General workflow for siRNA screening at ICCB-Longwood

Complete online RNAi screen application

Application on ICCB-L website (Forms)

ICCB-L Director will contact you for initial meeting

Jennifer Smith, ICCB-L Director, Jennifer_Smith@hms.harvard.edu
Caroline Shamu, Faculty Director, Caroline_Shamu@hms.harvard.edu

Discuss: specifics and goals of your screen
general screening process at ICCB-Longwood
steps to move forward

Sign and return RNAi User Agreement

PDF on ICCB-L website (Forms)

Receive equipment training

This needs to occur prior to initiating work at ICCB-Longwood.
Initial assay development frequently occurs outside ICCB-Longwood.

Complete Training Request on ICCB-L website (Forms)

Complete billing information form

PDF on ICCB-L website (Resources, Screening Documents)

Assay Optimization

This is an iterative process. Screeners generally start by developing an assay suitable for siRNA screening, then optimize transfection conditions for their cells, and finally work to optimize the fully automated screening protocol including cell transfection and assay readout methods.

Refer to Resources, Helpful Publications and Links on ICCB-L website

Important parameters to optimize:

- Plate type
  - Dependent on assay readout and cell type
  - Primarily 384-well
  - Corning plates most frequently utilized
- # cells/well
  - Determine robust way to consistently count cells during screen
    - Lower cell # for image-based assays (500 – 1,500 cells/well)
    - Range from 500 – 5,000+ cells/well
  - Final volume ~50 uL/well (40 – 60 uL/well)
- Cell passage #: can impact transfection efficiency and phenotype
- Transfection reagent
  - Panel available at ICCB-Longwood for testing on small scale
    - For more information, contact Jennifer Smith
- Type of transfection – reverse or forward
  - Reverse transfection is utilized for most screens at ICCB-Longwood
  - Reverse transfection requires fewer plates and tips (saves $ and time)
- Goals when optimizing transfection efficiency:
  - Maximize siRNA uptake
  - Minimize toxicity
  - Determine level of knockdown
  - siRNA concentration (20 – 50 nM)
- Choose positive and negative controls
  - No universal negative control works for all cell lines/assays
  - Dharmacon non-targeting siRNAs available at ICCB-Longwood to test on small scale
    - For more information, contact Jennifer Smith
  - Advantageous to have positive controls of different strengths – strong, medium
- Time of siRNA knockdown
  - Typically 72 h
  - Range 48 – 96 h, 120 h maximum
- Practice with equipment at ICCB-Longwood
- Automate transfection protocol
  - Reagent volumes and concentrations
  - Timing
  - Addition of assay-specific controls – ideally using automation, can be manual
- Assay readout – can optimize this step independent of RNAi transfection

Success in assay optimization is generally monitored by assessing assay robustness factors, including the Z' factor. See below for more details.

Please contact ICCB-Longwood staff at any point with questions or issues you would like to discuss.

Z' factor determination

Additional information on the ICCB-L website (Resources, Data Analysis/Informatics)
Perform this test using automation protocol developed for your assay
Options are:
1/2 plate positive control and 1/2 plate negative control
1/3 plate each of 2 different strength positive controls, 1/3 plate negative control (***preferred***)
Same # of replicates (typically 3) as will be utilized in screen
Look for signs of edge effects

Z' factor acceptable? Ideally $\geq$ 0.5
Lower Z' factors (eg 0.2) can be acceptable in RNAi screens

Yes

Set up appointment with data team
Jennifer_Splaine@hms.harvard.edu
David_Wrobel@hms.harvard.edu

No

Consult Jennifer Smith
Jennifer_Smith@hms.harvard.edu
Continue assay optimization

Pilot screen

Human Pilot 1 (Plate #51,083) and additional plate from Human 4
First plate in mouse siRNA genome (Plate #50,025) and additional plate from Mouse 4

