Agencourt® RNAdvance® Blood

Total RNA Isolation from PAXgene Preserved Blood

Please refer to http://www.agencourt.com/technical for updated protocols and refer to MSDS instructions http://www.beckmancoulter.com/customersupport/msds/msds.asp when handling or shipping any chemical hazards.

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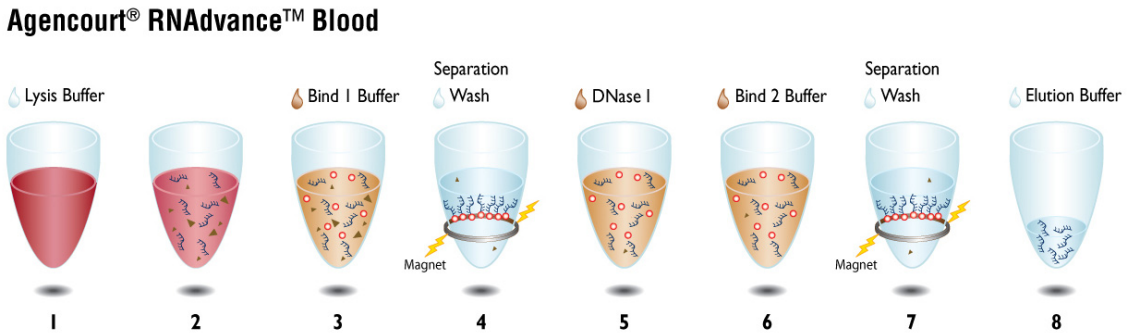
Agencourt RNAdvance Blood: Table of Contents

Introduction 1
Process Overview 2
Kit Specifications 2
Working Under RNase Free Conditions 2
Materials Supplied in the Kit 3
Materials Supplied by the User 4
Calculation of Yield 4
Procedure 5
Part A - Reagent Preparation 5
Part B - Agencourt RNAdvance Blood 96 Well Plate Protocol 6
Part C - Agencourt RNAdvance Blood 2 mL Tube Protocol 8

Introduction

The Agencourt RNAdvance Blood total RNA purification kit utilizes Agencourt's patented Agencourt SPRI® paramagnetic bead-based technology to isolate total RNA from PAXgene[1] preserved blood. The protocol can be performed in both 96-well and single tube formats. Purification begins with lysis and protein digestion. Following lysis, the RNA is immobilized onto the magnetic particles allowing separation from contaminants using a magnetic field. The RNA is then treated with DNase and the contaminants rinsed away using a simple wash procedure. The Agencourt RNAdvance Blood kit is amenable to automation as it utilizes magnetic separation, thus eliminating the need for vacuum filtration or centrifugation. The following protocols are used for the isolation of total RNA from 400 µL of PAXgene preserved blood per well in 96 well and 2 mL tube formats.

Process Overview



1. **Add Lysis buffer to PAXgene blood and mix**
2. **Lysis and Proteinase K digestion**
3. **Addition of Bind 1 Buffer**
4. **Magnetic Separation of beads from supernatant, wash with Wash Buffer and Ethanol**
5. **DNase I reaction**
6. **Rebinding with Bind 2 Buffer**
7. **Magnetic Separation of beads from supernatant, wash with Ethanol**
8. **Elution**

Kit Specifications

The Agencourt RNAdvance Blood kit is manufactured under RNase-free conditions and has been tested and certified not to contain contaminating nucleases. The Agencourt RNAdvance Tissue kit can be used in 96 well and single tube formats.

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 Blood 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 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.

