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Product Information

Target ID Library

Catalog Number MREH01
Storage temperature –20 ˚C

TECHNICAL BULLETIN

Product Description
The Target ID Library is designed to assist in discovery and identification of microRNA (miRNA) targets.

The Target ID Library is a plasmid-based, genome-wide collection of cloned cDNA. Each cDNA is cloned into the 3'UTR after a dual-selection fusion protein, TK-ZEO, which encodes a thymidine kinase-zeocin fusion protein. Dual-selection allows the user to transfect cells and screen the entire library at once, selecting first for stable transformants and secondly, after introducing a miRNA of interest, for cDNAs containing the miRNA's target. Selected cDNAs are identified by sequencing (see Figure 1-3 for Target ID Library Workflow and details).

The cDNA inserts in the Target ID Library were prepared via oligo-dT priming with a pool of total RNA prepared from multiple human tissues and cell lines to give broad coverage of the human transcriptome. This cDNA was cloned into the p3´TKzeo dual-selection plasmid (see Figure 4 for plasmid map). cDNA inserts in the Target ID Library range from 0.5 to 4 kb, with an average size of 1.2 kb. The gene targets represented in the library can be found on the Sigma-Aldrich webpage.

As with all miRNA target identification methods, we highly recommended that targets identified by screening the Target ID Library be validated by a second method, such as via microarray, qRT-PCR, reporter assay (i.e., luciferase), or Western Analysis.

Components/Reagents

Product	Quantity	Volume (Concentration)	Cat. No.
MISSION® miRNA Target ID Library	1	100 µL (approx 1 µg/µL) (See Certificate of Analysis).	LMREH01
MISSION Target ID Amplification Primer 1*	1	200 µL (25 µM)	T4830
MISSION Target ID Amplification Primer 2*	1	200 µL (25 µM)	T4955

*Primer Sequences can be found in Figure 4

Reagents Required but not Provided
Transfection and Cell Culture
- Growth medium optimized for the specific cell line
- Transfection reagent:
  - Cell specific Nucleofection Reagent plus Nucleofector (Amaxa)
  - Lipofectamine 2000, Invitrogen Catalog Number 11668
- Zeocin, Invitrogen Catalog Number R250-01
- Ganciclovir, Sigma Catalog Number G2536

MicroRNA Expression Plasmid
- pCMV-MIR, MicroRNA Expression Plasmids, Origene Catalog Number PCMVMIR
- pBABE-Puro (Plasmid 1764; Addgene)
- Antibiotic selection

Genomic DNA Purification and PCR Reagents
- GenElute™ Mammalian Genomic DNA Miniprep Kit, Sigma Catalog Number G1N10
- JumpStart™ REDTaq® ReadyMix™ Reaction Mix - for PCR, Sigma Catalog Number P0982
- JumpStart™ Taq ReadyMix™, Sigma Catalog Number P2893

PCR Cloning

- TOPO TA SEQ, Invitrogen Catalog Number K4575-01 or equivalent

Storage
Store at −10 °C to −25 °C

Precautions and Disclaimer
This product is for R&D use only, not for drug, household, or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

Figure 1. Workflow for Target ID Library
Sections A-C: Refer to sections in the Procedure. Each step is illustrated and described in detail in Figs 2 and 3.

