PURY RNA Plus

For purification of total RNA from animal cells and tissues using PURY RNA Mini Column and gDNA Removal Column

P2030

Storage

Store at RT

Contents

- Product Manual
- PURY Cell & Tissue Lysis (PCT) Buffer — P2030-PCT
- PURY Wash Buffer 1 — P2030-W1
- PURY Wash Buffer 2 — P2030-W2
- RNase Free Water — P2030-RW
- PURY RNA Mini Column (blue ring) in Collection Tube — P2030-RC1
- gDNA Removal Column (yellow ring) in Collection Tube — P2030-GC2
- Collection Tube — P2030-C2

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Introduction

The PURY RNA Plus is designed to purify RNA from small amounts (up to $1X10^7$ cells) of a variety of cells and easy-to-lyse tissue types. This kit supplies the gDNA Removal Columns, a spin column that quickly and effectively removes genomic DNA without the need of DNase digestion. One of the most important aspects during the isolation of RNA is to prevent degradation of the RNA. Cells and tissues are first lysed by incubation in a chaotropic ion PURY Cell & Tissue Lysis Buffer, which immediately inactivates RNases. The lysate is added to the gDNA Removal Column (yellow rings) to clarify the lysate and to remove contaminating gDNA. After the addition of ethanol with the flow-through, the RNA is bound to the PURY RNA Mini Column (blue rings). Subsequent wash steps removes salts, metabolites, and macromolecular cellular components. High quality RNA is eluted with RNase-free H_2O. The RNA preparation using PURY RNA Plus can be performed at room temperature. The eluate should be treated with care because RNA is very sensitive to trace contaminations of RNases, often found on general labware, fingerprints, and dust. Keep RNA frozen at –20 °C for short term or –70 °C for long term storage to ensure RNA stability.

Equipments and Reagents to Be Supplied by User

For all protocols

*** Optional:** 14.3M 2-mercaptoethanol (β-ME)
- Sterile, RNase-free pipet tips
- Microcentrifuge (with rotor for 2 ml tubes)
- RNase Free 1.5ml or 2.0ml microcentrifuge tube
- 96–100% ethanol
- Disposable gloves

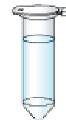
For tissue samples

- RNA Guardian Solution or liquid nitrogen

Equipment for sample disruption and homogenization.

Depending on the method chosen, one or more of the following are required.
- Trypsin and PBS
- PURY Shredder Spin Column
- Blunt-ended needle and syringe
- Mortar and pestle
- Tissue homogenizer

Schematic overview



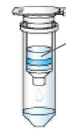
Disrupt and homogenize using PURY Cell & Tissue lysis (PCT) Buffer (300 or 600 µl).

1. Optional: if purifying RNA from cell lines (or tissue) rich in Ribonucleases, add fresh β-ME to 1% (v/v) on the PCT buffer before use. (ex: 3 or 6 µl β-ME/ 300 or 600µl PCT buffer)

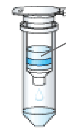
* Cell: Incubate 10 min at RT.
* Tissue: Each depending on the homogenization protocol.

Transfer the homogenized lysate to a gDNA Removal Column placed in a 2 ml collection tube (supplied). Centrifuge for 30 sec at 11,000 x g. Discard the column, and save the flow-through.

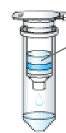
Add 1 volume (usually 300 or 600 µl) of 96-100% ethanol to the flow-through, and mix well by pipetting.



Transfer up to 700 µl of the sample to an PURY RNA Mini Column placed in a 2 ml collection tube (supplied). and centrifuge for 30 sec at 11,000 x g. Discard the flow-through. Place the PURY RNA Mini Column in a new collection tube.

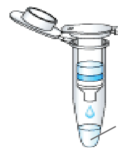


Add 600 µl PURY Wash Buffer 1 to the PURY RNA Mini column and centrifuge for 30 sec at 11,000 x g to wash the spin column membrane. Discard the flow-through. Place the PURY RNA Mini Column in a new collection tube.



Add 500 µl PURY Wash Buffer 2 to the PURY RNA Mini column and centrifuge for 30 sec at 11,000 x g to wash the spin column membrane. Discard the flow-through. Repeat above step twice in total. (2X)

Place the PURY RNA Mini column in a 1.5ml tube. Centrifuge at full speed (20,000 xg) for 1 min.



Place the PURY RNA Mini Column in a new 1.5 ml tube. Add 30–50 µl RNase-free water directly to the spin column membrane. Centrifuge for 1 min at 11,000 x g to elute the RNA.

Quality Control

In accordance with GenDEPOT's Quality Assurance System, each lot of PURY RNA Plus is tested against pre-determined specifications to ensure consistent product quality.

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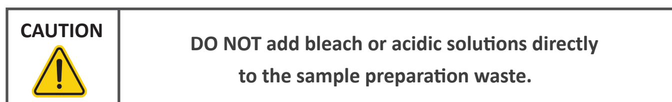
Safety Information

When working with chemicals, always wear a suitable lab coat, disposable gloves and protective goggles. For more information, please consult the appropriate safety data sheets (SDSs). These are available online in convenient and compact PDF format at www.gendepot.com where you can find, view and print the SDS for each GenDEPOT kit and kit component.



