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EXPERIMENTAL PROCEDURE FOR THE DETECTION OF A RARE HUMAN mRNA WITH THE DIG SYSTEM

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ABSTRACT

Newcomers to the DIG System often inquire about the possibility of performing Northern blot hybridizations with nonradioactive techniques. With the following examples, we would like to share our protocol for performing highly sensitive Northern blots. This procedure strictly adheres to the standard procedures detailed in our manuals and pack inserts, and there are no special "tricks" required.

As a target, we have used total human skeletal muscle RNA (Clontech). We selected two probes: β-actin and a probe comprising the cDNA of the transcription factor CTF1, which expresses a low abundant mRNA. We used *in vitro* transcribed RNAs exclusively as probes because, during the development of the DIG System, we have found that RNA probes exhibit a 10-100-fold higher sensitivity with RNA targets than do DNA probes. They are also less prone to background problems caused by probe concentrations that are too high. For DNA probes, we recommend an optimal probe concentration of 25 ng/ml. Using a probe concentration that is even slightly too high (e.g., 1.5 fold) will dramatically increase the background. For RNA probes, we recommend an optimal probe concentration of 100 ng/ml, which will not lead to background problems. In the following examples, we describe all experimental details, starting from the gel run for the blot.

MATERIALS AND METHODS

Electrophoresis conditions and sample preparation

See Table I for required solutions. Prepare all solutions with sterile dimethyldicarbonate-treated distilled water, then autoclave; we use dimethyldicarbonate instead of diethylpyrocarbonate because of DMDC's lower toxicity. When preparing Northern blots, use sterile solutions and sterile trays as much as possible. Treat gel boxes with ethanol prior to the run.

1. Prepare a formaldehyde/agarose gel. We use MOPS-formaldehyde (2% final concentration) agarose gels exclusively and typically prepare them with 1-1.5% Agarose MP.

2. Prepare fresh formamide/formaldehyde RNA loading buffer each day. If not prepared fresh for each use, the formaldehyde will react with the formamide during storage. The bromophenol blue will turn from blue to green during storage and degrade the target.

3. Denature the RNA target sample for 10 min. at 65°C in RNA loading buffer, and place on ice immediately. For example, to 5 µl (100 ng, 50 ng, 5 ng) of target RNA, add 10 µl RNA loading buffer.

We do not use glyoxal denaturation of RNA because (i) glyoxal must be freshly deionized prior to use for best results, and (ii) it has been reported that glyoxal may react with RNA to form complexes that are inhibitory to efficient transfer.

4. Load the dry gels, add electrophoresis buffer to the edges of the gel, allow the RNA to move into the gel at high voltage, and then add electrophoresis buffer to submerge the gel.

Note that our RNA loading buffer does not contain Ficoll or glycerol, which are generally used to simplify loading.

5. Run the gel with 1X MOPS buffer at 25 V overnight under a fume hood. Running gels overnight produces better separation. We include 1 mg/ml ethidium bromide in our running buffer to stain the RNA during the separation; ethidium bromide does not interfere with successful Northern blot hybridizations as has been occasionally reported. However, when running gels at high speed for a short time, we have sometimes observed that one half of the blot stains black after detection. This is probably a result of ethidium bromide running in the opposite direction of the RNA. In a short run, ethidium bromide does not pass through the total gel, and the "running front" becomes visible on the blot.

RNA transfer

1. Pre-equilibrate the gel in sterile 20X SSC for 10 min. This removes any remaining formaldehyde.

2. Transfer the RNA to a nylon membrane, using sterile 20X SSC as transfer buffer. To obtain the highest possible sensitivity, a positively charged nylon membrane is required. We always use the positively charged nylon membrane from Boehringer Mannheim, which has been specially developed to give the highest sensitivity with the DIG System. It is tested for a lower and an upper limit of charge, which is extremely important when working with nonradioactive probes at the high probe concentrations required for optimal results. Each lot is also quality controlled with DIG-labeled probes in a dot blot detection. Uncharged membranes can be used if highest sensitivity is not required.

