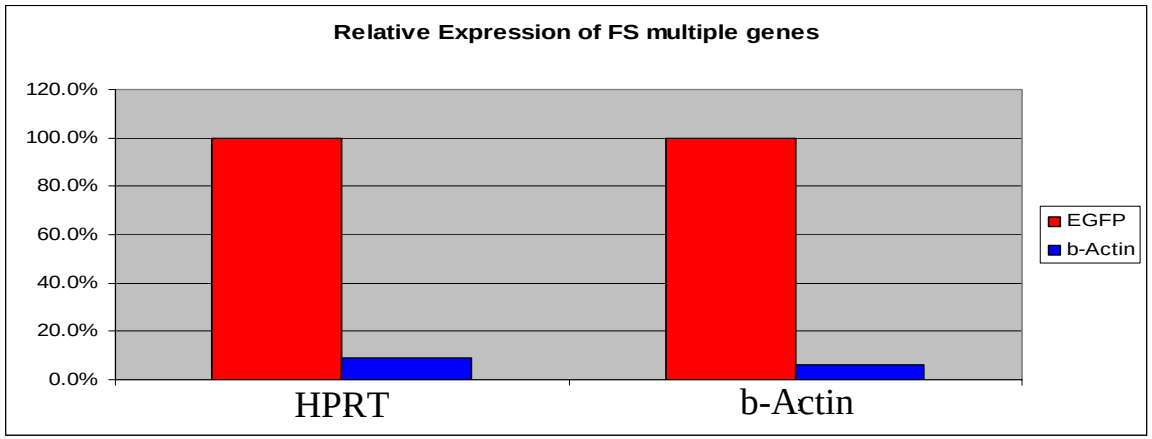
- Silencing HPRT and β-actin genes with the same transfection in FS cells.

FS HPRT



FS β-Actin

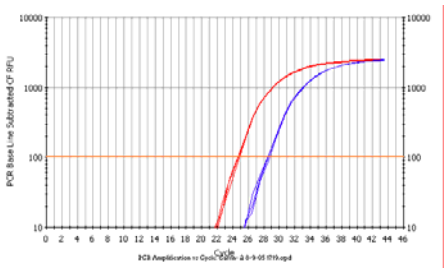


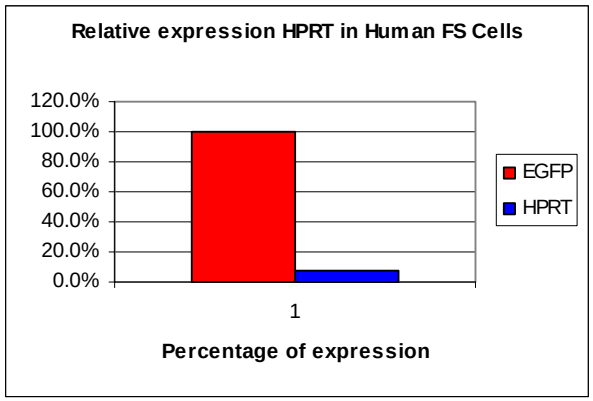


Universal siRNA: Mouse vs Human

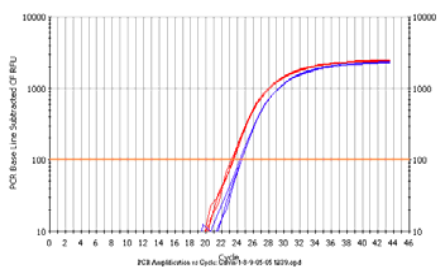
- Tested a universal HPRT in Mouse and Human cells
  - The siRNA worked considerably better in humans than mouse
  - A new sequence may have to be generated

