Chapter 14

High-Throughput Screening of Tyrosine Kinase Inhibitor Resistant Genes in CML

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Abstract

Genome-wide RNA interference (RNAi) screening in mammalian cells has proven to be a powerful tool for identifying new genes and molecular pathways relevant to many cellular processes and diseases. For example, screening for genes that, when inactivated, lead to resistance to cancer therapeutic drugs can reveal new mechanisms for how resistance develops and identify potential targetable strategies to overcome drug resistance. Here, we describe a detailed procedure for performing a high-throughput RNAi screen using a genome-wide human short hairpin RNA (shRNA) library for identifying tyrosine kinase inhibitor (TKI)-resistance genes in a human CML cell line model.

Key words Genome-wide, shRNA screen, RNAi, Tyrosine kinase inhibitor, CML, Imatinib, Drug resistance

1 Introduction

RNA interference (RNAi) is a mechanism by which expression of a specific gene is posttranscriptionally inhibited. RNAi is mediated by a double-stranded RNA, which recognizes and induces sequence-specific degradation of the corresponding mRNA, resulting in silencing or "knocking down" the gene. The development of RNAi as a genetic tool initially held great promise for analyzing loss-of-function phenotypes in mammalian cells using high-throughput, unbiased, function-based genetic screens. However, it was not until the development of RNAi expression libraries about 10 years ago [1–4] that such large-scale screens became feasible. Using these and other RNAi libraries, many studies have uncovered previously unappreciated genes and mechanisms involved in diverse biological processes in mammalian cells, particularly in the context of cancer, such as unknown tumor suppressor genes [5, 6], synthetic lethal interactions [7], and potential drug targets [8, 9].

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Today, a wide variety of RNAi libraries are commercially available. These libraries typically use a lentiviral vector to express a chimeric short hairpin RNA (shRNA), which is subsequently processed into a small interfering RNA (siRNA) to induce target mRNA degradation [10, 11]. Lentiviral-based libraries have high titers and broad trophism, making them suitable for use in a variety of mammalian cell types. Each shRNA in the library contains an easily sequenced barcode, which enable unambiguous identification of the shRNA. The genome-wide screen described in this protocol uses the human lentiviral shRNA library from The RNAi Consortium (TRC), which has ~85,000 shRNA constructs with 3–5 shRNAs per gene, targeting ~22,000 human genes [10, 12]. To facilitate screening such a large number of shRNAs, we have divided the library into "pools," with ~5000 shRNAs per pool for a total of 22 pools. It is worth noting that if other commercially available libraries are used, the protocol below should be modified accordingly (particularly for Subheading 3.5).

BCR-ABL mutation-independent resistance to tyrosine kinase inhibitors (TKIs) frequently occurs in CML [13–15]. Identifying TKI resistance-related genes helps increase our knowledge of how resistance occurs and may lead to the development of better therapeutic strategies for CML. Here we describe a genome-wide screening strategy in a human CML cell line to identify genes involved in resistance to the first-generation TKI, imatinib [16]. Because shRNA-mediated gene knockdown usually takes a few days to lead to the desired biological consequence, CML cells are transduced with the shRNA library and cultured under selective conditions for 1 week. Following an empirically determined lethal dose of imatinib, cells transduced with no shRNA or with an shRNA that does not confer TKI resistance will be killed. Live cells are then collected to identify shRNAs by sequencing, thereby identifying the corresponding genes related to TKI resistance. Here, we describe all the basics for a genome-wide shRNA screen, including shRNA library virus preparation and transduction, drug selection, shRNA identification either using TA cloning and conventional Sanger sequencing or deep sequencing, and essential procedures to validate the target genes.

2 Materials

Prepare all solutions using ultrapure double-distilled water (ddH_2O). Store all commercially obtained reagents according to the manufacturer's instructions.

2.1 Cell Lines and Culture Conditions

1. Cell lines: 293T cells (*see* **Note 1**) and human K562 CML cells (both from American Tissue Culture Collection).
2. Cell culture media: Add 50 mL of fetal bovine serum (FBS) into a 500 mL bottle of DMEM high glucose medium

(for 293T cells) or RPMI-1640 medium (for K562 cells), and add 500 μL of Penicillin–Streptomycin (1000×). Mix well and store at 4 °C. Prior to starting the cell culture experiment, warm the bottle in a 37 °C water bath for about 15 min.

