HiPer® Northern Blotting Teaching Kit

Product Code: HTBM028
Number of experiments that can be performed: 5

Duration of Experiment: 3 days
Day 1: Agarose Gel Electrophoresis, Capillary transfer of RNA
Day 2: UV Cross linking and Hybridization
Day 3: Detection and Result

Storage Instructions:
- The kit is stable for 6 months from the date of manufacture
- Store Total RNA and Biotinylated Probe at -20 °C
- Store 5X RNA Gel Loading Buffer, wash Buffers, Hybridization Buffer, Blocking Powder, Streptavidin HRP conjugate, Conjugate dilution buffer and TMB/H₂O₂ at 2-8 °C
- Other kit contents can be stored at room temperature (15-25 °C)
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Aim:

To learn the technique of Northern Blotting for the detection of a specific RNA fragment in a sample.

Introduction:

Northern blotting or Northern hybridization is a widely used technique in molecular biology to determine the molecular weight of mRNA and to measure relative amounts of mRNA present in different samples and for identifying alternatively spliced transcripts and multigene family members. Northern Blotting involves separation of RNA samples according to size by agarose gel electrophoresis and detection with hybridization probe complementary to part of or the entire target sequence. Northern Blot refers to capillary transfer of RNA from the electrophoresis gel to the blotting membranes.

Principle:

Northern blotting is a commonly used method to study gene expression by detection of RNA (or isolated mRNA) in samples. Northern blot technique was developed by James Alwine and George Starck and was named such by analogy to Southern blotting. In Northern Blotting the total RNA or mRNA is isolated from an organism of interest, and then electrophoresed on denaturing agarose gel, which separates the fragments on the basis of size. The next step is to transfer fragments from the gel onto nitrocellulose filter or nylon membrane. This can be performed by the simple capillary method. The transfer or a subsequent treatment results in immobilization of the RNA fragments, so the membrane carries a semi permanent reproduction of the banding pattern of the gel. The RNA is bound irreversibly to the membrane by baking at high temperature (80°C) or by UV crosslinking. For the detection of a specific RNA sequence, a hybridization probe is used. A hybridization probe is a short (100-500bp), single stranded nucleic acid either DNA or RNA probe that will bind to a complementary piece of RNA. Hybridization probes are labeled with a marker (radioactive or non-radioactive) so that they can be detected after hybridization. In non-radioactive detection the probe is labeled with biotin or dioxigenin. The membrane is washed to remove non-specifically bound probe and the hybridized probe is detected by treating the membrane with a conjugated enzyme, followed by incubation with the chromogenic substrate solution. As a result a visible band can be seen on the membrane where the probe is bound to the RNA sample. The entire procedure can be divided into following steps:

A] Denaturing Agarose Gel Electrophoresis:
Denaturing Agarose gel electrophoresis is a technique used for separation of RNA molecules according to their molecular size. In this gel electrophoresis, formaldehyde (a denaturant) is used along with MOPS electrophoresis buffer. RNA has high degree of secondary structures, making it necessary to use denaturing gels. Formaldehyde in the gel disrupts the secondary RNA structures so that RNA molecules can be separated by their charge migration. For analysis of RNA molecules, 1 to 1.2% agarose gels are used depending on the size of RNA to be separated. The position of RNA in the agarose gel is visualized by staining with low concentration of fluorescent intercalating dyes, such as Ethidium bromide. This can be added either in the gels or in the RNA sample before loading for better resolution. The integrity and size distribution of total RNA can be checked by observing the stained RNA.

B] Northern Blotting:
Northern blotting is the capillary transfer of resolved RNA fragments from the denaturing agarose gel to the nitrocellulose/nylon membrane. In this upward capillary transfer procedure, a support is used which is placed in a reservoir of transfer buffer to elevate the entire assembly. The wet wicks are placed on the support with both ends completely dipped in transfer buffer. A gel is placed on the wicks with RNA transferred side facing down. Over the gel, a wet positively charged nylon membrane is placed. A stack of paper towel is kept on filter papers as shown in figure 1. A small weight is placed over this entire assembly and is kept overnight.
During capillary transfer the RNA bands are transferred to positively charged nylon membrane in presence of a specific buffer. The resolved RNA fragments are transferred to the corresponding positions on the nylon membrane after the capillary transfer. The RNA is then immobilized on the membrane either by baking at high temperature or UV crosslinking. This results in the covalent linkage of RNA to the membrane, which prevents the nucleic acid from being washed away during the subsequent processing. This is followed by hybridization with labelled DNA or RNA probe and then the RNA of interest is detected on the membrane.

