Short Technical Reports

Highly Sensitive Northern Hybridization Using a Rapid Protocol for Downward Alkaline Blotting of RNA

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ABSTRACT

A simple and fast RNA gel blot procedure is described that uses 50 mM NaOH to simultaneously transfer and fix RNA to a positively charged nylon membrane. The RNA is transferred in a downward direction, and transfer is routinely completed within 2.5 h. The resulting blots give increased sensitivity over existing methods without affecting RNA integrity and can be used in both radioactive and nonradioactive detection procedures.

INTRODUCTION

RNA gel blot analysis, also called Northern blot analysis, is commonly used in molecular biology studies concerning gene expression (1). It allows quantification of steady-state mRNA levels and, at the same time, gives information related to the presence, size and integrity of discrete mRNA species. In recent years, alternative and more sensitive techniques to detect and quantify mRNAs have become available, including ribonuclease protection assay (6) and reverse transcriptase polymerase chain reaction (RT-PCR) (8). These techniques complement, rather than replace, Northern blot analysis, which can still be considered a standard procedure for mRNA studies because of its relative ease and highly informative character.

Perhaps the most commonly used Northern blot procedure involves size-fractionation of denatured RNA in an agarose-formaldehyde gel with 3-(*N*-morpholino)propanesulfonic acid (MOPS) as electrophoresis buffer. The RNA is then transferred to a membrane support by upward capillary transfer at neutral pH using 10× or 20× standard saline citrate (SSC). After blotting, the RNA is fixed to the membrane by baking at 80°C or by exposure to UV light (1). The membrane is then ready for hybridization. There are several variations of this basic protocol, mostly involving the composition of the gel and electrophoresis buffer, the type of membrane used and the conditions of hybridization. A significant improvement was reported by Beckers et al. (2) who showed that for positively charged nylon membranes, fixing the RNA by mild alkaline treatment is superior to baking or UV cross-linking in terms of detection sensitivity.

We are routinely using Southern and Northern hybridization in our study of virus-resistant transgenic sugarcane plants and found that downward alkaline Southern blotting of DNA using 0.4 M NaOH as transfer buffer indeed results in sharper and stronger signals compared to 20× SSC upward-transferred blots, as described (5). Here, we show that this procedure can be adapted to Northern blotting by replacing the 0.4 M NaOH solution with 50 mM NaOH. The downward alkaline capillary transfer of RNA is fast and easy, and it gives an additional 1.6-fold to 2.1-fold higher sensitivity than the procedure of Beckers et al.

MATERIALS AND METHODS

Denaturing Agarose Gel Electrophoresis

The sugarcane cultivars CP65-357 and CP72-1210 were transformed with a chimeric gene consisting of the maize *ubiquitin* promoter fused to the coding sequence of the sorghum mosaic virus strain H (SrMV-SCH) coat protein (CP) gene and followed by the 3′ untranslated sequences of the *nopaline synthase* gene. Total RNA was isolated from leaves as described (4) and resuspended in diethyl pyrocarbonate (DEPC)-treated water at a final concentration of 1.5 µg/µL. For the blots in Figure 1A, 12 µg RNA in Sample Buffer 1 (20 mM MOPS, pH 7.0, 5 mM sodium acetate, 1 mM EDTA, 50% formamide, 2.1 M formaldehyde) are denatured by heating 5 min at 65°C, chilled on ice and 1/10 vol of gel loading buffer is added (50% glycerol, 1 mM EDTA, pH 8.0, 0.25% bromophenol blue). The denatured RNA was separated on a 1.2% agarose gel containing 20 mM MOPS, pH 7.0, 5 mM sodium acetate, 1 mM EDTA and 2.1 M formaldehyde. The