2.2 Virus Preparation and Transduction

1. 6-well clear tissue culture plates.
2. TRC Lentiviral Human Genome shRNA Library (GE Dharmacon) divided into 22 pools (*see* **Note 2**) and a corresponding control nonsilencing (also called nontargeting) shRNA.
3. Lentiviral packaging plasmids pMD2.G (Addgene plasmid #12259) and psPAX2 (Addgene plasmid #12260).
4. Effectene Transfection Reagent kit (QIAGEN), which includes Effectene reagent, Enhancer, and EC buffer.
5. 0.45 μM filters.
6. Polybrene (1 μg/μL) (*see* **Note 3**).
7. Puromycin (5 mg/mL).
8. Phosphate buffered saline (PBS; 10×): 25.6 g $Na_2HPO_4 \cdot 7 H_2O$, 80 g NaCl, 2 g KCl, 2 g KH_2PO_4, ddH_2O to 1 L. Autoclave prior to use. Store at room temperature.
9. Crystal violet colony staining solution (0.05 %): Mix 0.5 g of crystal violet, 27 mL 37 % formaldehyde, 100 mL 10× PBS, 10 mL methanol, 863 mL ddH_2O. Store at room temperature.

2.3 Cell Viability (Imatinib Resistance) Assay

1. T25 tissue culture flasks.
2. Imatinib (10 mM): Weigh 24.68 mg of imatinib free base (MW: 493.60) powder (LC Laboratories) and transfer it into a 1.5 mL microcentrifuge tube. Dissolve with 1 mL of DMSO to reach a 50 mM (5×) stock solution. Dilute the 5× stock solution with DMSO into a 10 mM (1×) working solution. Store at −20 °C.
3. Trypan Blue.
4. Hemocytometer.
5. Dead Cell Removal Kit (Miltenyi Biotec), which contains ready-to-use MicroBeads and 20× Binding Buffer.
6. LS MACS columns and magnetic cell separator (Miltenyi Biotec).
7. Corning® clear bottom 96-well plates.
8. CellTiter 96® Aqueous One Solution Cell Proliferation Assay kit (Promega).

2.4 Genomic DNA Preparation for shRNA Identification

2.4.1 Sanger Sequencing

1. Cell lysis buffer: 0.5 % SDS, 200 μg/mL of protease K, 10 mM Tris–HCl, pH 8.0, 10 mM EDTA. Store at room temperature.
2. Phenol:chloroform:isoamyl alcohol 25:24:1 saturated with 10 mM Tris, pH 8.0, 1 mM EDTA. Store at 4 °C.
3. Chloroform. Store at room temperature.
4. Glycogen (20 mg/mL).
5. Sodium acetate (3 M): Dissolve 408.1 g of sodium acetate·$3H_2O$ (MW 136) in 800 mL of ddH_2O. Adjust the pH to 5.2 with glacial acetic acid. Store at room temperature.
6. Ethanol (95 and 70 %).
7. Buffer EB (QIAGEN).
8. Taq PCR buffer (10×): 100 mM KCl, 100 mM $(NH_4)_2SO_4$, 200 mM Tris–HCl, pH 8.75, 20 mM $MgSO_4$, 1 % Triton X-100, 0.1 % BSA. Store at −20 °C. Alternatively, it can be purchased.
9. dNTP set (100 mM each A, C, G, T).
10. Primers: Primer1 (MF18; 10 μM), TACGATACAAGGCTGT TAGAGAG; Primer2 (MF19; 10 μM), CGAACCGCAAGG AACCTTC; Sequencing primer (M22; 5 μM), AAACCC AGGGCTGCCTTGGAAAAG.
11. Taq and Pfu DNA polymerases.
12. Dimethyl sulfoxide (DMSO).
13. pGEM®-T Easy Vector Systems (Promega), which includes TA vector (50 ng/μL), T4 DNA ligase (3 U/μL), and 2× rapid ligation buffer.
14. DH5α competent cells. Store at −80 °C.
15. 2× LB broth: Dissolve 20 g of peptone, 10 g of yeast extract, and 5 g of NaCl in 1 L of ddH_2O. Autoclave prior to use. Store at room temperature.
16. Isopropyl-ß-D-thiogalactopyranoside (IPTG; 1 M). Dissolve 1 g of IPTG in 4196 μL ddH_2O. Filter sterilize with syringe and 0.22 μm filter. Store at −20 °C.
17. 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (BCIG or X-gal; 50 mg/mL): Dissolve 50 mg of X-gal in dimethylformamide (DMF). Store at −20 °C, protected from the light.
18. LB Amp plates: Add 15 g of agar to 1 L of 2× LB broth and autoclave for 25 min. Cool down and add ampicillin (100 μg/mL). Pour onto 10 cm dishes, let solidify, and store at 4 °C.