Image analysis if necessary

Send raw numerical data to Data Scientist

Jennifer Splaine, Jennifer_Splaine@hms.harvard.edu

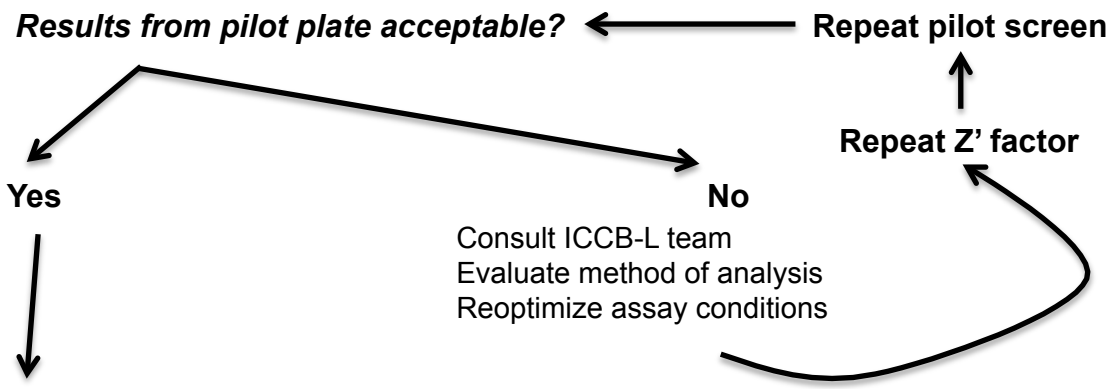
Jen will: format data into standard ICCB-Longwood format;
generate graphs to visualize quality control parameters (utilizing Dotmatics Vortex software);
calculate Z' factor based upon assay-specific positive and negative controls; and
email formatted data and graphs back to screener

Review and analyze data

See below for more information about data analysis
Refer to Birmingham *et. al* (Nat. Methods, 2009, 6(8):569-575)
Additional information on ICCB-L website (Resources, Helpful Publications and Links)

Analyze data
Acceptable Z' factor?
Potential hits?
Do the data make biological sense?

Evaluate graphs provided by Jen Splaine
Good correlation of replicates?
Clear separation of positive and negative controls?
Any visible patterns or edge effects?



To ensure quality and consistency of data, use the same assay protocol throughout your entire screen!

Primary screen

Send data to Jen Splaine as screening sessions are complete
Pay attention to Z' factor and positive/negative controls on each assay plate
Immediately catch any problems or changes in screen robustness!
Analyze data on a regular basis
Cherry picks can be performed at any point throughout primary screen

Analyze screen data: quality control and identifying potential screening positives

Accept there may be a large # of potential positives from primary screen (eg 1-3% of genome)
Helpful to categorize potential hits as strong, medium, weak
Refer to Birmingham *et. al* (Nat. Methods, 2009, 6(8):569-575)

Potential methods of data analysis frequently used at ICCB-Longwood:

1. Z-score – frequently used and is an easily applied method for plate normalization. The raw data distribution of sample values is converted to a standard normal distribution with a mean of 0 and standard deviation of 1. Each z-score for a well replicate represents the number of standard deviations above or below the plate mean of the experimental wells

$z = (x - \mu)/\sigma$

x is the raw data value of the well to be standardized
μ is the mean of the experimental well population of plate
σ is the standard deviation of the experimental well population of plate

2. Robust Z-score – variation of z-score. ***This is the recommended method of analysis***. Rather than using the mean and standard deviaiton, which are sensitive to outliers, the median and median absolute deviation (MAD) are utilized. This method is often preferable for RNAi screens.

$z^* = (x - median)/MAD * 1.4826$

x is the raw data value of the well to be standardized
median of the experimental well population of plate
MAD is median absolute deviation of the experimental well population of plate

Analyze screen data (continued)

3. Percent of control (or sample) – divide each well value by the plate mean of either negative controls or positive controls, or by the plate average of samples (experimental wells). The plate median for experimental wells is typically used, in which case the average result value for all sample wells will be ~1.0 (unless the raw data distribution is very skewed). Calculating percent of control or plate sample average does not account for plate variation to the same extent as the z-score. This type of analysis should only be performed when there is an adequate number of controls and a small coefficient of variation (ideal < 10%).

