ITQB-Nova - Universidade Nova de Lisboa	EPP-SOP-ITQB02
	Version 1.0

EPP - Standard Operating Procedure

(only for selected experiments intended to transfer results from one lab to the other)

Title: Polyacrylamide Northern Blot

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Instruction

Detection of small RNAs by blot hybridization.

1 Introduction

This protocol describes the detection of small RNAs by blot hybridization. The RNA samples are separated by denaturing polyacrylamide gel electrophoresis followed by electrophoretic transfer to positively charged nylon membranes. After transfer, the immobilized RNAs are subjected to hybridization with a ^{32}P-radiolabeled DNA or RNA probe and detected by phosphorimaging.

2 Equipment and chemicals

2.1 Equipment

Water bath
Vertical electrophoresis system
Electrophoresis Power supply
Blotting system
UV crosslinker
Hybridization oven
Fuji PhosphoImager

2.2 Chemicals

Tris Base
Boric Acid
EDTA
Acetic Acid
Urea
Polyacrylamide AA 40% 19:1 (RNA)
Ammonium persulfate
Tetramethylethylenediamine (TEMED)
Formamide
Mixed Bed Resin
Xylene cyanol
BromoPhenol Blue
Sodium Chloride
Sodium Citrate
Sodium lauryl sulfate
Radioactivity ɣ-^{32}P ATP

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3 **Media and Buffers**

3.1 **TBE 10x**

Tris Base	108g
Boric Acid	55g
EDTA	9,3g
Milli-Q Water	To 1000mL

3.2 **TAE 20x**

Tris Base	48,4g
Acetic Acid	11,4mL
EDTA 0,5M	20mL
Milli-Q Water	To 500mL

Note: Add some Milli-Q water before adding the acetic acid to prevent the Tris Base from becoming rock solid.

3.3 **PAA 10% Solution**

Urea	210g
TBE 10x	50mL
PAA 40% 19:1 (RNA)	125mL

Add ~20mL of Milli-Q Water autoclaved and leave the solution in a water bath at 42ºC to dissolve the Urea. After all the Urea is dissolved add water to 500mL.
Filter the solution with a 0,45µ filter and cover the flask with aluminium foil. Keep the solution at 4ºC.

3.4 **Deionized Formamide**

1g of Mixed Bed Resin for 10mL of formamide

3.5 **Loading Buffer**

Deionized Formamide	5mL
EDTA 0.5M (pH 8)	100µL
Xylene cyanol	0,005g
BromoPhenol Blue	0,005g

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3.6 **APS 10x**

0,1g of Ammonium persulfate dissolved in 1mL of Milli-Q Water.

3.7 **SSC 20X buffer**

NaCl	175,3 g
Na_3Cit	88,2g
Milli-Q Water	To 1000 ml

Note: Adjust pH to 7.0

3.8 **Washing Solutions**

	Solution I	Solution II	Solution III
SSC 20x	100mL	50mL	25mL
SDS 10%	10mL	10mL	10mL
bi-distilled Water	890mL	940mL	965mL

4 Procedures

4.1 Probe labeling

In a 1.5ml eppendorf tube add:

0.5 µl Specific Primer for sRNA 10nM
3 µl T4 PNK Reaction buffer
23.5 µl MQ water
2 µl ɣ-^{32}P ATP
1 µl T4 PNK
Total volume of 30µl
Incubate at 37ºC for at least 1h.
Purify the probe with a G25 column.
Store at -20ºC.

4.2 **Prepare glass plates for gel**

Wash plates with cold water, dishwashing liquid, and liquid cream (RNase Free). Do not scratch plates. Rinse with tap water until all soap is removed. The water should form an even sheet over the plate. Rinse with ethanol and wipe dry, make sure all dust is gone.
Also wash spacers and comb with soap and water. Rinse with ethanol.

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Put 1.5 mm thick spacers on the glass plate, put the notched glass plate on top of the spacers. Seal the glasses with agarose (above 1.5%) and clamp the plates together on both sides and bottom with large binder clips.

4.3 **Pour gel**

Each gel takes about 50ml of PAA solution. So, for 2 gels we prepare 100ml of PAA (Concentration depends on the size of the transcript that we are analysing) with 1ml of APS (10%) and 100µl of TEMED.

Hold the plates at approximately 45 degrees while pouring. Insert comb and slowly lower the plates down (put a pipette tip boxes under gel). Clamp the comb with binder clips.
Leave gel to polymerize overnight.

4.4 **Pre-running the gel**

Carefully remove comb and spacer on bottom and rinse off any excess acrylamide and urea around comb and on the glasses.
Stand the gel in the lower buffer reservoir so that the notched glass plate faces the top reservoir. Clamp the gel with the strong binder clips at the top reservoir on both sides. Make sure that the end of the wells is slightly below the rubber of the top reservoir.
Add 1x TBE buffer in both reservoirs. Rinse out the wells with 1x TBE buffer, using a syringe and with a syringe remove all air bubbles form the bottom of the gel.
Pre-run the gel at constant 420 V and limiting 24W per gel, (i.e. for 2 gels limiting 48W) for about 1 hr.