2.4.2 Deep Sequencing

1. DNeasy Blood & Tissue Kit (QIAGEN).
2. TaKaRa Ex Taq™ Polymerase (EMD Millipore), supplied with 10× Ex Taq Reaction Buffer and 2.5 mM dNTP mix.

3. P5-ORF primer mix. Dissolve all 16 P5-ORF primers (*see* Table 1) individually and mix them at an equal molar ratio to reach 5 μM.
4. P7 index primers (5 μM): Primer A, CAAGCAGAAGACGGCATACGAGATCGGTTCAAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTCTACTATTCTTTCCCCTGCACTGT; Primer B, CAAGCAGAAGACGGCATACGAGATATTGGATTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTCTACTATTCTTTCCCCTGCACTGT.
5. QIAquick Gel Extraction Kit (QIAGEN).
6. Bioanalyzer Lab-on-a-chip system (Agilent Technologies).
7. Deep sequencing instrument.

3 Methods

Carry out all experiments in an ultraviolet-sterilized vacuum hood. Incubate cells in a 5 % CO_2 incubator at 37 °C.

3.1 shRNA Lentivirus Preparation

1. Thaw early passage 293T cells (i.e., propagated for less than five passages) and grow them in DMEM high glucose medium for two more passages.
2. On day 1, plate 5×10^5 293T cells in each well of a 6-well plate. Use one well for each of the 22 pools, plus one more for the control nonsilencing (NS) shRNA. Shake the plates well to make sure the cells are evenly spread. Incubate at 37 °C for 24 h.
3. On day 2, aspirate old medium and add 2 mL of prewarmed fresh medium onto the cells (*see* **Note 4**). Incubate the cells at 37 °C until the transfection mixture is added. To make the transfection mixture, mix 1 μg of pooled shRNA plasmids (or control nonsilencing shRNA plasmid), 0.5 μg of pMD2.G (VSV-G envelope expressing plasmid), and 1 μg of psPAX2 (lentiviral packaging plasmid) in 100 μL of EC buffer. Add 3.2 μL of Enhancer, mix well by brief vortexing, and let sit at room temperature for 5 min. Add 10 μL of Effectene, vortex and let sit at room temperature for another 15 min. Dispense 0.5 mL of fresh medium to the transfection mixture and, while holding the plate still, gently dispense the entire mixture evenly on top of the cells.
4. On day 3, aspirate all of the medium and add 2.5 mL of prewarmed fresh medium. Incubate at 37 °C for 48 h.
5. On day 5, collect supernatant with a syringe and dispense it through a 0.45 μm filter to remove cell debris. Aliquot the virus supernatant into 1.5 mL microcentrifuge tubes. Store at −80 °C if not using immediately (*see* **Note 5**).

Table 1
List of primers used to prepare the P5-ORF primer mix

Primer name	Primer sequence (5′ → 3′)
Primer 1	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTTCTTGTGGAAAGGACGA
Primer 2	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTATCTTGTGGAAAGGACGA
Primer 3	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTGATCTTGTGGAAAGGACGA
Primer 4	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTCGATCTTGTGGAAAGGACGA
Primer 5	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTACGATCTTGTGGAAAGGACGA
Primer 6	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTCTAGAATCTTGTGGAAAGGACGA
Primer7	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTGACGACATCTTGTGGAAAGGACGA
Primer 8	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTTGGACACATCTTGTGGAAAGGACGA
Primer 9	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTTCTTGTGGAAAGGACGA
Primer 10	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTCTCTTGTGGAAAGGACGA
Primer 11	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTAGTCTTGTGGAAAGGACGA
Primer 12	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTGCCTCTTGTGGAAAGGACGA
Primer 13	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTAAGCTCTTGTGGAAAGGACGA
Primer 14	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTGAAACATCTTGTGGAAAGGACGA
Primer 15	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTCGAGAAATCTTGTGGAAAGGACGA
Primer 16	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTTTGAAGCATCTTGTGGAAAGGACGA