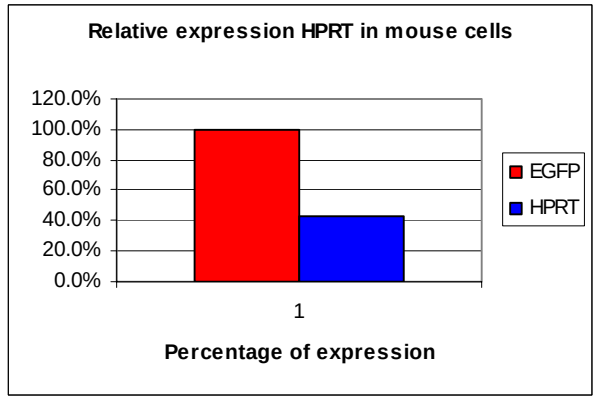
Human FS Cells



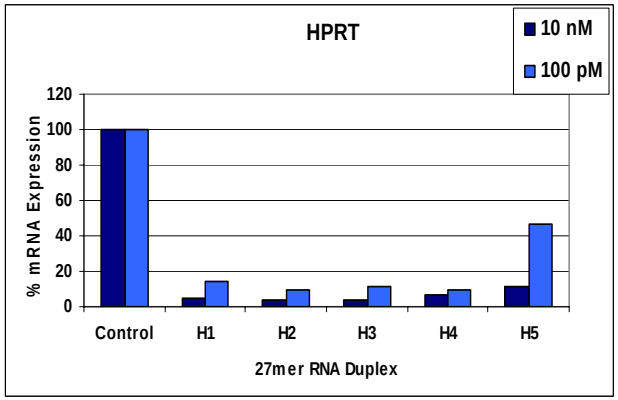


Mouse 3T3 Cells

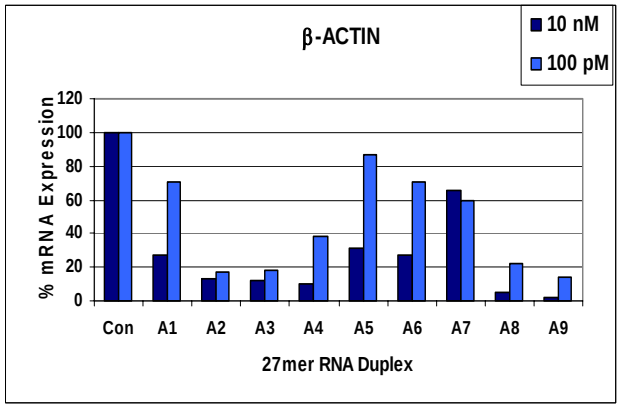




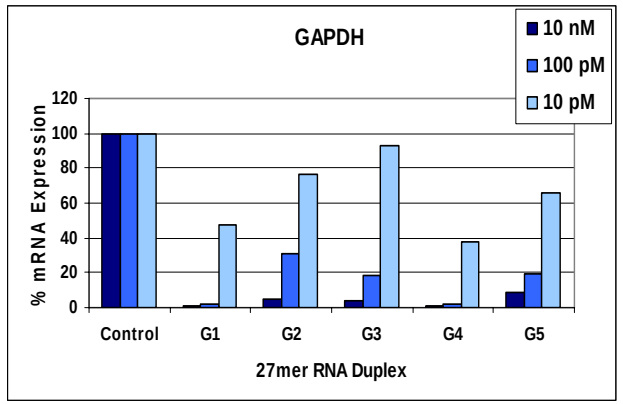
Silencing of endogenous mRNAs at low 27mer siRNA concentrations



