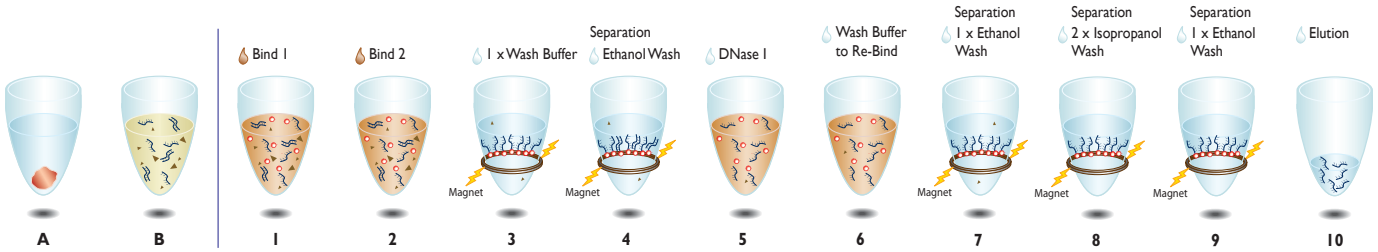
AGENCOURT® FormaPure® Kit Supplemental Protocol

FOR MICRO-RNA AND TOTAL RNA ISOLATION FROM FORMALIN-FIXED, PARAFFIN-EMBEDDED TISSUE

PROCESS OVERVIEW



Introduction

The Beckman Coulter's Agencourt® FormaPure® nucleic acid purification kit utilizes Beckman Coulter's patented SPRI® paramagnetic bead-based technology and is specially designed to isolate total nucleic acids or total RNA from a maximum input of 5 sections of 10 µm slices of formalin-fixed, paraffin-embedded tissue. 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® FormaPure® procedure does not require vacuum filtration or centrifugation.

Notice for miRNA and total RNA extraction

- If you plan to use the FormaPure kit for miRNA isolation, **do not add 100% Isopropanol directly to the Wash Buffer bottle**. See page 2 for Wash Buffer preparation. A 1:1 ratio of Wash Buffer: 100% Isopropanol is used for miRNA, plus total RNA isolation.
- 400 µL of Isopropanol was added in the Bind2 at step 9 instead of 300 µL.
- 85% ethanol instead of 75% ethanol was used in all ethanol washes.

miRNA and total RNA - Materials Supplied by the User

Consumables and Hardware:

- 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).
- Microtome for tissue sectioning.
- 1.7 mL microcentrifuge tubes (Fisher Scientific, Cat #NC9448938) for tube format
- 96-Well Riplate-2.2 mL (Ritter Medical, 43001-0200) for plate format.
- 72°C and 55°C water baths or heat plates.
- 37°C water bath or heat block for DNase incubation.

***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) & DNase I Buffer** (2 U/µL); Ambion #AM2224 or #AM2222 http://www.ambion.com
- **Reagent grade water, nuclease-free** (Ambion #AM9932; http://www.ambion.com)

miRNA and total RNA Extraction Using Agencourt FormaPure 96-well & Tube Protocol

If you have already made the following preparations during a previous experiment, please start at Step 1.

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AAG-666SP11.14-A

General Remarks on Handling RNA:

RNases are ubiquitous and general precautions should be followed in order to avoid the introduction of contaminating nucleases during the Agencourt FormaPure miRNA procedure (AAG-666SP11.14-A). 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.

Reagent Preparation

- **Preheat water baths or heat plate to 72°C and 55°C.**
- **Ensure that Agencourt FormaPure Proteinase K and Wash Buffer have been assembled according to the following instructions:**
- **Proteinase K (40 mg/mL):** Add PK Buffer directly to the PK vial based on the chart in the chart below. Write the date of assembly on the vial. Mix well by gently shaking the vial. Store the PK solution at -20°C when not in use.

	50 Prep Kit Part # A33341	96 Prep Kit Part # A33342	384 Prep Kit Part # A33343
Volume of PK Buffer to Add	1.2 mL	2.3 mL	2.3 mL/vial

- **Wash Buffer Solution preparation:**

For miRNA and total RNA extraction, prepare the Wash Buffer solution as follows:

Add 100% Isopropanol to the Wash Buffer in a proportion of 1:1 (Wash Buffer: Isopropanol).

Example: To make 10 mL of Wash Buffer solution, add 5mL of 100% Isopropanol with 5 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 1.75: 1 (Wash Buffer: Isopropanol). To make 10 mL of Wash Buffer solution, add 3.6 mL of 100% Isopropanol with 6.4 mL of Wash Buffer in a 15 mL conical tube, and vortex thoroughly for 5 s.

1. **Transfer FFPE tissue sections (up to 5 sections of 10 µm each) into a 1.7 mL microcentrifuge tube.**

   Beckman Coulter recommends performing the upfront deparaffinization and digestions steps in tubes for ease in handling sections. Once completed, the magnetic extraction can take place in tubes or 96 well plates.

   The optimal amount of starting material should be between 1-5 slices of the10 µm tissue. For large samples, trim away excess paraffin around tissue. If sections are attached to glass slides, wet the sections with 20 µL of Lysis buffer, *then* remove sections using a clean single-edge razor blade.

2. **Add 200 µL of Lysis buffer to each sample. Use the pipette tip to push the sections down into buffer until they are completely submerged.**

3. **Incubate the tube in a 70°C to 72°C water bath for 60 minutes.**

   This step breaks down paraffin and releases the tissue. Remove samples promptly as prolonged incubation at 70°C may damage RNA. Quick spin to collect condensation.

