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RNAi Screening of Leukemia Cells Using Electroporation

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Abstract

RNAi-mediated screening has been an integral tool for biological discovery for the past 15 years. A variety of approaches have been employed for implementation of this technique, including pooled, depletion/enrichment screening with lentiviral shRNAs, and segregated screening of panels of individual siRNAs. The latter approach of siRNA panel screening requires efficient methods for transfection of siRNAs into the target cells. In the case of suspension leukemia cell lines and primary cells, many of the conventional transfection techniques using liposomal or calcium phosphate-mediated transfection provide very low efficiency. In this case, electroporation is the only transfection technique offering high efficiency transfection of siRNAs into the target leukemia cells. Here, we describe methods for optimization and implementation of siRNA electroporation into leukemia cell lines and primary patient specimens, and we further offer suggested electroporation settings for some commonly used leukemia cell lines.

Keywords

siRNA screening; Leukemia; Electroporation

1 Introduction

Since the inception of RNAi, a number of techniques have emerged that employ the ubiquitous gene-silencing capacity of short interfering RNA (siRNA) and short hairpin RNA (shRNA) to functionally assess the biological relevance of genes in a variety of contexts, including many of the functional screens that have been conducted for target discovery in cancer [1–15]. There are two basic strategies for these screens depending on whether the siRNA or shRNA are delivered together in a single pool to the target cells, or whether they are segregated and delivered individually. In the former strategy, a panel of shRNAs coupled to identifiable barcodes in lentiviral vectors is delivered as a single pool to target cells. By comparing the initial frequency of each shRNA barcode with the frequency after a treatment or culture period (or comparing pools of cells where shRNA expression has been induced versus uninduced), target genes that are biologically important for the cellular phenotype under interrogation can be elucidated. In the latter, segregated strategy, a panel of siRNAs is distributed across multi-well plates where each well contains siRNA targeting a single gene. These siRNAs are then batch transfected into target cells that are subsequently cultured to allow for biological examination of the impact of each siRNA on the cellular phenotype in question (cell growth, survival, differentiation, etc.).

Both of these screening modalities have been successfully used to drive many important biological discoveries. Here we will focus on the segregated screening approach, and specifically on the methods for application of this approach on difficult-to-transfect leukemia cells (both cell lines and primary cells). Because leukemia cells are recalcitrant to transfection with standard liposomal or calcium phosphate methods, we have extensively used electroporation as a technique for delivery of both individual siRNAs as well as panels of siRNAs into leukemia cell lines and primary patient specimens. This chapter will describe the methodologies that we have developed for optimization of electroporation settings to balance the highest possible transfection efficiency with robust cell viability and growth post-electroporation [3–5, 16–18]. We additionally provide settings we have determined using this optimization approach for a variety of commonly used leukemia cell lines as well as primary patient specimens.

2 Materials

1. *siRNA reconstitution buffer*: dilute 5× siRNA buffer (GE Dharmacon) with Nuclease Free water.
2. *Lyophilized siRNA library* (e.g. siGENOME SMARTPool library from GE Dharmacon) in 96-well plates: Library plates are commonly comprised of siRNAs targeting 94 individual genes, one nonsilencing siRNA, and one well kept empty for a no cell, blank control (*see Note 1*).
3. *Culture media for cells*: For cell lines: use media according to the specific culture requirements of each cell line.

For primary myeloid leukemia samples: RPMI with 10 % FBS, 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin+βME.

For primary B-cell lymphoid leukemia samples: RPMI with 20 % FBS, 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin + βME.

For primary T-cell lymphoid leukemia samples: Same as B-cell lymphoid sample media with the addition of 1× Insulin Transferrin Selenium.

βME concentration is 10⁻⁴ M (dilute 3 µl of βME into 500 ml media); no βME is required for cell lines.
4. 96-well tissue culture plates.
5. 96-well PCR plates with conical bottom.
6. 200 µl filtered tips.

¹Use A1 corner well of a 96-well plate as a blank well and check orientation of plates while aliquoting the library. Additionally after aliquoting, check if each well contains siRNA. For consistency the library aliquoting could be performed using a liquid handler such as EpMotion.

