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Introduction

For the most sensitive and reliable Nuclease Protection Assays (NPAs), it is often suggested that the probes used be full-length and purified. While precipitations and spin columns remove free-nucleotides and DNase treatments can remove template DNA, these techniques do not allow qualitative analysis of the probe. Only by gel assessment can one reliably verify probe integrity. Gel purification is perhaps the only practical method for easily removing DNA template when making single-stranded DNA probes (e.g. by primer extension). For both RNA and DNA probe preparations, gel purification, in a single step, removes free nucleotides, DNA template, buffer components, and enzymes, as well as prematurely terminated products.

Prematurely terminated products can form during extension or transcription if the polymerase encounters significant secondary structure or a homopolymeric stretch of one nucleotide, or if there is an inadequate amount of limiting nucleotide (typically the labeled nucleotide in the reaction). Prematurely terminated products, when not removed, will increase background bands and smearing on the NPA gel after hybridization and digestion. We therefore suggest all RNA probes to be used in nuclease protection assays be gel purified using the Poly-Gel RNA Purification Kit.

Principle

The E.Z.N.A.[®] Poly-Gel RNA Extraction Kit is a straight forward and simple system for recovering RNA probes from denaturing acrylamide gels. After the in vitro transcription reaction, the product is run on a denaturing polyacrylamide gel (a mini protein gel apparatus can be used) to separate the DNA template, full-length RNA probe, any prematurely terminated products and free-nucleotides, by size. The gel is exposed to film and stained or UV shadowed (depending on the nature and quantity of the probe made, e.g. radioactive, non-isotopic or unlabeled probe). Full-length probe is identified and the band is cut from the gel. The probe is eluted by passive diffusion from the gel fragment and further purified using an HiBind[®] RNA spin-column. The procedure can produce enough probe, ready for hybridization in just 1-4 hours.

Storage and Stability

All components of the Poly-Gel RNA Purification Kit are stable for at least 24 months from the date of purchase when stored at 22-25°C. Once diluted, RWB Wash Buffer Concentrate must not be refrigerated. During shipment and storage under cool ambient conditions, precipitates may form in certain buffers. Simply warm to re-dissolve.

Kit Contents

Product	R6376-00	R6376-01	R6376-02
Purification	5	20	50
HiBind [®] RNA Mini column	5	20	50
Poly-Gel Filter Units	5	20	50
2 ml Collection Tubes	15	60	150
Buffer RFE	5 ml	10 ml	20 ml
Buffer RB	5 ml	20 ml	50 ml
RWB Wash Buffer	2 ml	5 ml	12 ml
User Manual	1	1	1

Before Starting

- Absolute ethanol
- Micro-centrifuge capable of 10,000 x g
- RNase-free microcentrifuge tubes and tips
- Disposable latex gloves
- DEPC-treated water

General Notes

Whenever working with RNA, always wear latex gloves to minimize RNase contamination. Use only clean RNase-free disposable plastic pipette tips when using the supplied reagents.

- During the Probe Clean Up (page X) work carefully but quickly.
- Under cool ambient conditions, crystals may form in certain buffers. This is normal and the bottle should be warmed to redissolve the salt.
- 2-mercaptoethanol (β -mercaptoethanol) is key in denaturing RNases and must be added to an aliquot of Buffer RB before use. Add 20 μ l of 2-mercaptoethanol per 1 ml of Buffer RB. This mixture can be stored for 1 week at room temperature.
- All centrifugation steps must be carried out at 22°C-25°C.

IMPORTANT

RWB Wash Buffer Concentrate must be diluted with absolute ethanol before use:

R6376-00 Add 8 ml absolute ethanol (96-100%)

R6376-01 Add 20 ml absolute ethanol (96-100%)

R6376-02 Add 48 ml absolute ethanol (96-100%)

Denaturing Acrylamide Gel Electrophoresis

We suggest using a small mini gel (e.g. BioRad Mini-Protean) for gel purification. Small gels offer the advantage that they are quick and easy to prepare (<45 min), run quickly (~30 min) and are inexpensive.

1. Prepare 5% Acrylamide/8M Urea denaturing polyacrylamide gel as follows (makes 15 ml, sufficient for a 13 cm x 15 cm x 0.75 mm thick gel).
Mix: 7.2 g urea
1.5 ml 10X TBE
1.875 ml 40% acrylamide (acrylamide: bis acrylamide of 19:1)
2. Add dH₂O to a final volume of 15 ml. Stir at room temperature until urea dissolves. Then add:
120 μ l 10% ammonium persulfate in dH₂O (fresh)
16 μ l TEMED
3. Mix briefly and pour. Allow to set (about 30 min).
4. To the sample, add an equal volume of Loading Buffer to the probe or, if the probe has been precipitated, resuspend directly in Loading Buffer. **Heat at 95°C for 3-5 minutes to denature any secondary structure, then place on ice to prevent renaturation.**
5. Flushing any urea from the wells, load the probe in the gel and run the gel until the faster blue dye front (bromophenol blue) reaches the bottom of the gel (approx. 30 minutes at 200 mA for minigels).

