Video Article

MISSION esiRNA for RNAi Screening in Mammalian Cells

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URL: http://www.jove.com/details.php?id=2008
DOI: 10.3791/2008
Citation: Theis M., Buchholz F. (2010). MISSION esiRNA for RNAi Screening in Mammalian Cells. JoVE. 39. http://www.jove.com/details.php?id=2008, doi: 10.3791/2008

Abstract

RNA interference (RNAi) is a basic cellular mechanism for the control of gene expression. RNAi is induced by short double-stranded RNAs also known as small interfering RNAs (siRNAs). The short double-stranded RNAs originate from longer double stranded precursors by the activity of Dicer, a protein of the RNase III family of endonucleases. The resulting fragments are components of the RNA-induced silencing complex (RISC), directing it to the cognate target mRNA. RISC cleaves the target mRNA thereby reducing the expression of the encoded protein[1,2,3]. RNAi has become a powerful and widely used experimental method for loss of gene function studies in mammalian cells utilizing small interfering RNAs.

Currently two main methods are available for the production of small interfering RNAs. One method involves chemical synthesis, whereas an alternative method employs endonucleolytic cleavage of target specific long double-stranded RNAs by RNase III *in vitro*. Thereby, a diverse pool of siRNA-like oligonucleotides is produced which is also known as endoribonuclease-prepared siRNA or esiRNA. A comparison of efficacy of chemically derived siRNAs and esiRNAs shows that both triggers are potent in target-gene silencing. Differences can, however, be seen when comparing specificity. Many single chemically synthesized siRNAs produce prominent off-target effects, whereas the complex mixture inherent in esiRNAs leads to a more specific knockdown[10].

In this study, we present the design of genome-scale MISSION esiRNA libraries and its utilization for RNAi screening exemplified by a DNA-content screen for the identification of genes involved in cell cycle progression. We show how to optimize the transfection protocol and the assay for screening in high throughput. We also demonstrate how large data-sets can be evaluated statistically and present methods to validate primary hits. Finally, we give potential starting points for further functional characterizations of validated hits.

Protocol

1. High-quality MISSION esiRNA libraries

1. For every gene of interest the most susceptible and specific target region for RNAi is chosen utilizing the DEQOR algorithm (http://cluster-1.mpi-cbg.de/Deqor/deqor.html).[4] DEQOR scores the silencing potential of all possible 21 nucleotides long siRNAs in a target-mRNA based on state-of-the art design-constraints[4]. In addition, each potential siRNA is analyzed for possible cross-reactivity with other genes by performing a BLAST search against the transcriptome of the organism studied. The program thereby provides an analysis of the overall quality and cross-silencing capacities of the potential esiRNA (300-600 bp length).
2. The chosen region of the target-gene cDNA is amplified by PCR using gene-specific primers flanked by bacteriophage RNA-polymerase-promoter sequences.
3. Every PCR product used for MISSION esiRNA production is verified for identity by sequencing and for purity by Caliper Labchip analysis.
4. Long double-stranded RNA is transcribed from the PCR-product by RNA polymerase, followed by an annealing of the transcribed strands.
5. esiRNAs are prepared by limited enzymatic digestion of the long double-stranded RNA using RNaseIII followed by purification via anion-exchange chromatography utilizing Q-sepharose spin columns.
6. esiRNAs are precipitated and resuspended in TE buffer. The yield is measured by UV-absorption measurements and the concentration is adjusted by dissolving in an appropriate volume of TE buffer. The overall quality of the final product is checked by Caliper Labchip analysis.

