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High-Throughput Functional MicroRNA Profiling Using Recombinant AAV-Based MicroRNA Sensor Arrays

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Abstract

There is a lack of methods for high-throughput functional microRNA (miRNA) profiling. In this chapter, we describe a recombinant adeno-associated virus-based miRNA sensor array (miRNA Asensor array), which is able to profile functional miRNAs in cultured cells. The preparation of an miRNA Asensor array and its usage are discussed.

Keywords

miRNA; miRNA activity; high-throughput; miRNA Asensor array

1 Introduction

The high-throughput profiling of microRNA (miRNA) expression and activity is critical for miRNA research. miRNA expression profiling can be achieved by microarray analysis [1], quantitative reverse transcription-polymerase chain reaction [2], and next-generation sequencing [3]. However, approaches for the high-throughput profiling of miRNA activity are lacking. miRNA activity, which is different from miRNA expression, correlates with mature miRNA function and is often affected by various factors, including the subcellular localization of miRNAs [4], the quantity of miRNA targets [5], and proteins that associate with the miRNA-induced silencing complex [6]. Therefore, functional miRNA profiling, which reflects miRNA activity, may confer some advantages over conventional miRNA profiling, if achievable.

Ideally, miRNA activity should be examined using the 3'-untranslated region (3'UTR) of its natural or predicted target genes as targets. However, individual miRNAs usually have more than one target gene, and the 3'UTR of genes often serve as targets for more than one miRNA [7]. Therefore, a perfect complementary sequence is often used in current assays of miRNA activity. This sequence is placed at the 3'UTR of a reporter expression cassette, forming a so-called miRNA sensor. miRNA activity is then determined by comparing the reporter expression level produced by the miRNA sensor with that produced by the "control sensor," which carries an identical reporter cassette lacking the miRNA complementary target [8, 9]. As a prerequisite, miRNA sensors must be efficiently transfected into cells for reporter gene expression. In many cases, this is difficult, particularly when high-throughput transfection is required. We have overcome this problem by establishing an adeno-associated

virus (AAV) reverse-infection array, which enables efficient and convenient high-throughput transduction for a variety of cell lines [10].

Based on our AAV reverse-infection array, we developed a dual-luciferase reporter system to examine miRNA activity in cultured cell lines in a high-throughput manner. This new system is referred to as an miRNA Asensor array [11]. Each miRNA Asensor contains two independent expression cassettes encoding a firefly luciferase (Fluc) and a *Gaussia* luciferase (Gluc). The former is used to calibrate the transduction efficiency, while the latter, which includes an miRNA perfect complementary target sequence in the 3' UTR, is used to monitor miRNA activity. The Asensor lacking the miRNA target sequence is designated as the control Asensor. The miRNA Asensor array is prepared by loading the Asensors into a 96-well plate. To calibrate the variation in loading of the different Asensors, the transduction coefficient (TC) of each miRNA Asensor must be determined. Using this array, we have acquired the functional profiles of 115 miRNAs for 12 cell lines. In this chapter, we describe the procedure for preparing an miRNA Asensor array and its application.

2 Materials

2.1 Vector Construction

1. pDRIVE-mPGK plasmid (InvivoGen, San Diego, CA, USA) (*see Note 1*).
2. Pyrobest DNA polymerase (Takara, Shiga, Japan).
3. Agarose.
4. TAE stock solution (50×): 2.5 M Tris-acetate, pH 8.0, 50 mM EDTA. Prepare 1× working solution by diluting TAE stock buffer (50×) with distilled water. Store at room temperature.
5. Power supply.
6. Mini horizontal gel electrophoresis cell.
7. Restriction enzymes *KpnI*, *BamHI*, *BglII*, *HindIII*, *XhoI*, and *EcoRI* (New England BioLabs, Inc., Ipswich, MA, USA).
8. Thermostatic water bath.
9. pGL4.14 (Promega Corp.) (*see Note 2*).
10. WD-9403E ultraviolet lamp.
11. DNA fragment purification kit. 12. T4 DNA ligase (New England BioLabs, Inc.)
12. T4 DNA ligase buffer (10×) (New England BioLabs, Inc.).
13. JM109 *Escherichia coli* competent cells (Takara).