3.2 Virus Titer Determination

1. Plate 10^4 293T cells in each well of a 6-well plate and incubate at 37 °C for ~16 h. Again, use one well for each of the 22 pools, plus one more for the control nonsilencing shRNA.
2. Thaw the virus supernatant (if previously frozen), and make a series of seven tenfold serial dilutions in DMEM medium. Mix 0.1 mL of diluted virus with 0.9 mL of fresh medium. Add polybrene to a final concentration of 2 μg/mL. Gently dispense the virus mixture on top of the 293T cells and incubate at 37 °C for 2 h. Aspirate the virus supernatant and add 2.5 mL of fresh medium. Incubate at 37 °C for 24 h.
3. Add 1 μg/mL of puromycin to each well and incubate at 37 °C until colonies form (usually about 10 days).
4. Wash colonies once with 1× PBS and stain with crystal violet staining solution at room temperature for 20 min. Wash the colonies multiple times with ddH_2O until the water runs colorless. Air-dry the plate and count the colonies. Calculate the virus titer using the following formula:

   Virus titer = colony number × dilution factor × 10

3.3 Determination of Optimum Imatinib Concentration for the Screen

1. Seed 1 × 10^6 K562 cells in 5 mL of RPMI-1640 medium in each of six T25 flasks, and add 1 μM, 2 μM, 5 μM, 10 μM, 20 μM, or 50 μM of imatinib to each flask.
2. Monitor cell viability every day for 7 days using a trypan blue assay according to the supplier's instructions. Briefly, mix the cells well by pipetting multiple times, and transfer 10 μL of cell culture to a 1.5 mL microcentrifuge tube and mix with 10 μL of trypan blue. Load the mixture onto the hemocytometer. Determine the cell number per mL using the following formula:

   Number of cells per mL = [number of cells in all four corner squares]/4 × 2 × 10^4

3. Identify the optimum concentration of imatinib that it is able to eliminate over 99 % of live cells in a week.

3.4 shRNA Library Transduction and Selection

1. Prepare the cell transduction mixture by mixing 2 × 10^6 K562 cells, 1 μg/mL of polybrene, shRNA virus stock (either a pool or the nonsilencingshRNA) at a multiplicity of infection (MOI) <1 (*see* **Note 5**), and fresh medium up to a total volume of 1 mL (*see* **Note 6**). Transfer the mixture to a well of a 6-well plate, and perform a spin-down infection at 3000 rpm (~2100 × *g*), at room temperature for 90 min in an Allegra X-12 plate centrifuge (Beckman Coulter), or equivalent.
2. Immediately after infection, add 2 mL of fresh medium to each well, and incubate at 37 °C for 48 h. Transfer the cells to T25 flasks, and add 1 μg/mL of puromycin to select for 3 days.

3. Split selected cells into two fractions. Freeze down one fraction in 100 % FBS with 7 % DMSO in liquid nitrogen as a backup. Treat the other fraction of the transduced cells with 20 μM of imatinib for 1 week. Monitor cell viability using a trypan blue assay every day. Stop the drug treatment when >99 % of control (nonsilencing) shRNA-treated cells die.
4. Isolate live cells using the Dead Cell Removal Kit according to the manufacturer's instructions. Briefly, pellet all the cells at 300 × *g* in a tabletop centrifuge. Remove the supernatant completely, and resuspend the cell pellet in 100 μL of MicroBeads per ~10^7 cells. Mix well and incubate at room temperature for 15 min. Meanwhile, place the LS MACS column on the magnetic stand and prepare the column by rinsing with 3 mL of 1× Binding Buffer. Load the cell suspension onto the column and rinse four times with 3 mL of 1× Binding Buffer. Collect the effluent as the live cell fraction.