Table 1. Solutions required for Northern blots.

RNA loading buffer	Prepare fresh solution daily. 250 µl formamide, deionized 83 µ1 formaldehyde, 37% (w/v), final conc. 7.7% 50 µl 10X MOPS buffer 0.01% (w/v) bromophenol blue
10X MOPS	200 mM morphol-inopropan-sulfonic acid 50 mM sodium acetate 10 mM EDTA pH 7.0 Make up in sterile H_20 and autoclave. After autoclaving, the solution will turn yellow. This does not interfere with the quality of the result, as has been occasionally believed.
20X SSC, sterile	3 M NaCl 300 mM sodium citrate, pH 7.0, autoclave
Low stringency wash buffer 1	2X SSC 0.1% sodium dodecyl sulfate (SDS), prepare a 10% stock solution, filter prior to use
High stringency wash buffer	0.1X SSC 0.1% SDS, prepare a 10% stock solution, filter prior to use
Washing buffer*	100 mM maleic acid, 150 mM NaCl, pH 7.5 (+20°C) 0.3%(v/v) Tween® 20
Maleic acid buffer*	100 mM maleic acid, 150 mM NaCl, pH 7.5 (+20°C)
Blocking solution*	1 % (w/v) Blocking reagent for nucleic acid hybridization dissolved in Maleic acid buffer and autoclaved. Blocking solution is cloudy and should not be filtered. It is stable for at least two weeks when stored at +4 °C, but must then be brought to room temperature before use.
Detection buffer	100 mM Tris-HCl 100 mM NACl, pH 9.5 (+20°C)

*Available in a ready-to-use form in the 010 Wash and Block Buffer Set (Cat. No. 1 585 762).

3. Fix the nucleic acids to the membrane. In our labs, we have almost exclusively switched to UV-cross linking (at 120 mJ) because of the speed and convenience (*i.e.*, with the Stratalinker auto-program). However, baking for 30 min at 120°C has proved equally efficient with the Boehringer Mannheim nylon membrane. When baking the membrane, wash it in 2X SSC or sterile water to remove the 20X SSC prior to baking.

4. After UV cross-linking, briefly wash the membrane in water, and air dry it. This removes residual 20X SSC, which is essential prior to hybridization. Alternatively, you may briefly wash the membrane in 2X SSC or sterile distilled water immediately after transfer (*i.e.*, prior to cross linking); however, this produces bands that are not as sharp.

Probe labeling

The β-actin RNA is available in DIG-labeled form (Cat. No. 1 498 045). For the CTF1 probe, we used the labeling procedure summarized here:

1. Linearize 1 µg template DNA with a restriction enzyme that generates 5' overhanging ends (1).

2. Treat linearized template DNA with phenol (2).

3. Perform *in vitro* transcription with T7 RNA polymerase according to the pack insert of the DIG RNA Labeling Kit.

4. Evaluate the efficiency of labeling reaction by a standard direct detection as described in The DIG System User's Guide for Filter Hybridization (3).

We recommend a final labeled probe concentration of 100 ng/ml hybridization solution.

Prehybridization, hybridization, and stringency washes

1. In a sealed plastic bag, prehybridize the blot in 25 ml hybridization solution (lacking the probe) for 30 min at 68°C. We use DIG Easy Hyb buffer for all blot applications. This buffer has been specially developed for nonradioactive probes and has many advantages. It is quality controlled in a Southern blot and a Northern blot hybridization and has been evaluated for all blot applications. It contains urea instead of formamide, so hybridization conditions corresponding to the presence of 50% formamide are applied.

This 68°C hybridization temperature has been empirically identified to be optimal for most RNA:RNA hybrids in the presence of 50% formamide. In rare cases when the prospective hybrid will have very high G+C content, this temperature may be increased.

2. Denature the probe for 5 min at 100°C, then immediately chill on ice.

3. Prewarm 3 ml (calculated for 67 cm^2) hybridization buffer to 68 °C, then add the probe to a concentration of 100 ng/ml.

