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An Improved, Rapid Northern Protocol

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We report a simple, sensitive and rapid method for performing Northern blotting analysis which avoids the use of toxic chemicals such as formamide and glyoxal. This technique allows sensitive detection of various transcripts from total RNA samples varying from 10μg to 2.5μg. These samples were probed for GAPDH and VCAM mRNA message using this technique and show similar results as conventional Northern blotting methods. In addition, this protocol can be accomplished much faster than the traditional formamide/formaldehyde or glyoxal protocols. This protocol can be easily implemented by most laboratories using inexpensive and reagents less toxic than those commonly used for RNA analysis.

Northern blotting remains the standard method for examining the induction/expression of specific genes (1). Northern blotting examines the relative amounts, and sizes of particular RNA species, and provides an assessment of the quality of a given RNA preparation. However, traditional Northern protocols have some disadvantages, including use of toxic chemicals such as formamide/formaldehyde, and are time-consuming due to repeated staining and gel equilibration procedures. Typical multi-step protocols also suffer from frequent RNase contamination with resulting degradation of the RNA.

Improved protocols incorporating the denatured glyoxal with agarose gel electrophoresis exhibit reduced reagent toxicity and greater ease of monitoring RNA throughout the procedure (2). However, protocols using glyoxal still have disadvantages: many deionizing steps must be carried out before the glyoxal can be used with RNA; and, prevention of glyoxitic acid formation requires that buffers be recirculated during the RNA electrophoresis.

Recently, a Northern protocol using a sodium phosphate buffer system was described (3) which involved using sodium phosphate as the buffering system for running and equilibrating agarose gels for the blotting steps. This protocol was found to be effective and comparable to formamide/formaldehyde gels, but still had some disadvantages. The use of a sodium phosphate buffer system also necessitates buffer recirculation due to its weak buffering capacity. In addition, gels must be equilibrated in appropriate transfer buffers before RNA transfer, which is time-consuming. Thus, there remains a need for a safer, simpler, and less cumbersome Northern protocol to improve the uniformity and accessibility of RNA analysis for many laboratories.

Here, we report the development of an improved Northern analysis method which is simple, rapid and less toxic than previously described methods.

MATERIALS AND METHODS

RNA isolation. ECV (ECV-1, human umbilical vein cells, transformed) cells and normal human umbilical endothelial cells (HUVEC) were used for this study. Total RNA was prepared from approximately $2x10^6$ cells using the Rneasy total RNA kit (Qiagen, Chatsworth, CA). RNA concentration was determined spectrophotometrically. A total of 2.5, 5 and 10 μg of total RNA was used for gel electrophoresis.

Electrophoresis. 15 μl RNA samples were mixed with 2.5μl of sterile 6X loading buffer [0.25% w/v bromophenol blue, 0.25% w/v xylene cyanol, 30% w/v glycerol, 1.2% SDS, 60mM sodium phosphate (pH 6.8)]. Samples were denatured at 75° C for 5 minutes and immediately loaded onto a 1.2 % agarose gel. The 1.2% agarose gel and running buffer were made with 1X TBE containing 1μg/ml of ethidium bromide (10μg/ml stock). The gel was electrophoresed at 7-10 V/cm (approximately 90-100 V) for 1 hour and 15 minutes. Gel electrophoresis was stopped and the gel photographed under U.V. light.

Equilibration and northern blotting. Zeta-Probe GT membrane (Bio-Rad, Hercules, CA) and transfer filter papers were equilibrated in 1X TBE for 10 minutes. The gel was then transferred to the nylon membrane using the Genie electrophoretic blotter (Idea Scientific, Minneapolis, MN) at 12 V for 1 hour and 30 minutes. Transfer efficiency was determined by examining the agarose gel and nylon membrane under U.V. light. Both 18 and 28S rRNA size markers were marked using a pencil. The membrane was then U.V. cross-linked for 5 minutes and incubated in 10 ml of pre-hybridization solution [0.25M Na_2HPO_4 (pH 7.2) and 7% SDS] for 30 minutes at 65 C.

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Northern probes. GAPDH (ATCC, Rockville, MD) and VCAM-1 (hereafter '*VCAM*') (4) probes were synthesized to a specific activity of at least $1x10^9$ cpm/μg using the Ready-to-go Random Primer Oligo kit (Pharmacia, Piscataway, NJ). Probes were purified using Sephadex G-50 spin columns (Pharmacia, Piscataway, NJ). Denatured probes were added to the pre-hybridization solution and left to hybridize at 55-60° C (depending on the Tm of the probe) for 4-8 hours. After hybridization, membranes were washed successively with vigorous agitation at room temperature for 15 minutes in each of the following solutions: 2X SSC/0.1% SDS, 0.5X SSC/0.1%SDS, 0.1X SSC/0.1%SDS. For single copy detection or high stringency, the last wash can be performed at 65° C. Membranes were then wrapped in cling-film (Saran wrap) and exposed to film for 12-24 hrs. at -70° C.