3.5 Genomic DNA Preparation and shRNA Identification

3.5.1 Method for Sanger Sequencing

1. Pellet 1–5 × 10^6 live cells per pool at (~300 × *g*) for 5 min in a tabletop centrifuge and then resuspend in 500 μL of cell lysis buffer.
2. Incubate the cell lysate at 55 °C for 2 h and then extract with an equal volume of phenol:chloroform:isoamyl alcohol. Transfer the aqueous phase into a new 1.5 mL microcentrifuge tube and extract again with an equal volume of chloroform.
3. Precipitate the DNA by adding 2 μL of glycogen, 0.1 volumes of 3 M sodium acetate, and two volumes of 95 % ethanol. Mix well by vortexing and leave at −80 °C for at least 1 h. Spin in a tabletop centrifuge at top speed at 4 °C for 30 min, and wash the pellet with 1 mL of 70 % ethanol. Aspirate ~900 μL of supernatant and use a pipette to remove the residual 100 μL to avoid losing the pellet. Air-dry the pellet for 3 min, dissolve it in 20 μL of Buffer EB, and measure the DNA concentration (*see* **Note 7**).
4. Assemble the following in a PCR reaction: ~100 ng genomic DNA, 2.5 μL 10× Taq buffer, 1 μL 10 mM dNTP, 1 μL MF18 primer, 1 μL MF19 primer, 1 μL DMSO, 0.5 μL Taq DNA polymerase, 18 μL ddH_2O.
5. Program a PCR machine with following cycling program and run the samples through:

   Step1 94 °C for 2 min

   Step2 94 °C for 30 s

   Step3 55 °C for 45 s

   Step4 72 °C for 1 min

   Step5 Go to **Step 2** for 29 additional cycles

   Step6 72 °C for 5 min

   Step7 4 °C indefinitely

6. To make sure the PCR reaction worked, load 3 μL of the reaction mixture on a 1 % agarose gel. A ~700 bp PCR product should be observed.
7. Ligate the PCR product into the TA cloning vector by setting up a ligation reaction as follows: 3 μL of PCR product, 1 μL of TA vector, 5 μL of 2× rapid ligation buffer, 1 μL of T4 DNA ligase. Incubate the ligation reaction at room temperature for 1 h followed by 4 °C for at least 12 h.
8. The next day, transform the ligation reaction into DH5α competent cells. Thaw cells on ice, and add 10 μL of the ligation reaction mixture into 100 μL of competent cells. Mix well by gently flicking the tube and incubate on ice for 30 min. Heat shock the cells at 42 °C for 1 min, then place on ice for 5 min. Add 900 μL fresh 2× LB medium and incubate at 37 °C for 30 min.
9. Meanwhile, mix 5 μL of 1 M IPTG and 50 μL of 50 mg/mL X-gal and spread on an LB Amp plate. Let sit at room temperature for a few minutes.
10. Spin down the cells in a table stop centrifuge at (~850 × *g*) for 2 min. Aspirate ~900 μL of the medium, and resuspend the cells in the remaining 100 μL medium. Spread on an LB Amp plate (*see* **Note 8**), and incubate at 37 °C for ~16 h.
11. The next day, aliquot 25 μL of ddH_2O into a series of PCR tubes, one for each colony to be picked (*see* **Note 9**). Pick a single white colony from the LB Amp plate using a pipette tip, place the tip in the PCR tube and mix well by pipetting. Remove 20 μL and discard.
12. Prepare a PCR master mix (by multiplying the following recipe by the number of colonies to be screened) and add 15 μL to each tube prepared in **step 11**: 2 μL of 10× Taq Buffer, 1 μL dNTPs, 0.5 μL M18 primer, 0.5 μL M19 primer, 0.125 μL Taq DNA polymerase, 0.125 μL Pfu DNA polymerase, 11 μL ddH_2O.
13. Program a PCR machine with the following cycling program and run the samples through:

    Step1 95 °C for 2 min

    Step2 94 °C for 1 min

    Step3 55 °C for 1 min

    Step4 72 °C for 3 min

    Step5 Go to **Step 2** for 34 additional cycles

    Step6 72 °C for 5 min

    Step7 4 °C indefinitely

14. To make sure the PCR reaction worked, load 3 μL of the reaction mixture on a 1 % agarose gel. Again, a ~700 bp PCR product should be observed.
15. Add 80 μL of ddH_2O to each tube and mix well by pipetting. Mix 2 μL of the diluted PCR product and 2 μL of M22 sequencing primer. Send for sequencing.
16. To identify shRNAs from the sequencing results, search for the sequence TTCAAAAA to find the beginning of the shRNA, CTCGAG to define the loop, and CCGGTG to define the end. Then map the shRNA sequence with the TRC shRNA library database (https://www.broadinstitute.org/rnai/trc/lib) to find the corresponding gene.