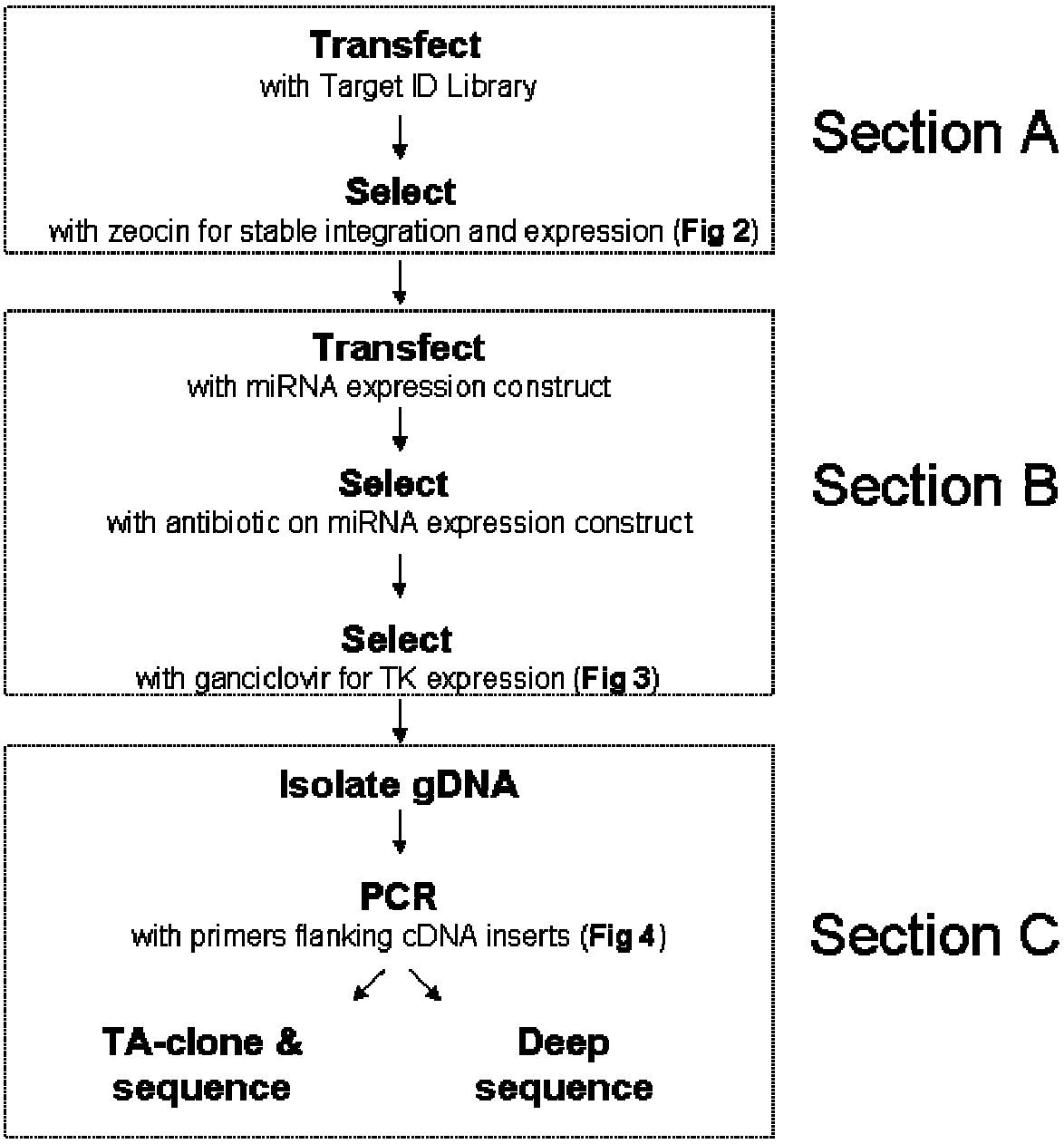


Fig 2. Transfection and Zeocin selection. The Target ID Library is a pool of plasmids (**A**), each with a human cDNA inserted into the 3'-UTR after a thymidine kinase-zeocin fusion protein (TKzeo; Fig 4). Cells are transfected with Target ID Library (**B**) and allowed to recover for 3-5 days. Constructs can integrate into the genome during this recovery period (**C**), and express the encoded transcript (**D**). After recovery, cells are exposed to zeocin (**E**). Cells expressing the TKzeo fusion protein from stably integrated Target ID constructs survive zeocin selection (**F**). Untransfected cells die (**G**). In addition, any cells containing a construct that is a target for an endogenous miRNA or any other factor expressed in the cell that inhibits expression of TKzeo from the Target ID construct will die (**H**).