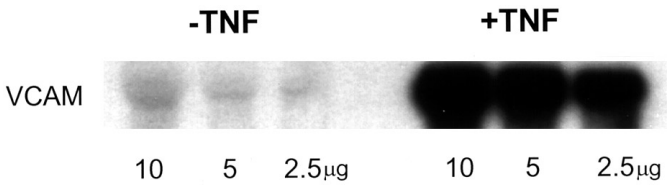

FIG. 2. Northern analysis for VCAM mRNA in HUVEC— Control human umbilical endothelial cells as well as TNF (1ng/ml) stimulated HUVEC were probed for VCAM mRNA. Control samples illustrate a faint band in the 10 and 5 g samples. TNF treated cells demonstrate VCAM mRNA in all RNA quantities.

RESULTS AND DISCUSSION

Figure 1 illustrates the ethidium bromide agarose gel and GAPDH Northern of ECV cell total RNA. Agarose gel electrophoresis demonstrated a typical banding pattern for total RNA gels (panel A). Varying concentrations of total RNA were also run to determine the sensitivity of Northern analysis. Panel B of figure 1 demonstrates a Northern blot for human GAPDH. A 1.4kb band can be seen in all samples regardless of RNA concentration. This protocol is quite sensitive to transcript levels since 2.5μg of total RNA yields a robust GAPDH band. Another important feature of this protocol is that very low backgrounds are achieved without the use of salmon sperm or calf thymus DNA to block blots. Lastly, these blots can be stripped by pouring boiling 0.1X SSC and 0.5% SDS solution on the membrane and shaking the blot vigorously.

We also examined the expression of VCAM mRNA.

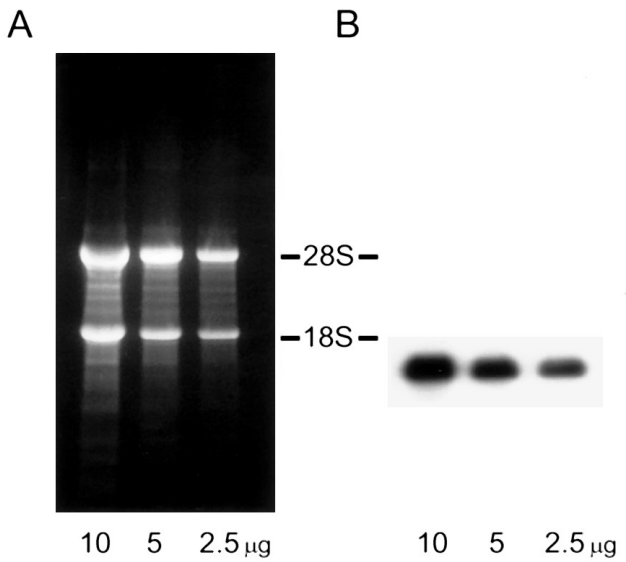

FIG. 1. Northern analysis of ECV total RNA— Panel A demonstrates the ethidium bromide s tained gel for ECV total RNA. 10,5, and 2.5 g of RNA were loaded as indicated. Panel B demonstrates the same samples probed for human GAPDH. All RNA concentrations demonstrate the presence of GAPDH.

VCAM mRNA has been shown to be present in human endothelial cells at low concentrations under basal conditions. Upon cytokine stimulation, the expression of VCAM message dramatically increases in HUVEC (4). Therefore, human umbilical vein endothelial cells were incubated with TNF (1ng/ml) for 4 hours and total RNA isolated. Figure 2 shows VCAM message levels in untreated and in TNFα treated HUVEC. A light band can be seen in both the 10 and 5μg samples of control cells again illustrating the sensitivity of this assay. Upon stimulation with TNFα all lanes demonstrated substantially higher amounts of VCAM mRNA. These data demonstrate that mRNA species that are not abundant can also readily be detected using this protocol. Indeed, we have previously shown with similar protocols that mRNA's with relatively low copy numbers like VEGF can be detected (5). It should be noted that the previous protocols used TAE buffers and were not transferred electrophoretically due to the relative instability of the TAE buffering system, therefore resulting in prolonged protocol time.

We demonstrate a Northern protocol that is inexpensive, sensitive, simpler and avoids the use of toxic aldehydes. Furthermore, a previous study demonstrated that a similar protocol is comparable to formamide/formaldehyde gels with similar banding patterns (3). This protocol utilizes the characteristics of strong ionic buffer systems (TBE), the benefits of non-toxic chemicals, and the advantage of rapid hybridization yielding highly efficient Northern analysis protocol. After optimization, we have typically performed the entire procedure in 24 hours. In addition, we show that this protocol works not only in control i.e. 'house-keeping' proteins but also inducible messages making examination of various transcripts possible. This method should make Northern analysis a more realistic and approachable tool for investigators who are not primarily working in the arena of molecular biology.

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