4. **Add 20 µL of PK solution (see page 2). Be sure to pierce through the paraffin layer at the top of the liquid to ensure the PK goes directly into the sample. Pipette mix three times with a mix volume of 200 µL.**

   The PK solution has a tendency to sink to the bottom of the sample, mix thoroughly to ensure good incorporation.

5. **Incubate the tube in a water bath at 55°C for 60 minutes.**

   This step digests the tissue and inactivates nucleases. The digestion can be extended overnight. Quick spin to collect condensation.

6. **Cool the tube on ice for 2 minutes to solidify any excess paraffin.**

7. **Transfer 200 µL of lysate to a new 96 well plate or 1.7 mL tube for nucleic acid extraction. Avoid any solidified paraffin on the side walls.**

   The transferred Agencourt FormaPure lysate can be stored at -20°C overnight for ease of processing.

8. **Add 150 µL of Bind 1 Buffer to each sample. Pipette mix 5 times with a mix volume of 300 µL.**

9. **Prepare Bind 2/Isopropanol solution: Shake or vortex Bind 2 Buffer vigorously to resuspend magnetic particles. For each individual isolation, combine 20 µL Bind 2 with 400 µL of 100% isopropanol in an RNase Free tube of suitable size. Mix well.**

   Example: For 10 isolations, combine 200 µL of Bind 2 with 4 mL of 100% isopropanol in a 15 mL conical tube. Unused Bind 2/Isopropanol solution should be discarded.

10. **Add 420 µL of Bind 2/Isopropanol solution (prepared in Step 9) to each sample. Pipette mix 5 times with a mix volume of 500 µL.**

11. **Incubate in 55°C water bath for 5 minutes.**

    Keep the incubator cover open since the plate is not sealed.

12. **Move the plate/tube onto the Agencourt Supermagnet plate (or SPRIStand for tubes) and separate for 10 minutes or until the solution turns clear. Slowly aspirate and discard the cleared supernatant.**

    Do not disturb the separated magnetic beads. If beads are drawn out, leave a few microliters of supernatant behind to avoid bead carryover.

13. **Off the magnet, add 300 µL of Wash Buffer (See Wash Buffer Solution preparation on page 2). Pipette mix 5 times to resuspend the beads. Incubate at room temperature for 1 minute.**

14. **Place plate/tube onto the magnet and separate for 10 minutes or until solution turns clear. Slowly aspirate the cleared solution from the plate/ tube and discard.**

    This step must be performed while the plate/ tube is situated on the magnet. Do not disturb the separated magnetic beads. If beads are drawn out, leave a few microliters of supernatant behind to avoid bead carryover

15. **Off the magnet, add 1000 µL of 85% ethanol. Pipette mix 5 times with a volume of 750 µL to wash the beads.**

16. **Separate on the magnet for 5 minutes. Carefully aspirate and discard the cleared supernatant.**

    If beads are drawn out, leave a few microliters of supernatant behind to avoid bead carryover.

17. **Let the beads air dry for 5 minutes. The plate/tube should air dry until the last visible traces of ethanol evaporate.**

18. **Prepare fresh DNase solution and discard any unused solution.**

    Combine 80 µL nuclease free water, 10 µL 10X DNase buffer and 10 µL of DNase I.

19. **Add 100 µL of DNase solution with the plate OFF the magnet. Pipette mix 5 times to resuspend the beads in the DNase solution.**

20. **Incubate plate/tube in a 37°C water bath for 15 minutes to facilitate digestion of DNA.**

    Keep the incubator cover open since the plate is not sealed.

21. ***DO NOT REMOVE THE DNase SOLUTION.*** **Add 550 µL of Wash Buffer Solution (See Wash Buffer Solution preparation on page 2), mix 5 times with a volume of 400 µL. Incubate at room temperature for 5 minutes.**

22. **Place plate/tube onto the magnet and separate for 10 minutes. Slowly aspirate the cleared solution from the plate/ tube and discard.**

    This step must be performed while the plate/ tube is situated on the magnet. Do not disturb the separated magnetic beads. If beads are drawn out, leave a few microliters of supernatant behind to avoid bead carryover.

23. **Off the magnet, add 750 µL of 85 % ethanol. Pipette mix 5 times with a volume of 500 µL to resuspend the beads.**

24. **Separate on the magnet for 5 minutes. Carefully aspirate and discard the cleared supernatant.**

    If beads are drawn out, leave a few microliters of supernatant behind to avoid bead carryover.

25. **Off the magnet, add 350 µL of 90% isopropanol. Pipette mix 5 times with a volume of 400 µL to resuspend the beads. Incubate in a 70°C water bath for 3 minutes.**

26. **Separate on the magnet for 3 minutes. Aspirate and discard the cleared supernatant.**

27. **Repeat steps 25-26 for a total of 2 heated isopropanol washes.**

28. **Off the magnet, add 750 µL of 85% ethanol. Pipette mix 5 times with a volume of 500 µL to resuspend the beads.**

29. **Separate on the magnet for 5 minutes, then carefully aspirate and discard the cleared supernatant.**

30. **Let the beads air dry for 10 minutes.**

    The plate/tube should air-dry until the last visible traces of ethanol evaporate. Over-drying the sample may result in a lower recovery.

31. **Off the magnet, add 40 µL of nuclease-free H2O. Resuspend the beads by pipette mixing 5 times. Incubate the plate/tube at room temperature for 1 minute.**

    Keep the incubator cover open since the plate is not sealed.

    The elution volume should be at least 40 µL to ensure complete elution. Samples that produce higher yields require larger elution volumes due to potential bead carryover during the final transfer. Optimal elution volumes may need to be determined experimentally.

32. **Separate on the magnet for 1 minute, then transfer the eluant to a suitable 96 well plate or a fresh tube for storage.**

    Wait for the solution to clear before transferring sample. When not in use, store the samples at -20°C.

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