HybSpeed™ RPA Catalog #1412

Protocol

A. Background: The Ribonuclease Protection Assay

The Ribonuclease Protection Assay (RPA) is an extremely sensitive procedure for the detection and quantitation of RNA species (usually mRNA) in a complex sample mixture of total or Poly(A) selected RNA. For the RPA, a labeled (nonisotopic or radioactive) RNA probe is synthesized that is complementary to part of the target RNA to be analyzed. This is done by placing the 3' end of the probe sequence adjacent to one of the phage polymerase promoters (T3, T7, or SP6) by cloning into a plasmid vector or by using a PCR primer that contains the promoter sequence. The corresponding T3, T7, or SP6 RNA polymerase is then used to generate an antisense RNA transcript by *in vitro* transcription. The labeled probe and sample RNA are incubated under conditions that favor hybridization of complementary sequences. After hybridization, the mixture is treated with ribonuclease to degrade unhybridized probe. Labeled probe that is hybridized to complementary RNA from the sample will be protected from ribonuclease digestion, and can be separated on a polyacrylamide gel and visualized either by autoradiography (radioactively-labeled probes) or by a secondary detection procedure (nonisotopically-labeled probes). When the probe is present in molar excess over the target in the hybridization reaction, the intensity of the protected fragment will be directly proportional to the amount of target RNA in the sample mixture. Ribonuclease protection assays are thus analogous to S1 nuclease protection assays, but ribonuclease is generally acknowledged to be easier to fine-tune and less prone to degrade double-stranded nucleic acid than S1 nuclease (Molecular Cloning, 1989; and Friedberg, 1990).

High-speed Hybridization

A major limitation in hybridization-based assays is that the hybridization step typically requires overnight or longer incubations; this is particularly true when the target molecule is present at very low levels (sub-picogram). Several approaches have been found to speed up nucleic acid hybridization reactions. One approach is to increase the probe concentration. However, this can result in unacceptably high levels of background signal (noise). The addition of neutral dextran polymers are known to increase rates about 10-fold (Wetmur, 1968), and dextran sulfate can increase apparent hybridization rates as much as 100-fold for Southern blots (Wahle et al., 1979). This increase can be attributed to the "volume exclusion principle", in which the concentration of the nucleic acid molecules is increased by reduction in the effective solvent volume as a consequence of hydration of the polymers. However, this approach also often leads to increased levels of background signal. Research at Ambion has lead to the development of the HybSpeed RPA kit which allows the hybridization step, normally requiring 12-24 hours, to go to completion in 10 minutes or less without the use of volume excluding polymers (patent pending). Figure 1 demonstrates the kinetics of the HybSpeed hybridization reaction. An additional benefit is that the HybSpeed RPA is 1.5 to 3 times more sensitive than the RPA II, and frequently yields sharper bands. This is presumably due to the increased stability of RNA duplexes in the hybridization buffer and the resulting reduction in nonspecific cleavage at RNase hypersensitive sites. The hybridization using the HybSpeed buffer is carried out at 68°C. This elevated temperature aids in reducing secondary structure of the mRNA and probe, resulting in less background due to undigested self-protected probe. Ambion's HybSpeed RPA kit combines the convenience of a single-tube assay (developed with the RPA II Kit) with the HybSpeed Technology to reduce the overnight hybridization time to ten minutes. The reduction in hybridization time will often allow the complete protection assay to often be performed in one day. The protected probe fragments are then analyzed using PAGE and autoradiography, phosphor imaging, direct imaging, or may be transferred to nitrocellulose or nylon membranes and detected using protocols recommended for the particular type of non-isotopically labeled probe used (Turnbow and Garner, 1993).

The HybSpeed RPA also allows multiprobe analysis within a single RNA sample, so that samples can be simultaneously hybridized with experimental probes and internal standards. Finally, the HybSpeed RPA includes a control vector for mouse β-actin and total RNA from mouse liver. These reagents allow researchers to self-test the quality of the kit and its protocol at any time.

Figure 1. Kinetics of Hybridization for Ambion's HybSpeed™ Technology.

Ten µgs of total mouse liver RNA probed with a 360 base antisense in vitro transcript for mouse β-actin. The coprecipitated RNA and probe were heat denatured in HybSpeed™ hybridization buffer and allowed to hybridize at 68°C for the indicated times. Digestion buffer was added to degrade unhybridized probe. The 250 bp RNA duplex was recovered and run on a 5% polyacrylamide/8M Urea/1X TBE denaturing gel and analyzed by autoradiography. The standard RPA II™ sample was hybridized at 42°C overnight in RPA II™ hybridization buffer.

B. Hybridization of Probe and Sample RNA

NOTE:

Samples with less than 50 µg of RNA; should be normalized to 50 µg with the yeast RNA provided. We generally recommend using gel purified probe or probe that has been determined to consist mainly of full-length transcript as assessed by gel electrophoresis, see Section V.A.

