AGENCOURT® RNACLEAN® XP

IN VITRO PRODUCED RNA AND CDNA PURIFICATION

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

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Introduction

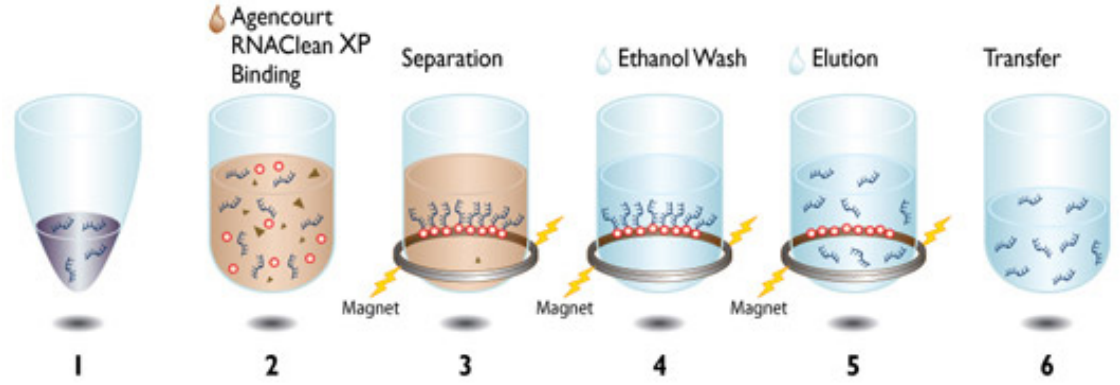
The Agencourt RNAClean XP system utilizes Agencourt's solid-phase paramagnetic bead technology for high-throughput purification of RNA or cDNA from in vitro applications such as transcription, antisense RNA (aRNA) amplification and RNA and cDNA probe synthesis. Agencourt RNAClean XP utilizes an optimized buffer to selectively bind RNA or cDNA to paramagnetic beads. Excess oligonucleotides, nucleotides, salts, and enzymes can be removed using a simple washing procedure. The resulting purified product can be used in the following applications:

- PCR[1] and RT-PCR
- Probes for microarray or macroarray
- RNase protection assays
- Transfection for RNAi experiments
- cDNA synthesis and labeling

[1] *The PCR process is covered by patents owned by Roche Molecular Systems, Inc., and F. Hoffman-La Roche, Ltd.*

The cleanup procedure is highly amenable to a variety of automation platforms because it utilizes magnetic separation and does not require centrifugation or vacuum filtration. More information on automating the Agencourt RNAClean XP kit can be obtained from Agencourt support at support@Agencourt.com or 1-800-773-9186.

Process Overview:



1. Enzymatic reaction
2. Binding of total RNA to magnetic beads
3. Separation of total RNA bound to magnetic beads from contaminants
4. Washing of RNA with Ethanol
5. Elution of RNA from the magnetic particles
6. Transfer away from the beads into a new plate

Kit Specifications:

The Agencourt RNAClean XP kit is manufactured under RNase-free conditions and has been tested and certified not to contain contaminating nucleases.

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 RNAClean XP 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.
- 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 Agencourt RNAClean XP Kit:

Agencourt RNAClean XP Magnetic Particle Solution

- Store at 4°C upon arrival, for up to 12 months. For best results shake the reagent well until all of the beads are completely in suspension and aliquot Agencourt RNAClean XP into RNase free containers. Do not pour remaining reagent back into the storage container.
- Mix Agencourt RNAClean XP well before use. The reagent should appear homogenous and consistent in color.
- DO NOT FREEZE.

Materials Supplied by the User:

Consumables and Hardware:

- **Agencourt SPRIPlate® magnetic plate:**
  - For 96 well format: Agencourt SPRIPlate 96 ring super magnetic plate [Agencourt #A32782; http://www.agencourt.com/]
  - For 384 well format: Agencourt SPRIPlate 384 magnetic plate [APN #A29165; http://www.agencourt.com/]
  - For single tube format: Agencourt SPRIstand magnetic tube rack [APN #A29182; http://www.agencourt.com/]
- **Reaction Plate:**
  - For 96 well format: 96 well 300µL round bottom microtiter plate [Costar #3795 http://www.vwrsp.com/] or 96 well PCR cycle plate [ABGene #AB-0800 http://www.abgene.com/]

For questions regarding this protocol, call Technical Support at Agencourt 1-800-773-9186

For 384 well format: 384 well 40µL PCR cycle plate [For Automation: MJ Research Hard-Shell™ PCR plate # HSP-3801; http://www.mjr.com/html/consumables/microplates/hard_shell.html]; or ABGene 384 well PCR cycle plate #AB-0937 (http://www.abgene.com/), will require manual intervention]