5 / 5 >85% at 10 nM
4 / 5 >85% at 100 pM



5 / 9 >85% at 10 nM
4 / 9 >85% at 100 pM



5 / 5 >85% at 10 nM
4 / 5 >85% at 100 pM

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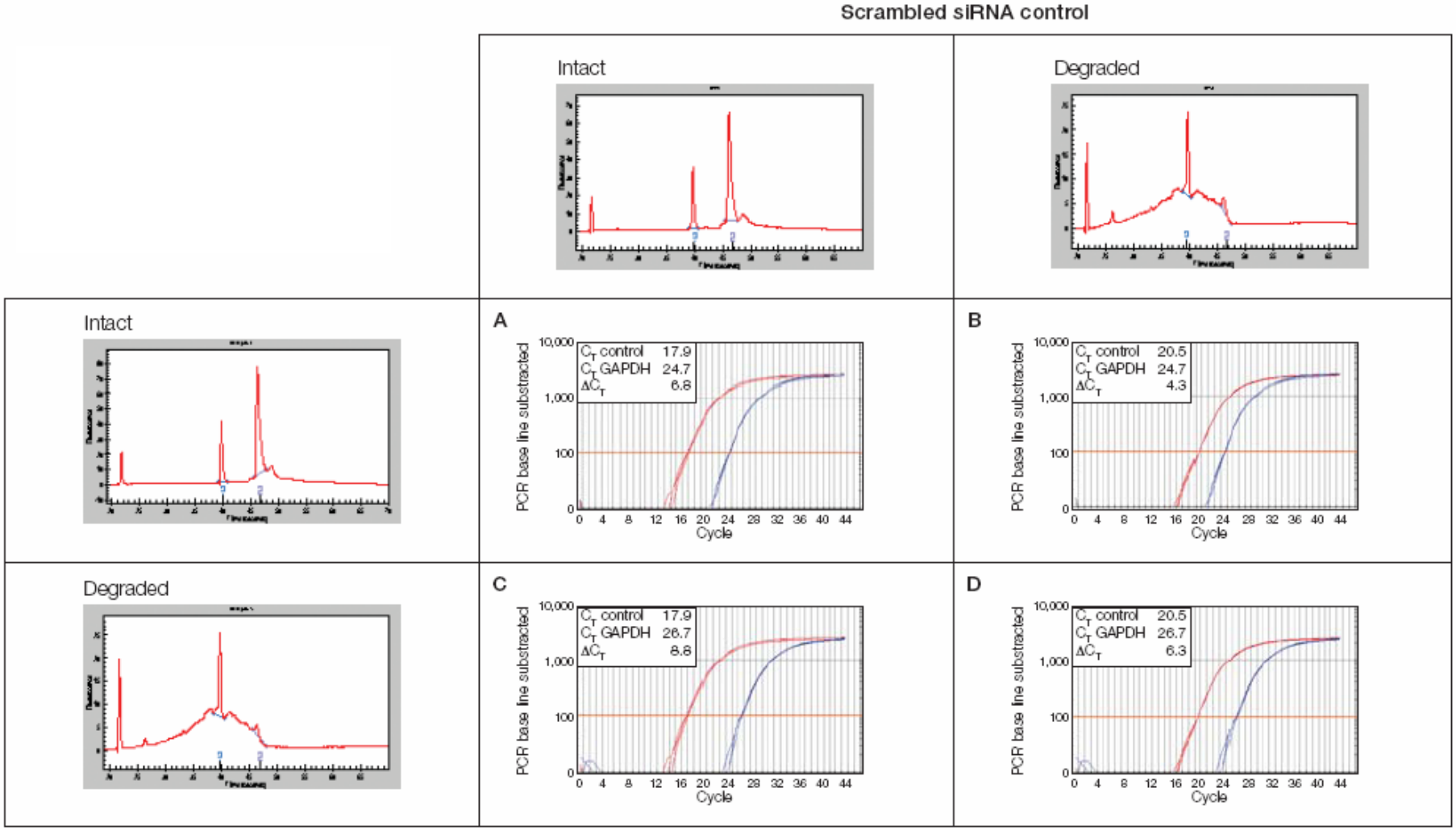
RNA Preparation

- Often taken for granted
- Importance of choosing a method that gives good yield is often underestimated
- Important to isolate highly pure nucleic acids since a lot of time and money are invested in downstream molecular biology applications
- Quality of the downstream results may be compromised without an effective method for nucleic acid purification

RNA Preparation

- Extract RNA (DNase treatment optional)
- Analyze RNA, careful quantification is necessary:
  - RiboGreen assay
  - Experion™ System

RNA Quality


Scrambled siRNA control
GAPDH siRNA
Intact
Degraded
Intact
Degraded
A
B
C
D
PCR base line substracted
Cycle
C_T control 17.9
C_T GAPDH 24.7
ΔC_T 6.8
C_T control 20.5
C_T GAPDH 24.7
ΔC_T 4.3
C_T control 17.9
C_T GAPDH 26.7
ΔC_T 8.6
C_T control 20.5
C_T GAPDH 26.7
ΔC_T 6.3

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Optimizing Reverse Transcription

Three Major Characteristics to Consider:

- For real time applications choose an RNase H^+ enzyme
- Choose a reverse transcriptase with a wide dynamic range – for good efficiency
- Primer choice (*i.e.*, Oligo(dT), random primers or gene specific) may impact the results differently

Importance of an RNase H$^+$ enzyme

- RNase H$^+$ enzymes result in accurate detection of low abundance message-- in as little as 100fg of input RNA
- RNase H degrades only those molecules that are in RNA:DNA hybrids -- giving clean cDNA for downstream qPCR
- RNase H$^-$ RT require an extra RNA degradation step before PCR = more expensive, more time consuming & increased risk of contamination

