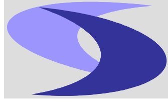

Signosis, Inc.
Innovative Plate Assay Solutions

Highly Sensitive MiRNA Northern Blot Assay Kit w/o Gel

Catalog Number NB-1002 **(For Research Use Only)**

Introduction

Abundance of mammalian miRNAs varies dramatically in tissues. For example, expression level of miR-133 is high in skeletal muscle, moderate in heart, and low in other tissues. To detect low expression of miRNAs requires the use of highly sensitive methods. **Signosis, Inc.'s** *Highly Sensitive miRNA Northern Blot Assay* is 10 to 100 times more sensitive than conventional biotin-based chemiluminescent detection.

Principle

RNA samples are separated through gel electrophoresis and transferred onto a membrane. Expression of a specific miRNA is detected with a biotin-labeled probe, containing two moieties – complementary sequence of the miRNA and a tag sequence. The tag sequence is then detected by an amplifier enriched with biotin molecules.

Materials provided with the kit

Component	Qty	Store At
Membrane	2	RT
Detection Sheet	2	RT
5X Hybridization Wash Buffer	80 mL	RT
5X Detection Wash Buffer	50 mL	RT
1X NB Hybridization Buffer	70 mL	RT
Blocking Buffer	60 mL	RT
Streptavidin-HRP Conjugate	60 µL	4°C
Substrate A	2.4 mL	4°C
Substrate B	2.4 mL	4°C
Gel Loading Buffer	90 µL	-20°C
Molecular Marker (20nt, 60nt, & 100nt)	30 µL	-20°C

Materials Required but Not Provided

- Biotin-labeled miRNA probe and amplifier (HP-XXXX)
- TBE
- Gel apparatus and related power supplies for Northern Blotting
- Gel filter paper and related gel transfer components
- Precast 15% TBE Urea-Gels or apparatus and materials for gel preparation
- Stratagene UV Cross-linker
- Hybridization oven
- Hybridization tubes
- Shaker
- Imaging system or X-ray film

Before Starting the Experiment Prepare the Following:

1. Add **160 mL** ddH_2O to **40 mL** *5X Hybridization Wash Buffer* to achieve *1X Hybridization Wash Buffe*r.
2. Add **200 mL** ddH_2O to **50 mL** *5X Detection Wash Buffer* to achieve *1X Detection Wash Buffer.*
3. Prewarm *1X NB Hybridization Buffer* and *1X Hybridization Wash Buffer*.
4. To prevent precipitate from forming in solution, these buffers need to be warmed up for 5 to 16 hours at 45°C. These reagents may also need to be stirred with a 5 mL or 10 mL pipette periodically. The buffers must have no visible precipitate before continuing.

Please Read the Assay Procedure Before You Begin

Assay Procedure

15% Acrylamide/Urea Gel Preparation

Note: The following steps are only an example on creating your own gel. Depending on the size of your samples, you may make changes to the preparation of gel as desired. Be aware: ***We do not include the materials for making your own gel in this kit.***

You may also choose to buy pre-cast 15% urea gels from your desired supplier. Note that precast gels can come in different sizes, so make sure it can fit into the apparatus you use.

For resolving 18-30mer oligonucleotides:

1. Combine the following in a beaker:

2.815 ml	40% acrylamide/bis-acrylamide (29:1)
3.6 g	Urea
0.75 ml	10x TBE Buffer

   Warm the mixture to 37°C to dissolve the Urea, but do not overheat or the urea will be destroyed.
2. Add water up to 7.5 ml and mix, then keep on ice for 5 minutes. Do not cool any longer or the urea will begin to precipitate. If this happens, rewarm the solution to dissolve the urea crystals.
3. Prepare your gel casting apparatus.
4. In your beaker, add the following and mix:

37.5 µl	10% ammonium persulfate (APS)
3.75 µl	TEMED

5. Immediately pour the gel after mixing, avoiding the introduction of air bubbles. Place the appropriate comb and allow the gel to sit untouched until after it has completely set.

Gel electrophoresis

6. Prepare and assemble your 15% TBE-urea gel and pre-run at **60V** for **30 minutes** using pre-chilled *0.5X TBE* as the running buffer.
7. While pre-running the gel, prepare RNA samples by mixing **3 µL** of *Gel Loading Buffer* with **7 µL** (5 µg) of total RNA in, heating at **70°C** for **5 minutes** and chill the samples on ice.
8. After the 30 minute pre-run of the gel is complete, rinse individual wells by pipetting the buffer up and down multiple times before loading RNA samples. Residual gel-packaging solution can disrupt sample loading if the wells are not sufficiently rinsed.
9. Carefully load **10 µL** RNA sample into one well of 15% pre-run urea-polyacrylamide gel. Load **5 µL Molecular Marker** next to the RNA sample. Run at **60V** until the bromophenol blue reaches approximately 3 cm away from the bottom of the gel.

<u>Note</u>: Different RNA samples can be loaded onto the gel for detection with a single miRNA probe or one RNA sample can be loaded onto different wells for the hybridization with different miRNA probes (see **Table 1** for recommended arrangement). An empty well between two different hybridization groups is recommended for easy excision after RNA transfer.