CAUTION	DO NOT add bleach or acidic solutions directly to the sample preparation waste.

PURY Cell & Tissue Lysis Buffer and PURY Wash Buffer 1 contain guanidine thiocyanate. This chemical can form highly reactive compounds when combined with bleach. If liquid containing these buffers is spilt, clean with suitable laboratory detergent and water. If the spilt liquid contains potentially infectious agents, clean the affected area first with laboratory detergent and water, and then with 1% (v/v) sodium hypochlorite.

Downstream Applications

The purified RNA is ready to use and is ideally suited for downstream applications that are sensitive to low amounts of DNA contamination, such as quantitative, real-time RT-PCR. The purified RNA can also be used in other applications, including: RT-PCR, Differential display, cDNA synthesis, Northern, dot blot, slot blot analyses, Primer extension, Poly A+ RNA selection, RNase/S1 nuclease protection, Microarrays.

With the PURY RNA Mini Plus procedure, all RNA molecules longer than 200 nucleotides are isolated. The procedure provides an enrichment for mRNA, since most RNAs <200 nucleotides (such as 5.8S rRNA, 5S rRNA and tRNAs, which together comprise 15–20% of total RNA) are selectively excluded. The size distribution of the purified RNA is comparable to that obtained by centrifugation through a CsCl cushion, where small RNAs do not sediment efficiently.

Important Notes

Determining the amount of starting material

It is essential to use the correct amount of starting material to obtain optimal RNA yield and purity. The maximum amount that can be used is limited by:

- The type of sample and its DNA and RNA content.
- The volume of PURY Cell & Tissue Lysis Buffer required for efficient lysis and the maximum loading volume of the PURY RNA Mini Column.
- The DNA removal capacity of the gDNA Removal Column.
- The RNA binding capacity of the PURY RNA Mini Column.

Tabel 1. PURY RNA Mini Column specification.

Table 2. Yield of total RNA with the PURY RNA Mini Plus.

- Perform all steps of the procedure at room temperature. During the procedure, work quickly.

- Perform all centrifugation steps at 20–25°C in a standard microcentrifuge. Ensure that the centrifuge does not cool below 20°C.

- Cell pellets can be stored at –70°C for later use or used directly in the procedure. Determine the number of cells before freezing. Frozen cell pellets should be thawed slightly so that they can be dislodged by flicking the tube in step 2. Homogenized cell lysates from step 3 can be stored at –70°C for several months. Frozen lysates should be incubated at 37°C in a water bath until completely thawed and salts are dissolved. Avoid prolonged incubation, which may compromise RNA integrity. If any insoluble material is visible, centrifuge for 5 min at 3000–5000 x g. Transfer supernatant to a new RNase-free glass or polypropylene tube, and continue with step 4.
Optional: Established the optimal lysis buffer composition in the user's test condition. If purifying RNA from cell lines (or tissue) rich in Ribonucleases, add fresh β-ME to 1% (v/v) on the PCT buffer before use. (ex: 3 or 6 µl β-ME/ 300 or 600µl PCT buffer)
- Dispense in a fume hood and wear appropriate protective clothing.
- PCT Buffer is stable at room temperature (15–25°C) for 1 month after addition of 2-ME.
- PURY Wash Buffer 1 and 2 is supplied as a concentrate. Before using for the first time, add ethanol (96–100%), as indicated on the bottle, to obtain a working solution.
- PCT Buffer may form a precipitate during storage.
If necessary, redissolve by warming, and then place at room temperature.
- PCT Buffer and PURY Wash Buffer 1 contain a guanidine salt and are therefore not compatible with disinfecting reagents containing bleach.

Table 1. PURY RNA Mini Column

Maximum binding capacity	100 ug RNA
Maximum loading volume	700 ul
RNA size distribution	RNA > 200 nucleotides
Minimum elution volume	30 ul
Maximum amount of starting material	
Animal cells	1 x 10^7 Cells
Animal tissues	30 mg

Table 2. Yileds of total RNA with PURY Total RNA Mini Plus

Cell cultures (1x 10^6 cells)	Average yield of total RNA (ug)
NIH/3T3	10
HeLa	15
COS-7	35
LMH	12
Huh	15
Mouse/rat tissues (10mg)	
Embryo (13 day)	25
Brain	5-10
Heart	4-8
Kidney	20-30
Liver	40-60
Lung	10-20

Amounts can vary due to factors , such as species, development al stage and growth conditions. Since the PURY Total RNA Mini Plus procedure enriches for mRNA and other RNA species >200 nucleotides, the total RNA yield does not include 5S rRNA, tRNA and other low-molecular -weight RNAs, which make up 15–20% of total cellular RNA.

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Purification of Total RNA from Animal Cells

It is essential to use the correct amount of starting material to obtain optimal RNA yield and purity. The minimum amount is generally 100 cells. RNA content can vary greatly between cell types (see table 3).