Dynamic Range of iScript

1.6×10^7

1.6×10^6

1.6×10^5

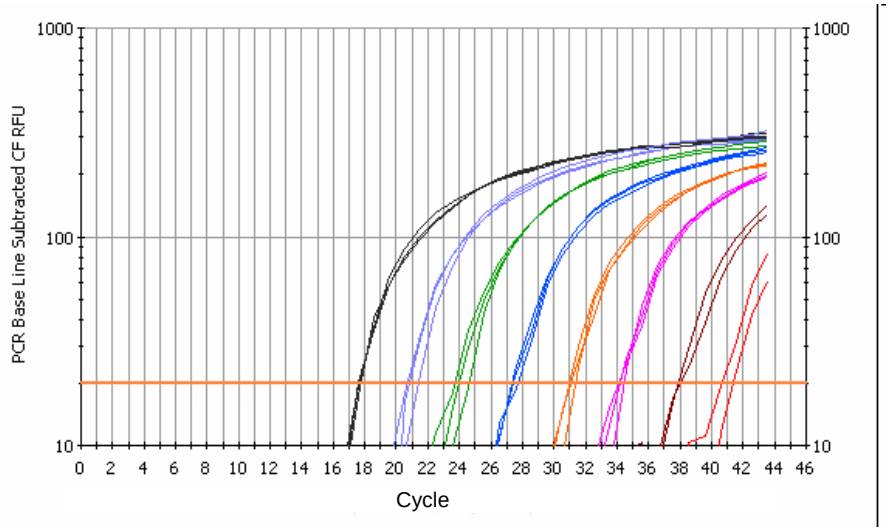
1.6×10^4

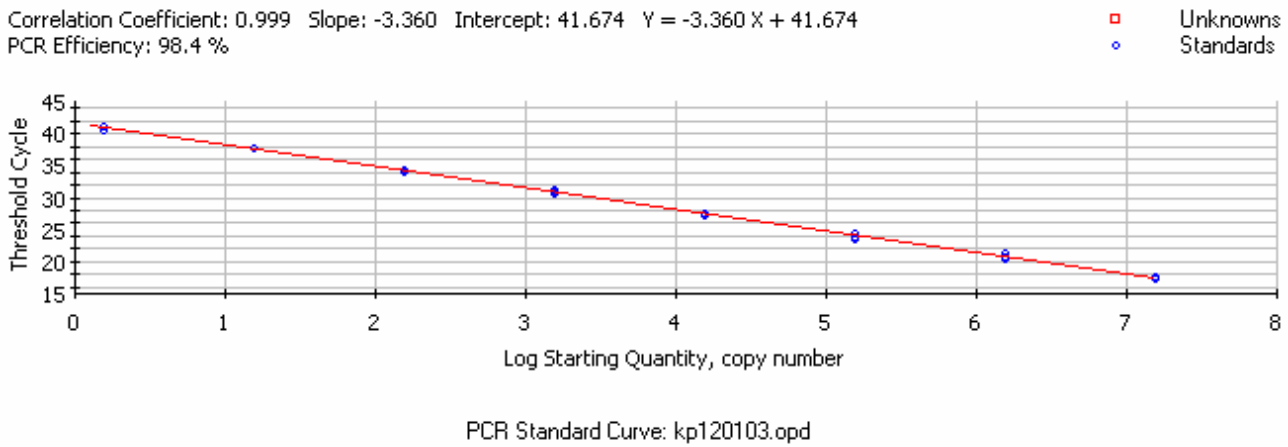
1.6×10^3

1.6×10^2

1.6×10^1

1.6×10^0





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What makes for a good qPCR?

- High Sensitivity
- Good Reproducibility
- Broad Dynamic Range

Optimization of multiplex

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Analytical Biochemistry 344 (2005) 33–42
ANALYTICAL BIOCHEMISTRY
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Four-color multiplex reverse transcription polymerase chain reaction—Overcoming its limitations

