EvoPure™ for Tissue RNA

Catalog Numbers: **R-907T-S, P-907T-M, P-907T-L**

Contents

Protocol Manual *Revision 2.12*

Please refer to http://www.alinebiosciences.com Support section for updated protocols and MSDS when handling or shipping any chemical hazards. The information provided is subject to change without notice.

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INTRODUCTION

The ALINE EvoPure™ Tissue RNA Kit utilizes ALINE's proprietary paramagnetic bead technology to isolate RNA from animal tissues such as muscles, liver, kidney, tails and ear lobes. This protocol provides instructions to extract DNA from fresh or frozen tissues. The protocol is performed in 96-well format. Purification begins by adding a lysis buffer and Proteinase K to digest tissue to release nucleic acids. Nucleic acids is then immobilized on magnetic particles. DNA is removed by subsequent DNase I digestion and rebinding. Contaminants can then be washed away using washing buffers, leaving the RNA ready for elution from the magnetic particles. The 96-well plate format procedure is highly amenable to automation due to the utilization of magnetic separation. Vacuum filtration or centrifugation is eliminated.

RNA prepared from the ALINE EvoPure™ Kit can be used in the following applications:

- RT-PCR amplification
- RNA expression analysis
- Membrane hybridizations (e.g., Southern and dot/slot blots).

Process Overview:

1. Lyse up to 10mg tissue specimen in Lysis Buffer, DTT, and Proteinase K.
2. Bind RNA to paramagnetic beads.
3. Separate beads from contaminants.
4. Wash the magnetic beads with an ethanol wash buffer to remove contaminants.
5. Elute DNA from magnetic particles.
6. Transfer eluted DNA to a new plate.

Reagents supplied in the kit:

The reagents have a shelf life of 12 months if stored as directed.

Lysis Buffer (clear)**:** Store at Room Temperature.
evoPURE Bind (magnetic beads): 2-8°C
Wash Buffer 1: Concentrated. Need to add 100% isopropanol as specified on the bottle before the first use. Store at Room Temperature.
Elution Buffer (EB): Store at Room Temperature.

Reagents to be supplied by users:
96-well plates 1.2mL
70% ethanol: Fresh made 70% is recommended. If75% for may be used for long term storage.

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Store at Room Temperature.
Proteinase K (40mg/mL): -20°C
□ **Optional: DNase I (RNase-free)** [(2 U/µL); cat# 2222, Ambion Inc., http://www.ambion.com]
□ **Optional: DNase I 10X buffer** (cat# 8170G, Ambion Inc., http://www.ambion.com)
□ **Reagent grade water, nuclease-free** (Ambion product # 9932; http://www.ambion.com)

Note:

□ **Make sure to add 100% isopropanol or ethanol to the concentrated Wash buffer and 70% ethanol Buffer before the first use according to the instructions on the bottle.**

□ Always work with gloved hands and change gloves frequently

□ Use RNase free, filtered pipette tips for pipetting whenever possible

PROCEDURE IN 96-WELL FORMAT

ALINE Biosciences strongly recommends using aerosol-barrier (filter) pipette tips when performing the protocol.

1. If proteinase K (PK) powder is supplied, add PK Buffer to PK tube according to the label on the bottle. Mix components by inverting the tube/ bottle several times. Do not vortex. Store PK solution at -20°C when not in use.

2. Add 100% isopropanol to Wash Buffer based on the label on the bottle. Mix thoroughly.

3. Prepare Lysis Buffer
Prepare this solution and use within 10 minutes – discard any unused solution.
For each sample combine 20 µL PK with 400 µL of Lysis Buffer. Example: for ten isolations mix 4.0mls of Lysis with 200uls of PK.

Note: Pipette enzyme directly into the liquid and pipette mix up and down to remove any residual enzyme from the inside of the tip. A light vortex can be done to ensure homogeneity, but avoid foaming.

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4. Prepare Bind Buffer
Prepare this solution fresh and per isolation – discard any unused solution.
For each sample combine 80ul Bind Buffer with 320ul of isopropanol for a total of 400ul.

5. If you wish to perform the *optional* DNase step:
Prepare DNase solution
Prepare this solution fresh and per isolation – discard any unused solution.
Combine 80 µL nuclease free water, 10 µL 10X DNase buffer, and 10 µL of DNase I.

