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Product Information

HiPer® Southern Blotting Teaching Kit

Product Code: HTBM027
Number of experiments that can be performed: 5

Duration of Experiment: 2 days
Day1: Agarose Gel Electrophoresis, Electro transfer and Hybridization
Day2: Detection and Result

Storage Instructions:

- The kit is stable for 12 months from the date of manufacture
- Store DNA Sample and Biotinylated Probe at -20 °C
- Store Tween 20, Agarose, 10XTBE, Nylon Membrane and Petri plate at room temperature (15-25 °C)
- Store the rest of materials at 2-8°C

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Aim:

To learn the technique of Southern Blotting for the detection of a specific DNA fragment

Introduction:

Southern blotting or Southern hybridization is a widely used technique in molecular biology for transfer of DNA molecules; usually restriction fragments, from an electrophoresis gel to a nitrocellulose or nylon membrane, and is carried out prior to detection of specific molecules by hybridization probing. In this method a DNA mixture is separated by agarose gel electrophoresis according to their size followed by transfer of the DNA bands to nitrocellulose/nylon membrane. Finally, the DNA of interest is probed for a specific sequence.

Principle:

Southern hybridization, also called Southern blotting, is a commonly used method for the identification of DNA fragments that are complementary to a known DNA sequence. It allows a comparison between the genome of a particular organism and that of an available gene or gene fragment. This technique also tells us whether an organism contains a particular gene, and provides information about the organism and restriction map of that gene. Southern hybridization was named after its inventor, Edward M. Southern, who developed the technique in 1975. As a result subsequent blotting techniques have used similar nomenclature, for example Northern blotting, the transfer of RNA; Western blotting, the transfer of proteins; and Southwestern blotting, for the characterization of proteins that bind DNA. In Southern Blotting the chromosomal DNA is isolated from an organism of interest, and digested with restriction enzyme. The restriction digested fragments are electrophoresed on an 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 either by electrotransfer i.e. electrophoresing the DNA out of the gel and onto a membrane or by the simple capillary method. The transfer or a subsequent treatment results in immobilization of the DNA fragments, so the membrane carries a semi permanent reproduction of the banding pattern of the gel. The DNA is bound irreversibly to the membrane by baking at high temperature (80°C) or by UV crosslinking. For the detection of a specific DNA sequence, a hybridization probe is used. A hybridization probe is a short (100-500bp), single stranded nucleic acid that will bind to a complementary piece of DNA. 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 DNA sample. The entire procedure can be divided into following steps:

I. Agarose Gel Electrophoresis: Agarose gel electrophoresis is a technique for separation of DNA molecules according to their molecular size. This is achieved when negatively charged nucleic acids migrate through an agarose gel matrix under the influence of an electric field (electrophoresis). Shorter molecules move faster and migrate farther than the larger ones. The position of DNA in the agarose gel is visualized by staining with low concentration of fluorescent intercalating dyes, such as Ethidium bromide.

II. Southern Blotting: Southern blotting is the electro transfer/capillary transfer of resolved DNA fragments from the agarose gel to the nitrocellulose/nylon membrane. For this transfer procedure, the gel is placed on the membrane and both of them are sandwiched between two filter papers as shown in Figure 1:

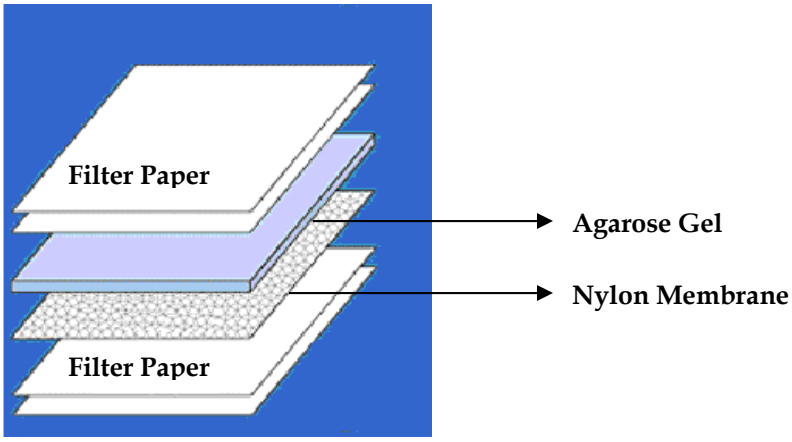


Fig 1: Arrangement of the gel and membrane for transfer

During electro transfer the DNA bands are transferred to positively charged nylon membrane in the presence of a specific buffer. First transfer the set (as shown in Fig 1) between two sponge pads and then place it in a plastic cassette. The entire set is then placed inside a gel tank filled with transfer buffer. The resolved DNA fragments are transferred to the corresponding positions on the nylon membrane after the electro transfer. The DNA of interest is detected on the membrane.

