Torrey Pines Biolabs, Inc.

RNase Protection Assay Kit I
(Catalog # 4910, for 200 samples)

KIT COMPONENTS		for 200 samples
1. 2× fast hybridization buffer	(2×fHB)	1.0 ml (Store at 4°C)
2. RNase A+T1		200 µl (Store at -20°C)
3. 10× RNase digestion buffer	(10×RDB)	1 ml ×2 (Store at R.T.)
4. Stop buffer	(1ml ×2, Add 20 ml 100% ethanol to 1ml Stop buffer upon arrival or before use. Store at -70°C)	
5. Loading buffer	(LB)	1 ml ×2 (Store at -20°C)
6. RNase-free H_2O		1 ml ×2 (Store at R.T.)

The whole kit can be stored in -20°C except stop buffer.

Equipment and reagents necessary but not supplied:

In vitro transcription system (including ^{32}P-UTP)
Benchtop microcentrifuge (15,000-18,000 rpm)
Scintillation counter
Power supply, Gel apparatus
6% sequencing gel
TEMED, 10% ammonium persulfate
5× TBE
90°C heat block (or PCR machine)

ASSAY PROTOCOL

I. Prepare the Probe

1. In vitro transcription of ^{32}P-UTP or ^{32}P-CTP labeled RNA probe according to manufacturer's protocol.
2. Count the radioactivity in liquid scintillation counter.

II. Hybridization

3. Add RNA sample (<4 µl), 1 µl of probe cocktail with 0.5-1.0×10^5cpm each probe, and 5 µl of hybridization buffer in a 0.65 ml RNase-free microtube, bring up to 10 µl with DEPC-treated water. Mix well with pipet tip.

   For example:

   If probe A has 2×10^5cpm/µl and probe B has 3×10^5cpm/µl. You have 10 samples with total RNA 2.5 µg/µl and you need 5 µg each sample. Mix 50 µl of 2×fHB, 5 µl of probe A and 3.3 µl of probe B and 21.7 µl of RNase-free water. Add 8 µl of the hybridization mixture to each tube, then add 2 µl of RNA to each tube and mix well. Each sample now has 1×10^5cpm per probe.

Torrey Pines Biolabs, Inc.
9073 Knight Road
Houston, TX 77054
web:www.chemokine.com
Tel: (713) 383-8144
Toll free: (866) 383-8144
Fax: (713) 383-8142
E-mail:tpbi@chemokine.com

4. Dilute 1 µl of hybridization mixture (without sample RNA) into 9 µl of DEPC-treated water for the future use as probe lane.
5. Hybridize at 90°C for 25 min (Can use heat block or PCR machine). There will be condensation on the cap of the tube. It's normal. **DON'T USE HOT COVER!** After 25 minutes, take microtubes out of heat block or PCR machine. Centrifuge briefly to collect the condensation to the bottom of the tubes.

III. RNase Digestion

6. Prepare RNase digestion solution cocktail as follows (for 10 samples):

Distilled water	900µl
10× RNase digestion buffer	100µl
RNaseA/T1	5µl

7. Add 100µl of digestion cocktail to each tube, vortex, centrifuge briefly and incubate at room temperature for 30 min.

IV. Precipitate RNA

8. Take the Stop Buffer out of -70°C. Invert to mix well before use. Add 200 µl of cold Stop Buffer to each tube and invert the tubes several times to mix the samples with Stop Buffer and incubate at -70° for 20 min. **It's critical to mix the stop buffer with the samples very well to get the good result. Put Stop Buffer back to -70° C.**
9. Take the samples out of -70°C. Centrifuge in microcentrifuge at maximal speed (>12,000 *g*) for 15 min. The white pellet can be seen at the bottom of the tubes. Discard the supernatant carefully (or you might lose the pellet).