¹The plasmid carries the mPGK promoter and is used as a template to amplify the fragment of mPGK promoter by PCR.

²The pGL4.14 plasmid includes the synthetic firefly *luc2* gene which has been codon-optimized for more efficient expression in mammalian cells. It is a basic vector with no promoter and contains a multiple cloning region for insertion of a promoter of choice. mPGK promoter was inserted into the multiple cloning region of pGL4.14 plasmid to obtain the *luc2* gene cassette.

14. Ampicillin stock solution: 100 mg/mL ampicillin. Dissolve 1 g of sodium ampicillin in 10 mL distilled water. Sterilize using a filter-sterilization unit. Store the solution in aliquots at -20°C .
15. LB culture medium: 1 % (w/v) tryptone, 0.5 % (w/v) yeast extract, and 1 % (w/v) NaCl. Sterilize by autoclaving for 20 min at 15 psi on the liquid cycle or using a filter-sterilization unit. Store at 4°C .
16. LB plate containing 100 $\mu\text{g/mL}$ ampicillin. Dissolve 15 g of agar in 1.0 L of LB culture medium and sterilize by autoclaving for 20 min at 15 psi on the liquid cycle. Cool to 50°C and add 1.0 mL of 100 mg/mL ampicillin. Pour into plates.
17. DNA ladder.
18. Benchtop incubator shaker.
19. Mini centrifuge.
20. Plasmid extraction kit.
21. Alkaline phosphatase, calf intestinal (CIP) (New England BioLabs, Inc.).
22. Annealing buffer (1 $\times$): 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA. Sterilize using a 0.2- μm filter and store at room temperature.
23. Qiagen plasmid midi kit (Qiagen, Inc., Valencia, CA).
24. TE buffer: 10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA. Sterilize by autoclaving for 20 min at 15 psi on the liquid cycle or using a filter-sterilization unit. Store at room temperature.

2.2 AAV Cell Lines

1. BHK-21 [C-13] cells (ATCC #CCL-10).
2. Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA).
3. DMEM medium.
4. Fetal bovine serum.
5. G418 sulfate. Prepare a 100 mg/mL solution using ddH₂O. Sterilize using a 0.2- μm filter. Store at -20°C .

2.3 Preparation of miRNA Asensor

1. A set of cosmids that contains the whole genome of HSV1 strain 17—SetC which is composed of cos6, cos28, cos14, cos56, and cos48 [12] (*see Note 3*).
2. pSub201 [13] (*see Note 4*).

³SetC cosmids were kindly provided by Dr. Davison (Glasgow University, UK). The digestion products of SetC cosmids were cotransfected into HSV1 sensitive cells (such as BHK-21) so that HSV1 was rescued.

3. Restriction enzyme (New England BioLabs, Inc.).
4. Lipofectamine 2000 transfection reagent (Invitrogen).
5. DMEM medium.
6. Fetal bovine serum.
7. DMEM/5 % FBS: 5 % FBS, 7.5 µg/mL human immunoglobulin in DMEM. Add 50 mL of FBS to 1.0 L of DMEM to obtain DMEM/5 % FBS. Then prepare DMEM/5 % FBS containing 7.5 µg/mL human immunoglobulin by adding 3 µL of 20 mg/mL pooled human immunoglobulin (Sigma) into 8 mL of DMEM/5 % FBS.
8. PBS²⁺: 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 1 mM CaCl₂, and 0.5 mM MgCl₂. Sterilize by autoclaving for 20 min at 15 psi on the liquid cycle or by filter-sterilization. Store at room temperature.
9. Methanol.
10. KaryoMax Giemsa Stain stock solution (Invitrogen).
11. Inverted microscope.
12. Chloroform.
13. NaCl.
14. PEG8000.
15. Benzonase nuclease (Merck) (*see Note 5*).
16. TE buffer (*see Subheading 2.1, item 25*).
17. 6-well plates.