4.5 **Load and run the gel**

Mix 5µl of each sample with 15µl of loading buffer (with BromoPhenol Blue and Xylene cyanol).
Heat at 80°C for 10 min, move to ice. After 2min on ice spin the samples.
Turn off power and rinse out the wells with 1x TBE using a syringe and needle, make sure all urea is rinsed out, load samples.
Run gel at constant 420 V and limiting 24W. The time of the running depends on the transcript that we are analysing. In a 10% PAA gel BromoPhenol Blue runs around 10nt and Xylene cyanol around 55nt.

4.6 **Electroblotting (Wet transfer)**

Prepare 4L of TAE 1x. Have at least 10 pieces of Whatman paper cut (18cmx15cm) per gel. Cut a Hybond N^+ 0.45µ membrane (18cmx15cm). Wash the transfer chamber, the gel cassette and the sponges with tap water. Wash two Tupperware and fill one with water and the other one with TAE 1x. Put the membrane in water (minimum 5min).

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Un-mount the gel:

1 Take out all buffer from top reservoir with syringe. Un-clamp gel.
2 Remove side spacers and pry apart glass plates with the help of a spacer. Cut off the wells from the gel and the side of the gel corresponding to the first well. Place a Whatman paper on top of the gel, flip over and remove other glass plate.
3 Saturate the sponges and the Whatman papers in TAE 1x and assemble in order: negative side of gel cassette (black)>WET sponge>4 pieces of WET Whatman paper>gel>Hybond membrane>5 pieces of WET Whatman paper>WET sponge>positive side of gel cassette (red). After each Whatman paper remove any bubbles (roll with a corex).

Insert the blot assembly into the transfer chamber (black with black and red with red).
Transfer at 24V for 1h45 at 4°C.
Disassemble the cassette and fix the RNA to the membrane with UV crosslink (1200µj/cm) for 3min.

4.7 **Pre-Hybridization**

Place the membrane in the hybridization bottle. Remove any bubbles between the membrane and the bottle.

Add about 15mL of hybridization solution to hybridization bottle and place the bottle in the hybridization oven rotating at a slow speed.
Pre-hybrid for at least 30 min at 42ºC in the hybridization oven.

4.8 **Hybridization**

Denature the probe by boiling it for 5min. Transfer the probe to ice for 2min.
Pipette 10µL (for strong probes) of probe to the hybridization bottle.
Hybridize overnight at 42ºC in the hybridization oven.

4.9 **Washing**

Wash times depend on your probe.
Pour out hybridization buffer in radioactive liquid waste. Turn off temperature in hybridization oven.
Add 50 ml wash solution 1, put hybridization bottle in the oven for 5 min rotating at maximum speed.
Pour out the washing solution into radioactive liquid waste.
Remove membrane from the bottle and check the signal.
Repeat washing step with washing solution 1 for 10 or 15 min. depending the intensity of the signal.
Pour out the washing solution and check again the signal. If the signal is very strong and there is still a lot of background repeat the washing with washing solution 2 for 15min.

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Pour out the washing solution and check again the signal. Repeat washing steps as many times as necessary until the background is around 3 or 4cps. If necessary, proceed the washing steps with washing solution 3 (stronger washing solution).

After the membrane is washed dry it in Whatman paper.

Place the dried membrane in a plastic folder and press to remove all air bubbles. Seal the edges and label the plastic folder with the date and probe.

4.10 **Exposure and image screening**

Place the membrane in a cassette and leave it to expose to a phosphorimage screen from several hours to several days depending on the intensity of your signal.

Scan the phosphoimage screen using the Fuji phosphoimager and quantify the northern using the ImageQuant Software.

5 Biosafety

When working with radioactivity follow these rules:

- Understand the nature of the hazard and get practical training. Never work with unprotected cuts or breaks in the skin, particularly on the hands or forearms. Never use any mouth operated equipment in any area where radioactive material is used. Always store compounds under the conditions recommended. Label all containers clearly indicating nuclide, compound, specific activity, total activity, date and name of user. Containers should be properly sealed.
- Distance yourself appropriately from sources of radiation. Doubling the distance from the source quarters the radiation dose (Inverse Square Law).
- Use appropriate shielding for the radiation 1 cm Perspex will stop all beta particles but beware from high energy beta emitters. Use lead acrylic or a suitable thickness of lead for X and gamma emitters.
- Contain radioactive materials in defined work areas Always keep active and inactive work separated as far as possible, preferably maintaining rooms used solely for radioactive work.
- Wear appropriate protective clothing and dosimeters. Laboratory coats, safety glasses and latex gloves must be worn at all times. Beware of static charge on gloves when handling fine powders. Local rules will define what dosimeters should be worn for work with high-energy isotopes.
- Monitor the work area frequently for contamination control in the event of a spill follow a decontamination protocol.
- Follow the local rules and safe ways of working. Do not eat, drink, smoke or apply cosmetics in an area where radioactive substances are handled. Use paper handkerchiefs and dispose of them appropriately. Never pipette radioactive solution by mouth. Always work carefully and tidily.

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- Minimize accumulation of waste and dispose of it by appropriate routes. Use the minimum quantity of radioactivity needed for the investigation. Disposal of all radioactive waste is subject to statutory control. Be aware of the requirements and use only authorized routes of disposal.
- After completion of work – monitor yourself, wash and monitor again. Never forget to do this.

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