siRNA is a hot topic!

Craig C. Mello, PhD,
Nobel Prize in Medicine 2006

New Technologies for RNAi Screening

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New Technologies for RNAi Screening

- Introduction
- siRNA Design
- Transfection of siRNA
- Critical factors for Screening Applications
- miRNA

Global Set-up With Three Headquarters in Major Markets

- Switzerland = Automation Center of Excellence
- Hamburg = Assay Center of Excellence
- California = Customer satisfaction center
- Singapore = Customer satisfaction center
- 30 Subs = Direct sales and marketing

Gaitherstown
~ 800 employees

Düsseldorf
~ 1,000 employees

Shanghai/Shenzhen
~ 400 employees
Mechanism of RNAi

- **Defense mechanism**
- **miRNA effects**
- **Target specificity**

New Technologies for RNAi Screening

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BioPred si:
An Algorithm Trained to Select Potent siRNA

Hall. et al. Nature Biotechnology July 2005

Evaluation of siRNA design algorithms (Matveeva, O. et al., NAR, 2007):
- Prediction rate true/ false positives + Prediction rate true/ false negatives
- Statistical analysis on the basis of independent publicly accessible siRNA data bases
- BioPredsi > ThermoComposition > DSIR

HP OnGuard siRNA Design
Reducing miRNA related off-target effects

3'UTR-Seed Region Analysis

- Seed region
  - Position 2-7 of miRNA / siRNA sequence
  - miRNA binding to mRNA through seed region
  - Presence of multiple seed region matches increases likelihood of off-target effects
**HP OnGuard siRNA Design**

- **SNP avoidance:**
  optimized to avoid all known single nucleotide polymorphisms (SNPs) using RefSNP database

- **Interferon motif avoidance**: screened for multiple sequence motifs known to result in an interferon response

*Hornung et al., Nature Medicine, 2005
Judge et al., Nature Biotech, 2005

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**QIAGEN siRNA Validation Project**

- Experimentally proven functionality
- Per siRNA 8 independent data points (replications etc.)
- Largest validated siRNA set (> 3700 siRNAs)
- Algorithm feeds validation, validation feeds algorithm
- QIAGEN algorithm based on ~ 8000 siRNAs (3000 genes)

Krüger et al., 2007; Insights into Effective RNAi Gained from Large-Scale siRNA Validation Screening. Oligonucleotides 17:237–250
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siRNA Transfection

Characteristics of HiPerFect Transfection Reagent:
- Minimal concentration of siRNA needed
- Minimal cytotoxicity
- Working also in many adherent primary cell types and some suspension cell lines
- Simple optimization/ tolerating broad range of cell densities
- Simple protocol/ easy to adapt to robotic systems
Example: Optimization of HiPerFect transfection for difficult-to-transfect cell type

Optimization = Balance between Efficiency and minimal OTE

![Graph showing relative norm. CDC2 expression over time for UASMC, fine tuning of protocol.

Minimal Off-target Effects with HiPerFect Reagent

Untransf.: HiPerFect

Vacuoles

Reagent L
Optimization of Transfection

High-Throughput-specific parameters for optimization:

- Stability of stock solutions (reproducibility)
- Interaction of reagent with material of flexible tubes etc.
- Liquid handling of reagent solutions
- Edge effects on culture plates
- ...

*Very specific for every system!*

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Screening for Survival Kinases: QIAGEN Kinase siRNA Library Screen

Day 1: Seed Cells  
Day 2: siRNA Transfection  
Day 4: Drug Treatment  
Day 5: Cell Death ELISA

MacKeigan, Murphy, and Blenis, Nature Cell Biology 2005

Quantify ELISA on plate reader

QIAGEN Human Genome siRNA Sets

Whole Genome v 4.0, launched in Jan 2008

18000 NMs

7000 genes

691 Kinases

205 Phosphatase

495 GPCR

Druggable Genome v4.0

Predicted Genes

5500 XMs

- 2 siRNA per target gene, 4 siRNA, pool
- 100 pmol, 250 pmol, 1 nmol
- 96 and 384 well
- Mouse WG, Rat DG

