

## Recommendations for using Ambion's RPA III™ and MAXIscript™ kits with PharMingen RiboQuant™ Multi-Probe Template Sets

### Advantages of Ambion's RPA III Kit

- One tube assay - PharMingen's kit requires 3 tubes per sample
- No messy oil overlay
- No Proteinase K digestion step
- No phenol/chloroform extraction of the protected RNA
- RNase is inactivated and precipitated at the end of the RPA in our exclusive Inactivation/Precipitation Mixture
- Use ordinary 12 X 15 cm gels - No sequencing gels
- Detailed Instruction manual with extensive troubleshooting section

Ambion is the RNase Protection Assay leader, our kits transform the RPA from a cumbersome and tricky technique to a routine and reliable assay. RPAs are an excellent way to compare and quantitate the expression level of a given message in different RNA pools. In order for such comparisons to be valid, the intensity of a protected fragment's signal should be normalized to that of an internal control whose expression remains invariant from sample to sample. In multiprobe RPAs, the intensity of different protected fragments' signals in a single sample will depend on their relative abundance, but other factors such as transcription efficiency, hybridization efficiency, secondary structure present in the probe and/or the target mRNA, and probe interactions or interference can also affect their intensity.

Ambion's technical service staff has received several inquiries about using PharMingen's RiboQuant™ multiprobe template sets with Ambion RPA III and MAXIscript Kits. We have tested the PharMingen templates in our kits and have found that with a few modifications to the standard protocols, good results can be obtained. Those modifications are outlined here; please refer to the MAXIscript and RPA III manuals for further details.

### A. Probe Synthesis

To use Ambion's T7 MAXIscript Kit, follow the manual instructions for synthesis of radioisotopically labeled probes. Ambion recommends using [<sup>32</sup>P]UTP or CTP 800 Ci/mmol, 10 mCi/ml (12.5 μM) while PharMingen recommends using [<sup>32</sup>P]UTP 3000 Ci/mmol, 10 mCi/ml (3.3 μM) as the radiolabeled NTP. We have successfully used both of these radiolabeled NTPs. Note, however, that transcription reactions typically require a minimum of 3 μM of the limiting (radiolabeled) NTP.

1. Assemble the transcription reaction as follows:

Nuclease-free dH <sub>2</sub> O	to 20 μl
RiboQuant multiprobe template set	1 μl
10 X Transcription Buffer	2 μl
10 mM ATP	1 μl
10 mM CTP	1 μl
10 mM GTP	1 μl
[ <sup>32</sup> P]UTP	5 μl
T7 RNA Polymerase + ribonuclease inhibitor	2 μl

2. Incubate for 1 hour at 37°C.

3. Add 1  $\mu\text{l}$  DNase I (2U/ $\mu\text{l}$ ) and incubate at 37°C for 30 minutes.
4. Add 80  $\mu\text{l}$  Nuclease-free dH<sub>2</sub>O. Extract the mixture with 100  $\mu\text{l}$  phenol:chloroform:IAA.
5. Precipitate with 10  $\mu\text{l}$  (1/10 volume) 5M NH<sub>4</sub>OAc and 275  $\mu\text{l}$  (2.5 volumes) EtOH. Chill reactions at -20°C for 15 -30 minutes or more and recover the transcripts by high speed centrifugation at 4°C for 15 - 30 minutes.
6. Carefully remove the supernatant and resuspend the pellet by vortexing in 50  $\mu\text{l}$  RPA III Hybridization Solution.
7. Count duplicate 1  $\mu\text{l}$  aliquots and dilute the probe to PharMingen's recommendation. You will need 2  $\mu\text{l}$  probe per RPA reaction.