4. Hybridize the blot at 68°C overnight in a sealed plastic bag.

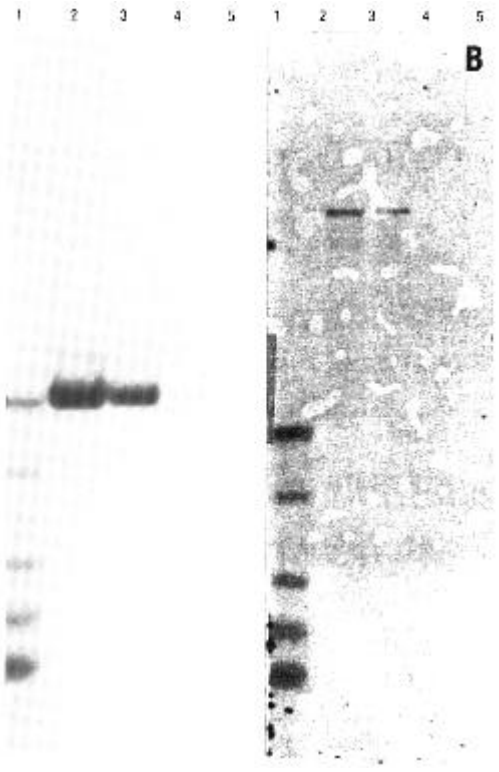

Figure 1. Northern blots detected with DIG-labeled RNA blots. The procedure described in the text and summarized in Table 2 was performed with CSPD chemiluminescent substrate, and the blot was exposed to X-ray film for 15 min
A. Hybridization with β-actin probe
B. Hybridization with CTF1 probe
Target:
Lane 1: 100 ng DIG-labeled RNA Molecular Weight Marker Ill
Lane 2: 100 ng human skeletal muscle total RNA
Lane 3: 50 ng human skeletal muscle total RNA
Lane 4: 5 ng human skeletal muscle total RNA
Lane 5: 1 ng human skeletal muscle total RNA

5. Remove the blots carefully from the bag. Immediately wash them twice, 5 min per wash, with shaking in low stringency wash buffer 1 (2X SSC, 0.1% SDS) at room temperature.

During the washes, shake the tray, and use excess amounts of buffer to prevent the membranes from sticking to the tray and partially drying. Never allow the blots to dry between prehybridization and the final washes.

6. Wash the blots twice, 15 min per wash, with shaking in prewarmed high stringency wash buffer 2 (0. lX SSC, 0.1% SDS) at 68°C.

It is extremely important to prewarm the washing buffer for these stringency washes.

After the stringency wash, the blot may be stored dry at 4°C (if the blot will not be stripped and reprobed) or placed in wash buffer for immediate immunological detection.

Chemiluminescent detection

1. Centrifuge the vial of antibody conjugate in a microcentrifuge for 5-10 min to pellet precipitates that form during storage. Use antibody conjugate from the surface of the solution only.

If not removed, the precipitates will lead to dark spots on the membrane during chemiluminescent detection. The antibody can be stored again at 4°C, but this centrifugation procedure must be repeated prior to the next use.

2. Perform chemiluminescent detection according to the pack insert, or apply the "Transparency Technique". See Table 2 for a summary of both methods of applying chemiluminescent substrate. We used the following "Transparency Technique" (steps 3-5 below) to apply the chemiluminescent substrate in our examples.

3. Place the membrane on a transparency. Add approximately 500 µl of diluted chemiluminescent substrate per 100 cm^2 membrane.

4. Cover the membrane with a second transparency sheet, and incubate for 5 mm at room temperature.

5. Allow excess liquid to drip off, and seal the "sandwich."

6. If CSPD® chemiluminescent substrate was used, preincubate the membrane for 10 min at 37°C.

This activates the decay of the unstable intermediate formed upon turnover of the substrate by alkaline phosphatase. If CDP-Star™ substrate was used, omit this step.

7. Place the "sandwich" in an X-ray cassette, and expose to X-ray film.

When using the CSPD substrate, exposures may also be performed at 37°C to obtain a faster result.