2.4 Determination of AAV Titer by Dot-Blotting

1. 4 M LiCl.
2. 95 % ethanol.
3. 75 % ethanol.
4. Mini centrifuge.
5. Benzonase nuclease.
6. 0.1 M EDTA.

⁴pSub201 plasmid was kindly provided by Dr. Samulski. It contains AAV2 *rep* and *cap* genes with their native promoters and polyA signals.

⁵Benzonase nuclease can degrade all forms of DNA and RNA while having no proteolytic activity. It is used to degrade any form of DNA and RNA that is not packaged into virions.

7. 10 mg/mL Proteinase K. Put 100 mg Proteinase K (Merck) into 8.0 mL distilled water. When completely dissolved, add more distilled water to the final volume of 10.0 mL. Store at -20°C .
8. 0.5 M NaOH.
9. Linearized plasmid (*see Note 6*).
10. WD-9403E Ultraviolet lamp.
11. Nylon membrane, positively charged (Millipore, Billerica, MA, USA).
12. PCR DIG Probe Synthesis Kit (Roche, Basel, Switzerland).
13. Anti-Digoxigenin-AP, Fab fragment from sheep (Roche).
14. BCIP 4-toluidine salt.
15. 4-Nitro blue tetrazolium chloride solution (NBT).
16. PBS²⁺ (*see Subheading 2.3, item 8*).
17. TE buffer (*see Subheading 2.1, item 25*).

2.5 Preparation of miRNA Asensor Array

1. BioLux *Gaussia* Luciferase Flex Assay Kit (New England BioLabs, Inc.).
2. Luciferase assay system (Promega).
3. Liquidator 96 Manual Benchtop Pipetting System (Rainin Instruments LLC., Oakland, CA, USA).
4. Laminar flow tissue culture hood.
5. CO₂ Incubator.
6. 96-well plates.

2.6 Profiling miRNA Activity Using an miRNA Asensor Array

1. Centrifuge.
2. BioLux *Gaussia* Luciferase Flex Assay Kit (New England BioLabs, Inc.).
3. 0.25 % trypsin.

3 Methods

The production of an miRNA Asensor array is illustrated in Fig. 1.

3.1 Vector Construction

A flowchart for vector construction is shown in Fig. 2. The sequences of the primers used in this protocol are given in Table 1.

⁶Linearized plasmid is prepared by digestion of the plasmid containing the probe binding sequence, which is used as DNA standard in determination of the titer of recombinant AAV.

3.1.1 Construction of the Control Asensor Plasmid

1. Amplify the core region of the mPGK promoter by PCR using pDRIVE-mPGK as a template [14] (see **Note 7**). Use Pyrobest DNA polymerase for PCR in a 50- μ L reaction system. Carry out the reaction at 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 62.2 °C for 30 s, and 72 °C for 40 s, with a final hold at 72 °C for 10 min. Load the products and DNA ladder in separate wells on an agarose gel and run at 150 V for 30 min. Excise the targeted DNA band and purify it using a DNA fragment purification kit.
2. Digest the purified PGK promoter fragment using *KpnI* and *BamHI*. In the meantime, digest the pGL4.14 plasmid with *KpnI* and *BglII*. Incubate for 2 h at 37 °C. Load the digested products in separate wells on an agarose gel and run at 150 V for 30 min. Excise the targeted DNA bands (digested PGK promoter and pGL4.14) and purify them using a DNA fragment purification kit.
3. Ligate the digested PGK promoter fragment with the digested pGL4.14 fragment using T4 DNA ligase at a molecular ratio of 3:1 (see **Note 8**). Incubate at 16 °C for 16 h. Transform the ligation product into JM109 *E. coli* competent cells following the manufacturer's instructions. Spread 200 μ L of transformed cells on LB plates containing 100 μ g/mL ampicillin. Incubate the plates overnight at 37 °C.
4. Pick single colonies from the plates and inoculate each colony in 5 mL of LB medium containing 100 μ g/mL ampicillin. Incubate at 37 °C for 12–16 h with vigorous shaking (220–300 rpm).
5. Harvest the bacterial cells and extract the plasmid using a plasmid extraction kit. Identify the plasmid by *KpnI* and *HindIII* digestion and confirm it by DNA sequencing. Designate the correct clone pGL4.14-PGK. Amplify the Luc2 gene cassette by PCR using pGL4.14-PGK as a template. Use Pyrobest DNA polymerase for PCR in a 50- μ L reaction system. Carry out the reaction at 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 58.7 °C for 30 s, and 72 °C for 160 s, with a final hold at 72 °C for 10 min. Load the products and DNA ladder in separate wells on an agarose gel and run at 150 V for 30 min. Excise the targeted DNA band (Luc2 gene cassette) and purify it using a DNA fragment purification kit.
6. Digest the Luc2 gene cassette and pAAV2neo-Gluc plasmid [15] with *XhoI*. Incubate the reactions at 37 °C for 2 h. Purify the digested products using a DNA fragment purification kit. Remove the 5' phosphate of the digested pAAV2neo-Gluc fragment using CIP and purify it using a DNA fragment purification kit.