The maximum volume of PURY Cell & Tissue Lysis (PCT) Buffer that can be used limits the maximum amount of starting material to 1 x 10^7 cells.

1. Harvest cells according to step 1a or 1b.

1a. Cells grown in suspension (do not use more than 1 x 10^7 cells):
Determine the number of cells. Pellet the appropriate number of cells by centrifuging for 5 min at 300 x g in a centrifuge tube (not supplied). Carefully remove all supernatant by aspiration, and proceed to step 2.

Note: Incomplete removal of cell-culture medium will inhibit lysis and dilute the lysate, affecting the conditions for DNA removal and RNA purification. Both effects may reduce RNA quality and yield.

1b. Cells grown in a monolayer (do not use more than 1 x 10^7 cells):
Cells grown in a monolayer in cell-culture vessels can be either lysed directly in the vessel (up to 10 cm diameter) or trypsinized and collected as a cell pellet prior to lysis. Cells grown in a monolayer in cell-culture flasks should always be trypsinized.

To lyse cells directly:
Determine the number of cells. Completely aspirate the cell-culture medium, and proceed immediately to step 2.

Note: Incomplete removal of cell-culture medium will inhibit lysis and dilute the lysate, affecting the conditions for DNA removal and RNA purification. Both effects may reduce RNA quality and yield.

To trypsinize and collect cells:
Determine the number of cells. Aspirate the medium, and wash the cells with PBS. Aspirate the PBS, and add 0.10–0.25% trypsin in PBS. After the cells detach from the dish or flask, add medium (containing serum to inactivate the trypsin), transfer the cells to an RNase-free glass or polypropylene centrifuge tube (not supplied), and centrifuge at 300 x g for 5 min. Completely aspirate the supernatant, and proceed to step 2.

Note: Incomplete removal of cell-culture medium will inhibit lysis and dilute the lysate, affecting the conditions for DNA removal and RNA purification. Both effects may reduce RNA quality and yield.

2. Disrupt the cells by adding PURY Cell & Tissue (PCT) Buffer.

For pelleted cells, loosen the cell pellet thoroughly by flicking the tube. Add the appropriate volume of PCT Buffer (see Table 3). Vortex or pipet to mix, and proceed to step 3.

Note: Incomplete loosening of the cell pellet may lead to inefficient lysis and reduced RNA yields. Ensure that β-ME is added to PCT Buffer before use (see "Important points before starting").

Table 3. Volumes of PURY Cell & Tissue Lysis (PCT) Buffer for pelleted cells

Number of pelleted cells	Volume of PCT Buffer
< 5 x10^6	300 ul
5 x 10^6 - 1 x 10^7	600 ul

For direct lysis of cells grown in a monolayer, add the appropriate volume of PCT Buffer (see Table 4) to the cell-culture dish. Collect the lysate with a rubber policeman. Pipet the lysate into a microcentrifuge tube (not supplied). Vortex or pipet to mix with incubating 10 min at RT (Room Temperature) and ensure that no cell clumps are visible before proceeding to step 3.

Note: Optional: β-ME is added to PCT Buffer before use (see "Important points before starting").

Table 4. Volumes of PURY Cell & Tissue Lysis (PCT) Buffer for pelleted cells

Dish diameter	Volume of PCT Buffer
< 6 cm	300 ul
6 - 10 cm	600 ul

Note: Regardless of the cell number, use the buffer volumes indicated to completely cover the surface of the dish.

3. Homogenize the lysate according to step 3a, 3b, 3c or 3d.

3a. If processing <1 x 10^5 cells, they can be homogenized by vortexing for 1 min. After homogenization, proceed to step 4.

3b. Pipet the lysate directly into PURY Shredder spin column (Cat No, P2040) placed in a 2 ml collection tube, and centrifuge for 2 min at maximum speed. Proceed to step 4.

3c. Homogenize the lysate for 30 sec using the D1000 Homogenizer (Cat No, D1000). Proceed to step 4.

3d. Pass the lysate at least 5 times through a 20-gauge needle (0.9 mm diameter) fitted to an RNase-free syringe. Proceed to step 4.

4. Transfer the homogenized lysate to a gDNA Removal Column placed in a 2 ml collection tube (supplied). Centrifuge for 30 sec at 11,000 x g. Discard the gDNA Removal Column, and save the flow-through.

Note: Make sure that no liquid remains on the column membrane after centrifugation. If necessary, repeat the centrifugation until all liquid has passed through the membrane.

5. Add 1 volume (usually 300 µl or 600 µl) of 96-100% ethanol to the flow-through, and mix well by pipetting. Do not centrifuge.

Note: If some lysate was lost during homogenization and DNA removal, adjust the volume of ethanol accordingly.

Note: When purifying RNA from certain cell lines, precipitates may be visible after addition of ethanol. This does not affect the procedure.

