Agencourt RNAdvance Blood Kit for Free Circulating DNA and miRNA/RNA Isolation from 200-300µL of Plasma and Serum

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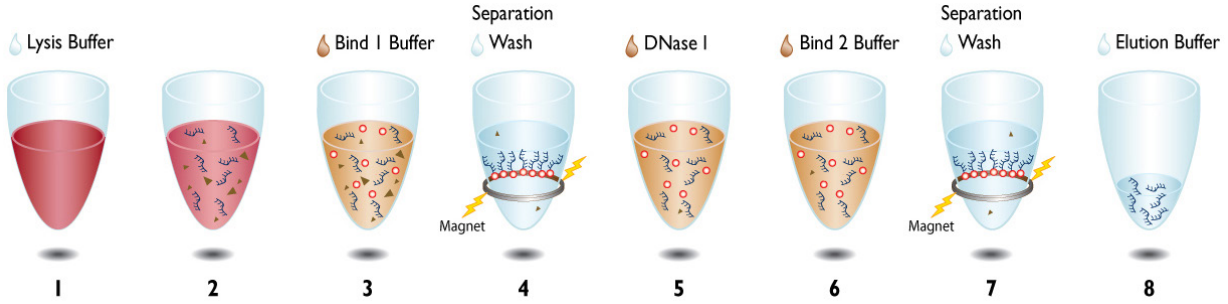
Process Overview



Introduction

The Agencourt RNAdvance Blood RNA purification kit utilizes Beckman Coulter's Agencourt SPRI paramagnetic bead-based technology to isolate total RNA from PAXgene preserved blood. The protocol is modified to isolate circulating cell free DNA (cfDNA), micro RNA (miRNA) and RNA from 200µL-300µL of plasma or serum per well in 96-well or 1.7mL tube formats.

Note for circulating DNA and miRNA/RNA extraction

If the RNAdvance Blood Kit is to be used for cfDNA and/or microRNA/RNA isolation:

- **100% Isopropanol must not be added directly to the Wash Buffer bottle.** See Reagent Preparation for Wash Buffer preparation.
- 500 µL of Isopropanol is added in the Bind1 buffer at step 4 instead of 400 µL (see Reagent Preparation step 3).
- 75% ethanol should be used instead of 70% ethanol in all ethanol washes.
- For faster magnetic bead separation, we recommend the use of the Magnum EX Universal Magnet Plate (ALPAQUA, P/N A000380)

Warnings and Precautions

Please refer to the RNAdvance Blood kit protocol, B66715, at www.beckmancoulter.com/ifu.

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General Remarks on Handling RNA

RNases are ubiquitous and general precautions should be followed in order to avoid their introduction to your sample during the RNAdvance Blood kit extraction procedure. 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 used 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 shared 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 from stock buffers. This lowers the risk of contaminating the stock solution.
- Wipe down work surfaces with commercial RNase-inhibiting surfactant solutions or 85% ethanol before starting work.

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)) or Magnum EX Universal Magnet Plate (ALPAQUA A000380)
- 1.7 mL microcentrifuge tubes (Eppendorf, 022364111) for tube format
- 96-Well Riplate-2.2mL (Ritter Medical, 43001-0200) for plate format
- 37°C and 55°C water bath or heat block for proteinase K digestion and DNase treatment
- Plate Seals (ThermoFisher #AB-580)

Reagents:

- 100% Isopropanol; American Bioanalytical AB07015 or equivalent
- 75% ethanol; freshly prepared/diluted from 100% ethanol (American Bioanalytical, AB00138 or equivalent)
- Reagent grade water, nuclease-free; Ambion AM9932
- RNase (Sigma R6513)

Reagent Preparation

Prepare the following reagents in advance for both the 96-well and 1.7 mL tube protocols:

1. **Add PK Buffer to the Proteinase K tube/bottle** (final concentration is 50mg/mL):
   For the 50 prep kit, add 1.2 mL of PK Buffer per tube of Proteinase K.
   For the 384 prep kit, add 10 mL of PK Buffer to the bottle of Proteinase K.
   Mix components by inverting the tube/bottle several times. To avoid foaming, do not vortex. The solution will appear cloudy immediately after mixing - let the solution sit for 5 minutes to clear prior to using. **Store the Proteinase K solution at -20°C when not in use.**

2. **Prepare Wash Buffer:**
   For circulating DNA, miRNA/RNA isolation: Prepare Wash Buffer in a new tube (not supplied) by adding 100% Isopropanol to the Wash Buffer in a proportion of 1:1 (Isopropanol: Wash Buffer). **Example:** To make 10 mL of Wash Buffer solution, add 5 mL of 100% Isopropanol with 5 mL of Wash Buffer in a 15 mL conical tube, and vortex thoroughly for 10 sec.
   For total RNA isolation only: Prepare Wash Buffer in a new tube (not supplied) by adding 100% Isopropanol to the Wash Buffer in a proportion of 1: 1.5 (Isopropanol: Wash Buffer). **Example:** To make 10 mL of Wash Buffer solution, add 4 mL of 100% Isopropanol with 6 mL of Wash Buffer in a 15 mL conical tube, and vortex thoroughly for 10 sec.

3. **Prepare Bind 1/Isopropanol Solution:**
   Shake the Bind 1 Bottle vigorously to resuspend the magnetic particles before aspirating.
   Prepare this solution fresh and per isolation – use within 15 minutes. Discard any unused solution.
   510 µL of Bind 1/Isopropanol Solution is required per sample. Vortex the tube/bottle containing the Bind 1 Buffer for at least 30 seconds to fully resuspend the beads. Combine 10 µL of Bind 1 Buffer with 500 µL 100% isopropanol and mix thoroughly.