⁷The core region of the mPGK promoter contains four Sp1 binding sites, a CCAAT box, a GC box, and a putative transcription start point, with a length of 553 nucleotides (nt).

⁸To increase the cloning efficiency, the ratio of insert to vector fragment should range from 3:1 to 10:1.

7. Ligate the digested Luc gene cassette with the digested pAAV2neo- Gluc using T4 DNA ligase at a molecular ratio of 3:1. Incubate the reaction at 16 °C for 16 h. Transform the ligation product into JM109 *E. coli* competent cells following the manufacturer's instructions. Spread 200 µL of transformed cells on LB plates containing 100 µg/mL ampicillin. Incubate the plates overnight at 37 °C.
8. Pick several colonies from the plates and inoculate each colony in 5 mL of LB medium containing 100 µg/mL ampicillin. Incubate at 37 °C for 12–16 h with vigorous shaking (220–300 rpm).
9. Harvest the bacterial cells and extract the plasmid using a plasmid extraction kit. Identify the plasmid by *KpnI* digestion and sequence it to confirm. Select a clone in which the orientation of the Luc2 gene cassette is opposite that of the Gluc gene cassette. Designate the clone as the control Asensor plasmid.

3.1.2 Construction of miRNA Asensor Plasmids

1. Obtain mature miRNA sequences from <http://www.mirbase.org>. Deduce the sequences of perfect complementary targets for the miRNAs based on the principle of Watson–Crick base pairing (see **Note 9**). Add “5' aatt3'” to the 5' end of the perfect complementary target to form the forward sequence, and add “5' gatc3'” to the 5' end of the mature miRNA sequence to form the reverse sequence. Synthesize oligonucleotides for the forward and reverse sequences.
2. Dissolve the synthesized oligonucleotides in 1× annealing buffer. Anneal the forward and reverse oligonucleotides to form the adaptor. Prepare the annealing reaction as follows:

Forward sequence solution (200 µM) 10 µL (100 µM final concentration).

Reverse sequence solution (200 µM) 10 µL (100 µM final concentration).

Carry out the reaction at 95 °C for 10 min, then gradually cool it to room temperature. Keep the reaction at room temperature for 2 h (see **Note 10**). Store the adaptor at –20 °C until needed.
3. Digest the control Asensor plasmid with *EcoRI* and *BglII*. Incubate the reaction at 37 °C for 2 h. Purify the digested product using a DNA fragment purification kit. Ligate the treated control Asensor plasmid with the adaptor as follows:

⁹In mammalian cells, most miRNA targets are not perfectly complementary to their mature sequences; one miRNA may have several targets. We use the perfect complementary target to investigate miRNA activity.

¹⁰Let the samples cool gradually to room temperature and keep them at room temperature for at least 1 h.

Treated control Asensor plasmid (40 ng/μL)	1 μL
Adaptor (100 μM)	1 μL
T4 DNA ligase buffer (10×)	2 μL
T4 DNA ligase (400 U/μL)	1 μL
ddH ₂ O	15 μL

4. Incubate the ligation reaction at 16 °C for 16 h. Transform the ligation product into JM109 *E. coli* competent cells following the manufacturer's instructions. Spread 200 μL of transformed cells on LB plates containing 100 μg/mL ampicillin. Incubate the plates overnight at 37 °C.
5. Pick several colonies from the plates and inoculate each in 5 mL LB medium containing 100 μg/mL ampicillin. Incubate for 12–16 h at 37 °C with vigorous shaking (220–300 rpm).
6. Harvest the bacterial cells and extract the plasmid using a plasmid extraction kit. Identify the plasmid by sequencing.