6. Transfer up to 700 µl of the sample, including any precipitate that may have formed, to a PURY RNA Mini Column placed in a 2 ml collection tube (supplied). Close the lid gently, and centrifuge for 30 sec at 11,000 x g. Discard the flow-through with collection tube. Place the PURY RNA Mini Column in a new 2ml collection tube(supplied). Proceed to step 7.

Note: If the sample volume exceeds 700 µl, centrifuge successive aliquots in the same PURY RNA Mini Column. Discard the flow-through after each centrifugation.

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7. Add 600 µl PURY Wash Buffer 1 to the PURY RNA Mini Column. Close the lid gently, and centrifuge for 30 sec at 11,000 x g to wash the spin column membrane. Discard the flow-through with collection tube. Place the PURY RNA Mini Column in a new 2ml collection tube (supplied). Proceed to step 8.

   Note: Opt.: After centrifugation, carefully remove the PURY RNA Mini Column from the collection tube so that the column does not contact the flow-through. Be sure to empty the collection tube completely.

   Note: PURY Wash Buffer 1 is supplied as a concentrate. Ensure that ethanol is added to Pury Wash Buffer 1 before use.

8. Add 500 µl PURY Wash Buffer 2 to the PURY RNA Mini Column. Close the lid gently, and centrifuge for 30 sec at 11,000 x g to wash the spin column membrane. Discard the flow-through with collectioin tube. Place the PURY RNA Mini Column in a 2ml new collection tube (supplied). Proceed to step 9.

   Note: PURY Wash Buffer 2 is supplied as a concentrate. Ensure that ethanol is added to PURY Wash Buffer 2 before use.

9. Add 500 µl PURY Wash Buffer 2 to the PURY RNA Mini Column. Close the lid gently, and centrifuge for 30 sec at 11,000 x g to wash the spin column membrane. Discard the flow-through with collection tube. Place the PURY RNA Mini Column in a RNase free 1.5ml microcentrifuge tube. Proceed to step 10.

10. Place the PURY RNA Mini Column in RNase free 1.5 ml microcentrifuge tube, and discard the old collection tube with the flow-through. Centrifuge at full speed for 1 min (20,000 x g).

    Note: Perform this step to eliminate any possible carryover of Wash Buffer, or if residual flow-through remains on the outside of PURY RNA Mini Column after step 9.

    Note: The long centrifugation dries the spin column membrane, ensuring that no ethanol is carried over during RNA elution. Residual ethanol may interfere with downstream reactions.

    Note: After centrifugation, carefully remove the PURY RNA Mini Column from the 1.5ml microcentrifuge tube so that the column does not contact the flow -through. Otherwise, carryover of ethanol will occur.

11. Place the PURY RNA Mini Column in a new RNase free 1.5 ml microcentrifuge tube. Add 30–50 µl RNase-free water directly to the spin column membrane. Close the lid gently, and centrifuge for 1 min at 11,000 x g to elute the RNA.

Note: Use pre-heated RNase free water (50-70°C) to increase the final yield of RNA. The pre-heated RNase free water will provide increased elution efficiently.

Note: Check the RNA concentration. The eluted RNA could be used immediately or stored at -80°C for later use.

12. If the expected RNA yield is >30 µg, repeat step 11 using another 30–50 µl of RNase-free water, or using the eluate from step 11 (if high RNA concentration is required). If using the eluate from step 11, the RNA yield will be 15–30% less than that obtained using second volume of RNase-free water, but the final RNA concentration will be higher.

Note: Eluted RNA should always be kept on ice during work for optimal stability. Contamination with almost omnipresent RNases (general labware, fingerprints, dust) may lead to degradation of isolated RNA. For short term storage, freeze RNA at –20°C, for long term storage freeze at –70°C.

Purification of Total RNA from Animal Tissues

1. Excise the tissue sample from the animal or remove it from storage. Remove RNA Guardian Solution (or equivalent Tissue RNA stabilizing reagent) stabilized tissues from the reagent using forceps. Determine the amount of tissue. Do not use more than 30 mg. Weighing tissue is the most accurate way to determine the amount.

   Note: For optimal results, stabilize harvested tissues immediately in RNA Guardian Solution. Tissues can be stored in the reagent for up to 1 day at 37°C, 7 days at 18–25°C, or 4 weeks at 2–8°C, or archived at –30°C to –15°Cor –80°C.

   Note: Fresh, frozen or RNA Guardian Solution stabilized tissues can be used. Tissues can be stored at –70°C for several months. Flash-freeze tissues in liquid nitrogen, and immediately transfer to –70°C. Do not allow tissues to thaw during weighing or handling prior to disruption in PCT Buffer.
   Homogenized tissue lysates from step 3 can also be stored at –70°C for several months. Incubate frozen lysates at 37°C in a water bath until completely thawed and salts are dissolved before continuing with step 4. Avoid prolonged incubation, which may compromise RNA integrity.

   Note: If desired, more than 30 mg tissue can be disrupted and homogenized at the start of the procedure (increase the volume of PCT Buffer). Use a portion of the homogenate corresponding to no more than 30 mg tissue for RNA purification, and store the rest at –80°C.

