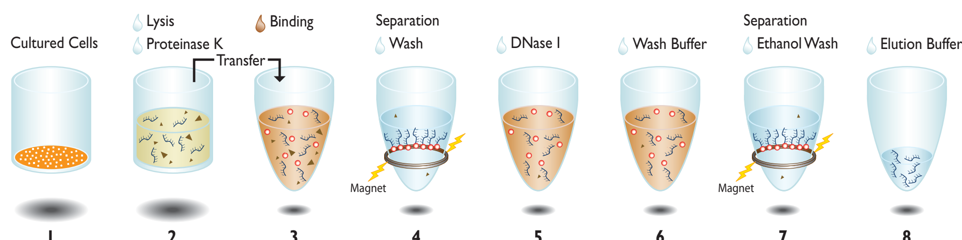
AGENCOURT® RNAdvance® CELL v2 Supplemental Protocol

FOR MICRO-RNA AND TOTAL RNA ISOLATION FROM CULTURED CELLS

PROCESS OVERVIEW



Introduction

The Agencourt® RNAdvance® Cell v2 kit utilizes Agencourt's patented SPRI® paramagnetic bead-based technology and is specially designed to isolate total RNA from 200-50,000 cultured eukaryotic cell lines. The total RNA extraction procedure was modified for micro-RNA (miRNA) purification by optimizing the formulation of the Bind Buffer, Wash Buffer and ethanol wash steps. This supplemental protocol enables recovery of miRNA, small RNA and total RNA, for applications such as RT-PCR. The protocol can be performed in a 96-well plate or in a 1.7 mL tube format. The Agencourt® RNAdvance® Cell v2 procedure does not require vacuum filtration or centrifugation.

Notice for miRNA and total RNA Isolation

- If you plan to use the RNAdvance Cell v2 kit for miRNA plus total RNA isolation, **do not add 100% Isopropanol directly to the Wash Buffer bottle**. See page 2 for Wash Buffer preparation. A 1:2 ratio of Wash Buffer: 100% Isopropanol is used for miRNA plus total RNA isolation.
- Binding buffer conditions: 80 µL of Bind buffer + 250 µL of Isopropanol. Use 330 µL in the Binding step (step 7).
- Use 85% ethanol for all ethanol washing steps.

Materials Supplied by the User:

Consumables and Hardware:

Magnetic Separator:
Agencourt SPRIPlate 96R – Ring Super Magnet Plate (Beckman Coulter Life Sciences, A32782) or Agencourt SPRIStand – Magentic 6-tube Stand (for 1.7 mL tubes) (Beckman Coulter Life Sciences, A29182)

Culture Plate:
Costar 96-Well EIA/RIA Plates Fisher Scientific #07-200-98

Prep Plate:
ABgene 1.2 mL 96-Well Storage Plate, Square Well, U-Bottomed (ABGene #1127; http:www.abgene.com) or Costar 96-Well EIA/RIA Plates (Round well); Fisher Scientific #07-200-105

*Reagents:

- **100% Isopropanol;** American Bioanalytical #AB07015; http://www.americanbio.com/
- **85% ethanol** made with nuclease free water *(Note:85% ethanol is hygroscopic. Fresh 85% ethanol should be prepared for optimal results).*
- **DNAse I(RNase-free)** (2U/µL); Ambion #AM2224 or #AM2222; http://www.ambion.com)
- **Nuclease-Free Water (not DEPC-Treated)** (Ambion #AM9932; http://www.ambion.com)

Working Under RNase Free Conditions:

RNases are ubiquitous and general precautions should be followed in order to avoid the introduction of contaminating nucleases during the Agencourt RNAdvance Cell v2 miRNA procedure (protocol IB-18266A). The most common sources of RNase contamination are hands, dust particles, and contaminated laboratory instruments, solutions and glassware. When working with RNA, the following procedures should be followed to limit RNase contamination:

- Always work with gloved hands and change gloves frequently.
- Use RNase free, filtered pipette tips for pipetting whenever possible.
- Use dedicated RNase free equipment, e.g. pipettes, pipette tips, gels boxes, etc.
- Avoid using reagents, consumables and equipment that are in common use for other general lab processes.
- When available, work in a separate room, fume hood or lab space.
- Use plastic, disposable consumables that are certified RNase free.
- Purchase reagents, such as commonly used buffers and water, that are certified RNase free. Prepare small individual aliquots of such buffers to avoid repeated transfer out of stock buffers. This lowers the risk of contaminating the stock solution.
- Wipe down work surfaces with commercial RNase inhibiting surfactant solutions or 70% ethanol before starting work.
- Treat electrophoresis gel boxes, including combs and gel trays, with 3% hydrogen peroxide for 10 minutes and rinse with DEPC treated water before use.

Procedure:

Agencourt RNAdvance Cell v2 was designed for routine extraction of miRNA plus total RNA from 200 to 50,000 cells per prep. The Agencourt RNAdvance Tissue kit is recommended for 50,000 – 2 million cells per prep.

1. **For each new kit, assemble Proteinase K once. Mark each tube or bottle with the date of assembly.**

	Proteinase K Solution (50 mg/mL)
	Volume of PK Buffer to add to lyophilized Proteinase K
96 Prep kit / A47942	400 µL
960 Prep kit / A47943	4 mL
Storage Condition	-20°C

2. **Preparation of Solutions** – It is generally recommended to prepare an additional 10% to account for pipetting error.

Wash Buffer Preparation:

For miRNA and total RNA isolation: Add 100% Isopropanol to the Wash Buffer in a proportion of 1:2 (Wash Buffer: Isopropanol). To make 10 mL of Wash Buffer solution, add 6.67 mL of 100% Isopropanol with 3.33 mL of Wash Buffer in a 15 mL conical tube and vortex thoroughly for 5 s.

For total RNA isolation only: Add 100% Isopropanol to the Wash Buffer in a proportion of 3.75: 1 (Wash Buffer: Isopropanol). To make 10 mL of Wash Buffer solution, add 2.1 mL of 100% Isopropanol with 7.9 mL of Wash Buffer in a 15 mL conical tube and vortex thoroughly for 5 s.

Prepare fresh 85% Ethanol with nuclease free water and vortex thoroughly for 5 s (Note: 85% ethanol is hygroscopic. Fresh 85% ethanol should be prepared for optimal results).

Prepare Bind Buffer Solution – Shake or vortex Bind Buffer to resuspend the magnetic particles. For each sample, combine 80 µL Bind Buffer with 250 µL of Isopropanol for a total of 330 µL Bind Buffer Solution.

Prepare DNase Solution – For each sample, combine 20 µl DiH2O, 2.5 µL 10X DNase I buffer and 2.5 µL DNase I, for a total of 25 µl DNase Solution.

Prepare Lysis/PK Solution – USE WITHIN 30 MINUTES – For each sample combine 3 µL PK (50 mg/mL) with 60 µL of Lysis Buffer, for a total of 63 µL Lysis/PK Solution. Mix gently to avoid creating bubbles.

3. **Remove the culture medium from the cells as completely as possible by pipetting.**

   For cell-culture plates, tip the plate slightly to one side and place the pipette tip in the corner of the well when aspirating. For cells grown in suspension, first pellet cells then carefully remove media.

4. **Add 63 µL of Lysis/PK Solution (prepared in Step 2) to each sample. Gently pipette tip mix 20 times at the bottom of the well to resuspend the cells.**

5. **Incubate the samples for 30 minutes at room temperature to complete the lysis and digestion.**

   Possible Stop Point: Once the 30 minute incubation is complete, the lysate can be frozen at -80°C and extracted at a later time. If freezing samples, seal the plate with an adhesive seal to prevent contamination. Thaw samples at room temperature before resuming the Agencourt RNAdvance Cell v2 process.