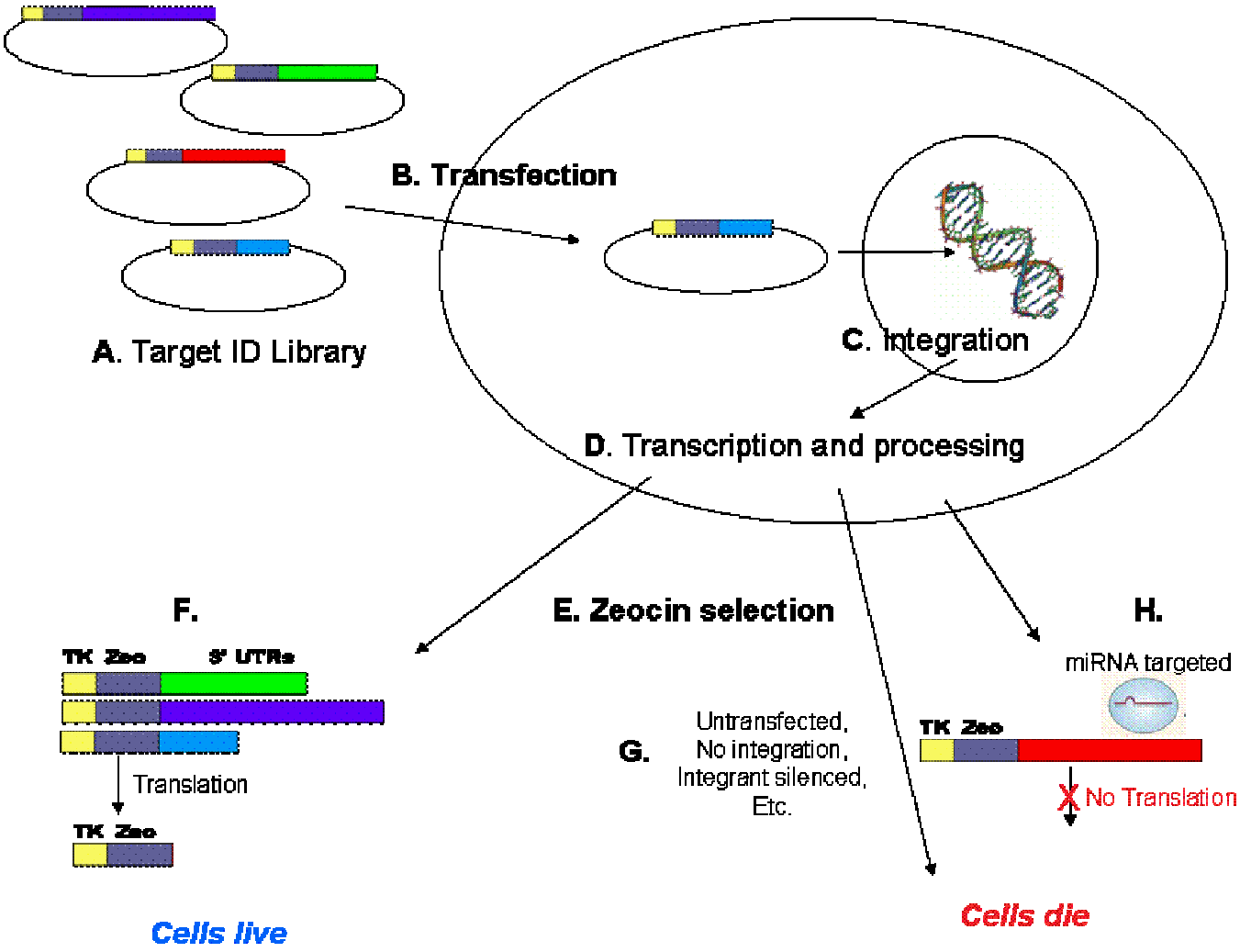


Fig 3. miRNA transfection and ganciclovir selection. Cells containing the Target ID Library (i.e., zeocin-selected cells) are transfected with a selectable microRNA expression construct (**A**). During recovery, the miRNA expression construct can integrate (**B**) and express the selectable marker encoded on the miRNA construct. After selection for stable integration (**C**) and cell expansion, cells are treated with ganciclovir (**D**). Cells producing thymidine kinase (TK) in the presence of ganciclovir (i.e., cells expressing TKzeo constructs not targeted by the miRNA) will die (**E**). On the other hand, cells containing Library constructs with miRNA target sites will not produce TK, and therefore, will survive ganciclovir selection (**F**). Surviving cells can be grown, gDNA isolated, and the cDNA containing miRNA target sites PCR-amplified using the Target ID Amplification primers. PCR products may be sequenced and aligned with the human genome to identify miRNA targets.