**C) Detection:**

After capillary transfer, RNA bands bound to the membrane are detected using a chromogen. The RNA of interest is hybridized with a biotinylated probe specific to it. The membrane is washed to remove excess unbound probe. It is then treated with Horseradish peroxidase (HRP)-conjugated streptavidin which attaches to the hybridized RNA. Finally, the membrane is incubated in a substrate solution containing TMB/ H₂O₂ (Tetramethyl benzidine H₂O₂ substrate) that reacts with HRP and as a result corresponding blue coloured RNA band develops on the nylon membrane as shown in Figure 2:
Kit Contents:
This kit can be used for the detection of a specific RNA in a given sample.

Table 1: Enlists the materials provided in this kit with their quantity and recommended storage

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Product Code</th>
<th>Materials Provided</th>
<th>Quantity</th>
<th>Storage</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>TKC306</td>
<td>Total RNA</td>
<td>0.060 ml</td>
<td>-20°C</td>
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<tr>
<td>2</td>
<td>TKC330</td>
<td>Hybridization Buffer</td>
<td>120 ml</td>
<td>2-8°C</td>
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<tr>
<td>3</td>
<td>TKC331</td>
<td>Biotinylated Probe</td>
<td>5 x 0.010 ml</td>
<td>-20°C</td>
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<td>4</td>
<td>ML048</td>
<td>5X RNA Loading Buffer</td>
<td>0.02 ml</td>
<td>2-8°C</td>
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<td>5</td>
<td>TKC333</td>
<td>Wash Buffer I</td>
<td>160 ml</td>
<td>2-8°C</td>
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<td>TKC334</td>
<td>Wash Buffer II</td>
<td>160 ml</td>
<td>2-8°C</td>
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<td>7</td>
<td>TKC323</td>
<td>Blocking Powder</td>
<td>2 g</td>
<td>2-8°C</td>
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<tr>
<td>8</td>
<td>TKC143</td>
<td>TMB/H2O2</td>
<td>30 ml</td>
<td>2-8°C</td>
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<tr>
<td>9</td>
<td>TKC382</td>
<td>Streptavidin HRP Conjugate</td>
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<td>TKC326</td>
<td>Conjugate Dilution Buffer</td>
<td>200 ml</td>
<td>2-8°C</td>
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<td>11</td>
<td>ML050</td>
<td>10X MOPS Electrophoresis Buffer</td>
<td>50 ml</td>
<td>RT</td>
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<tr>
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<td>TKC335</td>
<td>10X Transfer Buffer</td>
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<tr>
<td>13</td>
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<td>Tween 20</td>
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<td>14</td>
<td>MB002</td>
<td>Agarose</td>
<td>1 g</td>
<td>RT</td>
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<td>15</td>
<td>MB059</td>
<td>Formaldehyde 37%</td>
<td>12 ml</td>
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<td>16</td>
<td>TKC336</td>
<td>Filter Paper</td>
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<td>TKC337</td>
<td>Wicks</td>
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<td>18</td>
<td>TKC338</td>
<td>Nylon Membrane</td>
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<td>19</td>
<td>TKC339</td>
<td>Blotting Sheet</td>
<td>4 Nos.</td>
<td>RT</td>
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<tr>
<td>20</td>
<td>PW001</td>
<td>Sterile Disposable Petri plates</td>
<td>2 Nos.</td>
<td>RT</td>
</tr>
</tbody>
</table>

Materials Required But Not Provided:

Glassware: Conical flasks, Beakers
Reagents: Distilled water, Ethidium bromide (10 mg/ml), Isopropanol, Ethanol
Other requirements: Glass plate, Plastic box, Plastic tray, Gel rocker, Micropipettes, Tips, Microwave/Burner/Hotplate, Hot Air Oven, Incubator Shaker (55°C and 65°C), Forceps, Crushed ice

Storage:

HiPer® Northern Blotting Teaching Kit is stable for 6 months from the date of manufacture without showing any reduction in performance. Store all the kit contents as mentioned above.
Important Instructions:

1. Read entire procedure carefully before starting the experiment.
2. Denaturing agarose electrophoresis should be carried only one time on purchase of the kit, to avoid repeated freezing and thawing of RNA as this releases toxic fumes due to use of formaldehyde.
3. **Preparation of Hybridization Buffer:** Add 0.1 g of blocking powder to 10 ml of hybridization buffer. Mix well before use.
4. **Preparation of Streptavidin HRP-Conjugate Buffer:** Add 9 μl of Tween 20 in 9 ml of conjugate dilution buffer. Add 3 μl of Streptavidin-HRP Conjugate in 9 ml of conjugate dilution buffer for each experiment just prior to use.
5. Bring the buffers provided to room temperature a day prior to use except for Streptavidin-HRP conjugate buffer and TMB/H₂O₂.
6. Ensure no air bubbles are present between any of the layers of filter paper, cut gel and nylon membrane during capillary transfer.
7. Ensure that the filter paper, membrane and blotting sheets are exactly of the same size of the excised gel piece.
8. Reuse the plastic petri plate 2-3 times after thorough cleaning with distilled water and drying.