Kent Persson, Keith Hamby, Luis A. Ugozzoli *

Gene Expression Division, Bio-Rad Laboratories, Hercules, CA 94547, USA

Received 7 March 2005
Available online 29 June 2005

Topics

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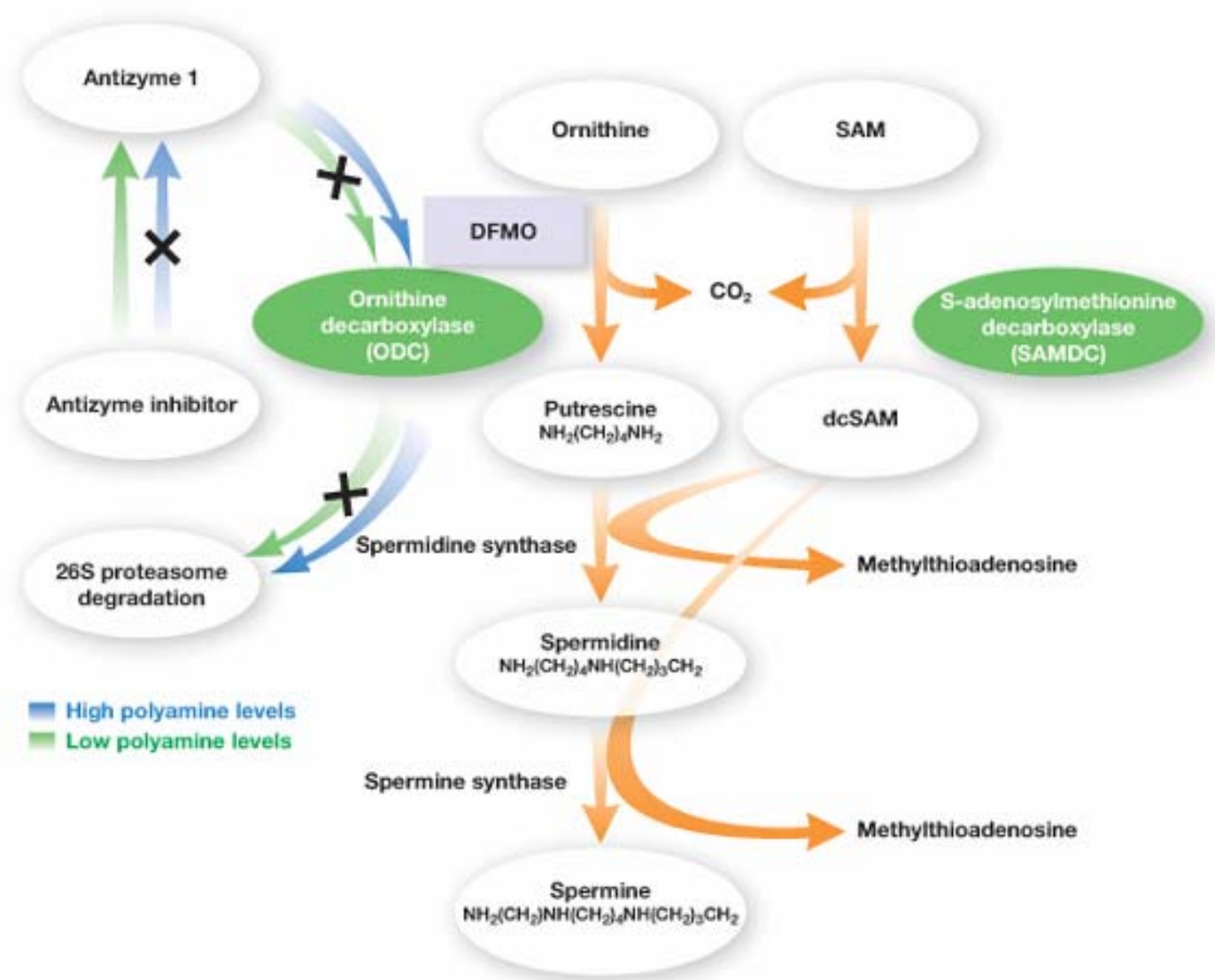
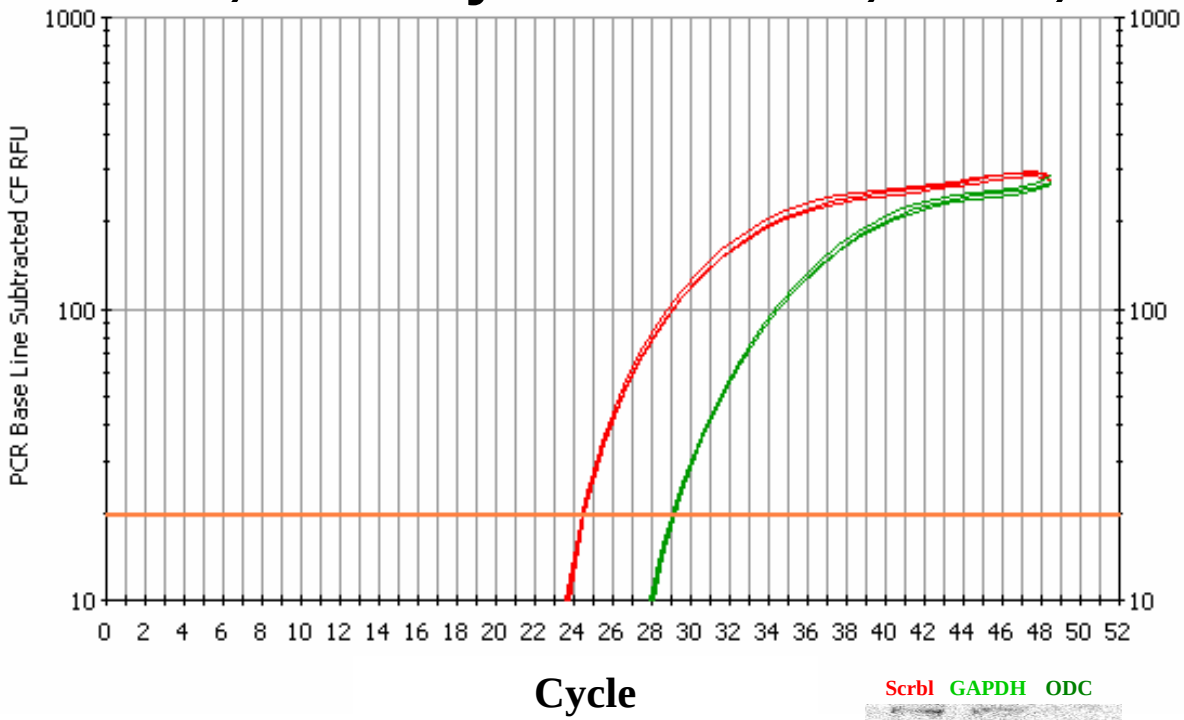
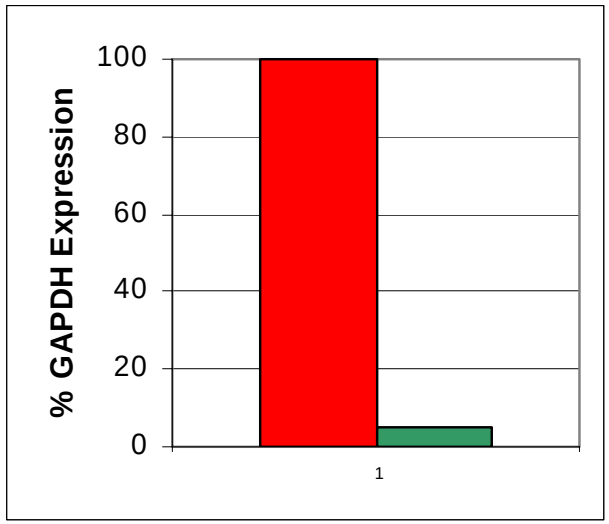
Case Study: Polyamine Pathway