III. Detection:

After electrotransfer, DNA bands bound to the membrane are detected chromogenically. A suitable blocking reagent is used to block the unoccupied sites on the membrane. Then the DNA 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 DNA. Finally, the membrane is incubated in a substrate solution containing TMB/ H_2O_2 (Tetramethyl benzidine H_2O_2 substrate) that reacts with HRP and as a result a blue coloured DNA band develops on the nylon membrane as shown in Figure 2.

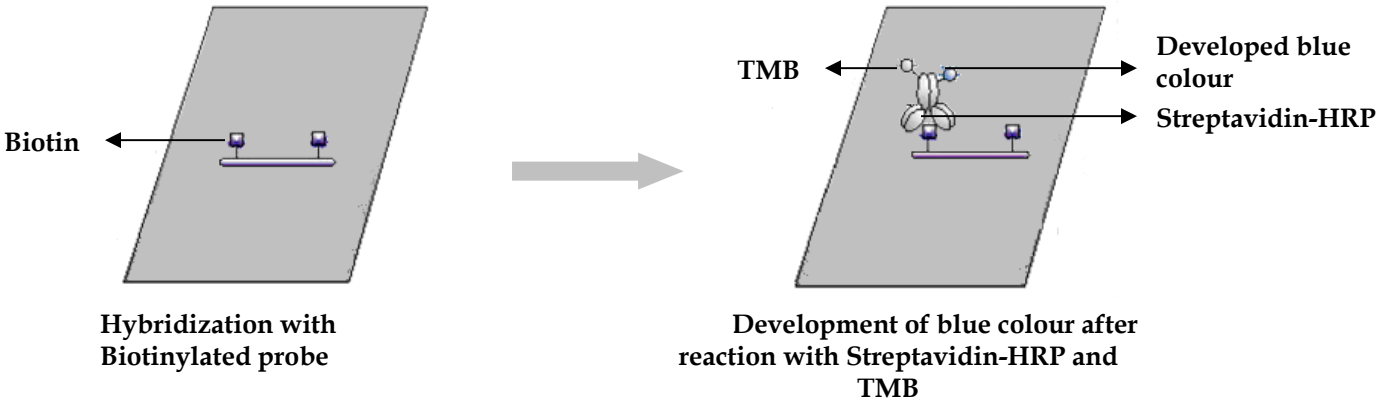


Fig 2: The hybridized DNA is detected after treatment with Streptavidin-HRP, followed by TMB substrate

Kit Contents:

This kit can be used for the detection of a specific DNA band in a given sample.
Table 1: Enlists the materials provided in this kit with their quantity and recommended storage

Sr. No.	Product Code	Materials Provided	Quantity	Storage
			5 expts	
1	TKC314	DNA Sample	0.06 ml	-20°C
2	TKC315	Prehybridisation Buffer	60 ml	2-8°C
3	TKC316	Hybridisation Buffer	60 ml	2-8°C
4	TKC317	Biotinylated Probe	5 x 0.015 ml	-20°C
5	TKC318	2X Wash Buffer I	80 ml	2-8°C
6	TKC319	2X Wash Buffer II	80 ml	2-8°C
7	TKC320	2X Wash Buffer III	80 ml	2-8°C
8	TKC321	2X Wash Buffer IV	80 ml	2-8°C
9	TKC322	Blocking Buffer	60 ml	2-8°C
10	TKC323	Blocking Powder	2 g	2-8°C
11	TKC324	10X Transfer Buffer	200 ml	2-8°C
12	TKC143	TMB/H_2O_2	30 ml	2-8°C
13	TKC325	Streptavidin HRP Conjugate	0.040 ml	2-8°C
14	TKC326	Conjugate Dilution Buffer	50 ml	2-8°C
15	MB067	Tween 20	0.05 ml	RT
16	MB002	Agarose	3 g	RT
17	ML011	10X TBE	300 ml	RT
18	TKC327	Nylon Membrane with filter paper	5 Nos.	RT
19	PW001	Sterile Disposable Petriplates	2 Nos.	RT