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- 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

Materials Supplied in the Kit

RNAdvance Blood reagents have a shelf life of 6 months when stored as directed:

Reagent	Storage Conditions on Arrival	Storage Conditions once In Use (Isopropanol, PK or buffer added)
Lysis	Room Temperature	Room Temperature
Bind 1	4°C	4°C
Wash	Room Temperature	Room Temperature
Bind 2	4°C	4°C
Proteinase K (PK)	-20°C	-20°C
PK Buffer	Room Temperature	-20°C (combined with PK)

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Materials Supplied by the User

Consumables and Hardware

***For 96 well format*:**

- **Agencourt SPRIPlate 96R Ring Super Magnet Plate** (Beckman #A32782/Agencourt #000322); (http://www.agencourt.com)
- **Processing Plates: 2.2 ml 96 well plate** (ABGene #AB-0661) **and**
  1.2 ml 96 well plate (ABGene #AB-1127; http://www.abgene.com)
- **Plate Seals;** (ABGene #0580; http://www.abgene.com)
- **Liquid handling robotics or a multi-channel hand pipette**
- **55ºC and 37ºC Water Baths**

For single tube format:

- **Agencourt SPRIStand (Tube kit only)** (Beckman #A29182/ Agencourt #001139; http://www.agencourt.com)
- **2.2ml microcentrifuge tubes** (Axygen Inc. #MCT-200-L-C; http://www.axygen.com/)
- **55ºC and 37ºC Water Baths**

Reagents (96 well and tube formats):

- **100% Isopropanol**
- **70% Ethanol made with nuclease free water** (*Note: 70% Ethanol is hygroscopic, prepare fresh 70% Ethanol regularly for optimal results*) American Bioanalytical # AB-00138; http://www.americanbio.com/]
- **DNase I (RNase-Free) & DNase I Buffer (**2 U/µL); Ambion #AM2224 (contains both components), http://www.ambion.com
  - **OR DNase I (RNase-free)** (2 U/µL); Ambion #AM2222 **AND DNase I 10X Buffer** Ambion #AM8107G, http://www.ambion.com
- **Reagent grade water, nuclease-free** (Ambion #AM9932; http://www.ambion.com)

Calculation of Yield

To determine yield of RNA, Agencourt recommends using OD260 measurement or visualization on agarose gel. These methods will give the most accurate quantitation of RNA. To determine RNA quality, dilute samples to 1-2 ng/µL for analysis using the Agilent 2100 Bioanalyzer PicoChip[1] assay.

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Procedure

Part A - Reagent Preparation

Prepare the following reagents in advance for both the 96 well and 2 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. 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. **Add 100% isopropanol to Wash Buffer bottle:**

   -For the 50 prep kit, add 30 mL of 100% isopropanol.
   -For the 384 prep kit, add 225 mL of 100% isopropanol.
   Following isopropanol addition, write the date on the label of the bottle and store the Wash Buffer at room temperature.

3. **Prepare Bind 1/Isopropanol Solution:**

   410 µL of Bind 1/Isopropanol Solution are required per sample. Vortex the tube containing Bind 1 Buffer *for at least 30 seconds* to fully resuspend the beads. Combine 10 µL Bind 1 Buffer with 400 µL 100% isopropanol and mix thoroughly. The Bind 1/Isopropanol Solution must be prepared fresh on the day of the experiment. Discard any unused portion.

4. **Prepare DNase Solution:**

   100 µL of 1X DNase solution are required per sample. Combine 85 µL nuclease-free water, 10 µL 10X DNase buffer, and 5 µL of DNase I. Make this solution fresh for each set of samples.

Part B - Agencourt RNAdvance Blood 96 Well Plate Protocol

Thaw frozen tubes of PAXgene blood at room temperature. **Cap the tubes tightly** then mix by inverting each tube several times or by vortexing.

1. **Aliquot 400 µL of PAXgene preserved blood into each well of a 2.2 mL processing plate.**

2. **Add Proteinase K and Lysis Buffer:**

   - Add 20 µL of Proteinase K (50mg/mL, prepared in Part A step 1)
   - Add 300 µL of Lysis Buffer

   Mix thoroughly by pipetting up and down 10 times.

3. **Lysis and Protein Digestion:**

   Seal plate with a plate seal. Incubate samples in water bath at 55°C for 15 minutes. Before proceeding to the next step, let the samples cool for 2 minutes to room temperature.