3.2 AAV Cell Lines

1. Extract the control and miRNA Asensors plasmid DNA using a Qiagen Plasmid Midi Kit.
2. Transfect the control and miRNA Asensors plasmid DNA into BHK-21 cells using Lipofectamine 2000 according to the manufacturer's instructions. After 24 h, split the cells 1:10 and culture in DMEM containing 10 % FBS and 400 μg/mL G418 (*see Note 11*). Change the medium every 3 days. After 15 days, neomycin-resistant colonies will be present (*see Note 12*). Expand the colonies to obtain stable cell lines harboring AAV provirus for miRNA Asensors.

3.3 Preparation of the AAV Helper Virus rHSV1-rc/ UL2

Prepare the AAV helper virus rHSV1-rc/ UL2 as reported previously [16] based on a set of cosmids that contains the whole genome of HSV1 strain 17—SetC which is composed of cos6, cos28, cos14, cos56, and cos48 [12]. rHSV1-rc/ UL2 contains the AAV2 *rep* and *cap* gene cassettes and is used to package AAV vectors into AAV2 capsids.

1. Excise the 4.3 kb fragment of AAV2 *rep* and *cap* genes under control of their native promoters from pSub201 [13] and insert it into *Xba*I site of cos6 generating cos6-rc UL2.
2. Digest cos6-rc UL2, cos28, cos14, cos56, and cos48 with *Pac*I, respectively. Precipitate the digested product with ethanol and dissolve it in TE buffer.

¹¹The final concentration of G418 in the DMEM 10 % FBS medium should be 400–800 μg/mL.

¹²If necessary, compare the rAAV yield from a number of colonies of AAV proviral cell lines and select the best cell line for future rAAV production.

3. Co-transfect the digested cos6-rc UL2, cos28, cos14, cos56, and cos48 into BHK-21 cells using Lipofectamine 2000 according to the manufacturer's instructions.
4. Replace the medium every 24 h until the cytopathic effect (CPE) appears. When full CPE is observed, collect the cells along with medium. Free and thaw the cells three times. Spin down the debris and collect the supernatant which contains rHSV1-rc/ UL2.
5. Propagate rHSV1-rc/ UL2 by infecting BHK-21 cells with the supernatant. When full CPE is observed, collect the cells along with medium. Free and thaw three times. Spin down the debris and collect the supernatant which contains rHSV1-rc/ UL2. Store the virus at -20°C .

3.4 Determination of the rHSV1-rc/ UL2 Titer

Determine the rHSV1-rc/ UL2 titer using a plaque assay, as described previously [17].

1. Split appropriate number of BHK-21 cells into 6-well plates (*see Note 13*) using DMEM/5 % FBS so that ~90 % confluence will be reached in 24 h.
2. Serially dilute prepared rHSV1-rc/ UL2 by tenfold from 10^{-1} to 10^{-8} using DMEM/5 % FBS (*see Note 14*).
3. Aspirate medium from BHK-21 cell culture. Add 500 μL of each dilution of rHSV1-rc/ UL2 to the corresponding well of cells.
4. Swirl the plate every 30 min to allow virus to absorb. 2 h later, replace medium with DMEM/5 % FBS containing 7.5 $\mu\text{g/mL}$ human immunoglobulin.
5. Incubate for ~2 days until plaques are visible.
6. Aspirate medium from plates. Rinse each well of monolayer cells twice with PBS²⁺ (*see Note 15*).
7. To fix the cells, add 500 μL of methanol to each well and leave it at room temperature for 5 min.
8. Dilute the KaryoMax Giemsa Stain stock solution 1:10 with distilled water. Aspirate the methanol and add 1 mL of the diluted Giesma stain to each well. Incubate for 20 min at room temperature.
9. Discard the stain solution. Rinse cells gently with cold tap water until the liquid is clear. Dry inverted plate on a paper towel.
10. Count plaques (circle or mark each plaque with a fine-point marker pen) viewing through an inverted light microscope at low (10 $\times$) magnification.