For single tube format: 1.7 ml tubes, RNase-free (ABGene #T5050G; http://www.abgene.com)

- Plate seals, adhesive or heat [for example: ABgene product # AB-3739; http://www.abgene.com/]
- Liquid handling robotics or a multi-channel hand pipette

Reagents:

- Fresh 70% ethanol, RNase free (*Note: 70% ethanol is hygroscopic. Fresh 70% ethanol should be prepared for optimal results)*
- Reagent grade water, RNase free [American Bioanalytical Product # AB02128; http://www.americanbio.com/]

Calculation of Percent Recovery:

To gauge percent recovery of RNA or cDNA, analyses of the samples pre-purification and post-purification are necessary. For RNA, Agencourt recommends either a RiboGreen®[2] assay or visualization on agarose gel. Spectrophotometric analysis using Optical Density (OD) at 260 nm is discouraged. At 260 nm both single and double-stranded nucleic acids will contribute to the overall absorbance reading. For the pre-purification sample, single-stranded primers, template DNA and rNTPs will contribute to the initial absorbance and give a falsely inflated reading of the quantity of RNA. By contrast, the RiboGreen assay uses an intercalating dye to quantitate RNA. When taking a RiboGreen reading pre-purification, primers and rNTPs will not falsely inflate the reading. However, RiboGreen will also fluoresce in the presence of DNA, so a reading should be taken only after the sample has been treated with a DNase I digestion step. This enables a more accurate quantization of recovery. For similar reasons, a PicoGreen® assay is recommended for analysis of DNA.

In addition to RiboGreen/PicoGreen readings, visualization of the sample pre- and post-purification on agarose gel with ethidium bromide is recommended, but will give a more subjective quantization. For most accurate results, run both pre- and post-purified samples on the same gel to minimize differences in electrophoresis parameters and imaging processes.

Procedure:

[2] RiboGreen & PicoGreen are available from Molecular Probes® http://www.probes.com/

96 Well Format for Small Volume Reactions (1-100 µL):

1. **Determine whether or not a plate transfer is necessary.**

   PCR plates generally have a maximum volume of 200 µL. If the reaction volume multiplied by 2.8 exceeds the volume of the PCR plate, a transfer to a 300µL round bottom plate is required. For 200 µL PCR plates the maximum reaction volume is 71µL with 127.8 µL Agencourt RNAClean XP reagent. Agencourt recommends the 300 µL Costar 3795 plate for use with the Agencourt RNAClean XP kit. A 300 µL microtiter plate can accommodate up to 100 µL of reaction volume and 180 µL of Agencourt RNAClean XP reagent. Reaction volumes larger than 100µL can be purified using the protocol "**96 Well Format for Large Volume Reactions (101-200 µL)**".

2. **Shake the Agencourt RNAClean XP bottle to resuspend any magnetic particles that may have settled. Add Agencourt RNAClean XP according to the reaction volume chart below:**

Reaction Volume (µL)	Agencourt RNAClean XP Volume (µL)
20	36
50	90
100	180

   The volume of Agencourt RNAClean XP for a given reaction can be derived from the following equation:

   (Volume of Agencourt RNAClean XP per reaction) = 1.8 x (Reaction Volume)

3. **Mix the Agencourt RNAClean XP and reaction thoroughly by pipette mixing 10 times or vortexing for 30 seconds.**

   This step binds RNA/cDNA products to the magnetic beads. Pipette mixing is preferable as it tends to be more reproducible. If vortexing is used, the plate must be sealed with a plate seal before vortexing. The color of the mixture should appear homogenous after mixing. Before moving the plate onto the magnet plate, let the mixed samples incubate for 3-5 minutes at room temperature for maximum recovery. For reactions 50 µL and larger, a 5 minutes incubation is strongly recommended and for the purification of single stranded DNA an incubation of up to 20 minutes can increase recovery.

4. **Place the reaction plate onto an Agencourt SPRIPlate 96 Super Magnet Plate for 5 to 10 minutes to separate beads from solution.**

   The separation time is dependent on the size of the reaction. Larger reaction sizes will require more time for separation. Wait for the solution to clear before proceeding to the next step.

5. **Slowly aspirate the cleared solution from the reaction plate and discard.**

   This step must be performed while the reaction plate is situated on the Agencourt SPRIPlate 96 Super Magnet Plate. Do not disturb the ring of separated magnetic beads.

