The Ups & Downs of Gene Expression:

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

Hilary Srere, Ph.D.

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.

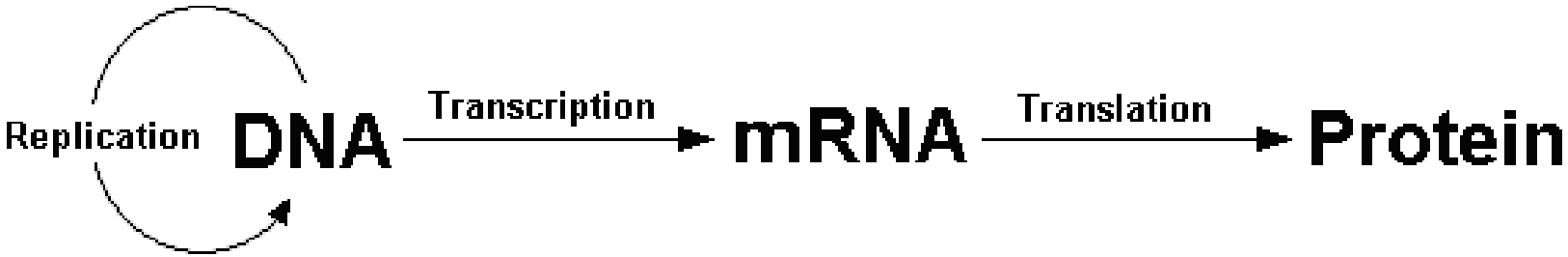
The Power of RNA Interference

Why is RNAi so powerful?

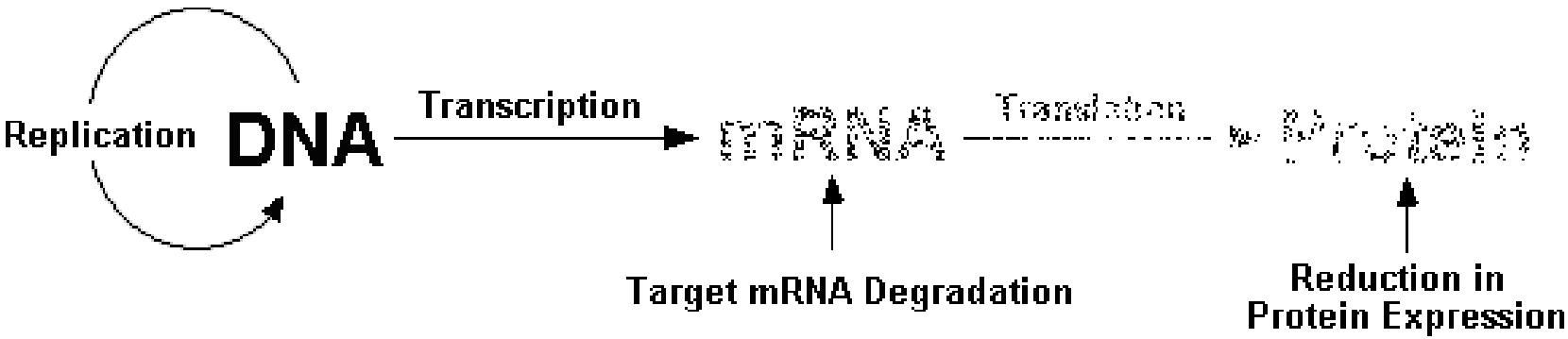
- Allows fast characterization of gene / protein function
- Enables study of pathways
- Facilitates rapid identification and validation of targets
- Therapeutic potential

Molecular Biology and RNAi

Central Dogma of Molecular Biology:



Basic RNA interference Mechanism:



Topics

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

RNAi: Challenge of Delivery

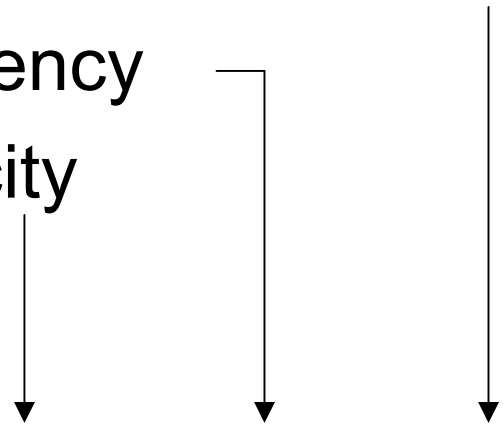
What delivery method is best?

- 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:

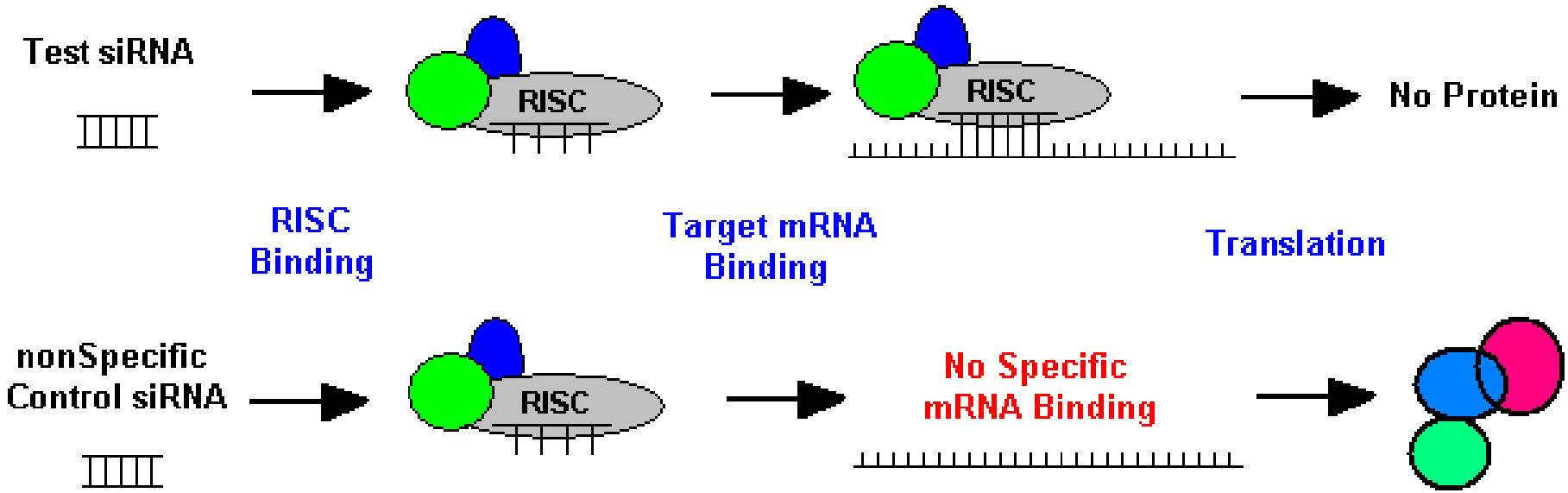
- Design / Development
- Efficiency
- Toxicity



Silencing (siRNA Activity)