Transfer

10. Dissemble the gel cast and remove the glass plates from each other to gain access to gel.
11. Transfer the gel to a glass tray filled with 0.5X TBE buffer.
12. Soak the *Membrane* and filter paper in 0.5X TBE.
13. Assemble the transfer unit in the following order on the black side of cassette: one fiber pad (or mesh sponge), one piece of filter paper, gel, membrane and one piece of filter paper, one fiber pad.
14. Make sure the gel at negative side and membrane at positive side and transfer cassette to BioRad Trans-Blot Cell and fill with pre-chilled 0.5X TBE.
15. Transfer at **60V** at for **l hour** in a cold room or put the tank on ice within an ice basket.
16. After transfer completes, immobilize the RNA to the *Membrane* with **Stratagene UV Cross-Linker** using the **Auto-Cross Link** function.
    - If you do not have a Stratagene UV Cross-Linker, you can expose the membrane to UV light with **120,000 µjoules** over the period of 60 seconds (**2,000 µjoules** per second).
17. Let the *Membrane* dry at **42ºC** for **15 minutes**.

Hybridization

18. Put the membrane into the *Hybridization tube* (Corning 50 mL disposable tube is recommended).
19. Soak the membrane with ddH_2O. Afterwards, discard the ddH_2O.

20. Add pre-warmed **4 mL** of *NB Hybridization Buffer* (pre-warmed to 42°C) into the bottle.
21. Rotate at **42°C** for **30 minutes**.
22. Replace the buffer with **4 mL** of fresh *NB Hybridization Buffer* pre-warmed at **42°C**.
23. Add **10 µL** of miRNA probe and rotate at **42°C** overnight.

After Overnight Probe Exposure:

24. Decant the probe-NB Hybridization solution and immediately rinse the membrane in the bottle with **20 mL** of *1X NB Hybridization Wash Buffer*.
25. After the *Membrane* has been rinsed, decant all remaining solution from the tube, and add pre-warmed **4 mL** of *NB Hybridization Buffer* (pre-warmed to 42°C) into the bottle.
26. Add **8 µL** of *Amplifier* and rotate at **42°C** for **2 hours**.
27. Rinse the *Membrane* in the bottle with **20 mL** of 1X *NB Hybridization Wash Buffer*.
28. Decant any remaining solution in the tube, and immediately add **20 mL** of *NB Hybridization Wash Buffer* again. Rotate at **42°C** for **30 minutes.**

Detection

29. Using forceps transfer the *Membrane* from the *Hybridization tube* to a container (with similar dimensions of the membrane). Each box could have one full membrane or two half size of the membranes. Do not overlap the membrane during any point of the following incubation steps.
30. Rinse the *Membrane* with **10 mL** of *1X Detection Wash Buffe*r.
31. Block the *Membrane* with **15 mL** of *Blocking Buffer* for **30 minutes** at room temperature with moderate shaking.
32. Take **1 mL** of the **used** *1X Blocking Buffer* that is currently blocking the *Membrane.* Dilute **15 µL** of *Streptavidin-HRP Conjugate* with **1 mL** of the **used** *1X Blocking Buffe*r. Dispense the diluted *Streptavidin-HRP Conjugate* into the container but do not directly dispense on the membrane.
33. Continue shaking the *Membrane* in the Streptavidin-Blocking Buffer solution for **45 minutes** at room temperature.
34. Decant the *Blocking Buffer* and wash the *Membrane* three times with **15 mL** of *1X Detection Wash Buffer*. Each wash should expose the membrane for 10 minutes.
35. Mix equal amounts of *Substrate A* and *B* to create a working *Substrate Solution*. Place the membrane on the bottom side of *Detection Sheet* on a flat surface, and overlay the *Membrane* with **1.2 mL** of *Substrate Solution*, ensuring that the substrate is evenly distributed over the *Membrane*. Gently place the top side of detection sheet over the membrane ensure that the substrates cover the entire surface of the *Membrane*, without trapping air bubbles on the *Membrane*. Incubate at room temperature for **5 minutes**.
36. Remove excess substrate by gently applying pressure over the top sheet using a paper towel.
37. Expose the *Membrane* using either Hyperfilm (2-10 minutes exposure) or a chemiluminescence imaging system (e.g. FluorChem Imager from Alpha Innotech). With either method, experiment with different exposure times.

Figure 1:
Sample of Highly sensitive miRNA Northern blot

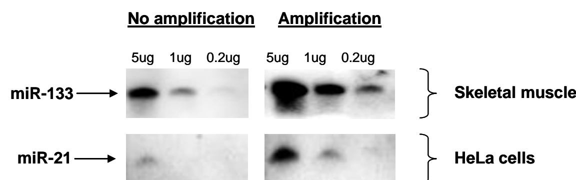

Table 1:

One Full Size Gel	Membrane Size	# Probes	Hybridization Tube	Detection	Substrate per Membrane
1 standard, 14 RNA samples	Full size membrane	1	1	1 membrane per container	1.2mL
1 standard, 6 samples (two duplicates with one blank well in between)	1/2 size membrane	2	2	2 membranes per container	0.7mL
1 standard, 2 RNA samples (three duplicates with one blank wells in between each duplicate)	1/3 size membrane	3	3	2 membranes per container	0.5mL