**Note:** If you are using PharMingen's transcription kit, prepare the probes according the PharMingen protocol.

## **B. RNA Preparation and Hybridization**

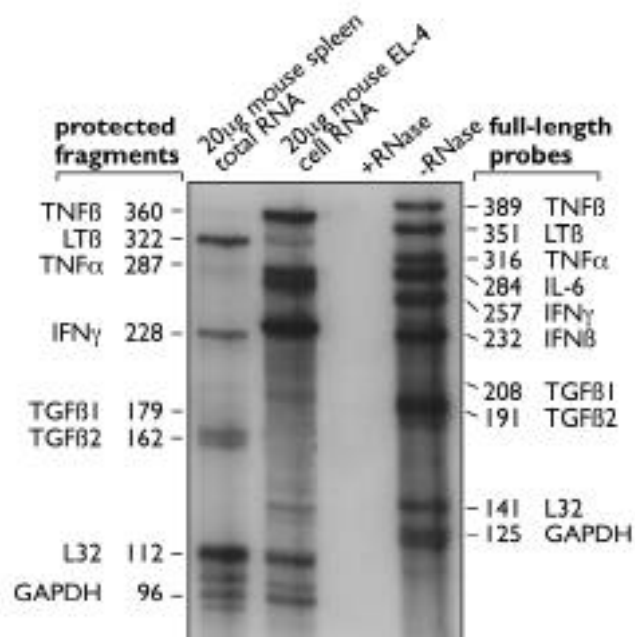
1. Input RNA should be pure, of high quality and solubilized in Nuclease-free water. Aliquot 1-20  $\mu\text{g}$  input RNA into 0.5 ml microcentrifuge tubes. Include 2 tubes containing 2  $\mu\text{l}$  (10  $\mu\text{g}$ ) of yeast RNA each to serve as controls. One yeast RNA tube will be treated with RNase while the other will not be RNase-treated.
2. Dry samples to completion in a vacuum evaporator centrifuge with no heat.
3. Resuspend samples in 8  $\mu\text{l}$  RPA III Hybridization Solution by vortexing.
4. Add 2  $\mu\text{l}$  diluted probe to each sample, heat for 2-3 minutes at 95°C and hybridize at 56°C overnight. Thermocyclers are ideal for incubation. We have found that the oil overlay required by PharMingen's protocol is not necessary.

## **C. RNase Digestion and Precipitation of Protected Fragments**

1. Dilute RNase A/T1 Mix in RNase Digestion III Buffer 1:1000.
2. One yeast RNA control sample should receive Digestion Buffer alone, without RNase. Add 100  $\mu\text{l}$  diluted RNase to each of the other tubes.
3. Vortex, touch spin and incubate at 30°C for 45 minutes.
4. Add 150  $\mu\text{l}$  Inactivation/Precipitation III Solution to each tube, vortex, touch spin and incubate at -20°C for 15-30 minutes.
5. Microcentrifuge at 4°C and high speed for 15-30 minutes.
6. Double aspirate supernatant and air dry pellets 5-10 minutes (*do not dry to completion in vacuum evaporator centrifuge*).

## D. Separation and Detection of Protected Fragments

1. Prepare a 5% acrylamide / 8M urea / 1X TBE gel at least 11 centimeters long measured from the bottom of the well.
2. Resuspend each pellet in 4-8  $\mu$ l Gel Loading Buffer II. The yeast RNA control sample without RNase should be resuspended in 40  $\mu$ l.
3. Heat all samples to 95°C for 3-5 minutes and quench on ice.
4. Rinse the wells of the gel and immediately load entire sample volume (except for the yeast RNA control sample without RNase, where only 4  $\mu$ l is loaded).
5. Run gel at 20-25 V/cm until the xylene cyanol band (the higher molecular weight band) has migrated about 5 cm from its position when the bromophenol blue (lower band) ran off the gel (this should take about one hour). Dry the gel and expose it to film overnight with an intensifying screen.



**Figure 1.** RPAs were performed using Ambion's RPA III™ Kit and <sup>32</sup>P-labeled RNA Probes generated from the RiboQuant™ Multiprobe Template Set, mCK3 using Ambion's MAXIscript™ Kit. The reactions were assessed on a 15 cm, 5% polyacrylamide/8M urea gel that was subsequently vacuum dried and exposed to film for 5 days at room temperature. (RiboQuant is a trademark of PharMingen.)