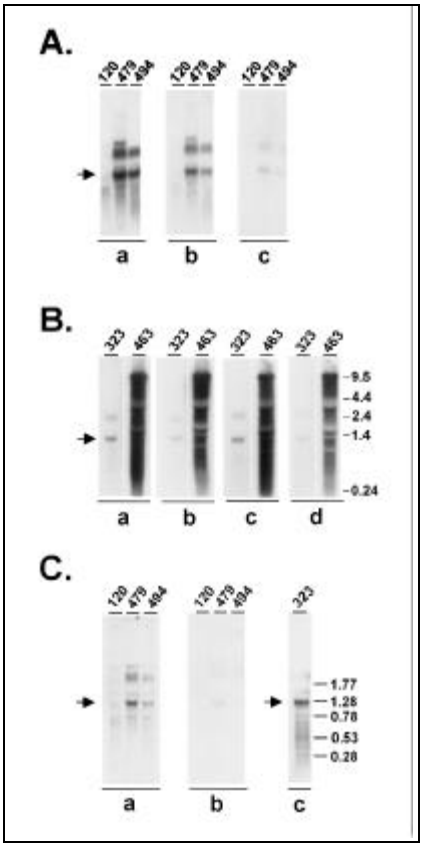

Figure 1. Influence of different blotting/fixation methods on detection of transgene coat protein mRNA or viral RNA levels in transgenic sugarcane leaf tissue. Numbers above the lanes refer to individual plants accumulating different levels of the coat protein mRNA or SrMV-SCH viral RNA (12 µg total RNA per lane). Blots in Panels A and B were hybridized with a radiolabeled riboprobe complementary to the SrMV-SCH CP mRNA. Blots in Panel C were hybridized with a fluorescein-labeled DNA probe corresponding to the SrMV-SCH CP coding region. RNA size markers are indicated on the right. The arrow indicates the 1.3-kb transgenic SrMV-SCH CP mRNA. (Panel A) Comparison of different blotting/fixation methods. a, downward alkaline blotting as described in Materials and Methods; b, 20× SSC blot fixed by alkaline treatment according to Beckers et al.; c, 20× SSC blots fixed by heat treatment. (Panel B) Comparison of two positively charged membranes in the alkaline blotting procedure and the procedure of Beckers et al. a and b, Hybond N+ membrane; c and d, Zeta-Probe GT membrane. a and c, alkaline blotting; b and d, procedure of Beckers et al. (Panel C) Comparison of the alkaline blotting procedure (a) vs. 20× SSC blots fixed by heat treatment (b) using a nonradioactive labeling and detection system. c, sample 323 was alkaline blotted.

composition of the electrophoresis buffer was 20 mM MOPS, pH 7.0, 5 mM sodium acetate and 1 mM EDTA. For the blots in Figure 1, B and C, 12 µg RNA in Sample Buffer 2 (20 mM HEPES, pH 7.4, 1 mM EDTA, pH 8.0, 50% formamide, 2.0 M formaldehyde) are denatured by heating 5 min at 65°C, chilled on ice and 1/10 vol of gel loading buffer is added (50% glycerol, 1 mM EDTA, pH 8.0, 0.25% bromophenol blue). The denatured RNA was fractionated on a 1.2% agarose gel containing 20 mM HEPES, pH 7.4, 1 mM EDTA, pH 8.0 and 0.45 M formaldehyde, which was also the composition of the electrophoresis buffer. Adding formaldehyde to the electrophoresis buffer greatly improves the resolution, as described (7). The 7.5- × 10.0- × 0.8-cm gels were run using a Mini-Gel unit (Sigma-Aldrich Chemical, St. Louis, MO, USA). The gels were run at 90 V for about 2 h with a 0.16–1.77- or 0.24–9.5-kb RNA ladder (Life Technologies, Gaithersburg, MD, USA) as size marker, and the buffer was mixed halfway through the run.

