The Ups & Downs of Gene Expression:

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

Teresa Rubio, Ph.D.
Hilary Srere, Ph.D., presenter
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:

![Diagram of DNA replication, transcription, mRNA, and protein translation]

Basic RNA interference Mechanism:

![Diagram showing RNA interference with target mRNA degradation and reduction in protein expression]
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)
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

Test siRNA

RISC Binding

RISC

Target mRNA Binding

Translation

No Protein

nonSpecific Control siRNA

RISC

No Specific mRNA Binding

Gene Expression Division
Experimental Design: Controls

How this will look as Data……..

[Graph showing Luciferase Expression (RLUs) vs. µl Lipid with two lines: one for Control (Low Tox) and one for Test.]
Experimental Design: Controls

How this will look as Data......

Luciferase Expression (RLUs)

µl Lipid

Control (Toxic)
Test
Toxicity Evaluations

Visual Analysis
- Morphology changes
- Detachment
- Lysis
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 Ct Difference
- Over 95% knockdown
- 1.25 µl siLentFect
- 10nM siRNA
Silencing Endogenous Genes in Multiple Cell lines

FS GAP

LNCaP GAP

HUVEC GAP

Relative Expression of GAPDH

Gene Expression Division
Several siRNAs in single transfection

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

**Relative Expression of FS multiple genes**

- EGFP
- b-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

Silencing of endogenous mRNAs at low 27mer siRNA concentrations:

- **HPRT**
  - 5/5 >85% at 10 nM
  - 4/5 >85% at 100 pM

- **β-ACTIN**
  - 5/9 >85% at 10 nM
  - 4/9 >85% at 100 pM

- **GAPDH**
  - 5/5 >85% at 10 nM
  - 4/5 >85% at 100 pM

Gene Expression Division

BIO-RAD
Topics

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

Intact

Degraded

GAPDH siRNA

Intact

Degraded

A

B

C

D

PCR base line substituted

PCR base line substituted

PCR base line substituted

PCR base line substituted

Gene Expression Division
Topics

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

Correlation Coefficient: 0.999  Slope: -3.360  Intercept: 41.674  \( Y = -3.360X + 41.674 \)
PCR Efficiency: 98.4%

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
Optimization of multiplex

Analytical Biochemistry 344 (2005) 33–42

Available online at www.sciencedirect.com

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

• What is RNAi?
• Methods of Delivery and Detection
• RNA Preparation
• Reverse Transcription
• qPCR Detection
• Case Study: ODC Pathway
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
Effect of ODC Down Regulation

ODC

SAMDC

OAZ

AZI

Control

Anti-ODC

25
38.5

21.2
21.7

27.3
24.7

24.4
23.8
Effect of DFMO Treatment

**ODC**

- Control: 25
- Anti-ODC: 38.5
- DFMO: 24.5

**OAZ**

- Control: 21.2
- Anti-ODC: 21.7
- DFMO: 21.3

**SAMDC**

- Control: 27.3
- Anti-ODC: 24.7
- DFMO: 24.2

**AZI**

- Control: 24.4
- Anti-ODC: 23.8
- DFMO: 24
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