¹³The titration is performed in duplicate to ensure accuracy.

¹⁴Make sure to use a clean, plugged pipet tip in each operation of the dilution process. Virus stocks are routinely of very high titer and repeated use of a pipet tip for dilution will affect the results.

¹⁵It is not necessary to operate under sterile conditions from this step of rHSV1-rc/ UL2 titration.

3.5 Preparation of AAV miRNA Asensors

1. Infect AAV cell lines of the control and miRNA Asensors with rHSV1-rc/UL2 at a multiplicity of infection (MOI) of 0.1 (*see Note 16*). When the cytopathic effect of the virus is nearly complete (~48 h later), aspirate the medium and harvest the cells with PBS²⁺.
2. Purify the AAV particles as reported previously [18]. For this purpose add 10 % (v/v) of chloroform to the collected cell suspension. Incubate at 37 °C with vigorous shaking (220– 260 rpm) for 1 h until all cells are lysed (*see Note 17*). Add solid NaCl to lysates till the final concentration is 1 M and constantly shake for 30 min at room temperature. Centrifuge at $12,000 \times g$ for 15 min at 4 °C. Harvest the supernatant.
3. Add an appropriate amount of solid PEG8000 to the supernatant to obtain a final concentration of 10 % (w/v). Cool the supernatant in ice water and let it stand still for 1 h. Recover the precipitated AAV particles by centrifugation at $10,000 \times g$ for 15 min at 4 °C. Dissolve the pellets with PBS²⁺ buffer. Add Benzonase nuclease to a final concentration of 200 U/mL and incubate at room temperature for 30 min.
4. Add equal volume of chloroform to suspension and vigorously shake for 2 min. Separate the organic and aqueous phases by centrifugation at $12,000 \times g$ for 5 min at 4 °C. Collect the aqueous phase containing the purified AAV particles.

3.6 Determination of the rAAV Titer by Dot-Blot Assay

1. Prepare a DIG-labeled CMV promoter probe using a PCR DIG probe synthesis kit. Set up the reaction as follows:

ddH ₂ O	41.25 µL
PCR DIG mix (10×)	5 µL
CMV-p-f (20 µM)	1 µL
CMV-p-r (20 µM)	1 µL
Enzyme mix	0.75 µL
Control Asensor plasmid (10 ng/µL)	1 µL

Carry out the reaction at 95 °C for 2 min, followed by 30 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 15 s, with a final hold at 72 °C for 7 min. Load 1 µL of the product onto an agarose gel and run it at 150 V for 30 min to verify the size. Add 5 µL of 4.0 M LiCl to the remaining product and mix. Next, add 150 µL of prechilled 95 % ethanol and incubate at

¹⁶The appropriate MOI of rHSV1-rc/ UL2 is critical for rAAV quality and production. Perform pilot studies to determine the best MOI, if possible.

¹⁷Complete cell lysis is determined by checking a drop of the culture under a microscope.

–20 °C for 2 h. Centrifuge at $12,000 \times g$ for 10 min. Wash the pellet twice with 75 % ethanol. Dry the pellet completely and dissolve it in 20 μ L of TE. Store at –20 °C until use.