2. Choosing a cell line and optimizing transfection for screening

1. Titrate amounts of esiRNA (use Eg5 as positive and renilla-luciferase as negative control) and increasing amounts of transfection reagent in a 384-well plate, add cells and incubate for 48 hours. Eg5 is a kinesin motor protein required for bipolar spindle assembly and depletion leads to a mitotic arrest. Repeat this procedure for different transfection reagents and cell lines. Here, we use HeLa cells cultured following standard procedures and oligofectamine (Invitrogen) as transfection reagent.
2. For choosing the optimal transfection conditions count the cells transfected with Eg5 esiRNAs that show a mitotic arrest (round shape) and the total number of cells transfected with renilla-luciferase (RLUC) esiRNA by light microscopy. Choose the condition with the least toxicity for RLUC and the most pronounced phenotype for Eg5.
   An alternative method for optimizing transfection conditions involves a stable cell line expressing EGFP (or another suitable reporter gene) under the control of a constitutive active promoter. After transfection of an esiRNA against EGFP (or another reporter gene) and RLUC (or another suitable negative control esiRNA) measure knock-down efficacy and toxicity by e.g. fluorescence assisted cell sorting (FACS). Make sure that the used esiRNA for EGFP is fully complementary to the target-mRNA.

3. Setting Up the Primary screen[5,6]

1. Optimize the pipetting accuracy for all used components on an automated pipetting station (e.g. Aquarius, Tecan and WellMate, Matrix). Because pipetting parameters change depending on the type of solution, volume and plate type this optimization has to be repeated for every step separately. If possible, the pipetting station should be placed under a laminar flow hood to avoid contaminations. All used components

should be sanitized before use either by autoclaving if applicable or by spraying with 75% ethanol. Optimize wash-protocols so that cross-contamination from well to well is excluded. Details for the optimization of automated pipetting cannot be give for space-constraints and depend largely on the utilized pipetting station. For further information refer to the manufactures protocol.
2. Transfect cells in 384-well format using the optimized conditions from above with esiRNAs for Eg5 and RLUC. Use an alternating pattern for Eg5 and RLUC esiRNA covering the whole plate.
3. After incubation for 48 hours fix the cells with cold 100% ethanol, rehydrate in PBS for 15 minutes, stain for 25 minutes in PBS containing 1 µg/ml DAPI and 100 µg/ml RNase A (Qiagen). Finally wash with PBS and store at 4°C.
4. Measure intensity of DAPI-fluorescence by microscopy e.g. on an Olympus ScanR system. Generate a histogram by plotting DAPI intensity against cell number and evaluate the cell cycle distribution by calculating the percentage of cells with the DNA-content 2N for G1-phase; < 2N > 4N for S-phase; 4N for G2/M-phase and > 4N for aneuploidy/polyploidy.
5. Evaluate the homogeneity of the results over the plate. Further optimization is needed if results differ significantly to exclude position effects. A common problem when using multi-well plates is enhanced evaporation at the edge wells during incubation time. This leads to different experimental conditions in different wells, which often translates to plate-position specific effects. For this reason, we recommend to leave all edge wells empty for a screen. To further minimize evaporation make sure that the incubators are kept at high humidity. We also routinely employ evaporation barriers such as Corning Breathable Sealing Tape which block evaporation. Also other resources for position effects have to be taken into consideration, e.g. technical variation of the readout system (microscope, plate reader, etc) or variations in liquid handling (gradients, inhomogeneity of solutions, etc).
6. The statistical differences between positive and negative controls provide a measure for the significance of the assay employed. A large difference between negative control (or background noise; mock-control) and sample has to be established before starting the actual screening. A good measure for the statistical significance is the Z-factor. The Z-factor is calculated following the equation:
$Z = 1- (3\ SD_{[sample]} + 3\ SD_{[mock]}) * (|Av_{[sample]} - Av_{[mock]}|)^{-1}$; with SD: standard deviation; Av: average. A Z-factor of $1 > Z > 0.5$ indicates a statistically significant separation of negative controls (and noise) from the positive controls[7].