4. **Prepare Lysis/PK Solution:** (use within 15 minutes and discard any unused solution):
   For each sample, combine 20 µL PK with 300 µL of Lysis Buffer. **Example:** For 10 isolations mix 3.0 mL of Lysis with 200 µL of proteinase K. (It is generally recommended to prepare an additional 10% to account for dead volume.)
   Note: Pipette enzyme directly into the liquid and mix up and down by pipetting to remove any residual enzyme from the inside of the tip. A light vortex can be done to ensure homogeneity, but avoid foaming.

Plasma and Serum miRNA Isolation Procedure

Prepare plasma supernatant:

For fresh plasma: centrifuge blood samples for 10 minutes at 1900xg (3000 rpm) at 4°C. Carefully aspirate plasma supernatant without disturbing the buffy coat layer. Centrifuge plasma samples for an additional 15 minutes at 16,000xg (in a fixed-angle rotor) at 4°C setting to remove any additional cellular debris and/or contamination of the circulating nucleic acids by genomic DNA and RNA derived from damaged blood cells.

For frozen plasma: thaw frozen tubes of supernatant cell free serum/plasma at room temperature. Centrifuge the plasma at 16,000xg (in a fixed-angle rotor) at 4°C for 5 minutes to remove any precipitates.

1. Aliquot 200-300 µL of plasma or serum into each well of a 2.2 mL processing plate or 1.7mL tube.

2. Add 320 µL of Lysis/PK Solution (see Reagent Preparation, step 4) to each sample, either in a tube or 96-well plate. Gently mix by pipetting up and down for 10 times at the bottom of the well to re-suspend the sample mix.

3. Seal the plate with a plate seal. Incubate the samples for 15 minutes at 55°C (or shake at 1000rpm for 30 minutes at room temperature) to complete the lysis and digestion.

   Before proceeding to the next step, let the samples cool for 2 minutes to room temperature if digestion was performed at 55°C.

   Note: *When using this plate in conjunction with a water bath, make sure the plate does not tip over and the seal does not get wet. Should the seal get wet or condensation form on the seal, spin the liquid down and very carefully remove the seal.*

4. Prepare Bind 1/Isopropanol Solution as described in Reagent Preparation, step 3. Add 510 µL of Bind 1/Isopropanol Solution to the samples and mix by pipetting up and down for 10 times. Incubate samples at room temperature for 5 minutes.

5. Place the 2.2 mL processing plate on an Agencourt SPRIPlate 96R –Ring Super Magnet Plate or Magnum EX Universal Magnet Plate (or place 1.7mL tubes on SPRIStand) and separate for 15 minutes, or wait for the solution to turn completely clear.

6. Fully remove supernatant from the 2.2 mL processing plate (or the 1.7mL tube) and discard.

   This step must be performed while the 2.2 mL processing plate or the tube is situated on the magnet.

7. Remove the 2.2 mL processing plate (or the 1.7mL tube) from the magnet and wash the beads by adding 800µL of Wash Buffer (see Reagent Preparation, step 2). Mix by pipetting up and down for10 times to re-suspend the magnetic beads.

8. Place the 2.2 mL processing plate (or the 1.7mL tube) on the magnet and separate for 8 minutes, or wait for the solution to turn completely clear.

9. Completely remove the supernatant from the 2.2 mL processing plate (or 1.7mL tube) and discard.

   This step must be performed while the plate is situated on the magnet. Do not disturb the ring of separated magnetic beads.

10. Remove the 2.2 mL processing plate (or the 1.7mL tube) from the magnet and add 800 µL of 75% ethanol. Mix by pipetting up and down for10 times to re-suspend the magnetic beads.

11. Return the 2.2 mL processing plate (or the 1.7mL tube) to the magnet for 5 minutes, or wait for the solution to turn completely clear. Remove the supernatant and discard.

12. Remove as much ethanol as possible and allow magnetic beads to dry for 5 minutes at room temperature.

    Optional: RNase digestion. ***Skip step 12 if no RNase treatment is required.***

    Remove the 2.2 mL processing plate from the magnet and add 100 µL of 20ng/uL RNase solution (Sigma R6513, prepare reagent to 20ng/uL). Mix by pipetting up and down for 5 times carefully to avoid bubbles and foaming.

    Seal plate with a plate seal and incubate 2.2 mL processing plate (or 1.7mL tube) in a water bath for 15 minutes at 37°C.

    DO NOT REMOVE THE RNase SOLUTION. Add 200 µL of Bind 2 Buffer plus 400 µL of 100% Isopropanol and mix by pipetting up and down for 10 times.

13. Repeat steps 10-11 twice for a total of three 75% ethanol washes.

14. Remove as much ethanol as possible and allow the magnetic beads to dry for 5 minutes at room temperature.

    Beads do not need to be completely dry, but the traces of liquid should be gone (i.e. droplets or puddles).

15. Remove 2.2 mL processing plate (or 1.7mL tube) from the magnet and elute nucleic acids by adding of 40 µL of nuclease free water. Mix by pipetting up and down for 10 times and incubate at room temperature for 2 minutes.

16. Return the plate to the magnet for 2 minutes and carefully transfer eluted nucleic acids away from the beads and into a fresh plate or tube and store samples at -80°C.

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