2. Follow either step 2a or 2b.

   2a. For RNA Guardian Solution stabilized tissues:

   If using the entire tissue, place it directly into a suitably sized vessel for disruption and homogenization, and proceed to step 3.

   If using only a portion of the tissue, cut it on a clean surface. Weigh the piece to be used, and place it into a suitably sized vessel for disruption and homogenization. Proceed to step 3.

   RNA in RNA Guardian Solution stabilized tissues is protected during cutting and weighing of tissues at ambient temperature (18–25°C). It is not necessary to cut the tissues on ice or dry ice or in a refrigerated room. Remaining tissues can be stored in RNA Guardian Solution. Previously stabilized tissues can be stored at –80°C without the reagent.

   2b. For unstabilized fresh or frozen tissues:

   If using the entire tissue, place it directly into a suitably sized vessel for disruption and homogenization, and proceed immediately to step 3.

   If using only a portion of the tissue, weigh the piece to be used, and place it into a suitably sized vessel for disruption and homogenization. Proceed immediately to step 3.

Note: RNA in harvested tissues is not protected until the tissues are treated with RNA Guardian Reagent, flash-frozen or disrupted and homogenized in step 3. Frozen tissues should not be allowed to thaw during handling. The relevant procedures should be carried out as quickly as possible.

Note: Remaining fresh tissues can be placed into RNA Guardian Solution to stabilize RNA. However, previously frozen tissues thaw too slowly in the reagent, preventing the reagent from diffusing into the tissues quickly enough to prevent RNA degradation.

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3. Disrupt the tissue and homogenize the lysate in PCT Buffer (do not use more than 30 mg tissue) according to step 3a, 3b, 3c or 3d.

Note: Ensure that β-ME is added to PCT Buffer before use.

Note: After storage in RNA Guardian Solution, tissues may become slightly harder than fresh or thawed tissues. Disruption and homogenization using standard methods is usually not a problem. For easier disruption and homogenization, we recommended using 600 µl PCT Buffer.

Note: Incomplete homogenization leads to significantly reduced RNA yields and can cause clogging of the PURY RNA Mini Column. Homogenization with the rotor–stator homogenizers generally results in higher RNA yields than with other methods.

Note: If processing fiber-rich tissues, complete disruption and homogenization may sometimes not be possible. However, small amounts of debris have no effect on subsequent RNA purification with PURY RNA Mini Plus and are usually digested in the proteinase K step.

Table 5. Volumes of PURY Cell & Tissue Lysis (PCT) Buffer for tissue

Amount of starting material	Volume of PCT Buffer
< 20 mg	300 or 600 ul
20 - 30 mg	600 ul

Note: Use 600 µl PCT Buffer for tissues stabilized in RNA Guardian Solution or for difficult-to-lyse tissues.

3a. Disruption and homogenization using the D1000 Homogenizer:
Place the weighed (fresh, frozen or RNA Guardian Stabilized) tissue in a suitably sized vessel. Add the appropriate volume of PCT Buffer (see Table 5). Immediately disrupt and homogenize the tissue until it is uniformly homogeneous (usually 20–40 s). Proceed to step 4.

3b. Disruption using a mortar and pestle followed by homogenization using a PURY Shredder homogenizer:
Immediately place the weighed (fresh, frozen or RNA Guardian stabilized) tissue in liquid nitrogen, and grind thoroughly with a mortar and pestle. Decant tissue powder and liquid nitrogen into an RNase-free, liquid-nitrogen–cooled, 2 ml microcentrifuge tube (not supplied). Allow the liquid nitrogen to evaporate, but do not allow the tissue to thaw. Add the appropriate volume of PCT Buffer (see Table 5). Pipet the lysate directly into a PURY Shredder Spin Column placed in a 2 ml collection tube, and centrifuge for 2 min at maximum speed. Proceed to step 4.

3c. Disruption and homogenization using PURY Micro Bead or other beads:
Place the tissues in 2 ml microcentrifuge tubes or 1.2 ml collection microtubes containing stainless steel bead (3–7 mm mean diameter).

Note: If handling fresh or frozen tissue samples, keep the tubes on dry ice. Place the tubes at room temperature (15–25°C). Immediately add the appropriate volume of PCT Buffer to tube.

Note: Do not use PCT Buffer with tungsten carbide beads, as these buffers can react with and damage the bead surface.

Place the tubes in the bead-milling homogenizer such as BEADBLASTER. Operate the Bead Homogenizer for 2 min at 20–30 Hz.

Note: The duration of disruption and homogenization depends on the tissue being processed and can be extended until no tissue debris is visible. Proceed to step 4.

4. Centrifuge the lysate for 3 min at maximum speed. Carefully remove the supernatant by pipetting, and transfer it to a gDNA Removal Column placed in a 2 ml collection tube (supplied). Centrifuge for 30 sec at 11.000 x g. Discard the column, and save the flow-through.

Note: This step is important, as it removes insoluble material that could clog the gDNA Removal Column and interfere with DNA removal. In some preparations, very small amounts of insoluble material will be present after the 3 min centrifugation, making the pellet invisible.