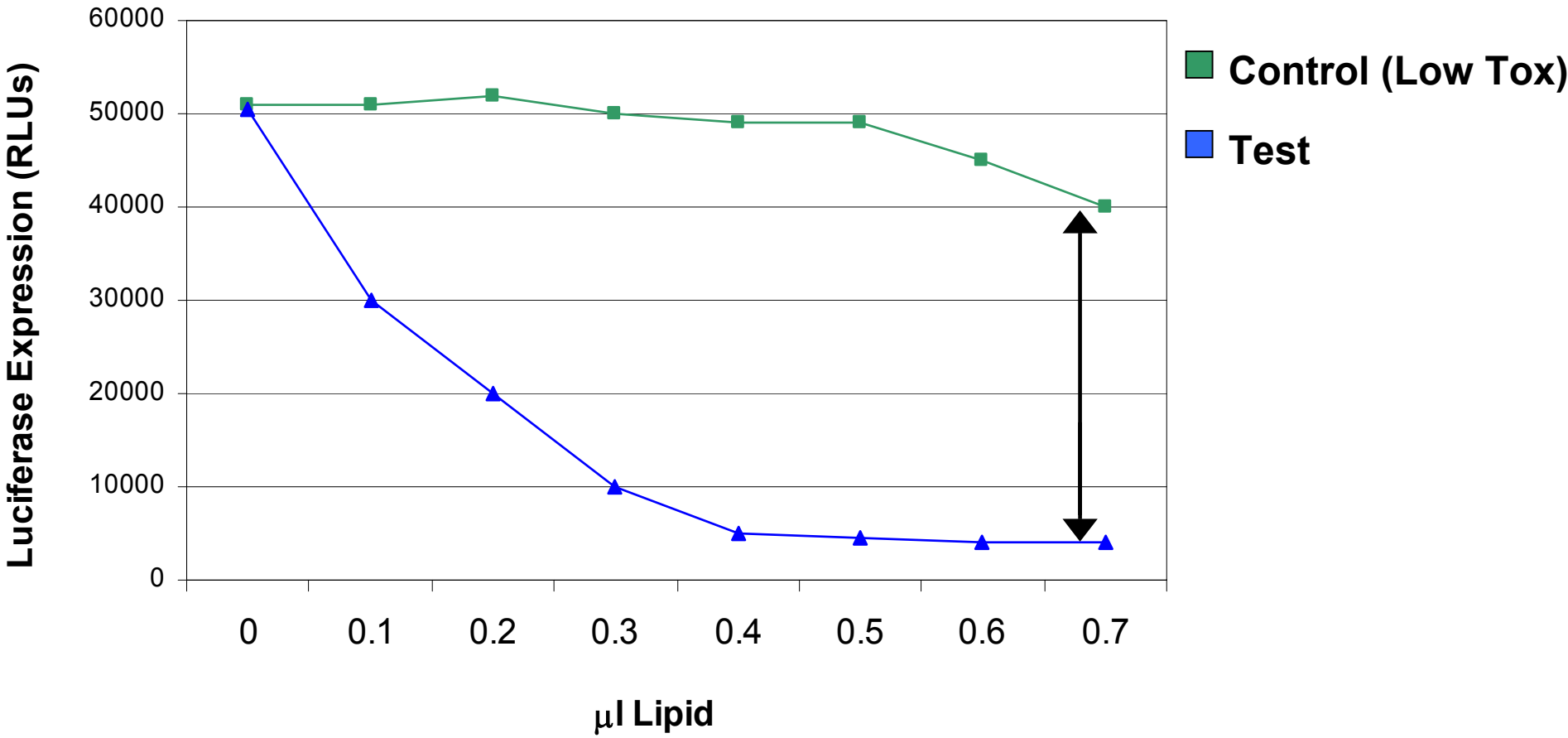
Experimental Design: Controls

Test siRNA vs. nonSpecific Control siRNA



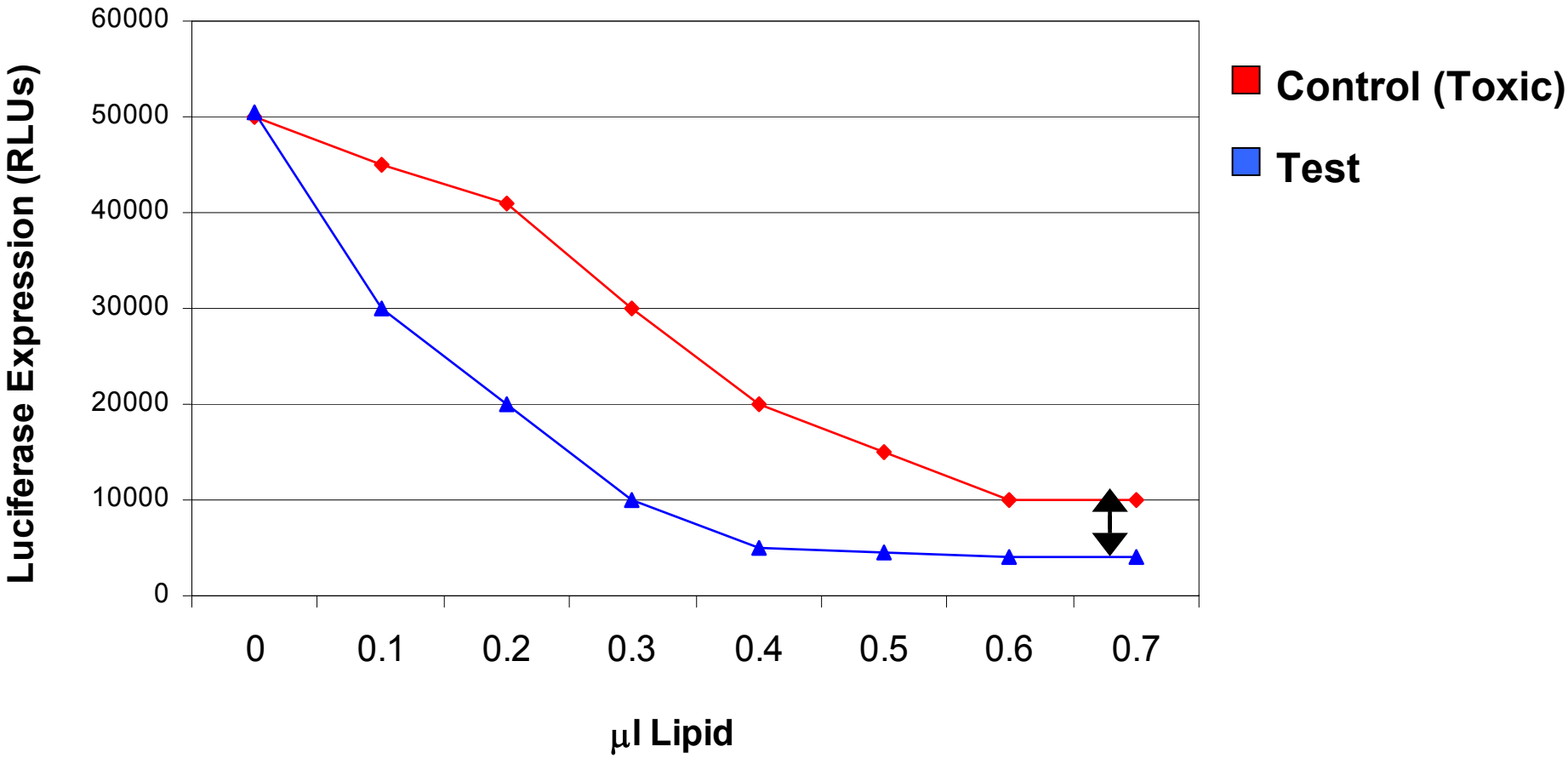
Experimental Design: Controls

How this will look as Data…….