1. For each experimental tube, mix labeled probe (approximately 100-800 pg of 250 nt or 1-10 fmol or 2 - 8 x 10^4 cpm high specific activity) with sample RNA (<1-20 µg) in a ***0.5 ml microfuge tube*** Set up 2 control tubes for every probe to be used, by mixing 10 µl Yeast RNA (50 µg yeast total RNA) with the same amount of labeled probe used for the experimental tubes.
2. Co-ethanol precipitate the probe and sample RNAs by adjusting the final NH_4OAc concentration to 0.5 M and adding 2.5 volumes of EtOH and mixing thoroughly. 5 M NH_4OAc is supplied with the kit for this purpose.
3. Place tubes in -20°C freezer for 15 minutes.
4. Pellet probe and sample RNA for 15 minutes at maximum speed of microcentrifuge, preferably at 4°C.
5. Carefully remove EtOH supernatant from each tube; allow samples to air dry for 5 minutes at room temperature.
6. To each pellet add 10 µl of HybSpeed Hybridization Buffer (pre-heated to 95°C) and immediately put the tube in a 95°C heating block or water bath. After all samples have HybSpeed Hybridization Buffer, thoroughly vortex each sample for 15 sec. and return them to the 95°-100°C bath. Repeat this vortexing Step **2-4** times, until all the pellets are completely dissolved. Do not be concerned about the foaming that may occur. Take care to keep the samples as close to 95°C as possible while resuspending the pellets, because RNA is not soluble in the HybSpeed Hybridization Buffer at temperatures below about 65°C. Complete solubilization of the co-precipitated probe + RNA is essential for maximizing the sensitivity of the HybSpeed System.
7. Heat all tubes at 95°C±5°C for 2-3 minutes after the last sample is resuspended.
8. Incubate tubes in a 68°C incubator (in a preheated tube rack) or submerge in 68°C water bath or heat block for 10 min. to permit hybridization of the probe and complementary mRNA in the sample RNA. Do not allow the samples to drop below 68°C. Transfer from the 95°C bath to the 68°C

incubation should take no more than 30 seconds.
For processing large numbers of samples it is best to split them into batches so that the suggested 10 minutes incubation period is not exceeded. See previous note

NOTE:
The 10 minute incubation may be extended to overnight for convenience. For very rare mRNAs, this may result in a slight increase of signal. However, there is a noticeable decrease in signal for hybridization times between approximately 15 minutes and 10 hours.

C. RNase Digestion of Hybridized Probe and Sample RNA

1. Prepare working dilutions of RNase A/T1 Mix in HybSpeed RNase Digestion Buffer. Standard dilution of RNase A/T1 Mix is 1:100. RNase A/T1 Mix should be vortexed and microfuged briefly before use. Keep at room temperature until used. ***Do not put on ice.***
   RNase T1 (alone) may be substituted for the RNase A/T1 Mix when using a probe from a different species than the sample RNA, or when the target sequence is very AU rich (See Section IV.B. and C. for more information on the use of RNase T1).
2. Add 100 µl of diluted RNase solution (RNase digestion buffer and RNases) to all experimental tubes (***one at a time as they are removed from the 68°C bath vortex and place at 37°C***) and to one tube of each pair of yeast RNA of control tubes. Do not microfuge the samples.
3. Add 100 µl HybSpeed RNase Digestion Buffer (without RNase) to the remaining yeast RNA control tube(s), one for each different probe used. Vortex tubes briefly.
4. Incubate tubes for 30 minutes at 37°C, ***re-vortexing*** samples after 15 minutes.
5. Add 150 µl HybSpeed Inactivation/Precipitation Mix to each tube. Vortex tubes briefly.
   Precipitation of very small fragments (less than 150 bases) can be improved by adding 50 µl of ethanol (for fragments of at least 100 bases) or 100 µl of ethanol (for fragments of at least 50 bases), in addition to 150 µl of HybSpeed Inactivation/Precipitation Mix.
6. Transfer tubes to -20°C freezer for at least 15 minutes. It is not necessary to add additional carrier during this precipitation.

D. Separation and Detection of Protected Fragments

1. Prepare a denaturing polyacrylamide gel suitable for separation of protected fragments of the expected size (typically a 5% polyacrylamide/± 8M urea/ 1 X TBE).
2. Remove tubes from freezer and microfuge for 15 minutes at maximum speed (at least 10,000 x g), preferably at 4°C. Carefully remove all supernatant from each tube. Residual supernatant will cause aberrant migration of bands in gel.
3. Dissolve each pellet in Gel Loading Buffer II by vigorous vortexing and brief microfuging. The volume of gel loading buffer is not critical; resolution of bands is optimal when the gel loading buffer forms a 2-3 mm layer in the well (usually 4-10 µl).
4. Heat all tubes for 3-4 minutes at 90°± 5°C.
5. Re-vortex and re-microfuge tubes briefly.
6. Load samples on polyacrylamide gel and run at approximately 250 volts for about 1 hour in 1X TBE buffer. (See Section VII. for buffer preparation.)
7. If radiolabeled probes were used:
   Transfer gel to filter paper, cover with plastic wrap, and expose to X-ray film room temperature or at -80°C with an intensifying screen.
   The gel can be fixed and dried on a gel dryer. Dried gels preclude diffusion of bands and give sharper resolution on autoradiographs.

If a nonisotopically labeled probe was used:

The gel must be transferred to a positively charged nylon membrane (electroblotting, See Section V.G.). The nonisotopic probe is then detected using an appropriate secondary detection procedure (e.g. Ambion's BioDetect Kit, Cat. #1925)