- Mouse Genome siRNA Sets: Identical to Human Sets
- Rat Druggable Genome Set
Critical factors for Screening Applications

- Pooling of siRNAs: Specificity versus cost effectiveness
- siRNA modifications
- RNAi rescue experiments: Identifying true positive hits
- Control siRNAs

Pooling of siRNAs

**Background:**

<table>
<thead>
<tr>
<th>siRNAs:</th>
<th>Separate siRNAs</th>
<th>Pools</th>
</tr>
</thead>
<tbody>
<tr>
<td>siRNA 1</td>
<td>siRNA 2</td>
<td>siRNA 3 (off target)</td>
</tr>
<tr>
<td>siRNA 2</td>
<td>siRNA 3 (off target)</td>
<td>siRNA 2</td>
</tr>
</tbody>
</table>

**Transfection:**

- Phenotype of siRNA is off target effect

**Interpretation:**

- Phenotype with all 4 siRNAs?
Reduction in False Positive Rate by Redundancy

Screen 1: DG1 2 duplexes per target, replicate txn
9% of single hits positive in validation
38% of double hits positive in validation

Screen 2: DG2 4 duplexes per target, replicate txn
>60% of hits with >1 hit positive in validation
>80% of hits with >2 hits positive in validation

Better design = more positive siRNA
Better Informatics = more specific siRNA

Data courtesy of S. Mousses, TGEN

What about pooling of siRNA?

High Throughput/Screening:

Low specificity of assay for analysis
⇒ many false positives
⇒ cost of validation is more relevant

Highly specific assay for analysis
⇒ less false positives
⇒ cost of screening is more relevant

⇒ Therefore, QIAGEN offers siRNA sets with separated siRNA, as well as with siRNA pools
2′-O-methyl modification: Risk of lower potency

MAPK-1 siRNA
Sense OMeU: U(m)G(m)CU(m)GACU(m)CCAAGCU(m)CU(m)GUU
Antisense OMe: CA(m)GAGCUUUGAGUCAGCAU

MAPK-14 siRNA
Sense OMeU: C(m)C(m)U(m)ACAGAGAAG(m)GCGGUU
Antisense OMe: CC(m)GCAGUUCUCUGUAGGUU

2′-O-methyl modification: No reduced induction of IFN related genes

Analysis of OAS2 expression 6h and 24h after transfection pIpC as positive control

siRNA is product of millions of years of evolution. It is a perfect molecule!
⇒ Any chemical changes mean a risk to its potency!
Rescue or Redundancy

Echeverri et al. (2006), Minimizing the risk of reporting false positives in large-scale RNAi screens. Nat Methods, 3(10):777-9

RNAi rescue experiments

⇒ QIAGene + siRNA targeting 3'-UTR
RNAi screening control guidance
www.qiagen.com/AllStars

<table>
<thead>
<tr>
<th>Negative control</th>
<th>AllStars Negative Control siRNA</th>
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<tbody>
<tr>
<td></td>
<td>- No phenotype in cell-based assays</td>
</tr>
<tr>
<td></td>
<td>- Lowest off-target profile in GeneChip analysis</td>
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<tr>
<td></td>
<td>- Shown to enter RISC</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Positive control</th>
<th>AllStars Hs Cell Death Control siRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>- Transfection and knockdown success visible by light microscopy</td>
</tr>
<tr>
<td></td>
<td>- Ubiquitous utility in all human cell types</td>
</tr>
<tr>
<td></td>
<td>- For optimization of transfection efficiency</td>
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<table>
<thead>
<tr>
<th>To monitor the experiment</th>
<th>AllStars Knockdown Level Controls</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>- Target human CDC2 gene</td>
</tr>
<tr>
<td></td>
<td>- 3 potency levels available: 98%, 75%, and 50%</td>
</tr>
<tr>
<td></td>
<td>- Real-time PCR assays and reagents available</td>
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</tbody>
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AllStars Negative Control siRNA
www.qiagen.com/AllStars