3.5.2 Method for Deep Sequencing

1. Pellet 1–5 × 10^6 live cells per pool at (~300 × *g*) for 5 min in a tabletop centrifuge. Isolate genomic DNA using a DNeasy Blood and Tissue Kit according to the manufacturer's protocol for cultured cells. Determine the genomic DNA concentration (*see* **Note 7**).
2. Assemble the following in a PCR reaction: 3.3 μg DNA from each pool (*see* **Note 10**), 10 μL Ex Taq Reaction Buffer, 8 μL dNTPs, 10 μL P5-ORF primer mix (*see* **Note 11**), 10 μL P7 primer (A or B) (*see* **Note 12**), 1.5 μL TaKaRa Ex Taq™ polymerase, and ddH_2O to a total volume of 100 μL.
3. Program a PCR machine with the following cycling program and run the samples through:

   Step1 95 °C for 1 min

   Step2 95 °C for 30 s

   Step3 53 °C for 30 s

   Step4 72 °C for 30 s

   Step5 Go to **Step 2** for 27 additional cycles

   Step6 72 °C for 10 min

   Step7 4 °C indefinitely
4. To make sure the PCR reaction worked, load 10 μL of the reaction mixture on a 2 % agarose gel. The PCR product, including the shRNA region and Illumina sequences, should be ~298 bp.
5. Run the remaining 90 μL of PCR product on a 2 % agarose gel and purify the band using a QIAquick Gel Extraction Kit following the manufacturer's protocol with the following exception: Prior to the PE wash step, wash the column with one volume of PB buffer. Measure the concentration of the isolated PCR product (*see* **Note 7**).
6. Run each PCR product on a Bioanalyzer Lab-on-chip system as a quality control.

7. Combine an equivalent amount of PCR product from each of the 22 reactions as the final library and perform deep sequencing with single end 50 bp reads (*see* **Note 13**).
8. To identify the shRNAs from the screen, assess the quality of the sequence libraries using FastQC, a free software quality control tool for high-throughput sequence data [17]. Prior to alignment of the sequence reads, use a custom Perl script to identify the first four bases flanking the informative sequence at the 5′ end. Extract the core 16–21 bp sequences and map them to the human genome(hg19), allowing up to two mismatches, using Bowtie1, a software program designed to align short DNA sequences [18]. Do not perform further analysis on reads that do not contain the first four bases of the 5′ sequence.

3.6 Validating Candidate Genes

1. Prepare individual virus supernatants for each shRNA clone identified from the screen, as described earlier in Subheading 3.1.
2. Infect 2×10^5 K562 cells with 0.5 mL of virus supernatant and 2 μg/mL of polybrene. Add 3 mL of fresh medium and culture for 24 h, then add 1 μg/mL of puromycin to select for 5 days. Cells may be divided and half frozen at −80 °C as a backup (*see* **Note 14**).
3. To quantify imatinib resistance, plate 2500 knockdown cells (counted by trypan blue assay) per well in triplicate in 96-well plates. Culture the cells in plain medium (200 μL total volume) and add either 10 μM imatinib or DMSO (control) for 4 days (*see* **Note 15**). Add 20 μL of CellTiter 96® Aqueous One Solution Cell Proliferation Assay MTT reagent to each well (*see* **Note 16**), mix by pipetting, and incubate at 37 °C for at least 1 h (*see* **Note 17**). Record the absorbance at 490 nm (A490) using a Victor3 or SpectroMaxM5 plate reader (*see* **Note 18**).
4. Determine the resistance to imatinib by calculating the ratio of relative cell viability of candidate gene knockdown cells to that of control cells (*see* **Notes 19** and **20**).

   Resistance = ([A_{490} Candidate shRNA Imatinib]/[A_{490} Candidate shRNA DMSO])/([A_{490} NS shRNA Imatinib]/[A_{490} NS shRNA DMSO]).
5. RNAi-mediated gene knockdown is known to have off-target effects [19, 20]. Therefore, for candidates that test positive, it is imperative to pick a second or third shRNA for the same candidate gene and test for knockdown efficiency using qRT-PCR and imatinib resistance. Any gene with two or more effective shRNAs that confer imatinib resistance can be considered a validated candidate for follow-up studies.