6. **Dispense 200 µL of 70% ethanol to each well of the reaction plate and incubate for 30 seconds at room temperature. Aspirate out the ethanol and discard. Repeat for a total of three washes.**

   It is important to perform these steps with the reaction plate situated on an Agencourt SPRIPlate 96 Super Magnet Plate. Do not disturb the separated magnetic beads. Be sure to remove all of the ethanol from the bottom of the well as it may contain residual contaminants.

7. **Let the reaction plate air-dry for 10 minutes.**

   The plate should air-dry until the last visible traces of ethanol evaporate. Over-drying the sample may result in a lower recovery. If the samples will be used immediately, proceed to Step 8 for elution. If the samples will not be used immediately, the dried plate may be sealed and stored at 4°C or -20°C. If long term freezing of the samples is required, Agencourt recommends transferring the samples away from the beads into a new plate, as the beads can shatter and fragments may no longer respond to the magnetic field.

8. **Add 40 µL of RNase-free water to each well of the reaction plate and seal to vortex 30 seconds or pipette mix 10 times.**

   The ring of magnetic beads forms approximately 30-40 µL from the bottom of the well. A greater volume of elution buffer can be used, but using less than 30µL will necessitate extra vortexing, to ensure the liquid comes into contact with the beads, and it may not be sufficient to fully elute the product. RNase free water is the recommended elution buffer. Elution is rapid and it is not necessary for the beads to go back into solution for complete elution to occur.

When setting up downstream reactions or transferring the samples, pipette the eluant from the plate while it is situated on the Agencourt SPRIPlate 96 Super Magnet Plate. This will prevent bead carry over.

96 Well Format for Large Volume Reactions (101-200 µL):

Considering the 300µL capacity of standard 96-well microtiter plates, the following protocol employs two serial Agencourt RNAClean XP binding reactions/separation events per sample. Both purifications occur within the same well. Reaction volumes smaller than 100µL can be purified using the protocol "**96 Well Format for Small Volume Reactions (1-100 µL)**".

1. **Transfer half the volume of the unpurified reaction into a 300 µL round bottom microtiter plate.**

   Agencourt recommends the Costar 3795 microtiter plate for use with the Agencourt RNAClean XP kit.

2. **Shake the Agencourt RNAClean XP bottle to resuspend any magnetic particles that may have settled. Based on the half-volume of the reaction, add Agencourt RNAClean XP according to the chart below:**

Half Reaction Volume (µL)	Agencourt RNAClean XP Volume (µL)
60	108
75	135
100	180

   The volume of Agencourt RNAClean XP for a given reaction can be derived from the following equation:

   (Volume of Agencourt RNAClean XP per reaction) = 1.8 x (Reaction Volume)

3. **Mix the Agencourt RNAClean XP and reaction thoroughly by pipette mixing 10 times or vortexing for 30 seconds. Let the plate incubate at room temperature for 5 minutes before proceeding to the next step.**

   This step binds RNA (or cDNA) products to the magnetic beads. Pipette mixing is preferable as it tends to be more reproducible. If vortexing is used, the plate must be sealed with a plate seal before vortexing. The color of the mixture should appear homogenous after mixing. For the

purification of single stranded DNA an incubation of up to 20 minutes can increase recovery.

4. **Place the reaction plate onto an Agencourt SPRIPlate 96 Super Magnet Plate for 5 to 10 minutes to separate beads from solution.**

   The separation time is dependent on the size of the reaction. Larger reaction sizes will require more time for separation. Wait for the solution to clear before proceeding to the next step.

5. **Slowly aspirate the cleared solution from the reaction plate and discard.**

   This step must be performed while the reaction plate is situated on the Agencourt SPRIPlate 96 Super Magnet Plate. Do not disturb the ring of separated magnetic beads.

6. **Dispense the second half of the unpurified reaction into the same wells as the first half of the reaction.**

   Some beads will be disturbed by the addition of the second half of the reaction. This is expected and will not cause a problem.

7. **REMOVE THE MICROTITER PLATE FROM THE Agencourt SPRIPlate 96 Super Magnet Plate. Add the appropriate volume of Agencourt RNAClean XP according to the chart in Step 2 (1.8 µL x [Volume of Second Half of Reaction]).**

8. **Mix the Agencourt RNAClean XP and reaction thoroughly by pipette mixing 10 times or vortexing for 30 seconds. Let the plate incubate at room temperature for 5 minutes before proceeding to the next step.**

   This step binds RNA/cDNA products to the magnetic beads. Pipette mixing is preferable as it tends to be more reproducible. If vortexing is used, the plate must be sealed with a plate seal before vortexing. The color of the mixture should appear homogenous after mixing.

