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Simplified riboprobe purification using translucent straws as gel tubes

Shahar Kol*[1], Izhar Ben-Shlomo[2], Eli Y. Adashi[3], Richard M. Rohan

Division of Reproductive Endocrinology, Department of Obstetrics and Gynecology, University of Maryland School of Medicine, Baltimore, MD 21201, USA

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Abstract

Gel purification of radioactive riboprobes enhances the quality of the ribonuclease protection assay. A simple and effective method for riboprobe purification is described. The method uses acrylamide gels in plastic tubes to achieve electrophoretic separation of the RNA polymerase products.

Keywords: Riboprobe purification; Ribonuclease protection assay; Radioactive riboprobe; Translucent straw

1. Introduction

The ribonuclease protection assay (RPA) is a powerful technique for quantifying the abundance of specific mRNAs. Gel purification of the full length riboprobe is highly recommended. High specific activity radioactive riboprobes (800 Ci/mmol [^{32}P]UTP) are required to gain the maximal sensitivity from RPA. However, synthesis of riboprobes in low concentrations of UTP (3 μmol) results in a high percentage of products that are smaller than full-length (Fig. 1). The amount of full-length product can range from as little as 8% (Interleukin-1α) to 67% (ribosomal protein large 19, (RPL-19)). Elimination of the substantial amounts of subfragments greatly increases the quality of the results and reduces the amount of radioactive probe that is used in the assay.

2. Materials and methods

To demonstrate the need for purification, RPL-19 riboprobe was transcribed to specific activities of 150, 50 and 16.7 Ci/mmol [^{32}P]UTP. Half of each reaction was gel-purified. Purified (4.7, 2.9 and 1.5 $\times$ 10^4 counts/min, respectively) or unpurified (1.5, 1.0 or 0.5 $\times$ 10^5 counts/min, respectively) products were used to probe equal amounts of rat ovarian cell RNA. Because of the presence of prematurely terminated products (Fig. 1), 3 times more unpurified probe was used in order to insure that each hybridization contained the same amount of full-length probe. Hybridization with purified probe produced a more intense protected fragment (Fig. 2). Phosphorimage analysis indicated a 4%, 25% and 40% (high to low specific activity) enhancement of signal detection using purified probes.

Previously, we had employed 0.4 mm thick sequencing-type gels for probe purification [1]. This method, however, requires the availability of a darkroom, and entails the process of alignment of the gel with the autoradiograph, a cumbersome and not a very accurate undertaking. Because of these difficulties,

* Corresponding author, 655 W. Baltimore Street, Room 11-007, Baltimore, Maryland, 21201. Tel.: 410 7064050; Fax: 410 7065747.

[1] On leave from the Department of Obstetrics and Gynecology, Rambam Medical Center, Haifa, Israel. Recipient of an American Physician Fellowship, and of a 1994 Merck Senior Fellow Award of the Endocrine Society.

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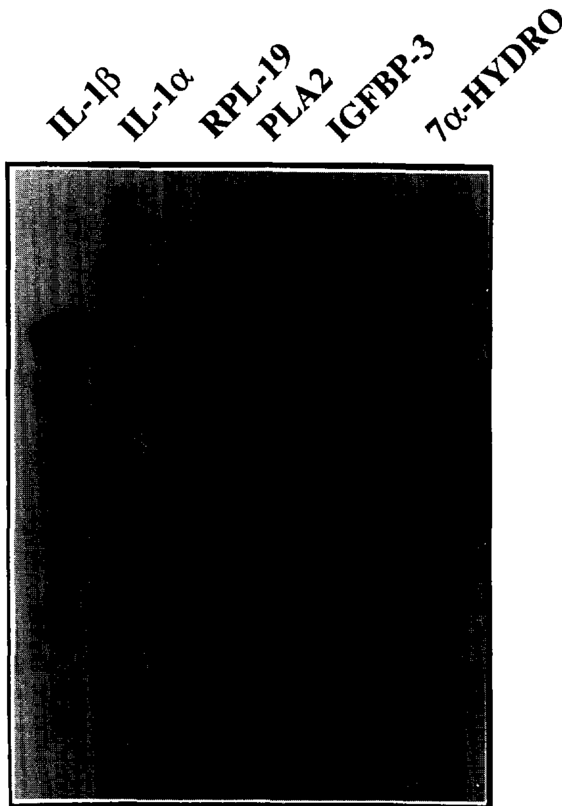