7. 10–20 µl filtered tips.
8. Adhesive breathable plate seals to cover 96-well plates and to avoid edge effect due to evaporation.
9. Multichannel pipettes.
10. Plastic trough for multichannel pipetting.
11. Cell strainer (40 µm).
12. Phosphate-buffered saline (PBS).
13. 96-well electroporation plates (Bio-Rad).
14. 96-well electroporation machine (such as Bio-Rad Gene Pulser MXCell).
15. Centrifuge to spin 96-well plates.
16. CellTiter 96® AQueous One Solution Cell Proliferation Assay reagent (Promega).
17. Cell viability reagent (e.g. propidium iodide, trypan blue).
18. Spectrophotometric plate reader.
19. Optional: Use an automated liquid handler to aliquot siRNA library and prepare media plates (Such as Epimotion, Eppendorf; Multidrop, ThermoScientific; etc.).
20. siPORT Buffer Recipe: The recipe for the siPORT buffer is listed in Table 1.

3 Methods

Perform all the steps at room temperature unless specified.

3.1 Reconstitute Library

1. Make 7.5 ml siRNA reconstitution buffer per one 96-well plate containing 1 nmol siRNA per well (mix 1.5 ml 5× siRNA buffer with 6 ml Nuclease Free water).
2. Spin down 96-well plate containing lyophilized 1 nmol siRNA library for 30 s at 2000 × *g* at room temperature.
3. Aliquote 54 µl reconstitution buffer per well in 96-well master siRNA plate, resulting in a stock siRNA concentration in each well of ~18.5 µM (*see* Note 1).
4. Vortex briefly (using Eppendorf MixMate or similar product) and let it sit at 4 °C for 10 min, vortex again and then spin before aliquoting.

5. Next from each plate aliquot 6 μ l/well into seven skirted PCR plates using multichannel pipette or automated liquid transfer device. Master plate will be saved as 8th plate. Thus, a single siRNA masterplate containing 1 μ mol per well siRNA library will create eight daughter libraries.
6. Seal the plates with PCR plate transparent seals and store at -80°C until ready to use.

3.2 Optimization of Electroporation Protocol

Before performing siRNA screening, optimize electroporation conditions for each new cell line or type of cells (Fig. 1).

1. Spin down 1×10^5 cells per condition for leukemia cell lines or 3×10^5 cells per condition for primary leukemia cells at $350 \times g$, for 5 min.
2. Resuspend in 1 ml PBS.
3. Spin down again.
4. Resuspend in 100 μ l siPORT Buffer per condition.
5. Optional—if you have a known positive control target gene that will reduce growth/viability of target cells (“killer siRNA”) add to a final concentration of 1 μ M nonspecific or killer siRNA per condition (6 μ l of 18.5 μ M siRNA+100 μ l of cells in siPORT buffer). In the absence of a known target gene, PLK1 siRNA can be used as a generic killer siRNA.
6. Save unelectroporated cells (equivalent to one condition) for control.
7. Transfer to 96-well electroporation plates with gap width of 4 mm (cuvettes of varying gap width can also be used, however, changing cuvette gap width will dramatically alter the effective pulse lengths).
8. Electroporate using Square Wave protocol using a combination of different voltages and pulse lengths, ranging from 150 to 450 V and 1.5–5.0 ms, respectively (Fig. 1) (see Note 2).

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²On a 96-well electroporation plate there are a total of 24 well sets, each well set comprises four wells. All four wells in an individual well set will always be electroporated with the same electroporation condition. Therefore, a total of 24 electroporation conditions can be tested simultaneously. It is recommended to optimize at least 12 electroporation conditions for a new cell line. Also, it is not necessary to use the whole plate at the same time and unused well sets can be utilized for later experiment. For example four cell lines can be tested using half of a plate or eight cell lines can be tested using a whole plate with 12 different electroporation conditions (though only half this number would be possible if using a nonspecific and killer siRNA or each cell line as this would require two rows for each cell line).

9. Transfer 10–12 μ l electroporated cells in triplicate in a 96-well plate containing 90 μ l media/well to perform MTS analysis at 96 h and culture left over cells in a 24-well plate with culture media in 1:10 ratio to analyze cell viability at 48 h (*see* Note 3).
10. Compare absolute percent cell viability via propidium iodide, trypan blue, or similar as well as cell growth relative to non-electroporated cells using MTS assay, CellTiterGlo or similar (Figs. 1 and 2). Electroporation conditions should maintain absolute cell viability at 80 % or higher with propidium iodide/trypan blue and reduction in growth should be no lower than 50 % of the non-electroporated cells by MTS assay (greater than 60 % relative to non-electroporated cells is preferred) (*see* Note 4, Table 2).