6. Homogenize up to 10 mg of tissue per 400 µL of Lysis Buffer using a rotor-stator homogenizer.
Mince tissue into small pieces. Large pieces can't get into the homogenizer and get shredded. Do not use more than 10mg tissue. Refer **Appendix 1** for sample homogenization details.
Homogenization may be scaled up to any volume using this ratio. *Only 400 µL of lysate may be processed at a time.* Complete homogenization and lysis of the tissue is a highly critical step in the isolation of high quality total RNA.

7. Transfer 400 µL of homogenized lysate to processing plate (AB-1127) and seal with plate seal or transfer to a 1.7ml microcentrifuge tube.

8. Incubate plate/ tube in water bath for 25 minutes at 37ºC.
Following incubation lysate may be frozen indefinitely at -80ºC.

Note: If there are chunks of undigested tissue, spin down at maximum speed and transfer clear supernatant to a new tube for processing.

9. Shake Bind Buffer to resuspend magnetic particles before using. Add 400 µL of Bind Buffer and slowly pipette mix 5 times. Incubate at room temperature for 5 minutes.

Note: Try to avoid bubbles while tip mixing. Some bead clumping may occur; it will not affect the quality or yield.

10. Place on magnet for 6 minutes. 96 well plate.
Wait for the solution to clear before proceeding to the next step.

11. Fully remove supernatant from the plate/ tube and discard.
This step must be performed while the plate/ tube is situated on the magnet. Avoid disturbing the separated magnetic beads by aiming the pipette tip for the center of the well/ tube bottom. If beads are drawn out, leave a few microliters of supernatant

behind. Colored liquid can be due to tissue homogenization.

12. REMOVE plate/ tube from the magnet and wash by adding 800 µL of Wash Buffer. Pipette mix 10 times. Try to avoid bubbles while tip mixing.

13. Return plate/ tube to the magnet for 5 minutes.
Wait for the solution to clear before proceeding to the next step.

14. Fully remove supernatant from the plate/ tube and discard.
This step must be performed while the plate/ tube is situated on the magnet. Avoid disturbing the separated magnetic beads by aiming the pipette tip for the center of the well/ tube bottom. If beads are drawn out, leave a few microliters of supernatant behind. Colored liquid can be due to tissue homogenization.

15. REMOVE plate/ tube from the magnet and wash by adding 800 µL of 70% ethanol per well and gently pipette mix 4 times.
Beads do not have to be fully resuspended in this step.

16. Return plate/ tube to the magnet for 5 minutes.
Wait for the solution to clear before proceeding to the next step.

17. Remove as much ethanol as possible, then REMOVE the plate/ tube from the magnet.
Pipette slowly to avoid aspirating beads. If too much ethanol is present (more than 5 µL), the DNase digestion will be inhibited.

18. *Optional:* Add 100 µL of DNase solution with the plate OFF the magnet. Incubate at room temperature for 1 minute without mixing to hydrate the beads.

Note: If you do not wish to perform the DNase step, skip to step 23. Perform only a total of 3 ethanol washes.

19. Pipette mix 5 times to resuspend the beads in the DNAse solution.

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

21. *DO NOT REMOVE THE DNase SOLUTION*. Add 550 µL of Wash Buffer and pipette mix 5 times. Incubate at room temperature for 4 minutes.

22. Place plate/ tube onto the magnet and separate for 7 minutes.
Wait for the solution to clear before proceeding to the next step.

23. Aspirate supernatant and wash by adding 600 µL of 70% Ethanol. Do not pipette mix.
This step is performed on the magnet.

24. Incubate for 2 minutes to allow beads to resettle. Remove ethanol and discard.

25. Repeat steps #23 and #24 for a total of 3 ethanol washes (including wash from #15 if omitting DNAse step).

26. Remove final ethanol wash completely and allow beads to dry for 10 minutes at room temperature.

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

27. Remove plate/ tube from the magnet and elute by adding a minimum of 40 µL ofnuclease free water. Pipette mix 10 times and incubate at room temperature for 2 minutes.

28. Return plate/ tube to the magnet for 2 minutes and carefully transfer eluted RNA away from the beads into fresh plate/ tube for storage.