4. Primary screen

1. Prepare 384-well tissue culture plates for transfection of esiRNAs utilizing the optimized conditions from above. Here, we use 15ng esiRNA per well dissolved in 5µl TE buffer. At least eight control positions per plate should be loaded with suitable positive and negative controls for the biological process studied (here: Eg5 and RLUC). To avoid position effects the edge wells are loaded with 5µl TE buffer only.
2. Add 5µl OptiMEM (Invitrogen) containing 0.2µl Oligofectamine per well, mix and incubate for 20 minutes at room temperature.
3. Add cell suspension onto the transfection mixture (here: 40µl of a HeLa cell suspension at 25 cells/µl concentration; equivalent to 1000 cells per well) and incubate for 72 hours.
4. Measure DNA-content on an automated microscope (Olympus ScanR, see above). Evaluate using Z-score statistics for hit selection:
Z-scores are calculated for all sample esiRNAs using the signal for the negative control as reference. Note, the Z-score is not the same as the Z-factor. Z-scores provide a statistical measure for significance of sample values in comparison to a (mock-) control. It is calculated following the equation:
$Z = (value_{[Sample]} - average_{[mock]}) * standard\text{-}deviation_{[mock]}^{-1}$.
For hit selection a threshold has to be applied. Typically, a significance criteria like: $2 < Z < -2$ is used, but depending on the quality of the dataset, the studied biological process or simply the scope of the screen, different thresholds may be applicable. Beside the fairly easy Z-score statistics also more elaborate mathematical evaluation methods can be used (a first inside into the wide field of statistical evaluation of screening data can be found in Malo et al.[8]).

5. Secondary screen and hit validation

1. A secondary screen follows the primary screen to eliminate false positives due to experimental errors or off-target effects. For the selected hits the same procedure as used in the primary screen should be repeated for a bigger number of replicates (3-5) to allow a better statistical evaluation.
2. For verified hits a secondary non-overlapping esiRNA (or another non-overlapping silencing trigger) against the targets identified in the initial screens should be used. The same assay and read-out should be applied as for the primary screen.
3. Ultimately the selected genes can be validated by cross-species RNAi rescue[9]. Thereby a bacterial artificial chromosome (BAC) encoding e.g. the mouse ortholog of the gene is stably transfected into cells. BAC-constructs preserve a gene in its genomic context and allow for a nearly-physiological expression. RNAi against the endogenous human gene will leave the mouse transgene expression unaltered which rescues the RNAi-phenotype. RNAi-rescue provides the gold-standard for the verification of RNAi phenotypes available to date.
4. Finally, the validated candidates are studied in more detail to eventually derive mechanistic understanding. In many cases RNAi can be used in these experiments, e.g. in secondary, more elaborate assays.

Discussion

RNA interference has become a standard technique to study loss-of-function phenotypes. Large-scale collections of RNAi-mediators are available from different suppliers and provide an easy, cost-effective and rapid method for gene-silencing. This opened the gate for systematic screening for key-players in many biological processes allowing a genome-scale perspective on a wide range of different species and cell types.

High false positive and false negative rates are a common challenge in RNAi screens. To address this problem, considerable efforts have been invested to improve the efficacy and in particular the specificity of the silencing triggers. An important discovery was that a pool of different siRNAs targeting the same transcript greatly enhances target specificity. Because a very complex pool of different siRNAs is produced by the endoribonuclease, esiRNAs are high target specificity triggers, reducing the false positive rate in RNAi screens. esiRNAs have also demonstrated to achieve efficient knockdowns, reducing also the false negative rate.

Because RNAi screens are technically demanding, they will likely remain challenging to perform on a routine basis for some time. However, as reagents and instruments get better and more laboratories share their expertise, the use of RNAi screens in modern biology are deemed to increase in the future.

Disclosures

Authors declare financial relationships with commercial entities that might have an interest in the submitted work.

Acknowledgements

We would like to acknowledge members of the Buchholz laboratory and the Sigma-Aldrich RNAi-team for discussion. We would like to thank the Max Planck Society and the Bundesministerium für Bildung und Forschung grands Go-Bio [0315105] for support.

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