Transfer and Fixation of RNA to Nylon Membrane

After electrophoresis, the gels were sliced in pieces for the different treatments. In the alkaline blotting procedure [Figure 1, A(a), B(a), B(c), C(a) and C(c)], the gel is equilibrated for exactly 10 min in a 50 mM NaOH solution, while a downward transfer setup was prepared exactly as described by Koetsier et al. (5) for downward alkaline Southern blotting of DNA, except that the transfer solution was 50 mM NaOH. The membrane, the 3-MM filter paper (Whatman International, Maidstone, England, UK) immediately below the membrane and the two on top of the gel and the blotting wicks were presoaked in 50 mM NaOH. Unless stated otherwise, the positively charged nylon membrane Hybond®-N+ (Amersham Pharmacia Biotech, Little Chalfont, Bucks, England, UK) was used. The transfer was stopped after 2.5 h, and the gel stained for 30 min in a 0.5 µg/mL ethidium bromide (EtdBr) solution to check that transfer was complete. The membrane was then briefly neutralized in DEPC-treated 2× SSC buffer (20× SSC is 3 M NaCl, 0.3 M sodium citrate, pH 7.0), air-dried and stored at room temperature until further use. The membrane can be incubated at 80°C for 15 min to speed up drying. In the procedure of Beckers et al. (2) [Figure 1, A(b), B(b) and B(d)], the gel is washed for 30 min in 1% (wt/vol) glycine buffer, 20 min in 50 mM NaOH solution and finally equilibrated for 40 min in 20× SSC. Blotting is done overnight in an upward direction using the conventional setup (1) with 20× SSC as transfer solution. The membrane was pre-wetted with 20× SSC before use. After transfer, the RNA was fixed by incubating the membrane for 5 min in a 50 mM NaOH solution on a rotary shaker, briefly washed in 2× SSC, air-dried and stored at room temperature until further use. In the third procedure [Figure 1, A(c) and C(b)], the gel is blotted overnight immediately after electrophoresis by upward capillary transfer using 20× SSC. After transfer, the membrane is briefly rinsed with 2× SSC, air-dried and the RNA is fixed for 2 h at 80°C in an oven without vacuum.

Hybridization, Washing and Autoradiography

Membranes for Figure 1, A and B were prehybridized in 15 mL hybridization solution [5× sodium chloride sodium phosphate EDTA (SSPE), 5× Denhardt's solution, 0.5% wt/vol sodium dodecyl sulfate (SDS) solution, 20 µg/mL denatured salmon sperm DNA] in a water bath for 2 h at 65°C. The solution was then replaced with fresh hybridization solution, and a riboprobe was added at $0.75–1.50 \times 10^6$ cpm/mL. Riboprobes were made using the SP6/T7 Transcription Kit (Boehringer Mannheim GmbH, Mannheim, Germany) with [α-^{32}P]CTP, 3000 Ci/mmol according to the manufactur-

Table 1. Summary of the cpm Values Obtained from Strips of the RNA Gel Blots

Membrane	RNA Sample	cpm Value 1[a]	cpm Value 2[b]	Factor Difference
Hybond-N+	323	264	140	1.9
	463	24 884	15 141	1.6
Zeta-Probe GT	323	185	90	2.1
	463	16 161	8942	1.8

The gel blots are shown in Figure 1B.

[a]Alkaline blots a and c.

[b]Blots b and d obtained as described by Beckers et al. (2).

er's instructions and purified on a Quick Spin™ column (Boehringer Mannheim GmbH). Hybridization was left overnight at 65°C. The membranes were washed with 3× SSC, 0.1% SDS at 68°C for 2 × 25 min; then with 1× SSC, 0.1% SDS at 68°C for 2× 20 min and finally with 0.1× SSC, 0.1% SDS at 55°C for 30 min. The membranes were exposed to BioMax MS™ X-ray Film (Eastman Kodak, Rochester, NY, USA) for 8–24 h at -80°C with enhancing screens. Membranes for Figure 1C were hybridized to a fluorescein-labeled DNA probe covering the SrMV-SCH CP coding region. Labeling, hybridization, washing and detection were according to the manufacturer's instructions (Gene Images System; Amersham Pharmacia Biotech). These membranes were exposed to BioMax MS X-ray film for 2–3 h at room temperature.

RESULTS AND DISCUSSION

The downward alkaline Southern blotting procedure is preferred by many researchers because it is faster and gives stronger and sharper hybridization signals than those obtained by conventional upward-transferred 20× SSC blots (5).

Downward capillary transfer of RNA from an agarose gel to a hybridization membrane using a transfer solution containing 3 M NaCl and 8 mM NaOH was previously demonstrated (3). While RNA can be hydrolyzed under certain alkaline conditions, the Northern blot procedure of Beckers et al. (2) does include a 20-min gel wash in 50 mM NaOH without apparent adverse effects on the final hybridization signals. Here, we describe a downward alkaline blotting procedure for RNA that uses 50 mM NaOH as transfer solution. In this procedure, the gel is incubated for 10 min in a 50 mM NaOH solution before a 2.5-h downward capillary transfer using the same solution. Since the RNA is fixed to the hybridization membrane during transfer, the fixation step can be omitted. Two positively charged nylon membranes were tested, Hybond-N+ and Zeta-Probe® GT from Bio-Rad (Hercules, CA, USA).