Fig. 1. An autoradiograph showing the products of riboprobe synthesis reactions with 6 different DNA templates from rat cDNAs (IL-1α, interleukin-1α; IL-1β, interleukin-1β; RPL-19, ribosomal protein large 19; PLA_2, phospholipase A_2 (group II, secretory type); IGFBP-3, insulin-like growth factor binding protein 3; 7α-HYDRO, hydroxycholesterol 7-α hydroxylase). Riboprobe synthesis was performed at a specific activity of 800 Ci/mmol UTP. The amount of radioactivity in each lane was quantified by phosphor image analysis (Molecular Dynamics).

we had set out to simplify the process of localizing and excising riboprobes after gel electrophoresis. The new purification method utilizes readily available, translucent, individually wrapped soft drink straws (SweetHeart, Chicago, IL) to form individual tube gels in a standard vertical gel apparatus (SE 400, Hoefer Scientific Instruments, San Francisco, CA; Protean II, BioRad, Alameda CA, and others). First, the gel plates are assembled with 3 mm spacers and set into the casting stand as would normally be done for a slab gel. The side spacer clamps are loosened slightly and straws are removed from their wrappers with gloved hands and squeezed into the gap between the plates. Each straw is inserted to within 2–3 mm of the bottom of the gel plates and then the excess is trimmed with a razor. The side clamps are then carefully tightened and the gel plates are sealed to the bottom rubber gasket as would normally be done. Ammonium persulfate (25 μl of 25% w/v) and TEMED (25 μl) are added to 10 ml denaturing gel solution (5%–8% acrylamide, 8.3 M urea, 1 × TBE) and the solution quickly poured into the plates outside of the straws. Additional gel solution (3 ml per straw) is prepared (1 μl 25% ammonium persulfate and 1 μl TEMED per ml of gel). After the plug is set, each straw is filled with the additional gel solution to within 10 mm of the top. Butanol is gently layered onto the top of the gel within each straw in order to form a flat surface.

After the gel has set, the gel form is attached to the electrophoresis chamber, the top of each tube gel is rinsed with buffer and the gels are pre-run. Riboprobe synthesis products are resuspended in denaturing loading solution (98% formamide, 0.1% SDS, 0.1 mM ETDA, 0.02% bromophenol blue and xylene cyanol) and