Procedure:

**Day 1:**

1. **Denaturing Agarose Gel Electrophoresis**

**Preparation of 1X MOPS Electrophoresis Buffer:** To prepare 500 ml of 1X MOPS Electrophoresis Buffer, add 50 ml of 10X MOPS Buffer to 440 ml of RNase free water and add 10 ml of Formaldehyde (37%). Mix well before use.

**Precautions to be taken while handling RNA:**

1. Prior to start the experiment, the electrophoresis tank should be cleaned with detergent solution (e.g., 0.5% SDS), thoroughly rinsed with RNase-free water, and then rinsed with ethanol and allowed to dry.
2. Tips, pipettes, electrophoresis unit etc to be used for the experiment must be UV treated for 15-20 minutes.
3. Use sterile, disposable plasticwares and micropipettes reserved for RNA work to prevent cross-contamination with RNases from shared equipments.
4. Use RNase-free water for diluting the solutions.

**Preparation of Denaturing Agarose gel:** To prepare 50 ml of 1.2 % agarose solution, mix 5 ml of 10X MOPS Electrophoresis Buffer with 45 ml of autoclaved deionized water in a glass beaker or flask. To this add 0.6 g of agarose. Heat the mixture in a microwave, burner or hot plate, swirling the glass beaker/ flask occasionally, until agarose dissolves completely (Ensure that the lid of the flask is loose to avoid buildup of pressure). Allow solution to cool to about 55-60°C. Add 0.9 ml of 37% Formaldehyde and mix well and add 1μl of EtBr (10 mg/ml), mix well and pour the gel solution into the gel tray sealed on both sides with adhesive tape. Allow the gel to solidify for about 30 minutes at room temperature (15-25°C).

**NOTE:** Before running the gel, equilibrate it in 1X MOPS Buffer for atleast 30 minutes.

**Loading of the RNA samples:** To prepare sample for electrophoresis, add 2 μl of 5X RNA gel loading buffer to 10 μl of total RNA samples. Mix well by pipetting and load the samples into 5 wells.
**Electrophoresis**: Connect the power cord to the electrophoretic power supply according to the conventions: Red-Anode and Black- Cathode. Electrophorese at 100 volts and 70 mA until dye markers have migrated an appropriate distance, depending on the size of RNA to be visualized.

**NOTE**: Toxic fumes are released on electrophoresis, hence it is necessary to run the gel in a fume hood.

**2. Capillary Transfer of RNA:**

1. After electrophoresis, soak the gel in RNase-free water for 5 minutes to remove formaldehyde.
2. Repeat the above step twice and then discard water.
3. Observe the gel under UV transilluminator to excise the gel piece using a gel cutter.

**NOTE**: Excise the entire piece of gel i.e. 5 lanes. Take care not to excise the individual lanes. Do not expose yourself to UV.

4. Set up capillary blot with transfer buffer as follows.
   1. Rinse the materials required for transfer with isopropanol followed with RNase-free water thoroughly.
   2. Fill the buffer reservoir with 200 ml 10X transfer buffer.
   3. Keep a support in the buffer reservoir.
   4. Wet the wicks with transfer buffer. Ensure both the ends of the wicks are completely dipped in the transfer buffer.
   5. Place the gel piece upside down i.e. RNA transferred side should face the wick.
   6. Wet the nylon membrane for few minutes in the transfer buffer. Place the membrane supplied on the gel. Ensure that no air bubbles are trapped between the gel and the membrane, as this will affect efficient transfer.
   7. Wet the filter paper and place over the membrane, ensure no air bubbles are trapped between the membrane and filter paper.
   8. Carefully place a weight over this stack. Ensure that the book is placed on the centre of this stack, such that even pressure is applied.