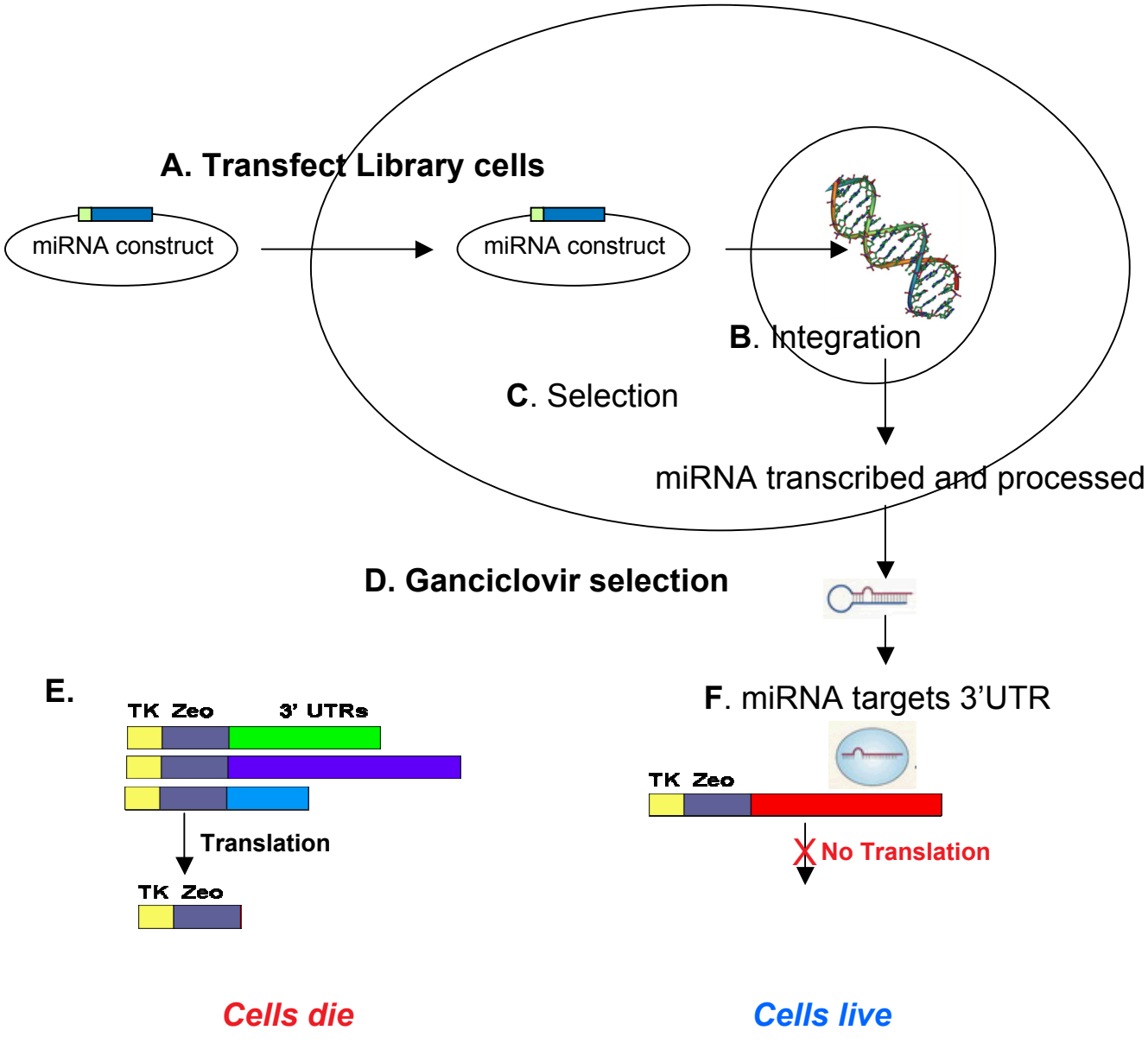
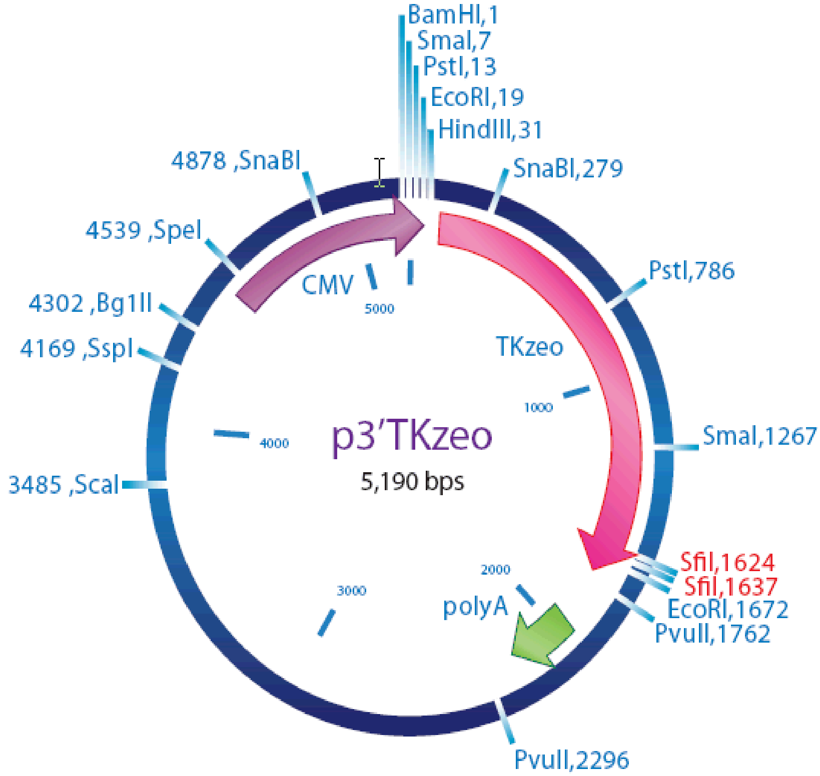


