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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 [α-^{32}P] 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	[α-^{32}P] UTP
1 μl	T7 RNA *polymerase (keep at -20ºC until use; return to -20ºC immediately).*

4. Mix by gentle pipetting or flicking.
5. Quick spin in a microfuge
6. Incubate at 37ºC 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 37ºC 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 ml 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 4ºC.
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 4ºC.
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 1μl of samples in a scintillation counter. Expect a maximum yield of 3 x 10^6 cpm/μl with an acceptable lower limit of 3 x 10^5 cpm/μl.
27. Store the probe at -20ºC until needed.

RNA PREPARATION AND HYBRIDIZATION

1. If target RNA has been stored in water, freeze the samples for 15 min at -70ºC.
2. Dry completely (~1hr) in a vacuum evaporator centrifuge (no heat).
3. Add 8μl 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 90ºC.
10. Immediately turn the temperature to 56ºC.
11. Incubate for 12-16 hr.

RNASE TREATMENTS

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

2.5 ml	RNase buffer
6 μl	RNase A + T1 mix

3. Remove the RNA samples from the heat block and pipet 100 μl 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 30ºC.
6. Before the RNase digestion is completed, prepare the Proteinase K cocktail (per 20 samples):

390 μl	Proteinase K buffer
30 μl	Proteinase K
30 μl	yeast tRNA

7. Mix and add 18 ml 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 37ºC.
12. Add 65 μl Tris-saturated phenol and 65 μl 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 μl 4 M ammonium acetate, invert to mix, and add 650 ml 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 4ºC.
20. Carefully remove the supernatant and add 100 μl ice-cold 90% ethanol.
21. Spin in the microfuge for 5 min at 4ºC.
22. Carefully remove the supernatant and air-dry the pellet completely *(do not dry in a vacuum evaporator centrifuge).*
23. Add 5 μl 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 90ºC.
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 50ºC.
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 80ºC.
15. Place the dried gel on film in a cassette with intensifying screen and develop at -70ºC.