

RNA Protection Assay (RPA)

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PROBE SYNTHESIS

1. Dry DNA riboprobe in speed vacuum in order to have a final volume of 1µl.
2. Bring the [⁻³²P] UTP, GACU nucleotide pool, DTT, 5X transcription buffer, and RPA template set to room temperature.
3. Add the following in order to a 1.5ml Eppendorf tube
 - 1 µl RNasin
 - 1 µl GACU pool
 - 2 µl DTT
 - 4 µl 5X transcription buffer
 - 1 µl RPA template set (or riboprobe DNA)
 - 10 µl [⁻³²P] UTP
 - 1 µl T7 RNA polymerase (*keep at -20°C until use; return to -20°C immediately*).
4. Mix by gentle pipetting or flicking.
5. Quick spin in a microfuge
6. Incubate at 37°C for 2 hrs.
7. Terminate the reaction by adding 2µl of DNase.
8. Mix by gentle flicking.
9. Quick spin in a microfuge.
10. Incubate at 37°C for 30 min.
11. Add the following reagents (in order) to each 1.5ml Eppendorf tube.
 - 26 µl 20mM EDTA
 - 25 µl Tris-Saturated phenol
 - 25 µl Chloroform:isoamyl alcohol (50:1)
 - 2 µl yeast tRNA
12. Mix by vortexing into an emulsion.
13. Spin in a microfuge for 5 min at room temp.
14. Transfer the upper aqueous phase to a new 1.5 ml Eppendorf tube and add 50 µl chloroform:isoamyl alcohol (50:1).
15. Mix by vortexing.
16. Spin in a microfuge for 2 min at room temperature.
17. Transfer the upper aqueous phase to a new 1.5 ml Eppendorf tube and add 50 µl 4M ammonium acetate, invert the tubes, and add 250 µl ice-cold 100% ethanol.
18. Invert the tubes to mix
19. Incubate for 5 - 10 min in liquid nitrogen.
20. Spin in a microfuge for 15 min at 4°C.
21. Carefully remove the supernatant and add 100µl of ice cold 90% ethanol to the pellet.
22. Spin in the microfuge for 5 min at 4°C.
23. Carefully remove all of the supernatant and air dry the pellet for 5-10 min. (*Do not dry in a vacuum evaporator centrifuge*)
24. Add 50µl of hybridization buffer and solubilize the pellet by gently vortexing for 20 sec.

25. Quick spin in a microfuge.
26. Quantitate 1 μ l of samples in a scintillation counter. Expect a maximum yield of 3×10^6 cpm/ μ l with an acceptable lower limit of 3×10^5 cpm/ μ l.
27. Store the probe at -20°C until needed.

RNA PREPARATION AND HYBRIDIZATION

1. If target RNA has been stored in water, freeze the samples for 15 min at -70°C .
2. Dry completely (\sim 1hr) in a vacuum evaporator centrifuge (no heat).
3. Add 8 μ l of hybridization buffer to each sample.
4. Solubilize the RNA by gently vortexing for 3-4 min and quick spin in the microfuge.
5. Dilute the probe to appropriate concentration. (*There is an optimal probe concentration for each standard multi-template set which is included in the Technical data sheet*)
6. Add diluted probe to each RNA sample and mix by pipetting.
7. Add a drop of mineral oil to each tube.
8. Quick spin in the microfuge.
9. Place the samples in a heat block pre-warmed to 90°C .
10. Immediately turn the temperature to 56°C .
11. Incubate for 12-16 hr.

RNASE TREATMENTS

1. Turn the heat block to 37°C for 15 min prior to the RNase treatments.
2. Prepare the RNase cocktail (per 20 samples):

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| 2.5 ml | RNase buffer |
| 6 μ l | RNase A + T1 mix |
3. Remove the RNA samples from the heat block and pipet 100 μ l of the RNase cocktail underneath the oil into the aqueous layer (bubble).
4. Spin in the microfuge for 10 sec
5. Incubate for 45 min at 30°C .
6. Before the RNase digestion is completed, prepare the Proteinase K cocktail (per 20 samples):

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|-------------|---------------------|
| 390 μ l | Proteinase K buffer |
| 30 μ l | Proteinase K |
| 30 μ l | yeast tRNA |
7. Mix and add 18 μ l aliquots of the cocktail to new eppendorf tubes.
8. Using a pipettor, extract the RNase digests from underneath the oil (try to avoid the oil) and transfer to the tubes containing the Proteinase K solution.
9. Quick vortex.
10. Quick spin in the microfuge.
11. Incubate for 15 min at 37°C .
12. Add 65 μ l Tris-saturated phenol and 65 μ l of chloroform:isoamyl alcohol (50:1).
13. Vortex into an emulsion.

14. Spin in the microfuge for 5 min at RT.
15. Carefully extract the upper aqueous phase and transfer to a new tube.
16. Add 120 μ l 4 M ammonium acetate, invert to mix, and add 650 μ l ice-cold 100% ethanol.
17. Mix by inverting the tubes.
18. Incubate for 5 - 10 min in liquid nitrogen.
19. Spin in the microfuge for 15 min at 4°C.
20. Carefully remove the supernatant and add 100 μ l ice-cold 90% ethanol.
21. Spin in the microfuge for 5 min at 4°C.
22. Carefully remove the supernatant and air-dry the pellet completely (*do not dry in a vacuum evaporator centrifuge*).
23. Add 5 μ l of 1X loading buffer.
24. Vortex for 2-3 min.
25. Quick spin in the microfuge.
26. Prior to loading the samples on the gel, heat the samples for 3 min at 90°C.
27. Place them immediately in an ice bath.

GEL RESOLUTION OF PROTECTED PROBES

1. Clean a set of gel plates thoroughly with water and detergent.
2. Wipe the plates with ethanol.
3. Wipe one side of one of the plates with Sigmacote (make sure you note the side you wipe the Sigmacote on).
4. Assemble the gel mold.
5. Pour immediately into the gel mold, remove any air bubbles, and add an appropriate comb.
6. After polymerization (~1hr) remove the comb and flush the wells thoroughly with 0.5X TBE.
7. Place gel in a vertical rig and pre-run at 40 watts constant power for ~45 min with 0.5X TBE as the running buffer. Gel temperature should 50°C.
8. Flush the wells again with 0.5X TBE and load samples.
9. Also load a dilution of the probe set in loading buffer to serve as size markers.
10. Run the gel at 40 watts constant power until the leading edge of the Bromophenol Blue reaches 30 cm.
11. Disassemble the gel mold, remove the plate that has the sigmacote on.
12. Adsorb the gel to a filter paper.
13. Cover the gel with Saran wrap and layer between two additional pieces of filter paper.
14. Place in the gel dryer under vacuum for ~1 hr at 80°C.
15. Place the dried gel on film in a cassette with intensifying screen and develop at -70°C.