Fig 4. Plasmid Map and Location of Amplification Primers



Amplification Primer 1

```
GGTCCGGGAC GACGTGACCC TGTTCATCAG CGCGGTCCAG GACCAGGTGG TGCCGGACAA CACCCTGGCC TGGGTGTGGG TGCGCGGCCT GGACGAGCTG
CCAGGCCCTG CTGCACTGGG ACAAGTAGTC GCGCCAGGTC CTGGTCCACC ACGGCCTGTT GTGGGACCGG ACCCACACCC ACGCGCCGGA CCTGCTCGAC
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(1309-1328)

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TACGCCGAGT GGTCGGAGGT CGTGTCCACG AACTTCCGGG ACGCCTCCGG GCCGGCCATG ACCGAGATCG GCGAGCAGCC GTGGGGGCGG GAGTTCGCCC
ATGCGGCTCA CCAGCCTCCA GCACAGGTGC TTGAAGGCCC TGCGGAGGCC CGGCCGGTAC TGGCTCTAGC CGCTCGTCGG CACCCCCGCC CTCAAGCGGG

TGCGCGACCC GGCCGGCAAC TGCGTGCACT TCGTGGCCGA GGAGCAGGAC TGACCGACGC CGACCAACAC CGCCGGTCCG ACGCGGCCCG ACGGGTCCGA
ACGCGCTGGG CCGGCCGTTG ACGCACGTGA AGCACCGGCT CCTCGTCCTG ACTGGCTGCG GCTGGTTGTG GCGGCCAGGC TGCGCCGGGC TGCCCAGGCT
```