4 Notes

1. 293T cells are used to prepare the shRNA viruses, as they transfect easily and support high-level expression of viral proteins.
2. To facilitate screening the genome-wide shRNA library, we generated 22 pools of ~5000 shRNAs each. To derive the pools, we grew bacterial stocks from 50 96-well plates and pooled aliquots of them together prior to plasmid preparation.
3. Polybrene improves overall transduction efficiency by enhancing receptor-independent virus absorption through the cell membrane. Usually, the more polybrene that is added, the higher the transduction efficiency. However, too much polybrene can be deleterious to target cells and may lead to increased cell size, reduced proliferation, or even cell death. Therefore, a preliminary experiment should be done to find the optimum tolerated amount of polybrene. Test a range of concentrations, from 1 to 10 μg/mL, and monitor cells for size, proliferation, and viability.
4. Before changing the medium, make sure cells have reached ~70 % confluency. Cells with low confluency will be more easily disturbed, which can cause a low virus titer.
5. Virus titer may drop significantly over time when frozen at −80 °C. Therefore, if the viral stocks are frozen, the titer of the virus needs to be retested before using for library screening.
6. With suspension cells, such as K562 cells, it is advisable to test transduction efficiency before performing an shRNA library infection. Empirically determine parameters such as the highest tolerated amount of polybrene and minimal amount of virus required to reach 50 % infection.
7. We use a NanoDrop spectrophotometer (Thermo Scientific) to measure DNA concentrations in solutions of precipitated DNA and PCR reactions.
8. On occasion, there might be too many colonies growing on the plate due to high competency of bacteria. To avoid this, plate two or three tenfold serial dilutions of the bacteria on different LB Amp plates.
9. The number of colonies to be picked for Sanger sequencing is estimated based on the percentage of live cells after drug treatment. As a starting point, pick 10–20 colonies per pool. Stop sequencing when the same shRNA identified more than once, indicating you have reached saturation.
10. We amplify genomic DNA from individual viral pools separately, so that if a pool fails to amplify it can be easily detected

and repeated. The amount of DNA used for each PCR reaction should be empirically determined based on the percentage of live cells after infection and drug selection. A typical mammalian diploid genomic DNA mass is ~6.6 pg. A representation of ~200 cells per shRNA with 5000 shRNAs per pool will require ~10^6 cells, and the total amount of genomic DNA required for PCR will be 6.6 μg. With drug selection killing >50 % of cells in majority of pools, we use 3.3 μg of DNA for each PCR reaction.

11. P5-ORF primer mix is a mixture of 16 different P5 primers with unique barcodes. Usually, at least 12 unique barcodes are required to aid in the cluster calling to sequence one sample.
12. Perform two separate PCR reactions for all 22 pools, one with P7 primer A and one with P7 primer B. Each primer has a unique barcode to serve as technical replicates.
13. For our deep sequencing we used a MiSeq Sequencing System (Illumina) and prepared samples using MiSeq® Reagent Kit v3 (Illumina) according to the manufacturer's instructions.
14. Try to freeze down the shRNA knockdown cells as early as possible (within a week of transduction) to prevent the effect of shRNA knockdown from getting lost over time.
15. MTT assays usually have a high background reading. Therefore, make sure to add control wells that contain only medium and DMSO.
16. Open the MTT reagent bottle in the cell culture hood to prevent contamination.
17. After adding the MTT reagent, test a series of incubation times (e.g., 30 min, 1 h, 2 h, 4 h) to determine an optimal time so that the color signal will not be saturated. Ideally, the reading should be in the signal range from 0.0 to 2.0. However, it is best to keep all the readings within the linear range and keep the highest absorbance close to 1.
18. Before loading the plate for reading, check if there are brown clumps clustering on the side of the well, which will compromise the accuracy of the absorbance reading and therefore the determination of cell viability. If present, try to break them up by pipetting a few times using multichannel pipette, then gently remove the bubbles.
19. Always use the same batch of cells to test the nonsilencing shRNA control as that used to test the candidate shRNA.
20. If there are greater than 50 % false-positive shRNA, it is likely that dead cells were not completely removed in Subheading 3.4 **step 4**. As a result, any contamination of shRNA-containing genomic DNA would lead to identification of contaminating shRNAs. If this is the case, thaw the backup cells stored in

Subheading 3.4 **step 3**, and perform the experiment again, this time using fluorescence-activated cell sorting (FACS) to directly sort live cells into lysis buffer, prior to DNA extraction.

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