9. **Place the reaction plate back onto the Agencourt SPRIPlate 96 Super Magnet Plate for 5 to 10 minutes to separate beads from solution.**

   The separation time is dependent on the size of the reaction. Larger reaction sizes will require more time for separation. Wait for the solution to clear before proceeding to the next step.

10. **Slowly aspirate the cleared solution from the reaction plate and discard.**

This step must be performed while the reaction plate is situated on the Agencourt SPRIPlate 96 Super Magnet Plate. Do not disturb the ring of separated magnetic beads.

11. **Dispense 200 µL of 70% ethanol to each well of the reaction plate and incubate for 30 seconds at room temperature. Aspirate out the ethanol and discard. Repeat for a total of three washes.**

    It is important to perform these steps with the reaction plate situated on a Agencourt SPRIPlate 96 Super Magnet Plate. Do not disturb the separated magnetic beads. Be sure to remove all of the ethanol from the bottom of the well as it may contain residual contaminants.

12. **Let the reaction plate air-dry for 10 minutes.**

    The plate should air-dry until the last visible traces of ethanol evaporate. Over-drying the sample may result in a lower recovery. If the samples will be used immediately, proceed to Step 13 for elution. If the samples will not be used immediately, the dried plate may be sealed and stored at 4°C or -20°C. If long term freezing of the samples is required, Agencourt recommends transferring the samples away from the beads into a new plate, as the beads can shatter and fragments may no longer respond to the magnetic field.

13. **Add 40 µL of RNase-free water to each well of the reaction plate and seal to vortex 30 seconds or pipette mix 10 times.**

    The ring of magnetic beads forms approximately 30-40 µL from the bottom of the well. A greater volume of elution buffer can be used, but using less than 30µL will necessitate extra vortexing, to ensure the liquid comes into contact with the beads, and it may not be sufficient to fully elute all of the product. RNase free water is the recommended elution buffer. Elution is rapid and it is not necessary for the beads to go back into solution for complete elution to occur.

    When setting up downstream reactions or transferring the samples, pipette the eluant from the plate while it is situated on the Agencourt SPRIPlate 96 Super Magnet Plate. This will prevent bead carry over.

Single Tube Format:

1. **Transfer the reactions into 1.7 mL RNase-free tubes.**

   Plastic labware is generally considered RNase-free if not touched by human hands. However, Agencourt recommends using 1.7 mL tubes from ABGene [product #T5050G] which are certified RNase free.

2. **Gently shake the Agencourt RNAClean XP bottle to resuspend any magnetic particles that may have settled. Add Agencourt RNAClean XP according to the reaction volume chart below:**

Reaction Volume (µL)	Agencourt RNAClean XP Volume (µL)
50	90
100	180
150	270
200	360

   The volume of Agencourt RNAClean XP for a given reaction can be derived from the following equation:

   (Volume of Agencourt RNAClean XP per reaction) = 1.8 x (Reaction Volume)

3. **Mix the Agencourt RNAClean XP and sample thoroughly by pipette mixing 15 times. Let the tube incubate at room temperature for 5 minutes before proceeding to the next step.**

   This step binds RNA/cDNA products to the magnetic beads. For maximum binding and recovery the tube must be removed from the Agencourt SPRIstand magnetic tube rack. Vortexing is not recommended. The color of the mixture should appear homogenous after mixing. For purification of single stranded DNA an incubation of up to 20 minutes can increase recovery.

4. **Place the tube onto the Agencourt SPRIstand magnetic tube rack for 5 minutes to separate the beads from solution.**

   The separation time is dependent on the size of the reaction. Larger reaction sizes will require more time for separation. Wait for the solution to be clear before proceeding to the next step.

5. **Slowly aspirate the cleared solution from the tube and discard.**

This step should be performed while the tube is situated on the Agencourt SPRIstand. Do not disturb the magnetic beads, which have formed a spot on the side of the tube.

6. **Dispense 500 – 1000 µL of 70% ethanol into the tube and incubate for 30 seconds at room temperature. Aspirate out the ethanol and discard. Repeat for a total of three washes.**

It is important to perform these steps with the tube situated on the Agencourt SPRIstand. Do not disturb the separated magnetic beads. Be sure to remove all of the ethanol from the bottom of the well as it may contain residual contaminants.

NOTE: The volume of ethanol required for washing will depend on the size of original reaction. The wash solution must completely cover the entire bead mass on the side of the tube.