Appendix 1 - Recommended Homogenization Methods
Complete homogenization of the tissue is a critical step in isolating high quality total RNA. Incomplete homogenization may result in lower yields and decreased purity of the isolated RNA. Several protocols for the homogenization of various tissues are commercially available. Selection of the best method for a particular range of uses should be determined experimentally. The homogenization protocols and equipment listed below have been used successfully in our research laboratory.

- Keep tissue frozen on dry ice as much as possible to minimize degradation.
- Keep plastics, forceps, and cutting tools on dry ice to minimize degradation.
- Weigh tube without tissue and re-weigh with tissue to make sure that the appropriate amount of Lysis Buffer and PK are added to the sample.
- We recommend doing the homogenization in a 15 mL or 50ml tube. In a smaller tube foaming can create a problem.
- If you have enough starting material, make up an extra sample to compensate for dead volume and the foaming that may occur during and post homogenization. For example if processing two tissues samples (20 mg) homogenize enough for 3 (30 mg).
- If using a roto stator homogenizer, homogenize using an up and down motion in the tube. Avoid heating up the sample because this can degrade the RNA.

RNAdvance Tissue Lysis Buffer contains RNAse inhibitors. RNA will begin to degrade when thawed if it is not in contact with Lysis Buffer. Therefore place the frozen tissue directly into the Lysis Buffer before it begins to thaw. Homogenize as quickly as possible to stabilize the entire piece of tissue.
For tube format: tissue dispersing device: IKA Ultra Turrax (http://www.ika.de/ika/home.html) using a 5 mm dispersing element or Brinkman Polytron homogenizer
For 96 well plate format: Plate vortexer (Troemner VX2400 Multitube Vortexer) and two 3.2 mm stainless steel beads (http://www.biospec.com/ part number 11079132ss) per well. 2 ml deepwell plate (http://www.abgene.com/ Product # AB-0661); plate seal (http://www.abgene.com/ Product # AB-0580)
In general tissues may be divided into three groups or types for the purpose of homogenization:
(A) soft tissues such as liver, kidney, and lung
(B) fibrous tissues, such as skeletal, cardiac and vascular smooth muscle
(C) lipid rich tissues, such as adipose and brain.

(1) Tube Format:

Up to 10 mg of tissue may be homogenized in 400 µL of Lysis Buffer/ PK in a 1.7ul microcentrifuge tube. Larger amounts of tissue may be homogenized simply by using a 15ml/ 50ml conical tube and scaling up the volume of the homogenization. Use an additional 400ul of Lysis/PK for ever additional 10mg of tissue. For volumes above 2 mL total, a larger tissue dispersing element should be used (8 to 10 mm) to ensure complete homogenization. Foaming of the sample during lysis can be minimized by keeping the dispersing element in a fixed location in the center of the tube and as close to the bottom as possible.
Soft tissue, tube format: Samples should be homogenized at the highest speed setting for about 2 minutes.
Fibrous tissue, tube format: Samples should be homogenized at the highest speed setting for about 5 minutes. In addition the 37°C lysis incubation can be extended to 45 minutes for particularly tough tissue such as vascular smooth muscle.
Lipid Rich Tissues, tube format: Samples should be homogenized at the highest speed setting for about 30 – 90 seconds. Special care should be taken to avoid excessive foaming of lipid rich tissues during homogenization.

(2) 96 Well Plate Format:

Homogenization of up to 10 mg of tissue in 400 µL of Lysis Buffer/ PK may be accomplished using either a tissue dispersing device or metal beads and agitation ("bead beating"). The bead beating method provides a higher throughput solution for 96 well plate isolations. Add 400 µL of Lysis Buffer per well and up to 10 mg of tissue. Seal the plate with a plastic plate seal and shake vigorously (2400 RPM) in a plate vortexer for homogenization. Incubate the deep well plate at 37°C without removing the metal beads.
Upon completion of the incubation the lysate should be transferred to a 1.2 mL plate for

processing.
Soft tissue, plate format: Samples should be homogenized about 10 minutes at 2400 RPM.
Fibrous tissue, plate format: vortex about 20 to 25 minutes at 2400 RPM. *Please note: some fibrous tissue may not be completely dispersed using metal beads* – specific homogenization requirements for some fibrous tissues may need to be determined experimentally.
Lipid Rich Tissues, plate format: vortex for about 10 minutes at 2400 RPM.

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If you have any technical questions, please feel free to contact support@alinebiosciences.com or 1-888-987-3677.

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