   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. **Add 410 µL Bind 1/Isopropanol Solution (prepared in Part A Step 3) to the samples and pipette mix 10 times. Incubate samples at room temperature for 5 minutes.**

   Shake or tipmix Bind 1/Isopropanol Solution to disperse beads before adding to sample.

5. **Place 2.2 mL processing plate on Agencourt SPRIPlate 96R Super Magnet Plate and separate for 10 minutes.**

6. **Fully remove supernatant from the 2.2 mL processing plate and discard.**

   This step must be performed while the 2.2 mL processing plate is situated on the magnet. **The following technique is recommended when working with opaque supernatant:** Place the pipette tip on the side of the well and carefully aspirate the liquid by following the liquid level down until approximately 200-250 µL remains in the well. Next, carefully place the pipette tip at the center of the bottom of the well and slowly aspirate the remaining liquid, revealing the ring of beads.

7. **Remove the 2.2 mL processing plate from the magnet and wash the beads by adding 800 µL of Wash Buffer. (Isopropanol must be added to Wash Buffer before using the kit for the first time – See Part A step 2).**

   Pipette mix 10 times to resuspend the magnetic beads.

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8. **Transfer the suspension to a 1.2 mL processing plate. Be sure to transfer all of the sample and magnetic beads to the new plate.**

   Transferring the samples to the smaller plate allows for easier pipetting in subsequent steps.

9. **Place 1.2 mL processing plate on the magnet and separate for 7 minutes.**

   Wait for the solution to clear before proceeding to the next step.

10. **Completely remove supernatant from the 1.2 mL processing plate and discard.**

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

11. **Remove the 1.2 mL processing plate from the magnet and add 800 µL 70% ethanol. Pipette mix 5 times to resuspend the magnetic beads.**

12. **Return 1.2 mL processing plate to the magnet for 3 minutes.**

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

    Pipette slowly to avoid disturbing the beads. If too much ethanol is present (more than 5 µL), the DNase digestion will be inhibited, thereby affecting downstream applications.

14. **Remove the 1.2 mL processing plate from the magnet and add 100 µL of DNase solution (prepared in Part A Step 4). Pipette mix 5 times carefully - avoid bubbles and foaming.**

15. **Seal plate with a plate seal and incubate 1.2 mL processing plate in water bath for 15 minutes at 37ºC.**

16. ***DO NOT REMOVE THE DNase SOLUTION*. Add 200 µL of Bind 2 Buffer and pipette mix 10 times. Incubate at room temperature for 5 minutes.**

    Note: *Do not mistake Bind 2 for Bind 1. Bind 2 does not contain magnetic beads or require isopropanol.*

17. **Place 1.2 mL processing plate onto the magnet for 5 minutes.**

    Wait for the solution to clear before proceeding to the next step.

18. **Remove supernatant and discard. Wash by adding 800 µL of 70% ethanol. Do Not Pipette Mix. Let sit for approximately one minute and then remove ethanol while processing plate remains situated on the magnet plate.**

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19. **Repeat step #18 one more time for a total of 2 ethanol washes.**

20. **Allow magnetic beads to dry for 10 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).

21. **Remove 1.2 mL processing plate from the magnet and elute RNA by adding a minimum of 20 µL of nuclease free water. Pipette mix 10 times and incubate at room temperature for 2 minutes.**

    On average, a 20 µL elution will produce a 20-50 ng/µL solution of RNA.

22. **Return the plate to the magnet for 2 minutes and carefully transfer eluted RNA away from the beads and into a fresh plate for storage.**

Part C - Agencourt RNAdvance Blood 2 mL Tube Protocol

Thaw frozen tubes of PAXgene blood at room temperature. **Cap the tubes tightly** then mix by inverting each tube several times or by vortexing.