Materials Required But Not Provided:

Glassware: Test tubes
Reagents: Distilled water, Ethidium bromide (10 mg/ml), deionized water
Other requirements: Gel transfer apparatus, Gel rocker, Micropipettes, Tips, Microwave/Burner/Hotplate, Hot Air Oven, Incubator Shaker (45°C), Forceps

Storage:

HiPer® Southern Blotting Teaching Kit is stable for 12 months from the date of manufacture without showing any reduction in performance. Store all the reagents as mentioned above.

Safety:

Precaution: UV light can damage the eyes and skin. Always wear suitable eye and face protection when working with a UV light source. UV light damages DNA. If DNA fragments are to be extracted from the gel, use a lower intensity UV source if possible and minimize exposure of the DNA to the UV light.

Disposal of Ethidium bromide waste: All items that were in contact with Ethidium bromide must be disposed

off in the designated waste container (marked with "Ethidium bromide waste") within the Gel-Doc-Area, including gels, tissue paper to clean UV table, and nitrile gloves.

Hazard: Ethidium bromide is a powerful mutagen and is very toxic. Appropriate safety precautions should be taken by wearing latex gloves; however, use of nitrile gloves is recommended.

Important Instructions:

1. Read entire procedure carefully before starting the experiment.
2. DNA sample provided is ready to use and can be loaded directly onto the agarose gel.
3. **Preparation of Prehybridisation Buffer:** Add 0.1g of blocking powder to 10 ml of prehybridisation buffer. Mix well before use.
4. **Preparation of Hybridization Buffer:** Add 0.1g of blocking powder to 10 ml of hybridization buffer. Mix well before use.
5. **Preparation of 1X Transfer Buffer:** To prepare 1 litre of 1X Transfer Buffer, take 100 ml of 10X transfer buffer and add 900 ml sterile distilled water*. Store at 2-8°C. Mix well before use. The 1X Transfer Buffer can be reused 2-3 times.
6. **Preparation of 1X Wash Buffer I:** Dilute 15 ml of 2X Wash Buffer I with 15 ml of autoclaved deionized water. Mix well.
7. **Preparation of 1X Wash Buffer II:** Dilute 15 ml of 2X Wash Buffer II with 15 ml of autoclaved deionized water. Mix well.
8. **Preparation of 1X Wash Buffer III:** Dilute 15 ml of 2X Wash Buffer III with 15 ml of autoclaved deionized water. Mix well.
9. **Preparation of 1X Wash Buffer IV:** Dilute 15 ml of 2X Wash Buffer IV with 15 ml of autoclaved deionized water. Mix well.
10. **Preparation of Blocking Buffer:** Add 0.1g of blocking powder to 10 ml of blocking buffer
11. **Preparation of Streptavidin HRP-Conjugate Buffer:** Add 9 µl of Tween 20 in 9 ml of conjugate dilution buffer. Add 6.0 µl of Streptavidin- HRP Conjugate in 9 ml of conjugate dilution buffer for each experiment just prior to use.
12. Bring the buffers provided to room temperature a day prior to use except for Streptavidin-HRP conjugate buffer and TMB/H_2O_2
13. Ensure no air bubbles are present between any of the layers of filter paper, cut gel and nylon membrane during electroblotting.
14. Reuse the plastic petri plate 2-3 times after thorough cleaning with distilled water and drying.

Procedure:

Day 1: Agarose Gel Electrophoresis

1. **Preparation of 1X TBE:** To prepare 500 ml of 1X TBE buffer, add 50 ml of 10X TBE Buffer to 450 ml of sterile distilled water*. Mix well before use.

2. **Preparation of agarose gel:** To prepare 50 ml of 1 % agarose gel, add 0.5 g agarose to 50 ml 1X TBE buffer in a glass beaker or flask. Heat the mixture on a microwave or hot plateswirling 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 0.5 µl Ethidium bromide, mix well and pour the gel solution into the gel tray. Allow the gel to solidify for about 30 minutes at room temperature.