RNA Visualization

2. 1. Radioisotopic probes (³²P-, ³³P-, or ³⁵S labeled): Separate the glass plates, leaving the

gel attached to the larger bottom plate. Wrap a piece of clean plastic wrap over the gel. If the glass and gel will not fit into the film cartridge, then the bottom glass plate should be carefully removed and the gel wrapped entirely in plastic wrap (for easier handling). The gel is ready to expose to film.

2. 2. Place the gel (sandwiched between the glass and plastic wrap) against the film so that the film is closest to the gel. The film can simply be aligned with one corner of the glass plate, the corners and sides of the glass plate marked directly on the film with a permanent marker or fluorescent stickers can be used for orientation. One corner of the film (e.g. bottom right corner) is usually snipped so that the glass and gel can be precisely aligned with the film after developing.

2. 3. Expose the gel to autoradiographic film, about 30 seconds for a high specific activity ³²P-labeled probe and 10 minutes for a low specific activity ³²P-labeled probe or high specific activity ³⁵S-labeled probe. The goal is to get an exposure of a light gray band so that a thin gel fragment can be excised from the gel. Realign the glass plate and gel with the developed film (using the guide marks made earlier) and carefully excise the band using a nuclease-free scalpel or razor blade. The smaller the size of this gel fragment, the better the elution efficiency. This band should be the most intense band present. The gel can be re-exposed to insure that the gel and film were properly aligned and that the probe was indeed removed.

Note: Run markers or a known size standard so that the appropriate band is selected. If no markers have been run, the bromophenol (dark blue) and xylene cyanol (light blue) dyes can serve as size references. In a denaturing 5% polyacrylamide gel, bromophenol blue runs at 35nt and xylene cyanol runs at 130 nt.

2. 1. Non-isotopic probes (unlabeled or biotin-labeled): The gel needs to be removed from both of the glass plates as glass blocks UV light and will therefore prevent visualization by either UV shadowing or staining. The gel is wrapped in plastic wrap to aid in handling and marking. Remove the top glass plate, and lay a sheet of plastic wrap over the gel, then flip the gel & glass plate over and carefully peel the gel away from the bottom glass plate. Wrap the gel entirely in the plastic wrap.

Note: Use just a single layer of plastic wrap and try to prevent any bubbles from forming between the gel and plastic wrap. These bubbles can scatter the UV light and make visualization difficult.

2. 2. With non-isotopic and unlabeled probes, the gel cannot be directly exposed to film as with radioisotopic probes. Note, however, that a much greater mass of probe is usually synthesized and the gel can either be UV-shadowed with a short-wavelength hand-held UV light source and fluor-coated TLC plate or stained with EtBr or acridine orange and held over a UV transilluminator to visualize the probe's location within the gel.

Note: Any surface that the gel comes in contact with should be treated to remove RNase-contamination.

UV shadowing

Place the gel on top of the dull white side of the fluor-coated TLC plate and remove the

plastic wrap on top of the gel. Hold a hand-held short-wavelength (254 nm) UV light source over the gel. (**Long wavelength UV light will not work**). The TLC plate beneath the gel should glow bright purple wherever nucleic acids are present. The limit of sensitivity is about 0.4 µg in a single band.

Note: UV shadowing works for RNA or DNA, labeled or unlabeled, so this technique has many other applications.

Staining

Either acridine orange or EtBr may be used since subsequent purification with the HiBind RNA column will remove these dyes. Remove the gel from the plastic wrap and place in a 2.0 µg/ml acridine orange solution for 15 minutes.

Destain the gel in distilled water for 10 minutes. Re-wrap the gel in plastic wrap for easier handling, and place the gel on a UV transilluminator to visualize the probe.

Carefully cut out (using a nuclease-free scalpel or razor blade) the smallest gel fragment possible which contains the probe (corresponds to bright purple band on the TLC plate or band in the gel). The smaller the size of this gel fragment, the better the elution efficiency. This band should be the most intense band present. If you are concerned that not all the probe was cut out, visualize the gel again with UV light to verify that the probe band is gone.

RNA Probe Elution and Purification

Please take a few minutes to read and familiarize yourself with the following procedure. Make sure all necessary reagents and equipment are ready before starting. RWB Wash Buffer Concentrate must be diluted with absolute ethanol as indicated in **Before Starting** on page 3 and stored at room temperature. All centrifugation steps are to be performed at room temperature.