2. Digest the virus particles to release the DNA, as reported previously [19]. Specifically, add 10 μ L purified AAV particle suspension in 50- μ L reaction system where the final concentration of Benzonase nuclease is 200 U/mL and incubate at 37 °C for 30 min (*see Note 18*). Stop the digestion by adding 10 μ L of 0.1 M EDTA to the reaction system and mixing. Release virion DNA by adding 60 μ L PBS²⁺ containing 200 μ g/mL protein-ase K and incubate 1 h at 50 °C. Denature viral DNA by adding 120 μ L of 0.5 M NaOH and incubate for 10 min at room temperature.
3. Prepare the standard and sample DNAs, as reported previously [19]. Specifically, prepare linearized plasmid (from the plasmid containing the probe sequence) at 1×10^{12} copies/mL for DNA concentration standards (*see Note 19*). Make twofold serial dilutions ranging from 1×10^{12} to 3.125×10^{10} copies/ mL. Denature DNA standards by adding equal volume of 0.5 M NaOH to each dilution.
4. Load the standard and sample DNAs on a positively charged nylon membrane. Allow the membrane to air-dry completely. Fix the DNA to the membrane by exposing the side with DNA to a UV light source (254 nm) for 15.0 s.
5. Perform pre-hybridization and hybridization according to the manufacturer's Southern blot protocol. Test the DIG- labeled probe using anti-digoxigenin alkaline phosphatase- conjugated antibodies. Detect alkaline phosphatase activity in a color reaction using BCIP/NBT as the substrate (*see Note 20*).

3.7 Preparation of the miRNA Asensor Array

1. Select miRNA Asensors according to the experimental objectives.
2. Dilute the control and miRNA Asensors to 1.25×10^{10} virus genomes (vg)/mL in PBS²⁺.
3. Load the control and miRNA Asensors onto a 96-well cell culture plate using the Liquidator 96 Manual Benchtop Pipetting System (Rainin Instruments LLC.) at 2.5×10^8 vg/20 μ L/well.
4. Evaporate the arrays in a laminar flow tissue culture hood then store them at 2–8 °C (*see Note 21*). Prepare multiple sets of miRNA Asensor array

¹⁸This step digests any DNA that is present outside of virions.

¹⁹Since AAV genome is single-strand DNA, AAV titer is twice of the corresponding concentration of linearized plasmid.

²⁰Add appropriate volume of prepared BCIP/NBT according to the blot. Avoid exposure to light and incubate at room temperature for 30 min to 1 h. Stop the reaction by putting the blot into distilled water.

²¹Prepared miRNA Asensor arrays can be stored at 2–8 °C for more than 1 year with only negligible loss of infectivity.

plates in a batch. Determine the transduction coefficients (TC) for each miRNA Asensor in an array (*see Note 22*). Different sets of miRNA Asensor array plates that are prepared in the same batch are considered to have similar TC values. To detect the TC value for each miRNA Asensor in the array, determine the relationship between Gluc and Fluc activity of the control Asensor when no miRNA repression occurs. Make serial twofold dilutions ranging from 5.00×10^8 to 1.56×10^7 vg/well of control Asensor in a 96-well plate. Load BHK-21 cells onto the plate at a density of 1×10^4 cells/well and culture them for 48 h (*see Note 23*). Measure the Gluc and Fluc activities using a Gluc and Fluc assay kit, following the manufacturers' instructions. Deduce the relationship between the levels of Gluc activity (G) and Fluc activity (F) (Fig. 3). Compute the TC, which is the ratio of G for the Asensor_{miRNA} (G_{miRNA}) to that for the Asensor_{control} (G_{control}) when no miRNA repression occurs, as follows:

$$TC = \frac{G_{\text{miRNA}}}{G_{\text{control}}} = \left(\frac{F_{\text{miRNA}}}{F_{\text{control}}} \right)^n,$$

where F_{control} and F_{miRNA} represent the F of the Asensor_{control} and Asensor_{miRNA}, respectively. In the equation, n represents the relationship between F and G deduced from Fig. 3, as exemplified by 1.32 in Fig. 3.

3.8 Profiling miRNA Activity Using an miRNA Asensor Array

1. For adherent cells, digest the cells with 0.25 % trypsin to create a single-cell suspension. Determine the cell number and dilute the single-cell suspension to an appropriate density (*see Note 24*). For suspension cells, centrifuge the cells at $500 \times g$ for 5 min and resuspend them in culture medium. Determine the cell number and dilute the single-cell suspension to an appropriate density (*see Note 24*).
2. Load 200 μL of single-cell suspension into each well of the miRNA Asensor array. Culture the cells at 37 °C in an incubator with 5 % CO_2 for 48 h (*see Notes 25 and 26*).
3. Collect a 20 μL aliquot of supernatant from each well for a Gluc activity assay (*see Note 27*). Test for Gluc activity using a Gluc activity assay kit, following the manufacturer's instructions (*see Note 28*).