3. **Loading of the DNA samples:** To prepare sample for electrophoresis, load 10 µl of DNA sample into the well.

4. **Electrophoresis:** Connect the power cord to the electrophoretic power supply according to the conventions: Red-Anode and Black- Cathode. Electrophorese at 100-120 volts and 90 mA until dye markers have migrated an

appropriate distance, depending on the size of DNA to be visualized.

5. Cut the DNA Marker from the agarose gel by viewing it under UV transilluminator. Cut the gel about 3 mm from the first band and 2 mm below the last band ensuring the gel measures about 4 to 4.5 cm.

* Molecular biology grade water is recommended (Product code: ML024).

6. Take out the nylon membrane with the filter paper (provided). Ensure there is no protrusion of the filter paper and membrane from the gel.

7. Wet the cut gel, nylon membrane, filter papers and the electrotransfer cassette in 1X Electrotransfer buffer.

Electroblotting:

1. Assemble the gel with nylon membrane and filter papers as shown in figure 1. This blotting sandwich is placed within the sponge and blotting cassette. Try to avoid air bubble between gel and nylon membrane by rolling a glass tube on the membrane.
   Note: Take out the transparent sheets carefully while using the nylon membrane.
2. Insert this cassette into the gel transfer apparatus filled with cold transfer buffer and then connect the transfer unit to power supply as per conventions.
3. Electrophoreses the sample at 100V, 90 mA for 2 hours for blotting.

Immobilization of DNA on membrane:

1. Remove the nylon membrane after transfer from the blotting cassette and place the membrane in petri plate on a UV transilluminator (expose the membrane containing transferred DNA to UV light) for 20 minutes. This helps in fixing the DNA on the membrane.
2. Turn off the UV transilluminator and incubate the plate containing membrane in hot air oven at 70 °C for 30 minutes. This ensures complete immobilization onto the membrane.

Hybridization:

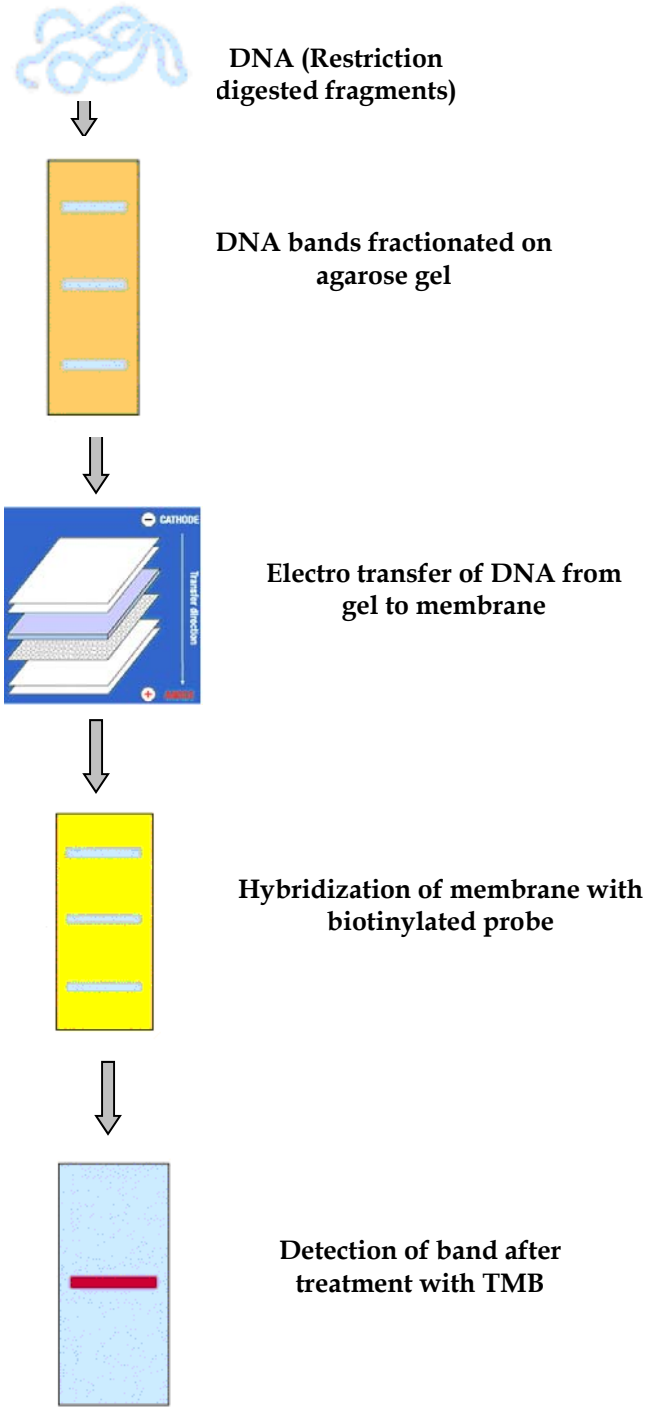
1. Bring the petri plate containing membrane to room temperature after incubation. Add 10 ml of prehybridisation buffer into it and incubate at 45 °C incubator shaker with mild shaking at 70-90 rpm for 45 minutes.
2. After incubation, discard the prehybridisation buffer. Care should be taken not to discard the membrane.
3. Add 10 ml of hybridisation buffer to the petri plate containing membrane.
4. Keep 1 vial of biotinylated probe for 10 minutes in boiling water bath and immediately chill by placing it on ice for 5-10 minutes. Add 15 µl of this probe to the hybridization buffer in the petri plate.
5. Incubate the petri plate at 45 °C incubator shaker with mild shaking at about 70-90 rpm for 16 hours.