3.3 Library Screening with siRNA in Leukemia Cells

1. Prepare three 96-well plates with 90 μ l of appropriate culture media in each well and store in incubator at 37 °C with 5 % CO₂ until use.
2. Reconstitute 3×10^7 cells from a primary patient or 1×10^7 cells from a cell line in 50 ml PBS for each 96-well plate siRNA library.
3. Spin the cells at $350 \times g$ for 10 min.
4. During the spin, thaw an aliquot of the 6 μ l siRNA library. Once thawed, spin at $2800 \times g$ for 15 s. leaving centrifuge break off and let slow down while waiting for cells to finish spinning.
5. Resuspend cells in 10.5 ml cold siPORT Buffer, mix the single cell suspension by pipetting and passing through a 40 μ m nylon cell strainer.
6. Transfer the cell suspension as quickly as possible to a disposable trough. Aliquot 100 μ l into each well of the siRNA library (except A1, blank well), making sure to mix thoroughly. Addition of 100 μ l of cells to the 6 μ l siRNA in each well results in a final siRNA concentration of $\sim 1 \mu$ M.

³Once cells are reconstituted in siPORT buffer and electroporated, cells need to be transferred to their destination plates immediately and quickly as prolonged exposure to siPORT buffer can have cytotoxic effects.

⁴If using a positive control siRNA, then the percent reduction in viability relative to the nonsilencing control should be used as an additional factor for selecting the optimal electroporation condition. The knockdown of the target can be validated by quantitative PCR and/or immunoblot. If utilizing fluorescently tagged siRNA, then the shift in mean fluorescent intensity of electroporated versus non-electroporated conditions (as assessed by flow cytometry) can be used in addition to cell growth/viability readings to select optimal conditions.

7. Transfer the entire contents from each well of the PCR plate to electroporation plate with multi-channel pipette set at 150 μ l.
8. Add 106 μ l of blank siPORT buffer to well A1 of the electroporation plate.
9. Inspect the plate to make sure the bottom of each well is fully covered in liquid by gently shaking plate; pop any remaining bubbles with pipette tips.
10. Select the appropriate protocol for electroporation as optimized in Subheading 3.2:
11. Place the plate in the plate handler, making sure to push down on plate until it is fully inserted. Leave the plate lid on and close the lid of the plate handler.
12. When ready, press pulse using the optimized electroporation program.
13. Remove the three plates with media from the incubator. For each row, mix thoroughly using a P200 multi-channel pipette set at 50 μ l and then transfer 12 μ l (see Note 5) from electroporation plate into each of the three plates with media using a P20 multi-channel pipette.
14. Seal all plates with adhesive and breathable plate sealer.
15. Incubate the plates at 37 °C for 4 days (96 h).
16. At day 4, add 20 μ l of MTS reagent to every well (including A1). Plates should be monitored throughout the day with readings taken when media is changing from yellow to brown. Generally with patient samples, plates should be visually checked after 1–2 h. If visible color change has occurred, then a reading should be taken. If not, take a longer reading (4–8 h later) and then it is a good idea to take another reading the next morning. Absorbances in the 0.1–1.0 range are preferred. If a sample has already achieved readings above 0.2, no further readings are needed.
17. For data analysis, subtract the blank well absorbance value from the individual well value. Take the full plate median and normalize each well absorbance value to the full plate median to calculate % growth. Then calculate the mean and standard deviation across all data points on the plate. Any

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⁵For slow growing cells, up to 18 μ l of electroporated cells per well can be transferred from the electroporation plate into the final cell culture plates containing cell culture media.

siRNA conditions with values lower than two standard deviations below the plate mean are candidate hits. Carefully examine data from all three plates for consistency and accuracy (*see* Note 6).