²²TC values are used to calibrate the variation in transduction efficiency among miRNA Asensors. The BHK-21 cell line is used to detect the TC due to its high susceptibility to AAV infection.

²³It is important to create single-cell suspensions so that the cells can be counted accurately and dispersed evenly after loading onto the plates.

²⁴To increase the sensitivity of the assay, perform a pilot study to determine the appropriate quantity of cells loaded per well for each line.

²⁵For cell lines with a slow growth rate, a longer culture time (4–5 days) is required so that the RIF can reach its peak value.

²⁶After loading the cells, place the miRNA Asensor array plate in a wet box to reduce evaporation of the culture medium.

²⁷Centrifuge the plates, then remove 20 μL aliquots of cell-free medium from each well for the Gluc activity assay.

²⁸Carry out the Gluc activity assay at 25 °C.

4. miRNA activity is represented as the relative inhibiting fold (RIF), which is the TC-calibrated ratio of G for the Asensor_{control} (G_{control}) to that for the Asensor_{miRNA} (G_{miRNA}). Compute the RIF value as follows:

$$RIF = \left(\frac{G_{\text{control}}}{G_{\text{miRNA}}} \right) \times TC.$$

5. Analyze the data. A sample miRNA activity profile for 12 cell lines is shown in Fig. 4.

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Fig. 1. Overview of the miRNA Asensor array approach. *SPTP* synthetic poly(A) signal/transcriptional pause site from the pGL4.14 vector, *miRT* perfect complementary miRNA target sequence. Adapted from ref. 15

Fig. 2.

The vector construction process. *mPGK promoter* mouse 3-phosphoglycerate kinase gene promoter region, *LacZ* *lacZ*-coding region, *SV40 poly(A)* SV40 polyA signal, *PGK promoter* core region of the mPGK promoter, *SPTP*, synthetic poly(A) signal/transcriptional pause site from the pGL4.14 vector, *Luc2* the firefly luciferase coding region, *SV40 late poly(A)* SV40 late-polyA signal, *ITR* AAV2 inverted terminal repeat, *CMV promoter* human cytomegalovirus promoter, *Gluc* *Gaussia* luciferase coding region, *BGH poly(A)* bovine growth hormone polyA signal. Adapted from ref. 15

Fig. 3.
Relationship between Fluc and Gluc activity in the control Asensor in BHK-21 cells. *RLU* relative light units. Adapted from ref. 15

Fig. 4.

Sample analysis of the miRNA activity profile for cell lines obtained from an miRNA Asensor array. The miRNA activity profiles of 12 cell lines are shown (a). Several miRNAs were selected and their activities in different cell lines were compared (b–g). *RIF* relative inhibiting fold. Adapted from ref. 15

Table 1

Primers used in this protocol

Primer name	5' → 3' Sequence	Function
PGK-f	CAGGGT <u>ACCA</u> TTCTACCGGGTAGGGGAGG	Forward primer for amplification of the PGK promoter
PGK-r	ATTGGATCCTCGAAAGGCCCGAGATGAG	Reverse primer for amplification of the PGK promoter
Luc-f	GA <u>ACTCGAG</u> TGGACAGGCCGCAAT	Forward primer for amplification of the Luc gene cassette
Luc-r	CA <u>ACTCGAG</u> GAGCGCCCAATACGC	Reverse primer for amplification of the Luc gene cassette
CMV-P-f	CCCATAAGGTCATGTACTGGGCAT	Forward primer for amplification of the DIG-label CMV promoter probe
CMV-P-r	GTTCCCATAGTAACGCCAATAGGG	Forward primer for amplification of the DIG-label CMV promoter probe

^aThe restriction enzyme site used to insert the amplified fragment into the vector is underlined in each primer. The restriction sites in PGK-f, PGK-r, Luc-f, and Luc-r were specific for *Kpn*I, *Bam*HI, *Xho*I, and *Xho*I, respectively