- Most thoroughly verified negative control siRNA available
- Multiple negative control siRNAs tested for non-specific effects with
  - Affymetrix GeneChip Arrays
  - Cell-based assays
    - □ Live-cell nucleic staining
    - □ Cell number
    - □ Nucleotide incorporation
    - □ Live-cell dye exclusion
    - □ DNA staining
    - □ RISC-incorporation analysis

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Affymetrix GeneChip Arrays

- Live-cell nucleic staining
- Cell number
- Nucleotide incorporation
- Live-cell dye exclusion
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AllStars Hs Cell Death Control siRNA

**Phenotype control**
- Blend of highly potent siRNAs
- siRNAs targeting genes indispensable for cell survival
- Gene knockdown induces high degree of cell death, visible by light microscopy

Primary normal human bronchial epithelial cells (NHBE) analysed 72h after transfection by microscopic inspection
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microRNAs - micromanagers of gene expression

- Characteristic of miRNAs
  - Endogenous small RNA
  - Regulation of at least 1/3 of the protein encoding genes
  - > 800 miRNAs in human (miRBase V12.0)
  - Mature miRNA ~ 22 nt long
  - miRNA Seed sequences (nt 2-7) crucial in target selection
  - Translational repression
  - Multiple target transcripts per miRNA

- Target mRNAs
  - miRNA binding sites typically at the 3'-UTR
  - Typically multiple miRNA binding sites
  - Binding sites for several different miRNAs in the same transcript
miScript System
Reverse transcription - principle

Detecting miRNAs & mRNAs from the same cDNA preparation
Detection of either mature miRNA or precursor miRNA

A complete offering for miRNA research

<table>
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<tr>
<th>Purification</th>
<th>Analysis</th>
<th>Functional studies</th>
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<tbody>
<tr>
<td>miRNeasy Mini Kit</td>
<td>miScript Reverse Transcription Kit</td>
<td>HiPerFect Transfection Reagent</td>
</tr>
<tr>
<td>miRNeasy 96 Kit</td>
<td>miScript SYBR® Green PCR Kit</td>
<td>Attractene Transfection Reagent</td>
</tr>
<tr>
<td>miRNeasy 97-98 Kit</td>
<td>miScript Primer Assays</td>
<td>miScript miRNA Mimics</td>
</tr>
<tr>
<td>miRNeasy Protect Animal Blood Kit</td>
<td>miRNeasy Protect Animal Blood Kit</td>
<td>miScript miRNA Inhibitors</td>
</tr>
<tr>
<td>PAXgene Tissue miRNA Kit</td>
<td>Custom miScript Prime Assays</td>
<td>Custom miScript miRNA Mimics and Inhibitors</td>
</tr>
<tr>
<td>PAXgene Blood miRNA Kit</td>
<td>miScript Controls</td>
<td>Human, Mouse, and Rat miScript Precursor Assays</td>
</tr>
<tr>
<td>miScript Primer Assay 96 and 384 Plates</td>
<td>miScript miRNA Mimics Set</td>
<td>Human miScript miRNA Inhibitor Set</td>
</tr>
<tr>
<td>miScript miRNA Control</td>
<td>miScript miRNA Inhibitor 96 and 384 Plates</td>
<td>Rotor-Gene Q</td>
</tr>
</tbody>
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QiAcube – miRNeasy & PAXgene protocols
**Leader in Genome Wide Libraries**

- QIAGEN **first** company to deliver both druggable and whole genome human siRNA sets

- **Largest user base** of Genome Set customers:
  - 12 'big' Pharma/Biotech Companies
  - Academic and Non Profit: Translational Genomics Institute, Scripps Institute, Dana Farber, Harvard University, GNF, Columbia University, University of Miami, CNRS, Max Planck Institute, Institute Curie, VTT Finland, AIST Japan, ...

- **Annual High-Throughput RNAi User Forum**

- **Scientific advisory board:**
  - Spyro Mousses (TGEN, US)
  - Sumit Chanda (Burnham Institute for Medical Research, US)
  - Natasha Caplen (National Cancer Institute, US)
  - John B. Hogenesch (The Scripps Research Institute, US)
  - Carl Novina (Dana-Farber Cancer Institute, Harvard Medical School, Boston, US)

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**QIAGEN RNAi Research Team**

Sample & Assay Technologies