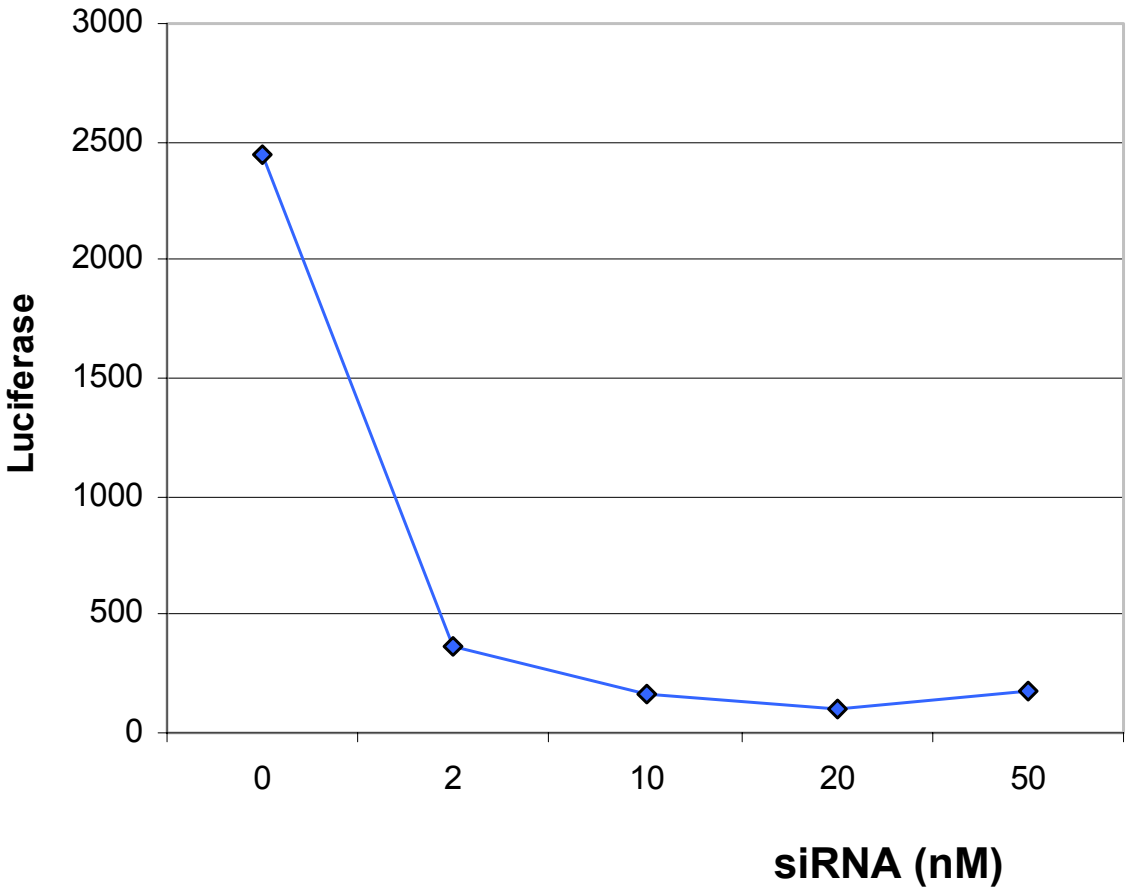
Experimental Design: Controls

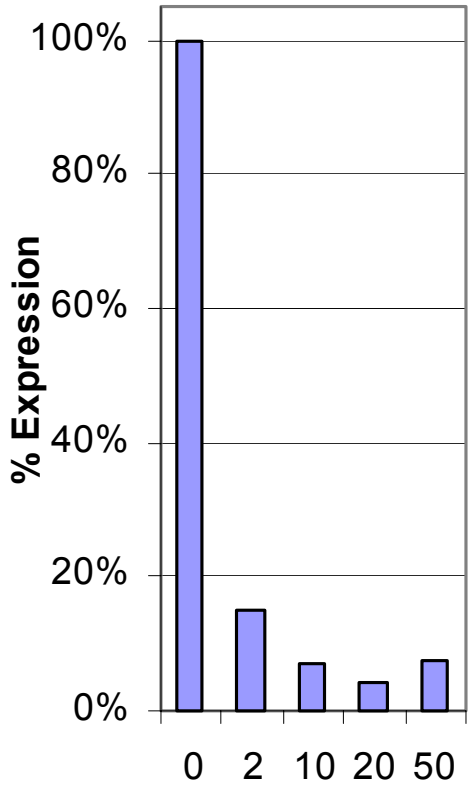
How this will look as Data…….



Efficiency: siRNA Amount

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



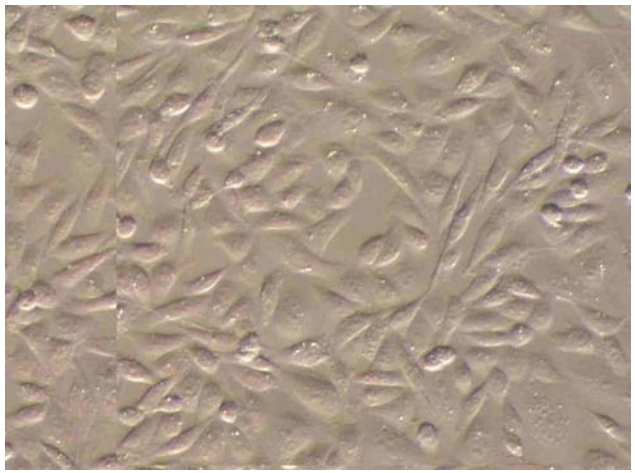


Toxicity Evaluations

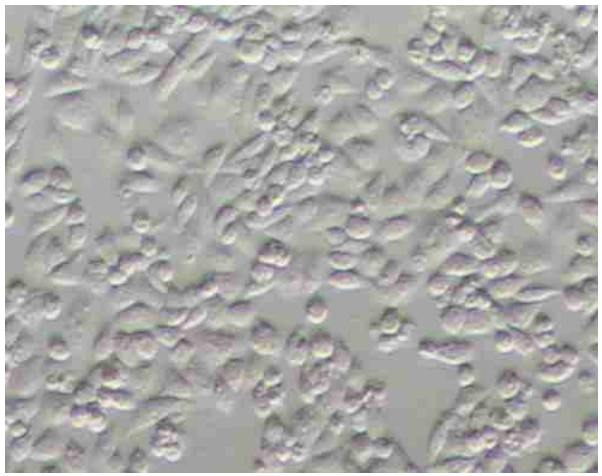
Visual Analysis

- Morphology changes