Note: Make sure that no liquid remains on the column membrane after centrifugation. If necessary, repeat the centrifugation until all liquid has passed through the membrane.

5. Add 1 volume (usually 300 µl or 600 µl) of 96-100% ethanol to the flow-through, and mix well by pipetting. Do not centrifuge. Proceed immediately to step 6.

Note: If some lysate was lost during homogenization and DNA removal, adjust the volume of ethanol accordingly.

Note: Precipitates may be visible after addition of ethanol, but this does not affect the procedure.

6. Transfer up to 700 µl of the sample, including any precipitate that may have formed, to a PURY RNA Mini Column placed in a 2 ml collection tube (supplied). Close the lid gently, and centrifuge for 30 sec at 11,000 x g. Discard the flow-through with collection tube. Place the Pury RNA Mini Column in a new 2ml collection tube (supplied). Proceed to step 7.

Note: If the sample volume exceeds 700 µl, centrifuge successive aliquots in the same PURY RNA Mini Column. Discard the flow-through after each centrifugation.

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7. Add 600 µl Pury Wash Buffer 1 to the PURY RNA Mini Column. Close the lid gently, and centrifuge for 30 sec at 11,000 x g to wash the spin column membrane. Discard the flow-through with collection tube. Place the PURY RNA Mini Column in a new 2ml collection tube (supplied). Proceed to step 8.

   Note: After centrifugation, carefully remove the PURY RNA Mini Column from the collection tube so that the column does not contact the flow-through. Be sure to empty the collection tube completely.

   Note: PURY Wash Buffer 1 is supplied as a concentrate. Ensure that ethanol is added to PURY Wash Buffer 1 before use.

8. Add 500 µl PURY Wash Buffer 2 to the PURY RNA Mini Column. Close the lid gently, and centrifuge for 30 sec at 11,000 x g to wash the spin column membrane. Discard the flow-through with collectioin tube. Place the PURY RNA Mini Column in a 2ml new collection tube (supplied). Proceed to step 9.

   Note: PURY Wash Buffer 2 is supplied as a concentrate. Ensure that ethanol is added to PURY Wash Buffer 2 before use.

9. Add 500 µl PURY Wash Buffer 2 to the PURY RNA Mini Column. Close the lid gently, and centrifuge for 30 sec at 11,000 x g to wash the spin column membrane. Discard the flow-through with collection tube. Place the PURY RNA Mini Column in a RNase free 1.5ml microcentrifuge tube. Proceed to step 10.

10. Place the PURY RNA Mini Column in RNase free 1.5 ml microcentrifuge tube, and discard the old collection tube with the flow-through. Centrifuge at full speed for 1 min (20,000 x g).

    Note: Perform this step to eliminate any possible carryover of Wash Buffer, or if residual flow-through remains on the outside of PURY RNA Mini Column after step 9.

    Note: The long centrifugation dries the spin column membrane, ensuring that no ethanol is carried over during RNA elution. Residual ethanol may interfere with downstream reactions.

    Note: After centrifugation, carefully remove the PURY RNA Mini Column from the 1.5ml microcentrifuge tube so that the column does not contact the flow -through. Otherwise, carryover of ethanol will occur.

11. Place the PURY RNA Mini Column in a new RNase free 1.5 ml microcentrifuge tube. Add 30–50 µl RNase-free water directly to the spin column membrane. Close the lid gently, and centrifuge for 1 min at 11,000 x g to elute the RNA.

Note: Use pre-heated RNase free water (50-70°C) to increase the final yield of RNA. The pre-heated RNase free water will provide increased elution efficiently.

Note: Check the RNA concentration. The eluted RNA could be used immediately or stored at -70°C for later use.

12. If the expected RNA yield is >30 µg, repeat step 11 using another 30–50 µl of RNase-free water, or using the eluate from step 11 (if high RNA concentration is required). If using the eluate from step 11, the RNA yield will be 15–30% less than that obtained using a second volume of RNase-free water, but the final RNA concentration will be higher.

Note: Eluted RNA should always be kept on ice during work for optimal stability. Contamination with almost omnipresent RNases (general lab ware, fingerprints, dust) may lead to degradation of isolated RNA. For short term storage, freeze RNA at –20°C, for long term storage freeze at –70°C.

Troubleshooting Guide

Clogged gDNA Removal Spin Column

Inefficient disruption and/or homogenization	Increase g-force and centrifugation time if necessary. In subsequent preparations, reduce the amount of starting material and/or increase the homogenization time.
Too much starting material	Reduce the amount of starting material. It is essential to use the correct amount of starting material.
Centrifugation temperature too low	The centrifugation temperature should be 20–25°C. Some centrifuges may cool to below 20°C even when set at 20°C. This can cause formation of precipitates that can clog the gDNA Removing Column. If this happens, set the centrifugation temperature to 25°C. Warm the lysate to 37°C before transferring it to the gDNA Removal Column.