3.4 Electroporation Plate Cleaning Protocol

Electroporation plates can be reused up to four times after following an extensive cleaning protocol as described below

1. Immediately after transferring electroporated cells to warmed media, shake the remaining liquid out of the electroporation plate.
2. Wash the plate twice with MiliQ water making sure to shake the water out of the plate after each wash.
3. Place in a tub with MiliQ water for 5–10 min.
4. Shake out the water and then place in a tub with 0.2 M HCl for 5–10 min (making sure not to leave the plate in for longer because the acid can corrode the metal).
5. Shake out the acid above the container and then wash with MiliQ water.
6. Using a 70 % EtOH spray bottle, spray each row and column at least 3 times.
7. Place the plate in the hood and let air dry.
8. Once dry, expose the plate and lid to a cell culture hood UV light source for 15 min; cover the plate with the lid and return to the original plate packaging until the next use.

References

1. Ngo VN, Davis RE, Lamy L, et al. A loss-of-function RNA interference screen for molecular targets in cancer. *Nature*. 2006; 441:106–110. [PubMed: 16572121]
2. Westbrook TF, Martin ES, Schlabach MR, et al. A genetic screen for candidate tumor suppressors identifies REST. *Cell*. 2005; 121:837–848. [PubMed: 15960972]
3. Tyner JW, Walters DK, Willis SG, et al. RNAi screening of the tyrosine kinome identifies therapeutic targets in acute myeloid leukemia. *Blood*. 2008; 111:2238–2245. [PubMed: 18025156]
4. Tyner JW, Deininger MW, Loriaux MM, et al. RNAi screen for rapid therapeutic target identification in leukemia patients. *Proc Natl Acad Sci U S A*. 2009; 106:8695–8700. [PubMed: 19433805]
5. Agarwal A, MacKenzie RJ, Eide CA, et al. Functional RNAi screen targeting cytokine and growth factor receptors reveals oncorequisite role for interleukin-2 gamma receptor in JAK3-mutation-positive leukemia. *Oncogene*. 2014; 34:2991–2999. [PubMed: 25109334]

⁶For data analysis, in addition to normalization of individual well values to plate median, values can also be normalized to the mean of each respective row and column. This can be done for each well for every row and column to eliminate positional artifacts and reduce run deviation.

6. Bric A, Miething C, Bialucha CU, et al. Functional identification of tumor-suppressor genes through an in vivo RNA interference screen in a mouse lymphoma model. *Cancer Cell*. 2009; 16:324–335. [PubMed: 19800577]
7. Hu G, Kim J, Xu Q, et al. A genome-wide RNAi screen identifies a new transcriptional module required for self-renewal. *Genes Dev*. 2009; 23:837–848. [PubMed: 19339689]
8. Luo J, Emanuele MJ, Li D, et al. A genome-wide RNAi screen identifies multiple synthetic lethal interactions with the Ras oncogene. *Cell*. 2009; 137:835–848. [PubMed: 19490893]
9. Schlabach MR, Luo J, Solimini NL, et al. Cancer proliferation gene discovery through functional genomics. *Science*. 2008; 319:620–624. [PubMed: 18239126]
10. Ebert BL, Pretz J, Bosco J, et al. Identification of RPS14 as a 5q-syndrome gene by RNA interference screen. *Nature*. 2008; 451:335–339. [PubMed: 18202658]
11. Lam LT, Davis RE, Ngo VN, et al. Compensatory IKKalpha activation of classical NF-kappaB signaling during IKKbeta inhibition identified by an RNA interference sensitization screen. *Proc Natl Acad Sci U S A*. 2008; 105:20798–20803. [PubMed: 19104039]
12. Silva JM, Mizuno H, Brady A, et al. RNA interference microarrays: high-throughput loss-of-function genetics in mammalian cells. *Proc Natl Acad Sci U S A*. 2004; 101:6548–6552. [PubMed: 15084744]
13. Zender L, Xue W, Zuber J, et al. An oncogenomics-based in vivo RNAi screen identifies tumor suppressors in liver cancer. *Cell*. 2008; 135:852–864. [PubMed: 19012953]
14. Hannon GJ, Rossi JJ. Unlocking the potential of the human genome with RNA interference. *Nature*. 2004; 431:371–378. [PubMed: 15372045]
15. Paddison PJ, Silva JM, Conklin DS, et al. A resource for large-scale RNA-interference-based screens in mammals. *Nature*. 2004; 428:427–431. [PubMed: 15042091]
16. Bicocca VT, Chang BH, Masouleh BK, et al. Crosstalk between ROR1 and the pre-B-cell receptor promotes survival of t(1;19) acute lymphoblastic leukemia. *Cancer Cell*. 2012; 22:656–667. [PubMed: 23153538]
17. Maxson JE, Gotlib J, Pollyea DA, et al. Oncogenic CSF3R mutations in chronic neutrophilic leukemia and atypical CML. *N Engl J Med*. 2013; 368:1781–1790. [PubMed: 23656643]
18. Sanda T, Tyner JW, Gutierrez A, et al. TYK2-STAT1-BCL2 pathway dependence in T-cell acute lymphoblastic leukemia. *Cancer Discov*. 2013; 3:564–577. [PubMed: 23471820]

Fig. 1.