- Detachment
- Lysis

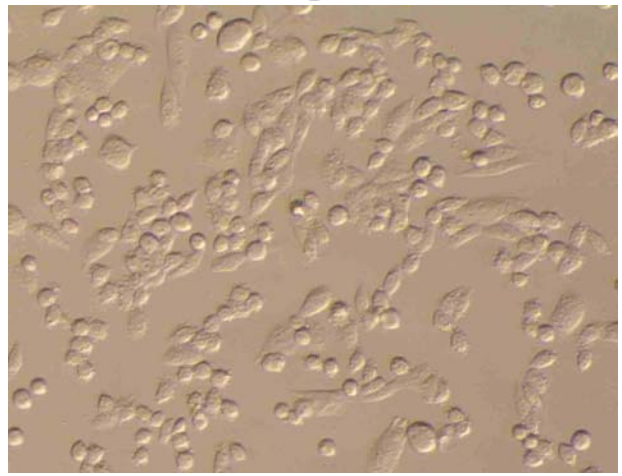
Low



Moderate

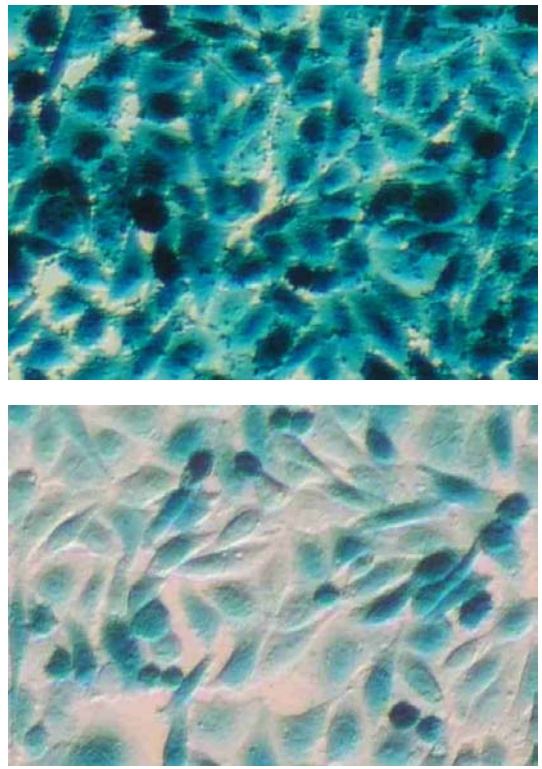


High



RNAi Detection Strategies

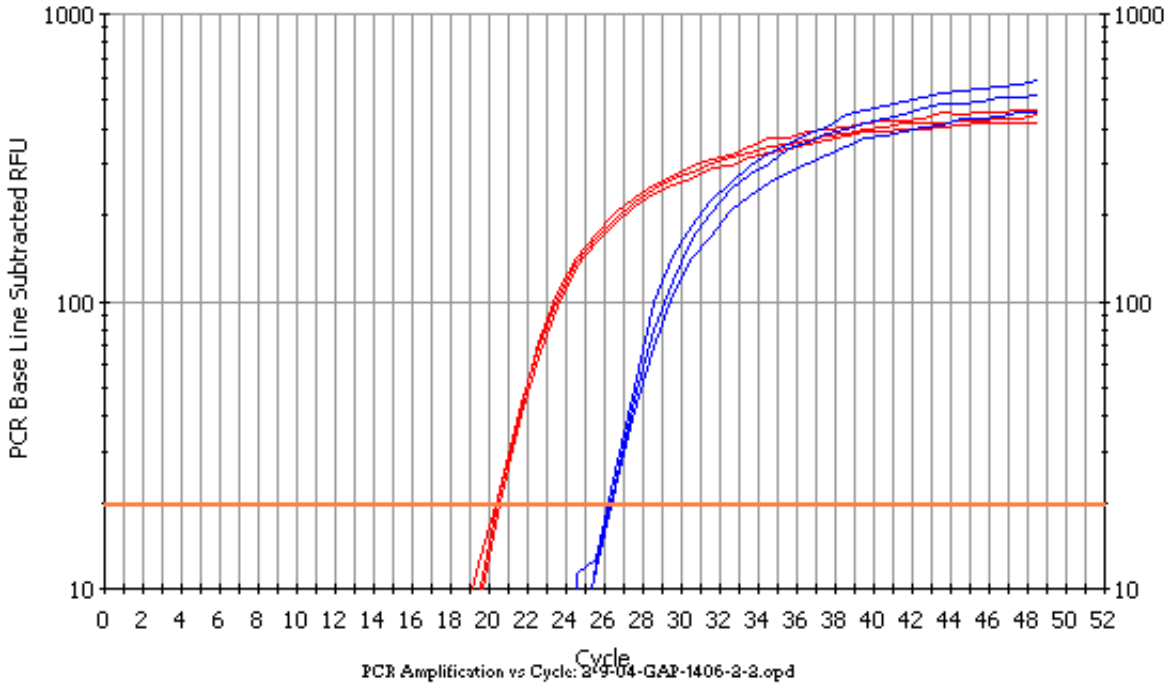
- Western Blots
- Northern Blots
- MicroArrays
- qPCR
  - **1.0 Cycle Threshold = 50% silencing**
  - **3.3 Cycle Threshold = 90% silencing**
  - **6.6 Cycle Threshold = 99% silencing**