1. **Aliquot 400 µL PAXgene preserved blood into a 2 mL microcentrifuge tube.**

2. **Add Proteinase K and Lysis Buffer:**

   - Add 20 µL of Proteinase K (50mg/mL, prepared in Part A step 1)
   - Add 300 µL of Lysis Buffer

   Mix thoroughly by pipetting up and down 10 times.

3. **Lysis and Protein Digestion:**

   Cap the tube. Incubate samples in water bath at 55°C for 15 minutes. Before proceeding to the next step, let the samples cool for 2 minutes to room temperature.

4. **Add 410 µL Bind 1/Isopropanol Solution (prepared in Part A Step 3) to the samples and mix by vortexing the tube. Incubate samples at room temperature for 5 minutes.**

   Shake or tipmix Bind 1/Isopropanol solution to disperse beads before adding to sample.

5. **Place tubes on an Agencourt SPRIstand (6 Position Tube Magnet) and separate for 10 minutes.**

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6. **Fully remove supernatant from tube and discard.**

   This step must be performed while the tube is situated on the magnet stand. **The following technique is recommended when working with opaque supernatant:** Place the pipette tip on the side of the tube opposite the magnet and carefully aspirate the liquid while following the liquid level down.

7. **REMOVE tube from the magnet stand and wash the beads by adding 800 µL Wash Buffer. (Isopropanol must be added to Wash Buffer before using the kit for the first time – See Part A step 2.)**

   Pipette mix 10 times to resuspend the magnetic beads.

8. **Transfer the suspension to a fresh 2 mL tube. Be sure to transfer all of the sample and magnetic beads to the new tube.**

   Transferring is important so later steps of the protocol won't be contaminated with blood solution that may have collected on the lid of the first tube.

9. **Place the tube on the magnet stand and separate for 5 minutes.**

   Wait for the solution to clear before proceeding to the next step.

10. **Completely remove supernatant from the tube and discard.**

    This step must be performed while the tube is situated on the magnet stand. Place the pipette tip at the bottom of the tube and carefully aspirate the liquid so the layer of separated magnetic beads on the side of the tube is not disturbed.

11. **Leave tube on the magnet stand and add 800 µL 70% ethanol. Let sit for approximately one minute and then remove ethanol while the tube is on the magnet stand.**

12. **Remove as much ethanol as possible from the bottom of the tube and dry for 5 minutes at room temperature.**

    Pipette slowly to avoid disturbing the beads. If too much ethanol is present (more than 5 µL), the DNase digestion will be inhibited thereby affecting downstream applications.

13. **Remove the tube from the magnet and add 100 µL of DNase solution (prepared in Part A Step 4). Pipette mix 5 times carefully - avoid bubbles and foaming.**

14. **Cap the tube. Incubate the samples in water bath at 37ºC for 15 minutes.**

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15. ***DO NOT REMOVE THE DNase SOLUTION*. Add 200 µL of Bind 2 Buffer and pipette mix 10 times. Incubate at room temperature for 5 minutes.**

    Note: *Do not mistake Bind 2 for Bind 1. Bind 2 does not contain magnetic beads or require isopropanol.*

16. **Place the tube on the magnet stand for 5 minutes.**

    Wait for the solution to clear before proceeding to the next step.

17. **Remove supernatant and discard. Wash by adding 800 µL of 70% ethanol. Do Not Pipette Mix. Let sit for approximately one minute and then remove ethanol while tube remains situated on the magnet stand.**

18. **Repeat step #16 one more time for a total of 2 ethanol washes.**

19. **Allow magnetic beads to dry for 10 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).

20. **Remove the tube from the magnet stand and elute RNA by dissolving the beads pellet in a minimum of 20 µL of nuclease free water. Pipette mix 10 times and incubate at room temperature for 2 minutes.**

    On average, a 20 µL elution will produce a 20-50 ng/µL solution of RNA.

21. **Return the tube to the magnet stand for 2 minutes and carefully transfer eluted RNA away from the beads and into a fresh tube for storage.**

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