6. **Transfer the entire lysate from the sample plate into a magnet-compatible 96 well round bottom plate. For manual processing, Abgene AB-1127 or costar 96-Well EIA/RIA Plates (Fisher Scientific 07-200-105) are recommended.**

7. **Shake or mix Bind Buffer Solution (prepared in Step 2) to resuspend magnetic particles. Add 330 µL of Bind Buffer Solution to each sample and pipette tip mix 10 times or until homogeneous.**

   During this step, nucleic acids bind to the magnetic particles. Isopropanol may float to the top of the liquid column so it is important to mix very well to incorporate the Bind Buffer Solution. For best results, use a mix volume that is slightly less than the total volume in the well.

8. **Incubate the samples for 5 minutes at room temperature to bind.**

9. **Place the sample plate on an Agencourt SPRIPlate 96R - Ring Super Magnet Plate for 5 minutes or until the solution clears. Carefully aspirate and discard the supernatant while the plate is situated on the magnet.**

   When aspirating, place the pipette at the center of the well to avoid disturbing the magnetic beads.

10. **Take the plate off the magnet. Add 300 µL of Wash Buffer (see page 2 for Wash Buffer preparation). Pipette tip mix 10 times, or until the magnetic particles are fully resuspended.**

    It is normal for a few beads clumps to remain after resuspension.

11. **Place the plate back on the magnet for 5 minutes, or until the solution clears. Fully remove and discard the supernatant while the plate is situated on the magnet.**

    When aspirating, place the pipette at the center of the well to avoid disturbing the magnetic beads.

12. **Take the plate off the magnet. Add 300 µL of 85% Ethanol. Gently pipette tip mix 5 times, or until beads are fully resuspended.**

13. **Place the plate back on the magnet for 5 minutes, or until the solution clears. Thoroughly remove and discard as much of the ethanol wash as possible.**

    Excess ethanol can reduce the activity of DNase during the next steps.

OPTIONAL DNAse treatment, Skip steps 14-20 if DNase treatment is not required

14. **Take the plate off the magnet. Add 25 µL of DNase Solution (prepared in Step 2) and pipette tip mix 10 times, or until the beads are fully resuspended.**

    The addition of aqueous DNase releases DNA and RNA from the beads. DNA will be digested and the RNA will need to be re-bound to the beads later in the protocol.

15. **Incubate the sample plate at room temperature for 15 minutes to complete the DNase digestion.**

16. **DO NOT REMOVE THE DNase SOLUTION. Add 165 µL of Wash Buffer to each sample and pipette tip mix 10 times, or until homogeneous.**

    During this step, Wash Buffer re-binds RNA to the beads. Additionally, the Wash Buffer helps to dissolve and rinse away proteins and other contaminants.

17. **Incubate the plate at room temperature for 5 minutes to bind.**

18. **Place the plate on the magnet for 5 minutes or until the solution clears. Remove and discard the supernatant.**

19. **Take the plate off the magnet. Wash the beads by adding 300 µL of 85% ethanol.**

    Pipette tip mix 5 times, or until beads are fully resuspended.

    Ethanol washes remove salt, Wash Buffer and any residual contaminants.

20. **Place the sample plate on the magnet for 5 minutes or until solution clears. Remove ethanol and discard.**

21. **Repeat steps 19-20 one more time for a total of 2 ethanol washes.**

22. **Remove as much of the final ethanol wash as possible. Allow the beads to dry for 10 minutes at room temperature while the sample plate is on the magnet.**

    Any droplets or puddles of liquid should be gone before continuing to the next step.

23. **Take the plate off the magnet. Elute the RNA by adding 40 µL of nuclease free water. Pipette tip mix 10 times and incubate at room temperature for 5 minutes to complete elution.**

24. **Place the plate back on the magnet for 2 minutes, or until the solution clears. Transfer the clear RNA solution to a new plate or new tubes for storage (-20°C).**

    If beads are aspirated during the transfer, dispense the eluant back into the well and let the plate sit longer to better compact the bead ring. Leave 5 µL of eluant behind to avoid bead carry-over. During the transfer, place the pipette tip in the center of the bead ring and aspirate slowly.

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