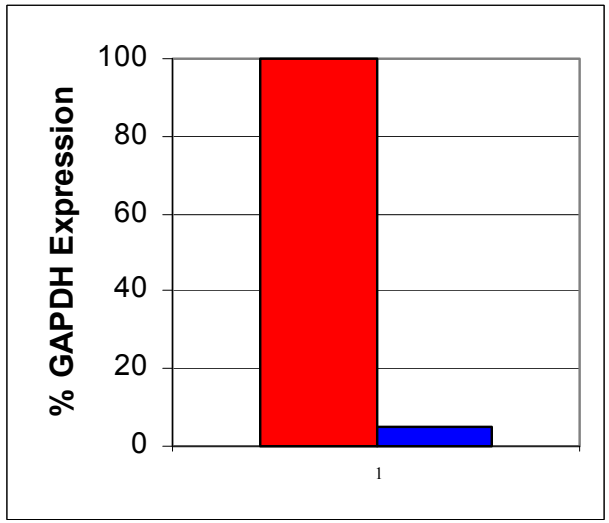


CHO-lacZ cells transfected with scrambled siRNA control (top) and beta-gal siRNA (bottom)

Detection: qPCR Analysis

GAPDH, Primary Fibroblasts, 48 hr, 6-well

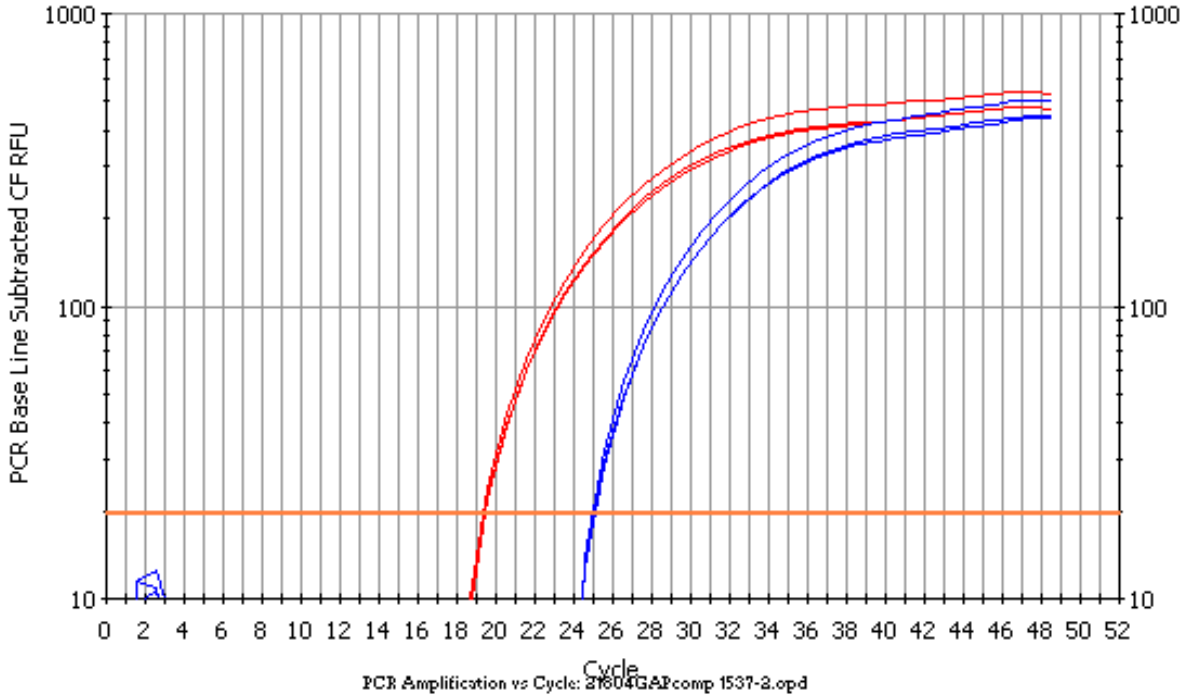


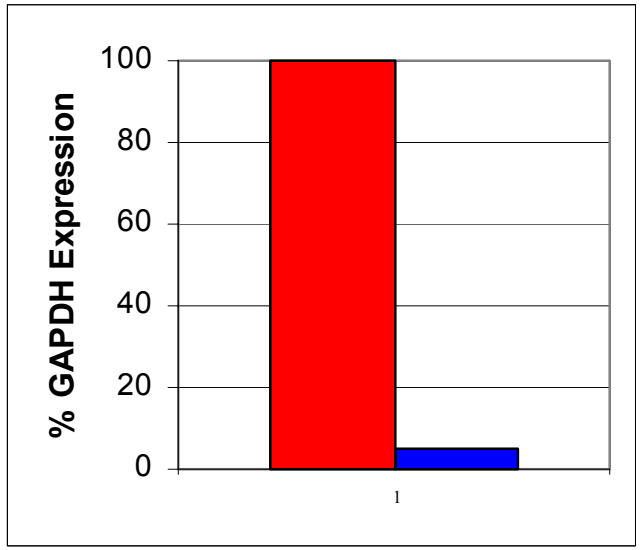


- 5.9 C_t Difference
- Over 95% knockdown
- 1.25 µl siLentFect
- 10 nM siRNA

Detection: qPCR Analysis

GAPDH, HeLa Cells, 48 hr, 6-well





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

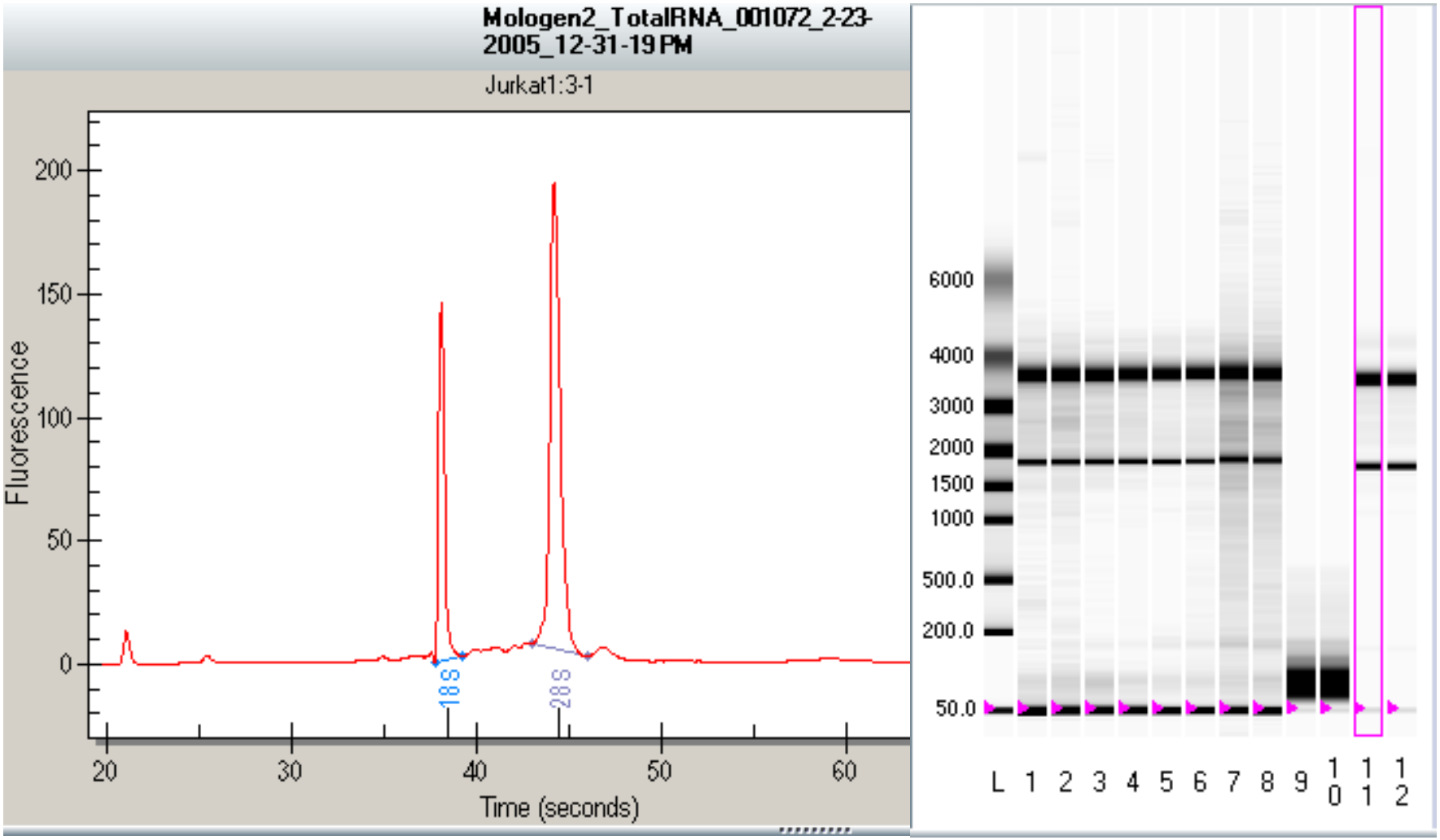
Topics

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

RNA Preparation

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

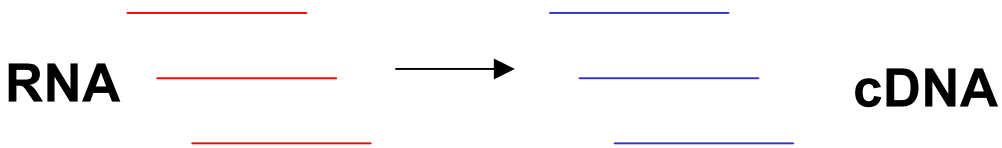
Experion System Data



Topics

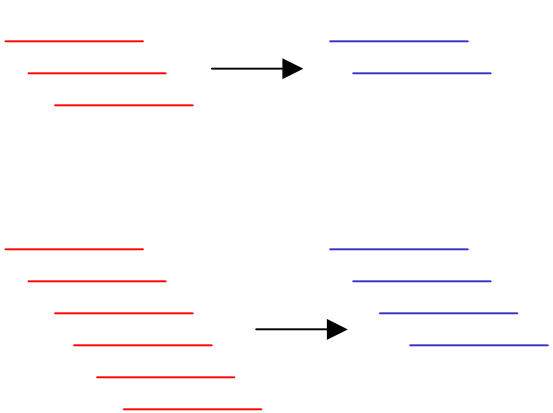
- What is RNAi?
- Methods of Delivery
- RNA Preparation
- Reverse Transcription
- qPCR Detection
- Case Study: ODC Pathway

Testing the Reverse Transcriptase

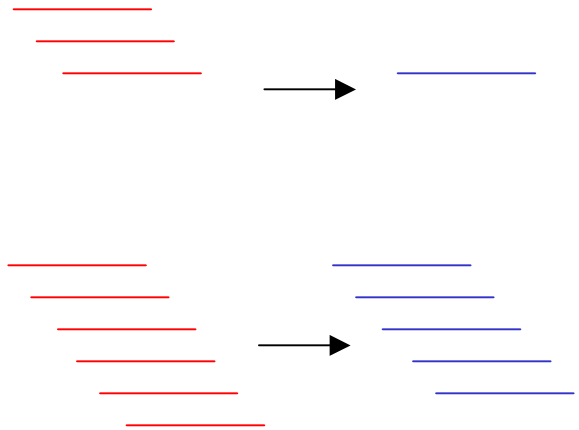


RT Efficiency: Its Effect on the Assay

Reproducible ?