Workflow for optimization of electroporation conditions for leukemia cells. **(a)** To determine optimal electroporation conditions, incubate cells with nonspecific siRNA or siRNA targeted to a gene that is necessary for maintenance of the growth/viability of the cells of interest. In the absence of a gene target that is specific to the cell line of interest, siRNA targeting PLK1 can be used as a generic “killer siRNA”. Electroporate cells using a variety of different voltages and pulse lengths, a good starting range for voltage is 150–450 V. Pulse length will vary widely depending on the gap width of cuvettes being used. For standard 4 mm gap width, a good starting range is 1.5–5.0 ms. **(b)** After electroporation, culture for 2 days and determine the percent cell viability using a method such as PI exclusion. **(c)** In parallel, plate cells in 96-well plates to conduct a proliferation/cell viability assay after 4 days using a tetrazolium-based assay, such as MTS. **(d)** To select the optimal conditions for electroporation, several factors must be balanced including PI exclusion cell viability readings exceeding at least 80 %, MTS cell viability readings of at least 60 % of unelectroporated control cells, and maximal impact of killer siRNA on cell viability compared with nonspecific control

Fig. 2. Exemplary PI exclusion and MTS cell viability readings from electroporation optimization experiments. MOLT-4 cells (1×10^5 cells/well) were electroporated using a square wave protocol with indicated voltages and pulse lengths for two pulses. 12 μ l of electroporated cells were transferred to 90 μ l of culture media for assessment of cell viability by PI exclusion at 48 h (a) and by MTS assay at 96 h in triplicate (b)

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Table 1

siPORT buffer recipe

Components	Stock (M)	Dilution	Final (mM)	For 250 ml	For 500 ml
Trehalose	1.2	0.1	120	25	50
HEPES	1	0.02	20	5	10
Myo-Inositol	0.1	0.01	1	2.5	5
KCl	0.1	0.01	1	2.5	5
MgCl ₂	0.1	0.01	1	2.5	5
K ₂ HPO ₄	0.1	0.01	1	2.5	5
KH ₂ PO ₄	0.04	0.01	0.4	2.5	5
KOH	0.214	0.01	2.14	2.5	5
Glutathione	0.1	0.01	1	2.5	5
Water				202.5	405

Table 2

Suggested electroporation conditions for several leukemia cell lines

Cell line	Electroporation condition	% viability via cell counting	% viability via MTS assay
MOLT4	250 V, 2.0 ms	86	79
JURKAT	200 V, 2.5 ms	81	62
GDM-1	250 V, 4.0 ms	95	63
Kasumi-2	200 V, 4.0 ms	81	53
LOUCY	250 V, 4.0 ms	83	87
MOLT-16	250 V, 2.0 ms	N/A	75
UT-7	350 V, 1.0 ms	N/A	77
SUPT-13	200 V, 4.5 ms	N/A	81
HBPALL	250 V, 3.5 ms	N/A	85
SKW-3	200 V, 4.5 ms	N/A	75
PEER	200 V, 4.5 ms	N/A	90
CCRF	250 V, 3.0 ms	N/A	89
RPMI-8402	250 V, 3.0 ms	N/A	60
PF-382	250 V, 3.0 ms	N/A	70
AML-1	250 V, 3.0 ms	N/A	53
ML-1	250 V, 3.0 ms	N/A	72
HL-60	250 V, 3.0 ms	N/A	63
TF-1	250 V, 3.0 ms	N/A	62
HT-93	300 V, 3.0 ms	N/A	80
Primary myeloid cells	275 V, 5.0 ms		
Primary lymphoid cells	250 V, 4.0 ms		

Note: The electroporation conditions might slightly vary from above suggested conditions because of variation in cell clones. Therefore it is recommended to optimize own conditions around the above-given parameters