Sfi I   Sfi I   **Amplification Primer 2**

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GGGGGGTCGA CCTCGAATCC TTAGGCCATT AAGGCCGGCC GCCTCGGCCC ACTTCGTGGG GTACCGAGCT CGAATTCACT GGCCGTCGTT TTACAACGTC
CCCCCCAGCT GGAGCTTAGG AATCCGGTAA TTCCGGCCGG CGGAGCCGGG TGAAGCACCC CATGGCTCGA GCTTAAGTGA CCGGCAGCAA AATGTTGCAG
```
(1674-1693)

Sfi I are the sites of cDNA Cloning.

Primer Sequences

MISSION® Target ID Amplification Primer 1
5´ ACGACGTGACCCTGTTCATC 3´

MISSION® Target ID Amplification Primer 2
5´ TAAAACGACGGCCAGTGAAT 3´

Procedure

Before starting the transfection procedures in Sections A and B below, test a range of antibiotic levels to determine the minimal amounts needed to kill your untransfected cell line, i.e., generate a "kill curve." Kill curves will be needed for zeocin and whatever antibiotic you will use to select for stable integration of your microRNA expression construct. A kill curve will also need to be performed for ganciclovir with cells stably expressing the Target ID Library. Cells not expressing the Library will be resistant to ganciclovir.

SECTION A. Transfect with Target ID Library and Select for Stable Cell Line

Zeocin kill curve: Zeocin is used to select stably transfected cells. However, excess zeocin can cause undesired phenotypic responses in most cell types. When the appropriate concentration of zeocin for a specific cell type is unknown, perform a kill curve experiment, i.e. expose untransfected cells to increasing amounts of zeocin to determine the minimum lethal dose.

1. Plate 1.6 x 10^4 cells into wells of a 96-well plate with 120 µL fresh media.
2. The next day add from 50 µg to 1 mg/ml of selection reagent to selected wells.
3. Examine viability every 2 days.

Replace the media containing selection reagent every 3 days. The minimum concentration of selection reagent that causes complete cell death after the desired time should be used for that cell type and experiment. We have found 500 µg/ml zeocin is optimum for A549, HeLa, and MCF7 cells.

Library Transfection and Selection

Nucleofection

Cell Prep:

1. Select a cell line that either does not express, or only expresses low levels of your microRNA of interest. The microRNA will be introduced in Section B for target selection after stable expression of the Target ID Library is achieved.
2. Culture/expand cells. We have obtained good results with 2 x 10^7 cells per Library transfection.
3. Trypsinize cells that are at >80% confluency, and transfer 2 x 10^7 cells to a 15 ml sterile screw topped tubes.
4. Pellet trypsinized cells at 200 x *g* for 5 min
5. Remove medium and wash cell pellet with Hanks Balanced Salt Solution or 1X PBS
6. Centrifuge at 200 x *g* for 5 min and then remove wash
7. Repeat wash step of cell pellet

Number of Cells per Nucleofection	Recommended # of Nucleofections
2 x 10^6	10

Nucleofection:

1. Pre-warm 6-well plates with 2 ml of complete medium at 37 °C
2. Add 2 µg of Target ID Library (not to exceed 10 µL) per 0.5 ml tube for each transfection.
3. Resuspend cells in the 15 ml tube from above with 100 µL of Amaxa Nucleofection Solution (cell specific) per 2 x 10^6 cells. For example: For 10 Nucleofections add 1ml of reagent
4. One reaction at a time, add 100 µL of cells to the 2 µg of plasmid. Mix with pipette.
5. Transfer mixture to a Nucleofector cuvette
6. Insert cuvette into Nucleofector instrument and run optimized program appropriate for the cell line (High Efficiency preferred over Cell viability)
7. Fill transfer pipette with pre-warmed medium. Take up cells in same pipette and transfer to 6-well plate. Repeat for each Nucleofection, one per well.
8. Return to growth chamber for overnight incubation
9. Replace medium and allow cells to recover for 3-5 days.
10. Replace medium with complete medium containing the appropriate level of Zeocin, as determined from the kill curve test performed before Library transfection.
11. Monitor cells for zeocin selection (cells dying)
12. Replace medium with zeocin every 2-3 days
13. Once confluent in 6-well plate, passage, pool and expand cells in larger flasks
14. Length of time for expansion of cells is user and cell line dependent, but it is highly recommended to expand zeocin resistant cells to generate cryo-stocks for future screening (~2-3 weeks; cell line dependent).

Lipofectamine 2000

Cell culture: (6-well format)

1. Adherent cells: One day before transfection, plate 5 x 10^5 to 1 x 10^6 in 2ml of complete medium in two 6-well plates
2. Incubate overnight