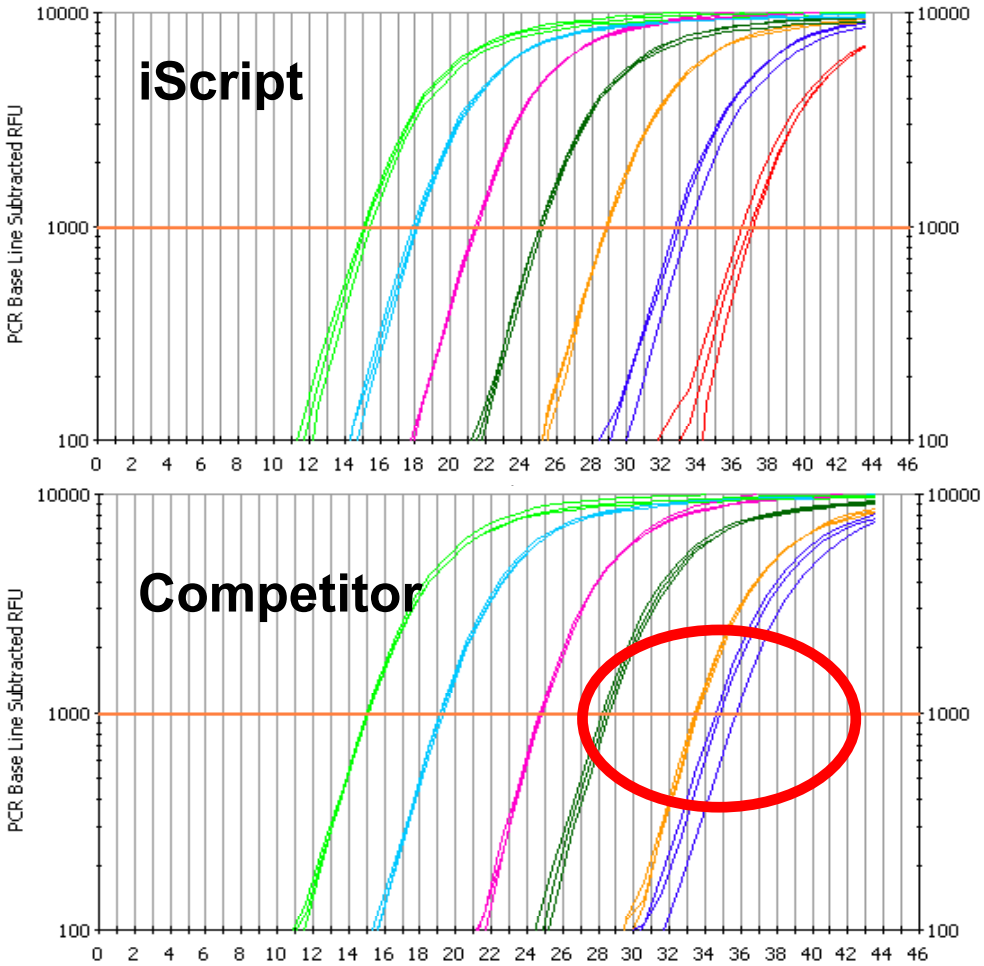


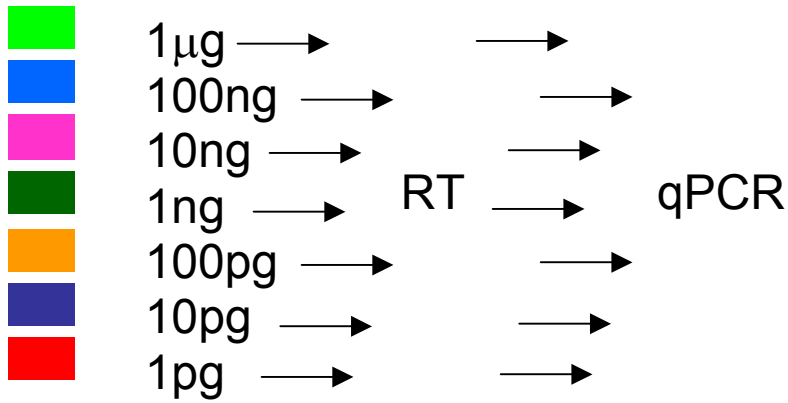
Reproducible Data



Not Reproducible

Reproducibility of RT





No discrimination at low Concentration

No detection at 1 pg

Dynamic Range of iScript

1.6×10^7

1.6×10^6

1.6×10^5

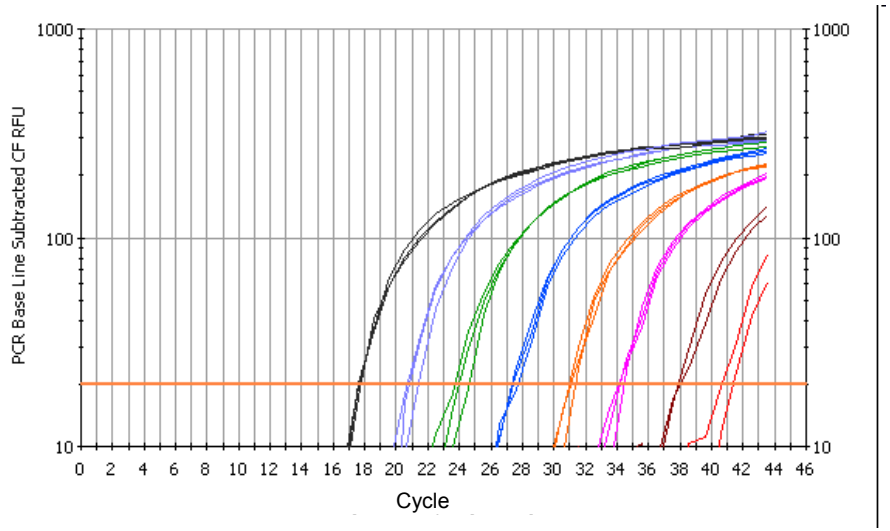
1.6×10^4

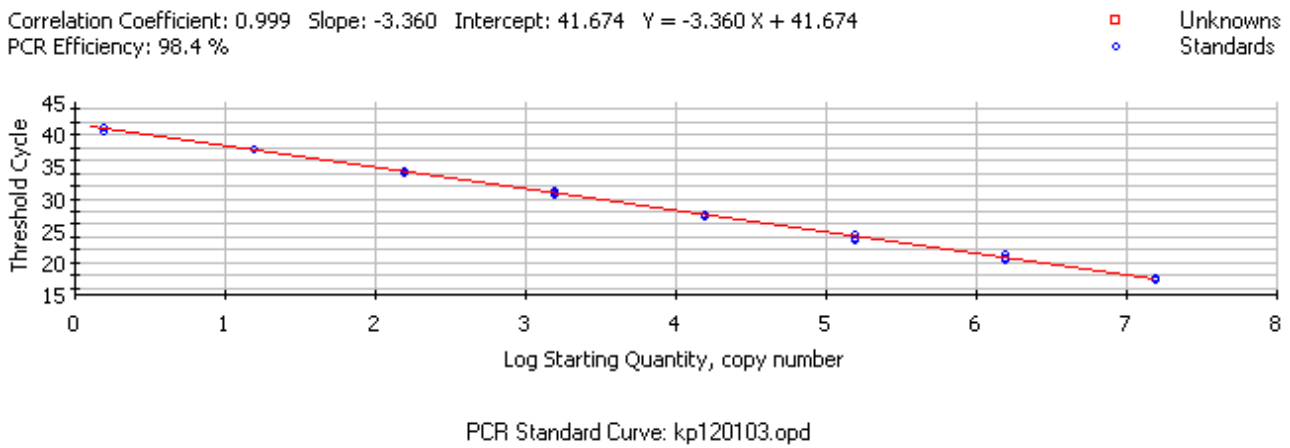
1.6×10^3

1.6×10^2

1.6×10^1

1.6×10^0





PCR Standard Curve: kp120103.opd

Topics

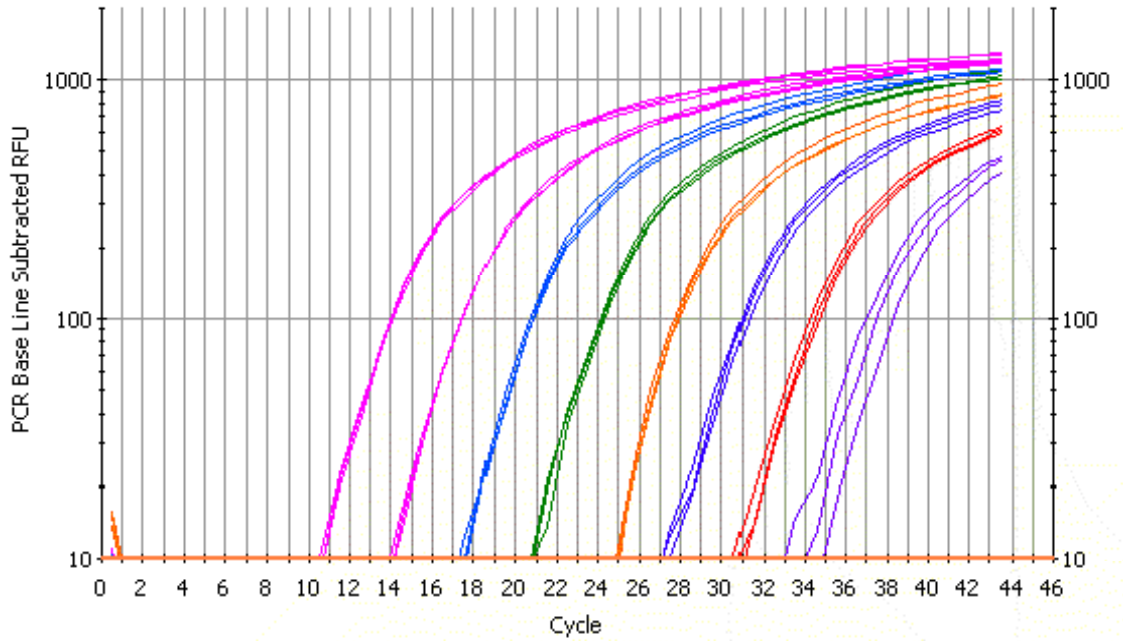
- What is RNAi?
- Methods of Delivery and Detection
- RNA Preparation
- Reverse Transcription
- qPCR Detection
- Case Study: ODC Pathway

What makes for a good qPCR?