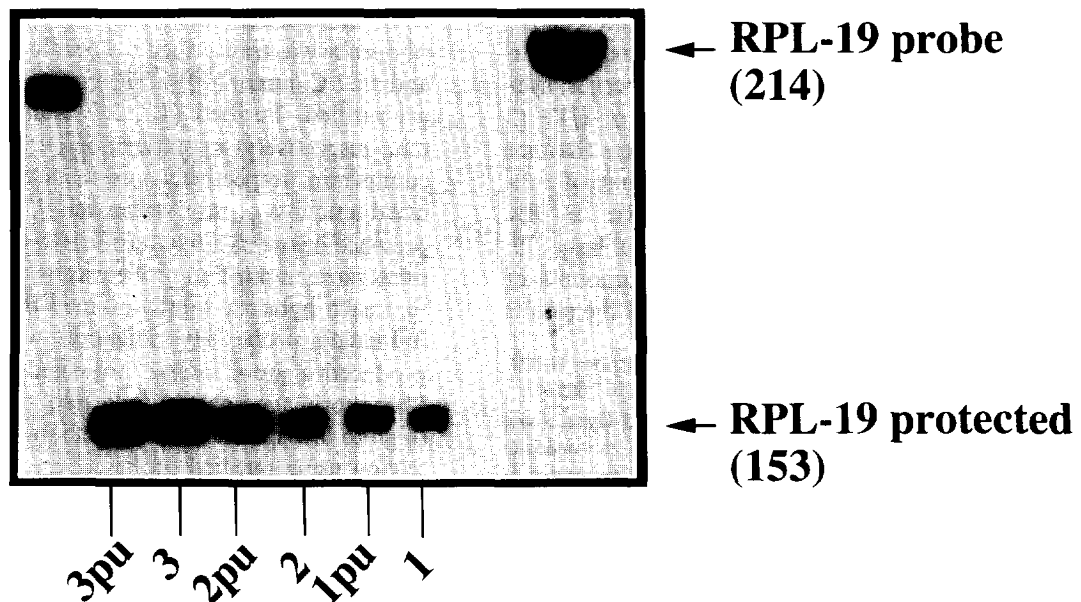

Fig. 2. Rat ovarian mRNA was hybridized to purified (pu) or unpurified RPL-19 riboprobe which had been transcribed to specific activities of 150 (3), 50 (2) or 16.7 (1) Ci/mmol [^{32}P]UTP. The position and size (214 nt) of probe, protected fragment (153 nt) and 200 nt size marker are indicated.

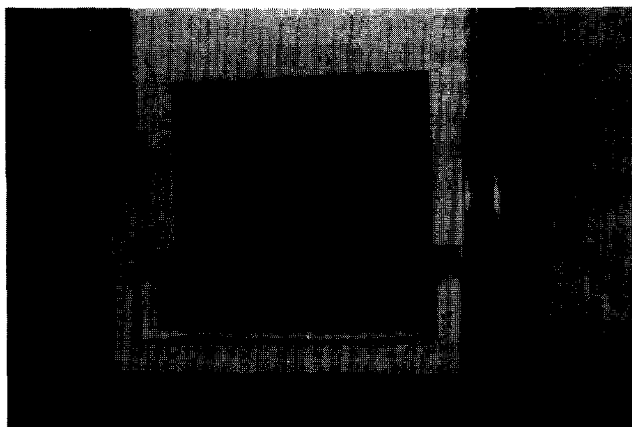

Fig. 3. A photograph of the SE 400 electrophoresis apparatus during a typical probe purification run. The loading buffer dye is easily seen through the translucent straws, enabling accurate termination of the electrophoresis.

layered onto the gel. Fig. 3 shows the SE 400 apparatus during a typical run.

Following completion of the electrophoretic separation, the glass plates are separated and the straws removed. Straws are taped onto a clean glass surface in a non-parallel arrangement (Fig. 4). The unambiguous geometry of gels can be accomplished with only 2 tubes, but is best done with 3 or more. An individually wrapped X-ray film (Kodak X-OmatART) is placed onto the straws for 2 min. The resultant autoradiograph image is aligned with the straws and used to mark the location of the full-length probe within each straw. Straws are cut with a razor blade by using a single, forceful downward motion on either side of the desired band. Forceps are used to hold the tube piece over the mouth of an open microfuge tube and a micropipette tip is used to push the gel slice out of the straw. Gel slices are immersed in 0.5M ammonium acetate, 1mM EDTA, 0.2% SDS at 37°C. The eluted probe can subsequently be concentrated by precipitation with 2.5 volumes of ethanol. Probes are typically eluted overnight. The elution process can be enhanced if the gel slices are macerated. However, this requires the subsequent removal of acrylamide fragments prior to precipitation. Microcentrifuge filtration (Z spin, Gelman, Ann Arbor, MI) has been found to be suitable for this purpose.