% Neg Control = x / Avg neg

% Exp Control = x / Avg sample

Submit analyzed data from primary screen in ICCB-L format and primary screen report

Email to David Wrobel (David_Wrobel@hms.harvard.edu)

Cherry pick deconvolution screen of individual siRNA duplexes

Submit cherry pick request to David Wrobel (David_Wrobel@hms.harvard.edu)

Cherry picks must be tested in same assay as primary screen
4 duplexes/gene are provided, each in its own well (4 wells/gene)

Analyze results of cherry pick

Negative controls are important since many siRNAs tested will score as hits at this stage.
We suggest at least 2 columns of negative control (32+ wells).
How many duplexes give the same phenotype as the SMARTpool?
Rank hits based upon # of duplexes that reconfirm SMARTpool phenotype
4/4 > 3/4 > 2/4 > 1/4 > 0/4

Prioritize hits based on re-test results and bioinformatics analysis

Bioinformatics analyses

After hits from the primary screen are preliminarily validated via deconvolution, many additional experiments are required to confirm their physiological relevance. The next two sections describes a few strategies that can be undertaken.

1. Bioinformatics analysis to identify probable transcripts impacted by off target effects (OTEs). Several methods have been developed to identify siRNA duplexes in which the seed sequence may target additional, unintended transcript(s).

Two that have been published and are available online:
Genome-wide enrichment of seed sequence matches (GESS)
http://www.flyrnai.org/gess/
Sigoillot, FD, et al. A bioinformatics method identifies prominent off-targetted transcripts in RNAi screens. Nat Methods. 2012. 9(4):363-6. doi: 10.1038/nmeth.1898

Haystack
http://rnai.nih.gov/haystack/
Buehler, E., et al. siRNA off-target effects in genome-wide screens identify signaling pathway members. Sci. Rep. 2012. 2, 428. dio: 10.1038/srep00428

Bioinformatics analyses (continued)

1. Bioinformatics analysis to identify probable transcripts impacted by off target effects (OTEs) continued.

In addition to GESS and Haystack, Dharmacon has a seed analysis tool to identify siRNAs that may be acting via OTEs in its libraries. David Wrobel at ICCB-Longwood can submit analysis requests to Dharmacon.

Please contact David Wrobel (david_wrobel@hms.harvard.edu) or Jennifer Smith (Jennifer_Smith@hms.harvard.edu) if you would like assistance in running one of the OTE analyses mentioned above.

2. Pathway analysis with the help of a bioinformatics collaborator is important at this point. ICCB-Longwood does not offer support in this area. Please contact Jennifer Smith (Jennifer_Smith@hms.harvard.edu) or Caroline Shamu (Caroline_Shamu@hms.harvard.edu) to identify resources in the area.

Follow-up experiments

Further follow-up experiments may be high or low throughput. Potential experiments include:

1. Test different reagents in original assay
   - Chemically modified siRNAs (same and/or different vendors)
   - shRNAs
   - esiRNAs

2. Test positive reagents in different assays

3. C911 – a mismatch siRNA design strategy to experimentally test whether an siiRNA phenotype is a consequence of OTE(s)
   - Publication: Buehler, E., et al. A Bench-Level Control for Sequence Specific siRNA Off-Target Effects. PLOS One. 2012. DOI: 10.1371/journal.pone.0051942
   - C911 calculator: http://rnai.nih.gov/haystack/C911Calc.html

4. Monitor level of mRNA/protein knockdown by qPCR/Western blot analysis

5. Knockdown targets via orthogonal technology – CRISPR, TALEN

6. Rescue siRNA phenotype by expressing RNAi-resistant mRNA

Publications related to your RNAi screen

We request that investigators acknowledge the ICCB-Longwood Screening Facility at Harvard Medical School in publications related to screening efforts or use of equipment at ICCB-L and, if appropriate, mention specific staff members with whom they worked.

It can also be helpful to specify sources of commercial compounds that were screened, as library vendors appreciate public mention and formal documentation of successful hit compounds.

Please contact Jennifer Smith (Jennifer_Smith@hms.harvard.edu) or Caroline Shamu (Caroline_Shamu@hms.harvard.edu) if interested in depositing the data from your screen into a public repository.

- PubChem Bioassay: http://www.ncbi.nlm.nih.gov/pcassay
- GenomeRNAi: http://www.genomernai.org/