- High Sensitivity
- Good Reproducibility
- Broad Dynamic Range

Dynamic Range of One-Step RT-qPCR

Beta-actin target, FAM-labeled probe, 1μg to 100fg input

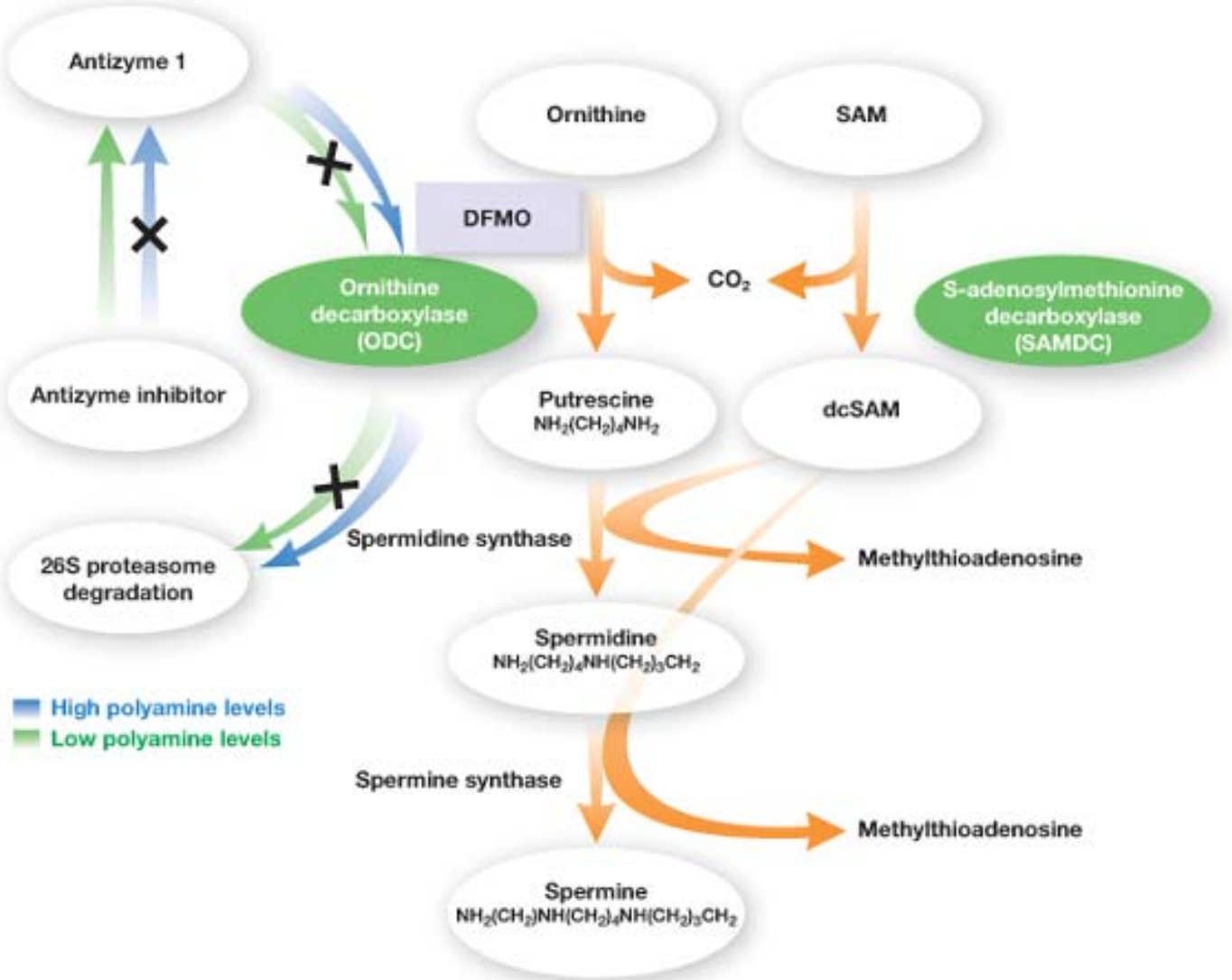


r = 1.000, slope = -3.39, efficiency = 97.2%

Topics

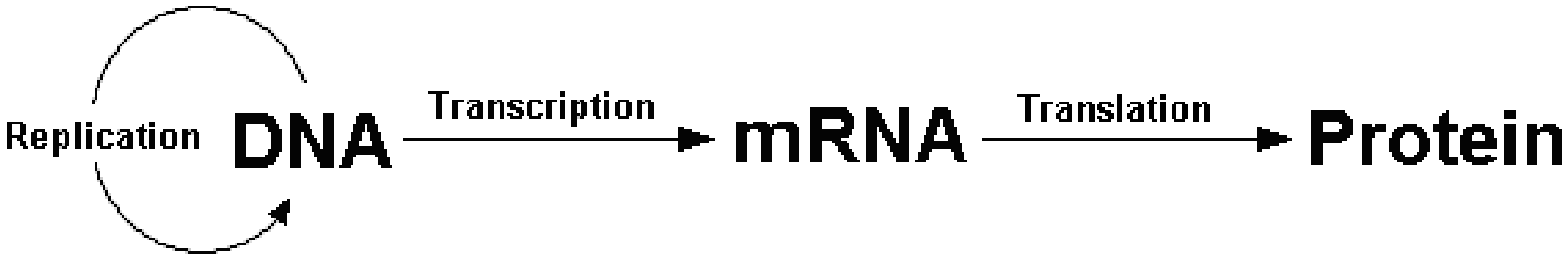
- What is RNAi?
- Methods of Delivery and Detection
- RNA Preparation
- Reverse Transcription
- qPCR Detection
- Case Study: ODC Pathway

Case Study: Polyamine Pathway



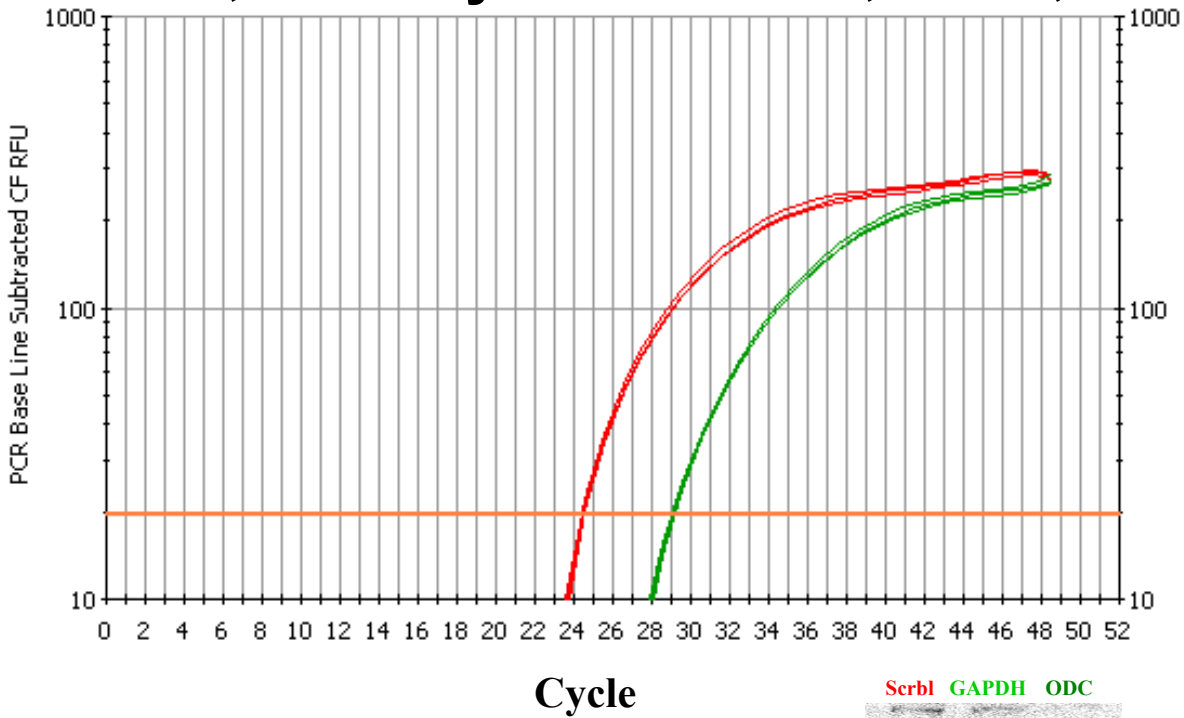
Points of Regulation

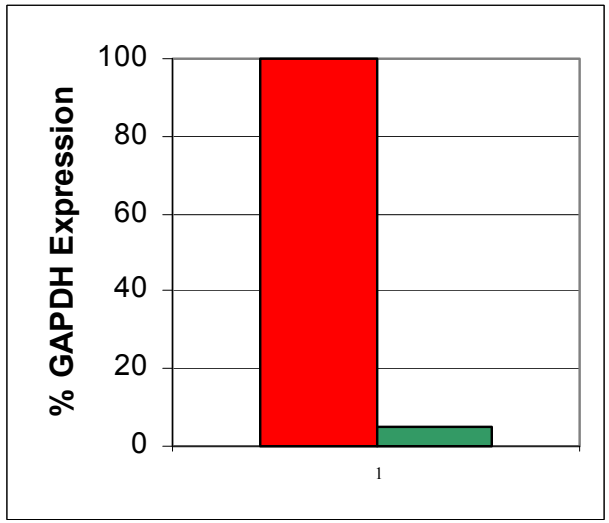
Central Dogma of Molecular Biology:

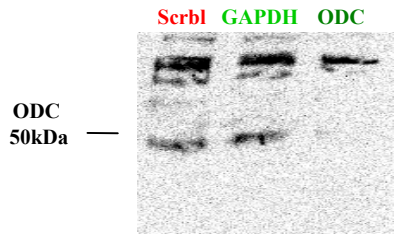


Down regulation of ODC

ODC, Primary Fibroblasts, 48 hr, 6-well







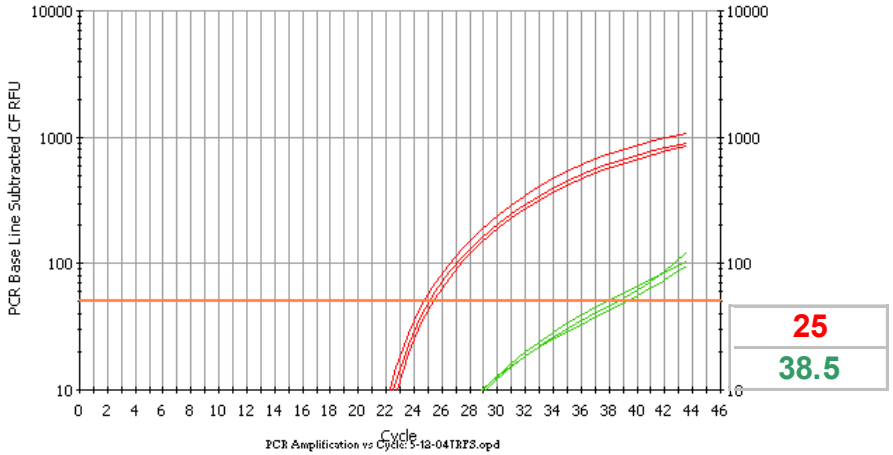
- 4.7 CT Difference
- Over 90% knockdown
- 2 µl siLentFect
- 10 nM siRNA