Table 2. Experimental procedure for detection of rare human mRNA on Northern blots.

Electrophoresis conditions and sample preparation	
Gel type	1.5% MOPS-formaldehyde (2%) agarose gel
Running buffer	1X MOPS
Running conditions	25 V overnight
RNA loading buffer	Formamide/formaldehyde
Sample denaturation	10 min at 65°C; place on ice immediately
RNA transfer	
Membrane	Boehringer Mannheim's Nylon membrane, positively charged, or uncharged
Gel equilibration	10 mm in sterile 20X SSC prior to transfer; remove formaldehyde
Transfer buffer	sterile 20X SSC
Fixation	UV-cross-linking at 120 mJ; wash membrane briefly in water; air dry
Probe labeling	
RNA probe	Linearize template DNA, phenolize, transcribe *in vitro*

Probe concentration and preparation
Denature probe for 5 min at 100 °C. Chill on ice immediately. Add DIG-labeled RNA to prewarmed DIG Easy Hyb to 100 ng probe/ml; this solution may be filtered through 0.45 mm cellulose acetate filter (Schleicher & Schuell, FP-03012) if desired.

Hybridization	
Pre hybridization	30 min at 68 °C in 25 ml DIG Easy Hyb in a sealed plastic bag
Hybridization buffer	3 ml (67 cm^2membrane) DIG Easy Hyb
Hybridization	68 °C (for RNA:RNA hybrids) overnight in sealed plastic bag or roller tubes
2 washes	Low stringency wash buffer 1 (2 X SSC, 0.1% SDS), 2 x 5 mm at room temperature; **shake**
2 washes	Prewarmed (68 °C) high stringency wash buffer 2 (0.1 X SSC, 0,1% SDS), 2 x 15 min 68 °C; **shake**
Possible stopping point	Transfer membrane to wash buffer for detection, or air-dry and store at 4 °C
Chemiluminescent detection	
Antibody conjugate	Centrifuge 5-10 min; take from surface only
Equilibration	5 min washing buffer
Blocking	30 min in l% blocking buffer
Antibody reaction	30 min with antibody diluted 1:10000 in 1% blocking buffer
2 washes	2 x15 min in wash buffer
Equilibration	2 min in Detection buffer
Alkaline phosphatase reaction	Dilute CSPD substrate 1:100 in detection buffer

Chemiluminescent detection (Choose from two substrate application methods)

1. Dipping technique

1. Incubate membrane shortly in 10 ml of diluted chemiluminescent substrate.
2. Briefly dry on Whatman 3MM paper.
3. Seal damp, and expose to X-ray film.

Diluted chemiluminescent substrate can be stored at 4 °C after filtration and addition of 0.1 mM sodium azide; these precautions are necessary to avoid bacterial contamination.

2. Transparency technique

1. Place membrane on transparency.
2. Add approx. 500 µl/100 cm^2 membrane.
3. Cover with second sheet of transparency, and incubate for 5 mm.
4. Let excess liquid drip off and seal.
5. Place in X-ray cassette, and expose to film.

RESULTS AND DISCUSSION

Using the procedure described here, we were able to detect the highly abundant mRNA for β-actin in as little as 5 ng of total RNA in a 15 min exposure (Figure 1A).This procedure also allowed us to detect the low abundant mRNA for the CTF1 transcription factor in 50 ng total RNA in just 15 min (Figure 1B). We have only used standard procedures as described in our DIG System User's Guide for Filter Hybridization and package inserts. We take several main precautions when working with RNA: keeping everything as sterile as possible, using non-powdered gloves, and handling the positively charged membrane with extreme care. We strictly follow the package insert instructions.

With the technique summarized in Table 2, we achieve an extraordinarily high sensitivity compared to radioactive hybridizations. In contrast to the 3-4-day exposures required to detect RNAs in at least 2-5 µg

poly(A)$^+$ RNA with a radioactive probe, the DIG System produces specific signals in 50 ng after just a 15 min exposure (Figure 1)

REFERENCES

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