7. Let the reaction tube air-dry 10 minutes on the Agencourt SPRIstand with the cap open.

The tube(s) should air-dry until the last visible traces of ethanol evaporate. Over drying the sample may result in a lower recovery. If samples will be used immediately, proceed to Step 8 for elution. If the samples will not be used immediately, the tubes should be capped and stored at 4°C or -20°C. If long term freezing of the samples is required Agencourt recommends transferring the samples away from the beads into a new tube or plate, as the beads can shatter and fragments may no longer respond to the magnetic field.

8. Elute the purified product from the beads with RNase-free water.

Add an elution volume of at least 30 µL to the tube and manually resuspend the beads by pipetting up and down several times. Expel the elution buffer down the side of the tube to ensure the entire bead mass comes into contact with the buffer. A greater volume of elution buffer can be used, and may be helpful for samples with a large quantity of magnetic particles. Using less than 15 µL may not be sufficient to fully elute the product. Elution is rapid and it is not necessary for the beads to go back into solution for complete elution to occur.

When setting up downstream reactions or transferring the samples, pipette the eluant from the tube while it is situated on the Agencourt SPRIstand. This will prevent bead carry over.

384 Well Format:

1. **Determine whether the total volume of sample plus Agencourt RNAClean XP will fit in the well of a 384 well PCR plate.**

   Generally 384 well thermal cycling plates have a maximum well volume of 40µL. Reactions of 14 µL or less can be purified in this type of plate (14 µL x 2.8 = 39.2 µL).

   For automation, Agencourt strongly recommends the MJ Research Hard-Shell PCR plate (HSP-3801). The design of this plate virtually eliminates warping caused by thermal cycling, making it easier for robotic systems to move the plates on and off of the Agencourt SPRIPlate 384 magnet. Other 384 well plates are compatible with the magnet, for example Marsh AB-0937, but will require manual intervention to move the plates on and off the Agencourt SPRIPlate384. For more information about the selection of plates contact the Agencourt support team at support@Agencourt.com or 1-800-773-9186.

2. **Gently shake the Agencourt RNAClean XP bottle to resuspend any magnetic particles that may have settled. Add Agencourt RNAClean XP according to the reaction volume chart below:**

Reaction Volume (µL)	Agencourt RNAClean XP Volume (µL)
10	18
14	25

   The volume of Agencourt RNAClean XP for a given reaction can be derived from the following equation:

   (Volume of Agencourt RNAClean XP per reaction) = 1.8 x (Reaction Volume)

3. **Mix the Agencourt RNAClean XP and reaction thoroughly by pipette mixing 15 times.**

   This step binds RNA (or cDNA) to the magnetic beads. Vortexing reactions in 384 well format is not recommended. The color of the mixture should appear homogenous after mixing.

4. **Place the reaction plate onto an Agencourt SPRIPlate 384 for 3 to 5 minutes separate the beads from solution.**

   The separation time is dependent on the size of the reaction. Larger reaction sizes will require more time for separation. The solution should be clear before proceeding to the next step.

5. **Slowly aspirate the cleared solution from the reaction plate and discard.**

   This step should be performed while the purification plate is situated on the Agencourt SPRIPlate 384. Do not touch the magnetic beads, which have formed a spot on the side of the well.

6. **Dispense 30 µL of 70% ethanol to each well of the reaction plate and incubate for 30 seconds at room temperature. Aspirate out the ethanol and discard. Repeat for a total of two washes.**

   It is important to perform these steps with the reaction plate situated on an Agencourt SPRIPlate 384. Do not disturb the separated magnetic beads. Be sure to remove all of the ethanol from the bottom of the well as it may contain residual contaminants.

7. **Let the reaction plate air-dry for 10 minutes.**

   The plate should air-dry until the last visible traces of ethanol evaporate. Over-drying the sample may result in a lower recovery. If the samples will be used immediately, proceed to Step 8 for elution. If the samples will not be used immediately, the dried plate may be sealed and stored at 4°C or -20°C. If long term freezing of the samples is required Agencourt recommends transferring the samples away from the beads into a new plate, as the beads can shatter and fragments may no longer respond to the magnetic field.

8. **Add 30 µL of RNase-free water to each well of the reaction plate and seal to vortex 30 seconds or pipette mix 10 times.**

   A 30 µL elution volume will ensure the liquid level is high enough to contact the magnetic beads. A greater volume of elution buffer can be used, but using less than 15 µL will necessitate extra vortexing, to ensure the liquid comes into contact with the beads, and may not fully elute the product. Elution is rapid and it is not necessary for the beads to go back into solution for complete elution.

When setting up downstream reactions or transferring samples, pipette the eluant from the plate while it is situated on the Agencourt SPRIPlate 384. This will prevent bead carry over.

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For questions regarding this protocol, call Technical Support at Agencourt 1-800-773-9186