V. Prepare Sample for Electrophoresis

10. Spin briefly and remove the remained liquid by pipetting.
11. Resuspend each pellet with 5 µl of Loading Buffer.
12. Take 1µl diluted probe from step 4, add 5 µl of Loading Buffer. This probe can be loaded at the far-left lane.
13. After heating samples at 90°C for 3 min, immediately transfer to wet ice.
14. Load samples onto the wells of 6% sequencing gel under standard conditions using 1× TBE buffer. Run gel at constant voltage or constant power. For example, if NOVEX 6% TBE-Urea gel is used, 150V constant voltage can get good result.
15. Transfer gel to filter paper, cover with plastic wrap, and dry for 30 minutes at 80°C.
16. Expose the dried gel to X-ray film or phosphor-screen.

THIS PRODUCT IS FOR RESEARCH USE ONLY, NOT FOR DIAGNOSTIC PROCEDURE.

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Time Table for Rapid RPA

1. Hybridization	20-25 min	90°C
2. RNase digestion	30 min	R.T.
3. Stop and precipitation	20 min	-70°C
4. Centrifugation	15 min	4°C or R.T.
5. Gel separation	30-40 min	R.T.

Total: Less than 3 hours.

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Troubleshooting:

A. Probe lane: No probe, probe lane high background, probe lane smear, probe degradation.
   1. Gel or running buffer (1× TBE) RNase contamination. Use gel apparatus for RNase protection assay only. Don't share with protein gel.
   2. RPA loading buffer RNase contamination. Aliquot loading buffer. One aliquot use 2 or 3 times only.
   3. Poor probe: 1) Only use [α-^{32}P]UTP within 2 weeks. The labeling won't be good beyond 1 month; 2) Don't use 'stabilized' [α-^{32}P]UTP (4°C storage); 3) Don't use labeled probe beyond 3 days. Probes longer than 400 bp are only good for 2 days. Choosing template 100-300 bp is strongly recommended unless you are an experienced user. 4) In vitro transcription: Make sure after adding all reagents, wait at least 1 minute before you add DNA template.

B. There is no protected band in the gel, especially the long probes.
   1. Bad RNA quality. Check RNA sample quality with agarose gel.
   2. RNA degradation during the assay. There are two steps that could cause the problem. 1) Hybridization. If you follow the strict rule of RNase-free operation, RNA degradation in this step can be avoided; 2) Stop RNase digestion. Invert to mix the Stop buffer well before use, but don't shake too hard. After adding stop buffer into sample tube, invert several times, make sure it has been mixed well.

C. Multi-bands are detected in the single probe assay.
   1. Samples are partial degraded. The same treatment as B.
   2. Different size transcripts probe. Usually, gel purification labeled probe is not necessary. But if the multi-bands are also shown in the probe-only lane, The probe needs to be gel purified.

D. Undigested probe. Because of the extra polylinker region, the band in the probe lane is always longer than the protected band. If you see a band on the sample lane with the size exactly the same as that on the probe lane, That's undigested probe. Usually, it is caused by:
   1. Use too much probe. If the probe is 100-400 bp, 0.5×10^5 cpm/sample is enough. One of the cause is the scintillation counter out of calibration.
   2. Low activity of RNase. It could be caused by repeat thaw-freeze. We suggest not repeat thaw-freeze more than 3 times. Small aliquot will help.

E. Weak signal even after 2-3 days exposure.
   1. Not enough sample RNA. For the tissue sample, 2-5 µg total RNA is necessary to run the assay, while for the cell line, 0.5 µg total RNA is the least to run the assay. If Qiagen's kit is used to extract the RNA, precipitation of RNA before the assay is strongly recommended.
   2. The specific target gene expression level is low. Always run housekeeping gene with the target gene. If the housekeeping gene is strong, and there is no sign of RNA degradation, this gene is either not expressed, or expressed at very low level in these cell or tissues. If that is the case, RPA might not be the best option.
   3. Check the DNA template to make sure that in vitro transcription will generate anti-sense riboprobe.
   4. Don't forget put the intensifying screen on the gel.

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