1. Transfer the gel fragment onto a nuclease-free microscope slide. With a second glass slide (or nuclease-free razor) mash and pulp the gel completely. Carefully transfer gel pulp to a nuclease-free microcentrifuge tube and add 200 µl Buffer RFE. This volume is usually enough to submerge a slice 2 mm x 5 mm x 0.75 mm. For a larger fragment adjust volume of Buffer RFE used until the gel is covered.
2. Incubate 1-2 h at 65°C. The elution time is dependent on the size of the gel fragment, RNA size and the temperature of the incubation. We find that about 90% of a 400 nt transcript elutes in 1 hr at 65°C. Larger fragments will take longer to elute. Proceed to step 3 for RNA clean up if downstream applications involve

enzymatic manipulation.

Note: It is not necessary to elute all the probe prior to hybridization, only what will be needed (i.e. $5-10 \times 10^4$ cpm of a high specific activity probe per RNase protection assay reaction, or $\sim 1 \times 10^7$ cpm for Northern Assays). An aliquot of Buffer RFE (containing some of the probe) can be removed at any time during the elution and used directly in the hybridization reaction. This allows setup of hybridization reactions on the same day as probe preparation. Note that elution of a high specific activity probe in 200 μ l of elution buffer should yield approximately $2-5 \times 10^4$ cpm/ μ l.

RNA Probe Clean Up

Note that all subsequent steps are required only if downstream application involve enzymatic reactions, such as reverse-transcription.

3. Transfer gel and buffer to a Poly-Gel filter unit mounted in a sterile 1.5 ml microcentrifuge tube. Use a blue pipette tip with the end cut to do this. Centrifuge at 10,000 x g for 5 min at room temperature to filter the sample.
4. To the eluate add 800 μ l Buffer RB/2-mercaptoethanol and vortex briefly to mix. With radioisotopic probes also add 5 μ g yeast tRNA in no more than 25 μ l as carrier.
5. **For RNA Probe >200nt**, add 500 μ l absolute ethanol and immediately vortex for 1 min. **For RNA Probe <200nt**, add 1.5 ml absolute ethanol and immediately vortex for 1 min. A precipitate may form on addition of ethanol. This will not interfere with the procedure and should be thoroughly resuspended. Proceed to the next step without delay.

Note: Remember to add 20 μ l 2-mercaptoethanol per 1 ml of Buffer RB before use. This mixture is stable at room temperature for 1 week.

6. Apply 700 μ l of sample from previous step onto HiBind[®] RNA Mini column assembled in a 2 ml collection tube (supplied). Centrifuge 20 seconds at 10,000 x g (approx 12,500 rpm on most micro-centrifuges).
7. Discard flow-through liquid from step 5. Apply remainder of sample and centrifuge as above. Again discard flow-through liquid and the collection tube.
8. Place column into a **NEW** 2ml collection tube (supplied). Pipet 500 μ l RWB Wash Buffer diluted with ethanol onto spin column. Centrifuge as above and discard flow-through.

Note: RWB Wash Buffer Concentrate must be diluted with absolute ethanol as described on page 3.

9. Again add 500 μ l RWB Wash Buffer to spin column and centrifuge. Discard liquid

and reuse 2 ml tube in next step. Spin empty column 2 min at 10,000 x g to dry HiBind[®] matrix. **This is critical for removing residual ethanol which may otherwise be carried over during elution and interfere with downstream applications.**

10. Transfer spin column to a new 1.5 ml microfuge tube. Elute the probe by pipetting 30-50 μ l DEPC-treated water (supplied) directly onto matrix. Centrifuge 1 minute at 8,000 x g (approx 10,000 rpm on most microcentrifuges). For non-isotopic probes (usually present in microgram quantities) repeat the elution with a second 30-50 μ l aliquot of DEPC-water to improve yield.

Probe Quantization

Radioisotopic Probes

Analyze the eluted probe by scintillation counting to determine the concentration (cpm/ml). A typical transcription reaction should yield approximately $2-6 \times 10^4$ cpm/ μ l in 100 μ l. Probe should be stored at -20°C or -70°C for greater stability.

Non-isotopic Probes

Measure the OD readings at 260 and 280 nm. The 260/280 ratio should be 1.9-2.0. Low 260/280 ratios indicate protein/acrylamide contamination which can lead to inaccurate concentration calculations. Based on the 260 reading and the conversion: 1 A260 unit = 40 μ g RNA, calculate the concentration of the probe. A typical 20 μ l transcription reaction (containing 0.5 mM of each NTP) and subsequent gel purification should yield about 4-8 μ g of RNA. The probe should be stored at -20°C or -70°C for greater stability. Degradation typically starts after 5-10 freeze-thaw cycles, so the probe should be stored as aliquots.