In Figure 1A, the downward alkaline blotting protocol (blot a) was compared to the method of Beckers et al., i.e., upward transfer using 20× SSC as transfer solution followed by alkaline fixation (blot b), and also to the conventional 20× SSC upward transfer followed by heat fixation (blot c). As shown, RNA fixation by alkaline treatment (blot b) indeed results in stronger hybridization signals compared to fixation by heat (blot c), as described (2). The hybridization pattern on the alkaline blot looks very similar to those obtained with the other procedures and, in fact, alkaline blotting results in an additional increase in sensitivity compared to the method of Beckers et al.

In Figure 1B, we compared the hybridization signal obtained with the alkaline blotting procedure vs. the procedure of Beckers et al. using two different positively charged membranes, Hybond-N+ (blots a and b) and Zeta-Probe GT (blots c and d). Two RNA samples were analyzed, one showing discrete mRNA bands between 1.3 and 2.4 kb (lane 323) and one showing a smear of viral RNA extending from about 10 kb to a few 100 bases (lane 463). Strips corresponding to each lane were cut from the membranes and radioactivity was quantified by liquid scintillation counting. Table 1 gives cpm values. As summarized in Table 1, the signals obtained after alkaline blotting are 1.6-fold to 2.1-fold stronger than when the procedure of Beckers et al. is used. The viral RNA lanes in Figure 1B indicate stronger hybridization signals, especially for the smaller RNAs, i.e., those below the 4.4-kb size marker. In agreement with this, the increase for the 323 sample (1.9-fold and 2.1-fold), which has only RNAs smaller than 2.4 kb, is slightly higher than for the viral RNA sample (1.6-fold and 1.8-fold).

Figure 1C shows that a nonradioactive labeling, hybridization and detection system (Gene Images) can be successfully used with blots obtained after downward alkaline transfer (blot a) and indicate a similar difference in sensitivity between the downward alkaline blot and the upward 20× SSC blot fixed by heat treatment (blot b). In addition, blot c shows that discrete RNA bands as small as 0.3 kb can be detected, indicating that exposure of the RNA to the al-

kaline solution during transfer does not result in detectable degradation.

In summary, a downward alkaline Northern blotting procedure is described that is simple, fast and sensitive without affecting RNA integrity. A single solution is required for gel equilibration and RNA transfer. RNA fixation occurs during transfer, which obviates the need for heat or UV fixation after blotting. The blots can be used in both radioactive and nonradioactive hybridization/detection procedures and give an additional 1.6-fold to 2.1-fold higher sensitivity compared with the method of Beckers et al. With the mini-gel system, gel electrophoresis, blotting and prehybridization can easily be accomplished on the same day with overnight hybridization. When the non-isotopic system is used, results can be obtained the next day.

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Limitations for Purification of Murine Interleukin-18 When Expressed as a Fusion Protein Containing the FLAG® Peptide

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ABSTRACT

As a strategy to purify recombinant murine Interleukin (IL)-18, we cloned the mature coding region of this protein into the pFLAG$^{\dot{O}}$-1 expression system. The intent was to use the FLAG peptide "tag" as an amino terminal addition to IL-18 so that purification of this fusion protein (FLAG-IL-18) on anti-FLAG$^{\dot{O}}$ antibody affinity columns could be performed. While significant amounts of recombinant IL-18 were present in E. coli *lysates, only a small portion of this material could be recovered on immunoaffinity columns conjugated with an anti-FLAG antibody. Surprisingly, the majority of recombinant IL-18 present in* E. coli *(strain JM83) bacterial lysates did not contain the FLAG peptide and therefore did not bind to immunoaffinity columns conjugated with an anti-FLAG antibody. However, we found that the BL21 strain of* E. coli, *which has reduced endogenous protease activity, could express the majority of recombinant IL-18 as the fusion protein, FLAG-IL-18. Taken together, these studies show that it is necessary to consider whether protease sites formed at the FLAG-protein junction can be easily cleaved by the bacterial strain used to express the fusion protein.*