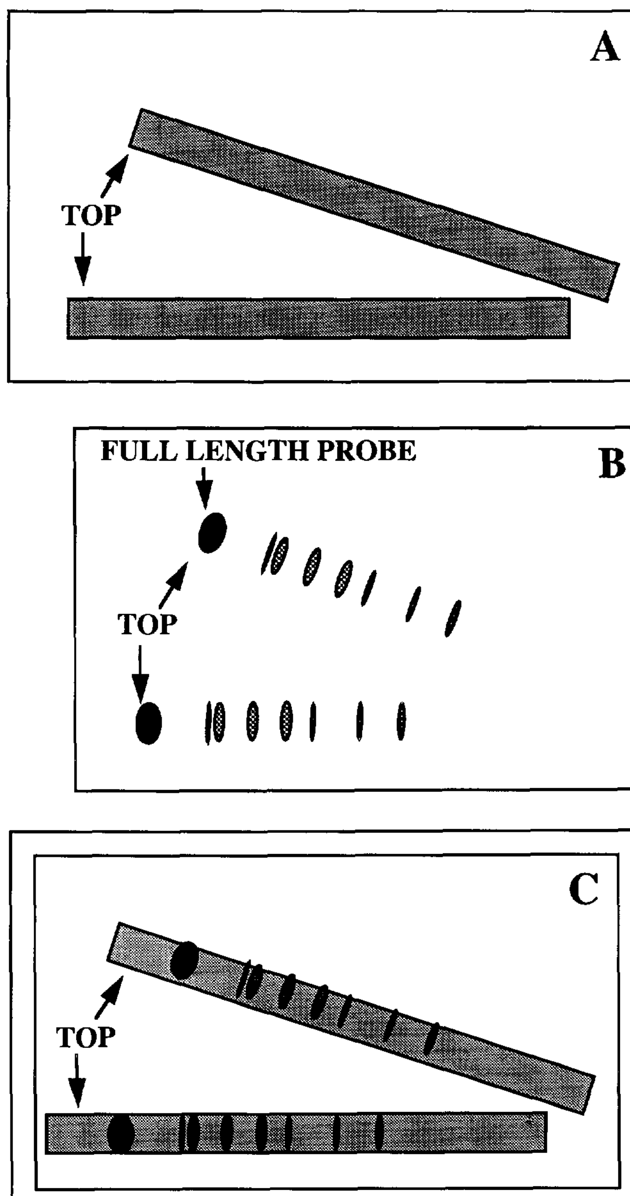

Fig. 4. A schematic illustration of probe purification. After electrophoresis the gel containing straws are taped onto a glass plate at an angle (A). An individually wrapped X-ray film is placed on the straws for 2 min. After developing, the full length probes with their subfragments are identified (B). The film is aligned with the straws (C), and the relevant segments containing the full length probes are excised.

3. Discussion

A major concern for the handling of RNA probes is the maintenance of RNase-free conditions. Individually machine-wrapped soft drink straws appear to meet this requirement since we routinely evaluate the quality of purified probes and have not observed any obvious degradation after purification (probe lane in Fig. 2).

Conducting electrophoresis in a confined tube such as a straw confers a significant advantage during handling of the gel. The straw can simply be pulled out of the apparatus without compromising the integrity of the gel. When isolating multiple probes, some of the straws can be stored behind a shield, so that the researcher can limit the amount of radioactivity which is handled at one time. In addition, the straws can be placed in a pattern that obviates the need for external markers in order to

align autoradiograph and gel. Prior to the development of the new technique described herein, slab gels had been transported to a darkroom so that physical marks could be placed on the gel and X-ray film. Other groups have advocated the use of highly radioactive dyes to aid the alignment. Asymmetric alignment of tubes eliminates these procedures allowing the procedure to be done at the electrophoresis bench. While taping the straws to the glass surface is currently the most cumbersome part of the procedure, it is anticipated that fabrication of a form which would precisely fit each straw would simplify this step. Cutting the straws and expressing the gel fragments are very straight forward and simplifies the handling of highly radioactive material. Finally, purification of riboprobes enhances results and reduces the amount of radioactivity that is used in the resultant RPA, thus reducing exposure to the researcher and contamination of laboratory equipment.

Reference

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