**Day 2:**

1. **Immobilization of RNA on membrane:**

   1. Carefully remove the stack of blotting paper and filter paper after overnight transfer.
   2. Observe the membrane and the gel piece under UV transilluminator to check whether complete transfer has taken place.
   3. Expose the membrane to UV light for 5 minutes (placed between the inner layers of tissue paper). This helps in fixing the RNA to the membrane.
   4. Switch off the UV, turn over the membrane and expose it to UV light for another 5 minutes.
   5. Bake the membrane at 70-80 °C for 30 minutes (place the membrane between the inner layers of tissue paper).
   6. Place the membrane in a ziplock or autoclaved petri plate at 4 °C.
   7. Mark the lanes on the membrane with a pencil, cut along the length of the membrane.
   8. Use one strip for hybridisation and developing and remaining strips can be stored at 4 °C until further use.
2. Hybridization:
1. Set an incubator shaker at 55°C, prior to placing the membrane for Prehybridization.
2. Place the membrane (RNA transferred side facing down) in a petri plate containing 10 ml of hybridization buffer.
3. Carry out Prehybridization at 55°C for 1 hour, with constant shaking (70-80 rpm).
4. Keep 1 vial of biotinylated probe for 5 minutes in boiling water bath and immediately chill by placing it on ice for 5-10 minutes.
5. Remove petri plate and discard the buffer.
6. Add 10 μl of this probe to the 10 ml of Hybridization buffer in the petri plate. Mix thoroughly and add drop wise to the petri plate. Make sure that you don’t add probe directly on the membrane.
7. Seal the petri plate and incubate at 55°C in an incubator shaker overnight with mild shaking at about 70-80 rpm.

Day 3:
1. Washes and Detection:
1. Transfer the membrane into a fresh petri plate containing 10 ml of Wash Buffer I.
2. Gently swirl the petri plate for 15 minutes at room temperature. Repeat the wash one more time. Discard the buffer after each wash.
3. Add 10 ml of prewarmed Wash Buffer II (65°C) and gently swirl the petri plate. Incubate at 65°C for 15 minutes in an incubator shaker and gently swirl. Repeat this step. Discard the buffer after each wash.
   NOTE: Do not let the membrane go dry at any step.
4. Add 9 ml of Streptavidin-HRP conjugate buffer (Refer Important instructions) to the petri plate and incubate at room temperature for 30 minutes with gentle rocking. Discard the conjugate buffer.
5. Take 30 ml of conjugate dilution buffer in autoclaved test tube and add 30 μl of Tween 20 to it and mix it thoroughly. This is to be used for further washes.
6. Carry out washes in a fresh and dried petri plate. Do not carry out washes in the petri plate used for conjugation. Use 10 ml of conjugate dilution buffer (See step 5) to carry out washes of 5 minutes at room temperature. Repeat the above step two more times.
7. Add 5 ml of TMB/H2O2 and gently swirl at room temperature until a blue colour band develops.
8. After blue colour band is seen stop the reaction by placing the membrane in distilled water.
Flow chart:

1. RNA Sample
2. RNA fractionated on agarose gel
3. Capillary transfer of RNA from gel to membrane
4. Bound RNA on the membrane
5. Biotinylated probe
6. Membrane incubated with biotinylated probe
7. Detection of RNA band after treatment with TMB
**Observation and Result:**

![Gel Image and Immunoblot of RNA Sample](image-url)

**Fig 3: Gel image and immunoblot of the RNA sample after Agarose Gel Electrophoresis and Northern blotting**

**Lane 1:** RNA sample on 1.2 % denaturing agarose gel

**Lane 2:** After Northern hybridization a blue band develops on the nylon membrane

**Interpretation:**

In non-radioactive Northern Hybridization a biotinylated probe is hybridized to the complementary target RNA (28s rRNA). The biotin of resulting hybridized complex binds to Streptavidin-HRP conjugate. In presence of TMB substrate HRP reacts with it and forms a blue colour which appears as a blue band on the nylon membrane. A few blue coloured bands can be seen below major 28s rRNA band due to non-specific binding of the probe to the total RNA bound on the membrane.

**Troubleshooting Guide:**

<table>
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<th>Sr.No</th>
<th>Problem</th>
<th>Probable Cause</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Low Sensitivity (faint bands or no bands)</td>
<td>Incomplete transfer</td>
<td>Following RNA transfer to membrane, view the gel on UV transilluminator to see if any RNA has remained in the gel</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Target RNA not effectively fixed on membrane</td>
<td>Check UV lamp or oven temperature</td>
</tr>
<tr>
<td>2</td>
<td>High background</td>
<td>Insufficient washing or contamination in buffer</td>
<td>Wash the membrane thoroughly as mentioned in the brochure</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Contaminated forceps</td>
<td>Always clean forceps after they are used with hybridization solutions containing labeled probe. Dirty forceps may deposit dye on membrane that will not wash away</td>
</tr>
<tr>
<td></td>
<td>Probe added onto membrane</td>
<td>Always add the probe to hybridization solution</td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>---------------------------</td>
<td>---------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Nonspecific bands seen</td>
<td>Membrane is exposed too long to the substrate solution</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Stop the reaction as soon as blue band develops</td>
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</tbody>
</table>

**Technical Assistance:**

At HiMedia we pride ourselves on the quality and availability of our technical support. For any kind of technical assistance mail at mb@himedialabs.com

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**Storage temperature**

15°C - 25°C

**Do not use if package is damaged**

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