Low RNA yield

Inefficient disruption and/or homogenization	Increase g-force and centrifugation time if necessary. In subsequent preparations, reduce the amount of starting material and/or increase the homogenization time.
Too much starting material	Reduce the amount of starting material. Overloading the PURY RNA Mini Column significantly reduce RNA yield.
Ethanol added to lysate before DNA removal	Pass the lysate through the gDNA Removal Column before adding ethanol to it.
RNA still bound to PURY RNA Mini Column membrane	Repeat RNA elution, but incubate the PURY RNA Mini Column on the benchtop for 10 min with RNase-free water before centrifuging.
Ethanol carryover	Perform the extended centrifugation to dry the PURY RNA Mini Column if any flow-through is present on the outside of the column.
Incomplete removal of cell-culture medium	When processing cultured cells, ensure complete removal of cell-culture medium after harvesting cells.

Low A_{260}/A_{280} value

Water used to dilute RNA for A_{260}/A_{280} measurement	Use 10 mM Tris·Cl, pH 7.5, not RNase-free water, to dilute the sample before measuring purity.

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RNA degraded

Inappropriate handling of starting material	Ensure that tissue samples are properly stabilized and stored in RNA Guardian Solution. For frozen cell pellets or frozen tissue samples, ensure that they were flash-frozen immediately in liquid nitrogen and properly stored at –70°C. Perform the RNA purification procedure quickly, especially the first few steps.
RNase contamination	Although all buffers have been tested and are guaranteed RNase-free, RNases can be introduced during use. Be certain not to introduce any RNases during the PURY RNA Mini Plus procedure or later handling.

DNA contamination in downstream experiments

Cell number too high	For some cell types, the efficiency of DNA removal may be reduced when processing very high cell numbers (containing more than 20 µg genomic DNA). If the eluted RNA contains substantial DNA contamination, try processing smaller cell numbers.
Incomplete removal of cell-culture medium or stabilization reagent	Be sure to remove any excess cell-culture medium or stabilization reagent to prevent significant dilution of the lysis buffer. The gDNA Removal Column will not work effectively if the lysis buffer is diluted.
Tissue has high DNA content	For certain tissues with extremely high DNA content (e.g., thymus), DNA may not be completely removed. Try using smaller samples (containing less than 20 µg genomic DNA), or perform DNase digestion of the eluted RNA followed by RNA cleanup.

RNA concentration too low

Elution volume too high	Elute RNA with less than 2 x 50 µl of water. Do not use less than 1 x 30 µl of water. Although eluting with less than 2 x 50 µl of water results in increased RNA concentrations, RNA yields may be reduced.

RNA does not perform well in downstream experiments

Salt carryover during elution	Ensure that PCT Buffer is at 20–30°C. When reusing collection tubes between washing steps, remove residual flow-through from the rim by blotting on clean paper towels.
Salt carryover during elution	Be sure to centrifuge at full speed (20,000x g) 1 min at 20–25°C to dry the PURY RNA Mini Column membrane. After centrifugation, carefully remove the column from the collection tube so that the column does not contact the flowthrough. Otherwise, carryover of ethanol will occur.

Appendix

Handling RNA

Ribonucleases (RNases) are very stable and active enzymes that generally do not require cofactors to function. Since RNases are difficult to inactivate and even minute amounts are sufficient to destroy RNA, do not use any plasticware or glassware without first eliminating possible RNase contamination. Great care should be taken to avoid inadvertently introducing RNases into the RNA sample during or after the purification procedure. To create and maintain an RNase-free environment, the following precautions must be taken during pretreatment and use of disposable and nondisposable vessels and solutions while working with RNA.

Proper microbiological, aseptic technique should always be used when working with RNA. Hands and dust particles may carry bacteria and molds and are the most common sources of RNase contamination. Always wear latex or vinyl gloves while handling reagents and RNA samples to prevent RNase contamination from the surface of the skin or from dusty laboratory equipment. Change gloves frequently and keep tubes closed whenever possible. Keep purified RNA on ice when aliquots are pipetted for downstream applications.

To remove RNase contamination from bench surfaces, nondisposable plasticware, and laboratory equipment (e.g., pipets and electrophoresis tanks), use of RNazor (Cat. No R7000) is recommended. RNase contamination can alternatively be removed using general laboratory reagents. To decontaminate plasticware, rinse with 0.1 M NaOH, 1 mM EDTA followed by RNase-free water, or rinse with chloroform if the plasticware is chloroform-resistant. To decontaminate electrophoresis tanks, clean with detergent (e.g., 0.5% SDS), rinse with RNase-free water, rinse with ethanol (if the tanks are ethanol-resistant), and allow to dry.

The use of sterile, disposable polypropylene tubes is recommended throughout the procedure. These tubes are generally RNase-free and do not require pretreatment to inactivate RNases.

Glassware should be treated before use to ensure that it is RNase-free. Glassware used for RNA work should be cleaned with a detergent, thoroughly rinsed and oven baked at 240°C for at least 4 hours (overnight, if more convenient) before use. Autoclaving alone will not fully inactivate many RNases. Alternatively, glassware can be treated with DEPC (diethyl pyrocarbonate), as described in below.