Transfection: (Per transfection; 12 replicates total)

1. Dilute 2 µg of Target ID Library plasmid in 250 µL of Opti-MEM I Reduced Serum Medium without serum. Mix gently
2. Mix Lipofectamine 2000 gently before use. Dilute 10 µL of Lipofectamine 2000 in 240 µL of Opti-MEM I Medium. Incubate for 5 min at room temperature
3. After the 5 min incubation, combine the diluted DNA with diluted Lipofectamine 2000 (total volume = 500 µL). Mix gently and incubate for 20 min at room temperature
4. During the incubation in step 3, remove medium from 6-well plates and replace with 1.5 ml of fresh medium
5. Add the 500 µL of complexes to each well. Mix by gently rocking the plate back and forth.
6. Incubate cells overnight.
7. Replace medium and allow cells to recover for 3-5 days.
8. Replace medium with complete medium containing the appropriate level of Zeocin, as determined from the kill curve test performed before Library transfection.
9. Monitor cells for zeocin selection (cells dying)
10. Replace medium with zeocin every 2-3 days
11. Once confluent in 6-well plate, passage, pool and expand cells in larger flasks
12. Length of time for expansion of cells is user and cell line dependent, but it is highly recommended to expand zeocin resistant cells to generate cryo-stocks for future screening (~2-3 weeks; cell line dependent).

SECTION B. Transfect Library Cells with miRNA-Expression Construct, Select for Stable Cell Line, and Select miRNA Targets

Note: Zeocin selection is no longer required nor desired. Exposure of cells to zeocin during miRNA expression and ganciclovir (target) selection may result in loss of miRNA targets.

Puro/G418 and Ganciclovir kill curves

Perform a kill curve for ganciclovir with cells stably expressing the Target ID Library and wild type cells for puromycin/G418

1. Plate 1.6 x 10^4 cells into wells of a 96-well plate with 120 µL fresh medium.
2. The next day add from 0.1 to 10 µg/ml of puromycin/G418, or 2-32 µM ganciclovir to selected wells.
3. Examine viability every 2 days. Replace the medium containing selection reagent every 3 days. The minimum concentration of selection reagent that causes complete cell death after the desired time should be used for that cell type and experiment. We have found 0.25 to 1 µg/ml puromycin is optimum for A549, HeLa, and MCF7 cells, 0.3 µg/ml G418 for MCF7 cells and 8-16 µM ganciclovir are optimum for A549, HeLa, and MCF7 cells.

Note: With ganciclovir selection, slow or no cell growth may be observed without complete cell death. Observe cells over several days' treatment to verify they are not actively dividing. One example, phenol red in the medium remains red.

microRNA Transfection and Target Selection

Nucleofection

Cell Prep:

1. Grow / expand Target ID Library cells to attain 2 x 10^7 cells, and trypsinize when cells are at >80% confluency.
2. Transfer 2 x 10^7 cells to a 15 ml sterile screw topped tube and pellet at 200 x *g* for 5 min
3. Remove medium and wash cell pellet with Hanks Balanced Salt Solution or 1X PBS
4. Centrifuge at 200 x *g* for 5 min and then remove wash
5. Repeat wash step of cell pellet

Number of Cells per Nucleofection	Recommended # of Nucleofections
2 x 10^6	10

Nucleofection:

1. Pre-warm 6-well plates with 2ml of complete medium per well at 37°C
2. Add 2 µg of microRNA expression plasmid (not to exceed 10 µL) per 0.5 ml tube for each transfection. Origene MicroRNA Expression Plasmids (Neomycin – G418 selection) or self-cloned microRNA genes in pBABE-Puro (Plasmid 1764; Addgene) have been used successfully. For the latter, the miRNA hairpin with ~ 200 bp on either side was PCR cloned from human DNA.
3. Resuspend cells in the 15 ml tube from above with 100 µL of Amaxa Nucleofection Solution (cell specific) per 2 x 10^6 cells. For example: For 10 Nucleofections, add 1ml of reagent
4. One reaction at a time, add 100 µL of cells to the 2 µg of plasmid. Mix with pipette.
5. Transfer mixture to a Nucleofector cuvette
6. Insert cuvette into Nucleofector instrument and run optimized program appropriate for the cell line (High Effiency preffered over Cell viability)
7. Fill transfer pipette with pre-warmed medium. Take up cells in same pipette and transfer to 6-well plate. Repeat for each Nucleofection, one per well.
8. Return to growth chamber for overnight incubation
9. Replace medium and allow cells to recover for 3-5 days.
10. Replace medium with complete medium containing the appropriate level of Puromycin or G418 as determined from the kill curve test performed before transfection with the miRNA construct.