Day 2: Blocking and Detection:

1. Decant the hybridization buffer, add 10 ml of 1X Wash Buffer I and gently swirl the petri plate for 5 minutes at room temperature. Repeat the washes twice (each wash for 5 minutes). Discard the buffer after each wash.
2. Add 10 ml of prewarmed 1X Wash Buffer II (70 °C) and gently swirl the petri plate. Incubate at 70 °C for 5 minutes in a hot air oven and gently swirl. Repeat the washes for another 2 times. Discard the buffer after each wash.
3. Add 10 ml of blocking buffer to the petri plate and incubate at room temperature for 1 hour with gentle rocking. Discard the blocking buffer.
4. Add 9 ml of diluted Streptavidin-HRP conjugate buffer to the petri plate and incubate at room temperature for 20 minutes with gentle rocking. Discard the conjugate buffer.

5. Add 10 ml of 1X Wash Buffer III to the petri plate and incubate at room temperature for 5 minutes each with gentle rocking. Repeat the washes two more times. Discard the buffer after each wash.
6. Add 10 ml of 1X Wash Buffer IV to the petri plate and incubate at room temperature for 5 minutes each with gentle rocking. Repeat the washes two more times. Discard the buffer after each wash.
7. Add 5 ml of TMB/H_2O_2 and gently swirl at room temperature for 15-20 minutes 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:



Observation and Result:

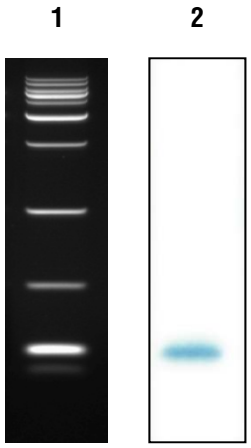


Fig 3: Gel image and immunoblot of the DNA sample after Agarose Gel Electrophoresis and Southern blotting

Lane 1: DNA sample after 1 % agarose gel electrophoresis
Lane 2: After Southern hybridization a blue band develops on the nylon membrane

Interpretation:

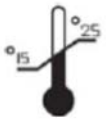
In non-radioactive Southern Hybridization a biotinylated probe is hybridized to the complementary target DNA. The biotin of resulting hybridized complex binds to Streptavidin-HRP conjugate. In presence of TMB substrate HRP reacts with it and forms a blue colour which appears as a blue band on the nylon membrane.

Troubleshooting Guide:

Sr.No	Problem	Probable Cause	Solution
1	Low Sensitivity (faint bands or no bands)	Incomplete transfer	Following DNA transfer to membrane, view the gel with UV transilluminator to see if any DNA has remained in the gel
		Target DNA not effectively fixed on membrane	Check UV lamp or oven temperature
2	High background	Insufficient washing or contamination in buffer	Wash the membrane thoroughly as mentioned in the brochure
		Contaminated forceps	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
		Probe added onto membrane	Always add the probe to hybridization solution

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



Storage temperature

Do not use if package is damaged

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