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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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.


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:
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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)
Experimental Design: Controls

Test siRNA vs. nonSpecific Control siRNA

- Test siRNA:
  - RISC Binding
  - Target mRNA Binding
  - No Protein

- nonSpecific Control siRNA:
  - RISC Binding
  - No Specific mRNA Binding
  - Translation
Experimental Design: Controls

How this will look as Data……..

- **Luciferase Expression (RLUs)**
- **µl Lipid**

Data representation:
- **Control (Low Tox)**
- **Test**
Experimental Design: Controls

How this will look as Data…….

![Graph showing Luciferase Expression (RLUs) vs. \( \mu \text{l Lipid} \)]

- Red line: Control (Toxic)
- Blue line: Test
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

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Detection: qPCR Analysis

GAPDH, HeLa Cells, 48 hr, 6-well

- 5.6 $C_t$ Difference
- Over 95% knockdown
- 1.25 $\mu$l siLentFect
- 10nM siRNA
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RNA Preparation

• Extract RNA (DNase treatment optional)

• Analyze RNA, careful quantification is necessary:
  RiboGreen assay
  Experion™ System
Experion System Data
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Testing the Reverse Transcriptase

RNA → cDNA
RT Efficiency: Its Effect on the Assay

RNA → cDNA

Reality?

Reproducible Data

Not Reproducible
Reproducibility of RT

No discrimination at low Concentration
No detection at 1 pg
Dynamic Range of iScript

1.6 x 10^7
1.6 x 10^6
1.6 x 10^5
1.6 x 10^4
1.6 x 10^3
1.6 x 10^2
1.6 x 10^1
1.6 x 10^0
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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%
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Case Study: Polyamine Pathway

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Points of Regulation

Central Dogma of Molecular Biology:

Replication → DNA → Transcription → mRNA → Translation → Protein

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Down regulation of ODC

ODC, Primary Fibroblasts, 48 hr, 6-well

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

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Effect of ODC Down Regulation

ODC

SAMDC

OAZ

AZI

Control

Anti-ODC

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Effect of DFMO Treatment

ODC

Control: 25, 38.5, 24.5
DFMO: 27.3, 24.7, 24.2

OAZ

DFMO: 24.4, 23.8, 24

SAMDC

AZI
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
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