11. Monitor cells for puromycin / G418 selection (cells dying)
12. Replace medium with puromycin / G418 every 2-3 days
13. Once confluent in 6-well plate, passage, pool and expand cells in larger flasks
14. Length of time for expansion of cells is user dependent, but it is highly recommended to expand puromycin / G418 resistant cells to generate cryo-stocks for future screening (~2-3 weeks; cell line dependent).
15. Replace medium with the appropriate levels of ganciclovir (GCV) and puromycin / G418, as determined from the kill curve test performed before transfection with the miRNA construct,
16. Monitor cells for selection (cells dying, miRNA targeting and knockdown of TK-ZEO)
17. Expand cells in the presence of GCV and puromycin / G418
18. Prepare Genomic DNA from the GCV selected cells.

19. PCR amplify inserts with kit primers (see PCR Amplification)
20. Clone into the TopoTA vector (Invitrogen) for standard sequencing or submit PCR product for deep sequencing

Lipofectamine 2000

Follow the same transfection protocol as above for creating a stable cell line with the plasmid Target ID Library. Use microRNA expression plasmids and select for Puromycin / G418 resistance cells and then proceed with GCV plus Puromycin / G418 selection.

SECTION C. PCR-Amplify Selected Library Inserts and Sequence

PCR Amplification
Note: This procedure is performed to PCR-amplify the library targets that have survived the Ganciclovir selection.

1. Collect ganciclovir selected cells to prepare chromosomal DNA using a GenElute Mammalian Genomic DNA Miniprep Kit (Catalog Number G1N10) or equivalent.

Note: We recommend preparing DNA from the parent cell line that does not contain Target ID Library to use as a negative control for comparison with DNA from target-selected cells in PCR.

2. PCR Amplify the genomic DNA with Amplification Primer 1 and Amplification Primer 2. Successful amplification has been obtained with JumpStart REDTaq ReadyMix Reaction Mix for PCR (Catalog Number P0982) and JumpStart Taq ReadyMix (Catalog Number P2893). Optimization of the conditions may be necessary if another polymerase is used.

Reagent	Amount / Reaction
Jumpstart REDTaq ReadyMix Reaction Mix	10 µL
Amplification Primer 1(25 µM)	0.2 µL
Amplification Primer 2 (25 µM)	0.2 µL
Genomic DNA	50-200 ng
Water, Molecular Biology Reagent, Catalog Number W4502	to 20 µL

Step	Temp.	Time	Cycles
Initial Denaturation	95 °C	5 min.	1
Denaturation	95 °C	30 sec.	40 cycles
Annealing*	62 °C	30 sec.	
Extension	68 °C	2 min.	
Final Extension	68 °C	5 min.	1
Hold	4 °C	Hold	

*Annealing temperatures may vary, but we have observed the best amplification products with annealing temperatures ranging between 53 and 64°C.

3. Resolve 2-5 µL of the PCR product on a 1% agarose gel. The expected product should be a smear with some visible DNA banding. Compare to control PCR product of genomic DNA from cells without the Target ID Library**.

4. Proceed with cloning and/or deep sequencing of PCR product.

** If no amplification product is observed or is identical to control DNA, optimize the PCR amplification conditions (i.e., primer concentration, annealing temperature and cycles).

Cloning and Sequencing

Note: We highly recommend cloning the PCR products and then performing standard sequencing from at least 96 of the clones using the Amplification Primers provided in the kit. This procedure has been performed successfully using 96-well overnight cultures and plasmid purification systems. Even if deep sequencing is desired, preliminary results from cloning and standard sequencing can be used as a quality check to determine whether the extra expense of deep sequencing is warranted.

1. Follow TA cloning kit manufacture's protocol for cloning PCR amplification products (above).
2. Transform clones into competent bacterial cells and select overnight on antibiotic containing medium.
3. Isolate and grow individual colonies in liquid culture containing appropriate antibiotic.
4. Purify plasmid DNA.
5. Perform sequencing reactions with Amplification Primers.
6. Identify gene targets by BLAST alignment of sequences with human transcriptome (for known transcripts) and human genome (for novel transcripts).*

*Cloning troubleshooting: If a high number of inserts from sequencing are plasmid sequence, optimize the cloning/sequencing conditions as such:

- PCR Amplification product (insert) quality and quantity
- Ligation reaction
- Plasmid preparation (quality and quantity)
- Sequencing reaction conditions