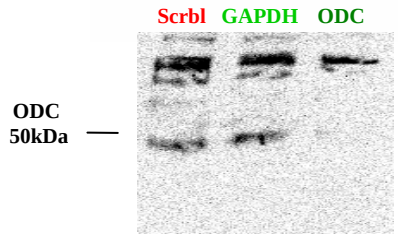


Down regulation of ODC

ODC, Primary Fibroblasts, 48 hr, 6-well







- 4.7 CT Difference
- Over 90% knockdown
- 2 µl siLentFect
- 10 nM siRNA

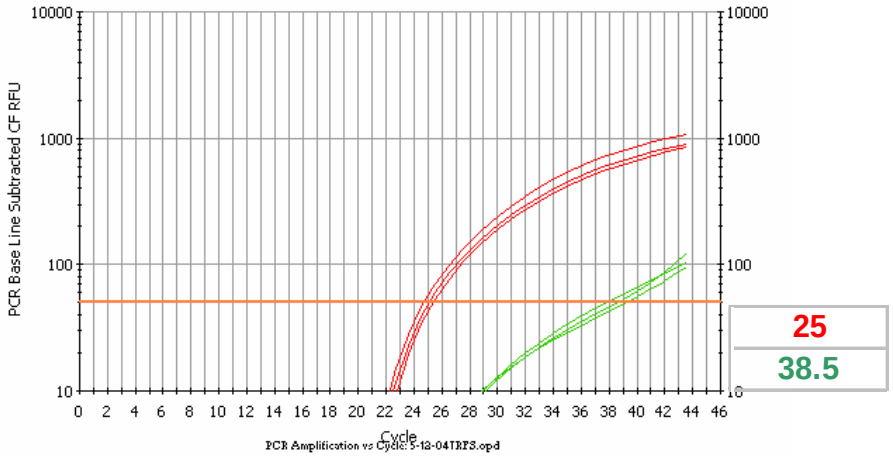
Gene Expression Division

Effect of ODC Down Regulation

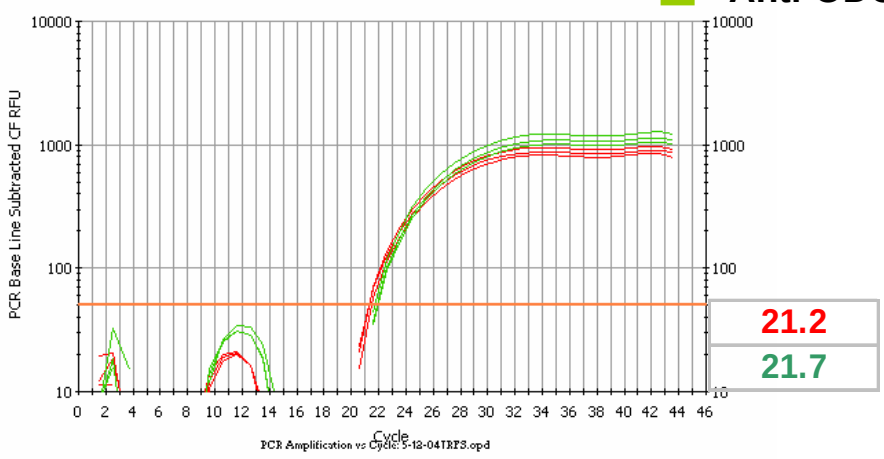
BIO-RAD

Control
Anti-ODC

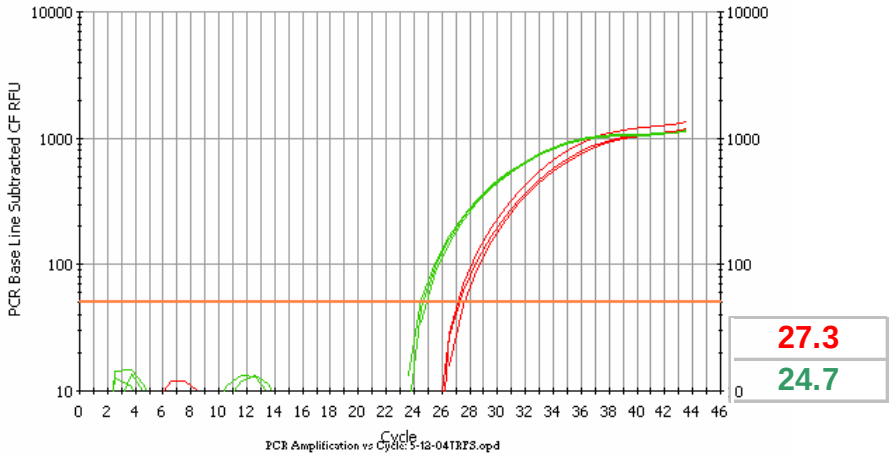
ODC



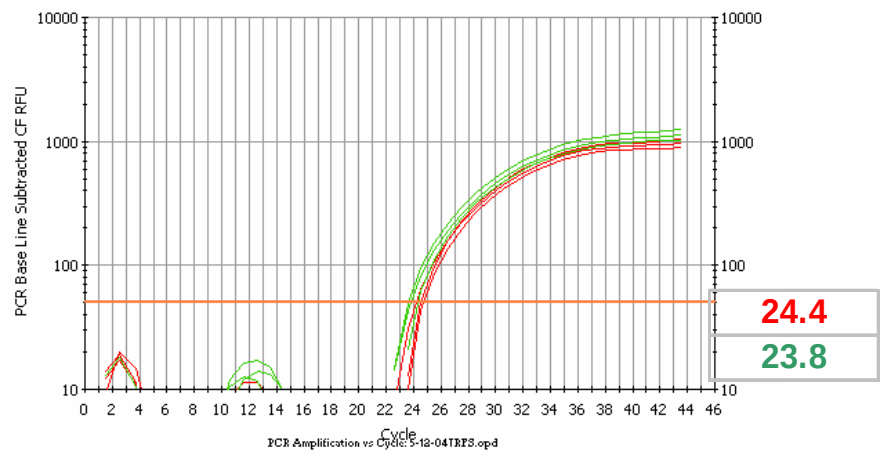
OAZ



SAMDC



AZI

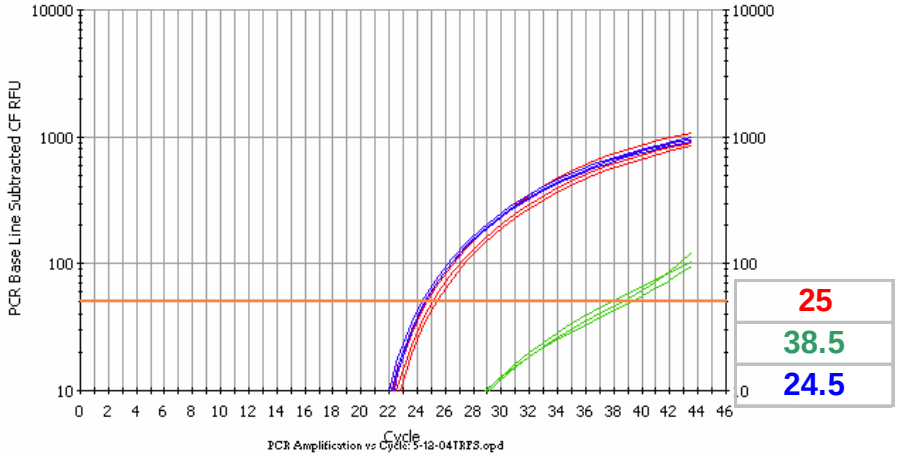


Gene Expression Division

Effect of DFMO Treatment

Control
Anti-ODC
DFMO

ODC



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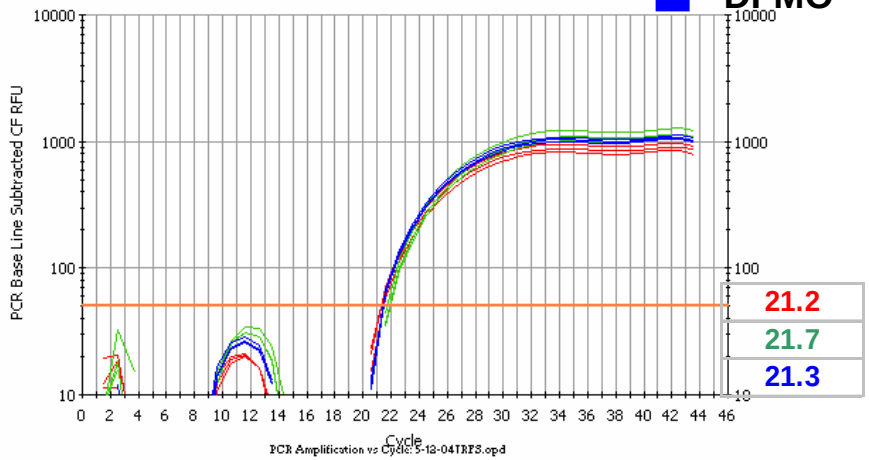
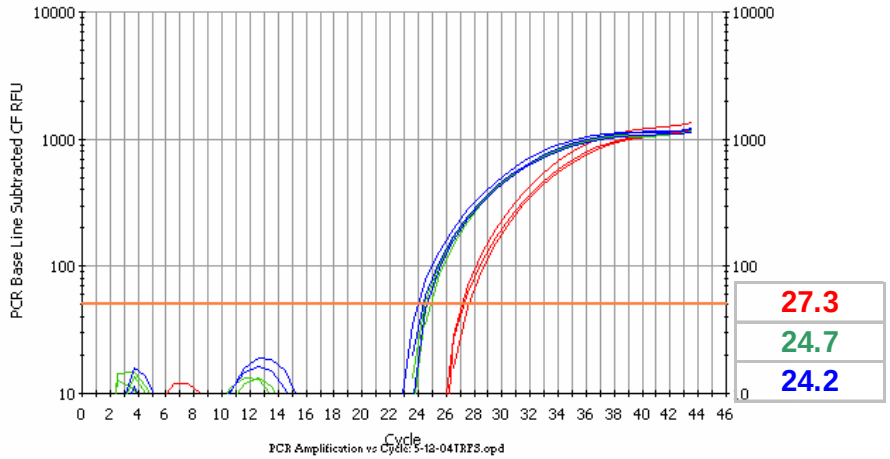
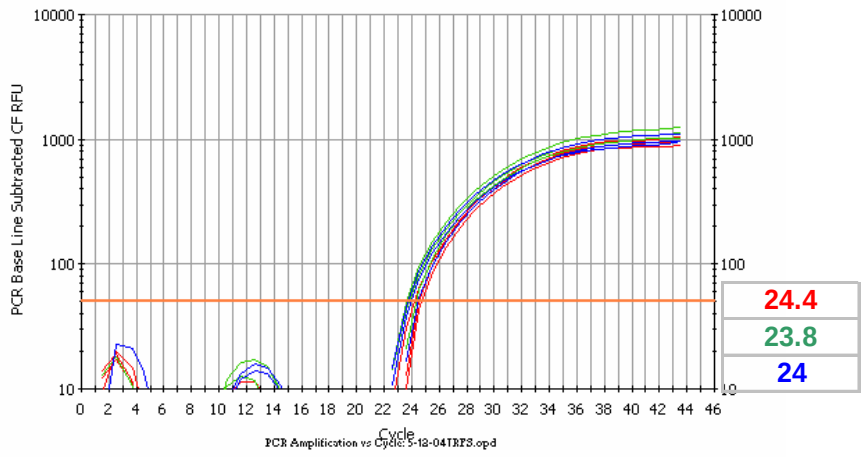


SAMDC



AZI



Gene Expression Division

Summary

- Transfection of primary fibroblasts with anti-ODC siRNA
  - results in a reduction of cellular ODC protein levels
  - results in up regulation of SAMDC transcript levels
  - regulatory enzymes OAZ and AZI were not affected (at the level of mRNA)

- Application of DFMO, which inactivates ODC protein
  - does not affect ODC transcript levels
  - results in the up regulation of SAMDC transcript levels

Summary continued

RNAi: Perfect Knockdown

- Choose a high quality RNA purification method (garbage in = garbage out)
- Good RT is critical to accurate transcript quantification
- Use a good, quantitative detection method: qPCR provides a fast, accurate, sensitive method for RNAi analysis