Effect of ODC Down Regulation

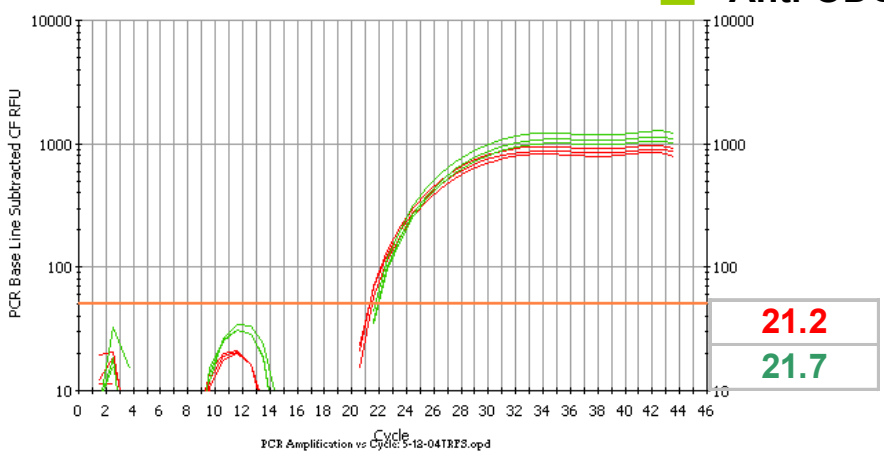
BIO-RAD

Control
Anti-ODC

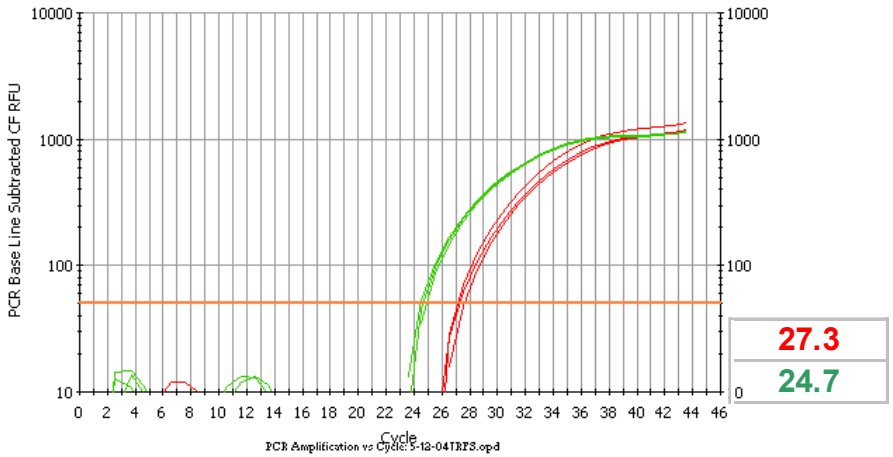
ODC



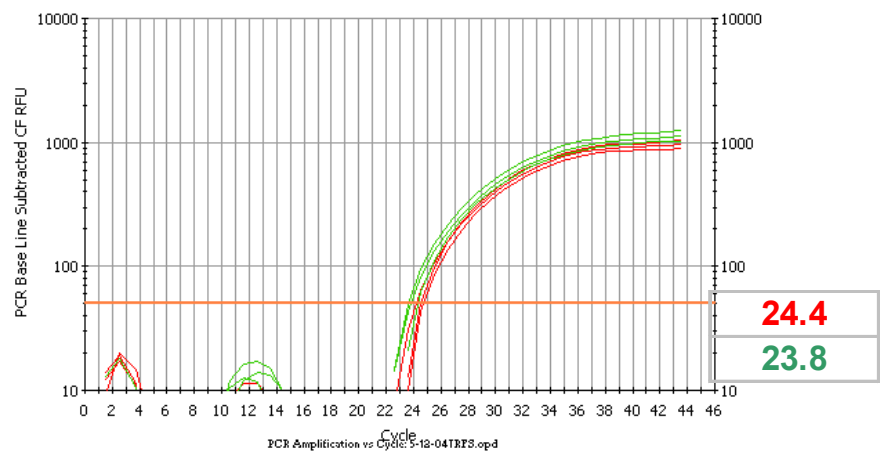
OAZ



SAMDC



AZI

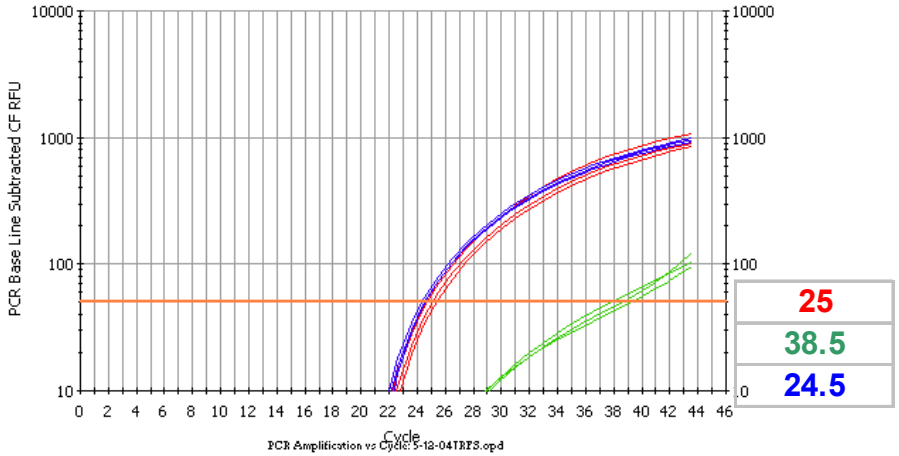


Gene Expression Division

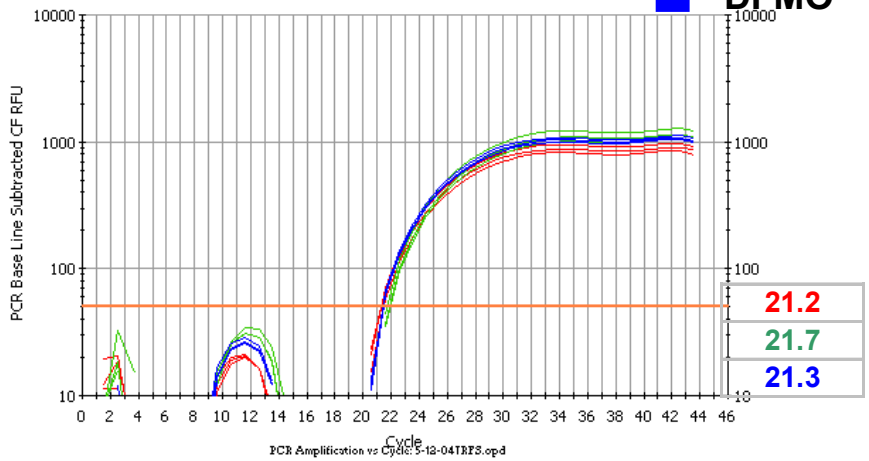
Effect of DFMO Treatment

Control
Anti-ODC
DFMO

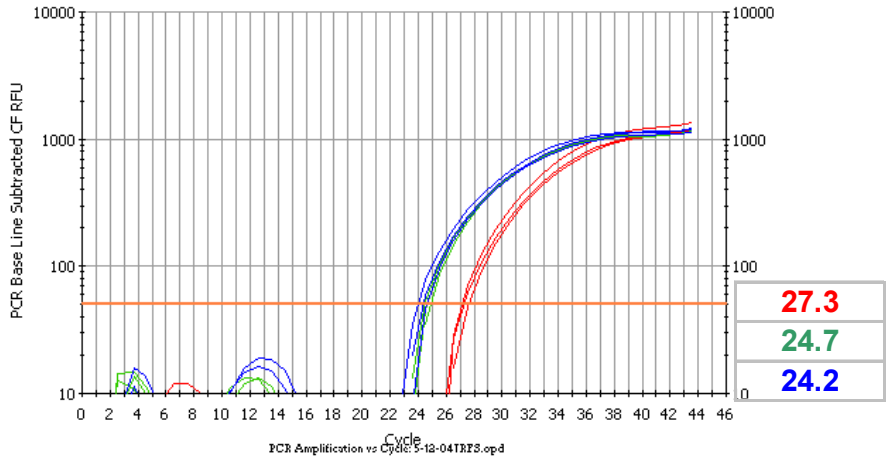
ODC



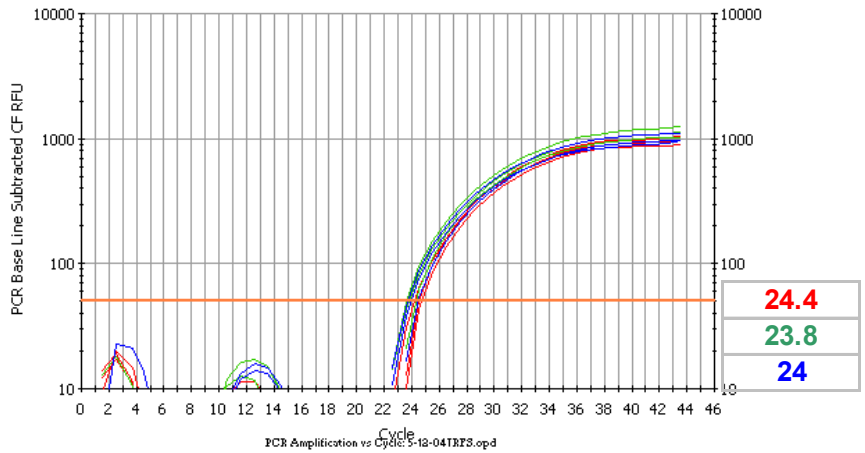
OAZ



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