Solutions (water and other solutions) should be treated with 0.1% DEPC. DEPC is a strong, but not absolute, inhibitor of RNases. It is commonly used at a concentration of 0.1% to inactivate RNases on glass or plasticware or to create RNase-free solutions and water. DEPC inactivates RNases by covalent modification. Add 0.1 ml DEPC to 100 ml of the solution to be treated and shake vigorously to bring the DEPC into solution. Let the solution incubate for 12 hours at 37°C. Autoclave for 15 minutes to remove any trace of DEPC. DEPC will react with primary amines and cannot be used directly to treat Tris buffers. DEPC is highly unstable in the presence of Tris buffers and decomposes rapidly into ethanol and CO_2. When preparing Tris buffers, treat water with DEPC first, and then dissolve Tris to make the appropriate buffer. Trace amounts of DEPC will modify purine residues in RNA by carbethoxylation. Carbethoxylated RNA is translated with very low efficiency in cell-free systems. However, its ability to form DNA:RNA or RNA:RNA hybrids is not seriously affected unless a large fraction of the purine residues has been modified. Residual DEPC must always be eliminated from solutions or vessels by autoclaving or heating to 100°C for 15 minutes.

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Storage, Quantification and Determination of Quality of RNA

Storage of RNA

Purified RNA may be stored at –30°C to –15°C or –70°C in RNase-free water. Under these conditions, no degradation of RNA is detectable after 1 year.

Quantification of RNA

The concentration of RNA should be determined by measuring the absorbance at 260 nm (A260) in a spectrophotometer. For small amounts of RNA, however, it may be difficult to determine amounts photometrically. Small amounts of RNA can be quantified using Agilent® 2100 Bioanalyzer, quantitative RT-PCR or fluorometric quantification.

Spectrophotometric quantification of RNA

To ensure significance, A_{260} readings should be greater than 0.15. An absorbance of 1 unit at 260 nm corresponds to 44 µg of RNA per ml (A_{260}=1 is 44 µg/ml). This relation is valid only for measurements at a neutral pH. Therefore, if it is necessary to dilute the RNA sample, this should be done in a buffer with neutral pH. The ratio between the absorbance values at 260 and 280 nm gives an estimate of RNA purity. When measuring RNA samples, be certain that cuvettes are RNase-free, especially if the RNA is to be recovered after spectrophotometry. This can be accomplished by washing cuvettes with 0.1 M NaOH, 1 mM EDTA, followed by washing with RNase-free water. Use the buffer in which the RNA is diluted to zero the spectrophotometer. An example of the calculation involved in RNA quantification is shown below:

Volume of RNA sample = 100 µl
Dilution = 10 µl of RNA sample + 490 µl of 10 mM Tris·Cl, pH 7.0 (1/50 dilution)
Measure absorbance of diluted sample in a 1 ml cuvette (RNase-free)
A_{260} = 0.2
Concentration of RNA sample = 44 µg/ml x A_{260} x dilution factor
= 44 µg/ml x 0.2 x 50
= 440 µg/ml
Total amount = concentration x volume in milliliters
= 440 µg/ml x 0.1 ml
= 44 µg of RNA

Purity of RNA

The ratio of the readings at 260 nm and 280 nm (A_{260}/A_{280}) provides an estimate of the purity of RNA with respect to contaminants that absorb in the UV spectrum, such as protein. However, the A_{260}/A_{280} ratio is influenced considerably by pH. Since water is not buffered, the pH and the resulting A_{260}/A_{280} ratio can vary greatly. Lower pH results in a lower A_{260}/A_{280} ratio and reduced sensitivity to protein contamination. For accurate values, we recommend measuring absorbance in 10 mM Tris·Cl, pH 7.5. Pure RNA has an A_{260}/A_{280} ratio of 1.9 - 2.1 in 10 mM Tris·Cl, pH 7.5. Always be sure to calibrate the spectrophotometer with the same solution used for dilution. For determination of RNA concentration, however, we recommend dilution of the sample in a buffer with neutral pH since the relationship between absorbance and concentration (A_{260} reading of 1 = 44 µg/ml RNA) is based on an extinction coefficient calculated for RNA at neutral pH.

Integrity of RNA

The integrity and size distribution of total RNA purified with PURY RNA Plus can be checked by denaturing agarose gel electrophoresis and ethidium bromide staining or by using the Agilent 2100 Bioanalyzer. The respective ribosomal RNAs should appear as sharp bands or peaks. The apparent ratio of 28S rRNA to 18S rRNA should be approximately 2:1. If the ribosomal bands or peaks of a specific sample are not sharp, but appear as a smear towards smaller sized RNAs, it is likely that the sample suffered from major degradation either before or during RNA purification. The Agilent 2100 Bioanalyzer also provides an RNA Integrity Number (RIN) as a useful measure of RNA integrity. Ideally, the RIN should be close to 10, but in many cases (particularly with tissue samples), RNA quality is greatly influenced by how well the original sample was preserved.

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