RNAprep Pure Micro Kit

For purification of total RNA from micro samples

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RNAprep Pure Micro Kit

(Spin Column)

Cat. no. GDP420

Kit Contents

	Contents	GDP420 (50 preps)
GDP420H	Buffer RL	30 ml
	Buffer RW1	40 ml
	Buffer RW	12 ml
	Carrier RNA	310 μg
	RNase-Free ddH_2O	1 ml
	RNase-Free ddH_2O	2×15 ml
	(RNase-Free Columns CR1 set)	50
	RNase-Free Centrifuge Tubes (1.5 ml)	50
	Handbook	1
GRT411	RNase-Free DNase I (1500 U)	1
	Buffer RDD (DNA Digest Buffer)	4 ml
	RNase-Free ddH_2O	1 ml

Note: GDP420H, GRT411 are shipped and packaged separatly.

Compatible Reagents

Proteinase K (Cat. no. GRT403-01/GRT403-02), Grinding Pestles, RNase-free Columns CS set

Storage

RNAprep Pure Micro Kit can be delivered at room temperature (15-30°C). When the kit has arrived, store RNase-free DNase I, Buffer RDD, and RNase-free ddH_2O at 2-8°C for up to 15 months. Other reagents can be stored at room temperature (15-30°C) for 15 months.

Introduction

RNAprep Pure Micro Kit adopts innovative technology to guarantee the isolation of RNA from micro amount of tissue and cell samples(as little as 10 cells). Unique component added in buffer system greatly enhances the capability of selective binding of RNA to silica membrane. RNase-free DNase I has been added to remove the trace DNA contamination. DNase I and other residual impurity will be removed in the following washing step. Eventually efficient purification of high-quality RNA in a small elution volume is guaranteed.

With the RNAprep Micro Pure Kit procedure, all RNA molecules longer than 200 bp are purified. The procedure enriches for mRNA, since most RNAs <200 bp such as 5.8S rRNA, 5S rRNA, and tRNAs are selectively excluded.

In the handbook, different protocols are provided for different starting materials. The protocols differ primarily in the disruption and homogenization of the sample. Once the sample is applied to RNase-free Spin Column CR1, the subsequent steps are similar.

Important Notes

1. Regents and consumables (not supplied):β-mercaptoethanol, ethanol (96%-100%), 1.5 ml RNase-free microcentrifuge tube.
2. Determining the amount of starting materials:

   It is essential to use the correct amount of starting material in order to obtain optimal RNA yield and purity. The maximum amount that can be used is determined by the type of sample and its RNA content. When processing samples containing high amounts of DNA or RNA, less than the maximum amount of starting material shown in Table 1 should be used, Table 2 shows typical RNA yields from various cells.

Table1. RNase-free Spin Column CR1 specifications

Maximum binding capacity	45 μg RNA
Maximum loading volume	700 μl
RNA size distribution	>200 bp
Minimum elution volume	10 μl
Maximum amount of starting material (animal cells)	5×10^5
Maximum amount of starting material (animal tissues)	5 mg

Note: Do not overload the RNase-free Spin Column CR1, as this will significantly reduce RNA yield and purity. Incomplete disruption also results in significantly reduced RNA yields.

Table 2. Typical yields of total RNA of HeLa cells using the Kit.

Cell-Culture vessel	Growth area (cm^2)	Number of cells
96-well	0.32–0.6	$4\text{-}5 \times 10^4$
48-well	1	1×10^5
24-well	2	2.5×10^5
12-well	4	5×10^5
6-well	9.5	1×10^6
Dishes (35 mm)	8	1×10^6
Flasks (40–50 ml)	25	3×10^6

3. Storage and treatment of samples

   In untreated tissues, RNA is easy to degrade in unprotected environment, so fresh tissues should be placed into liquid nitrogen and stored at -70°C immediately. Do not freeze-thaw the tissues. Buffer RL can be added to tissue samples after homogenization, and then stored at -70°C for several months.

4. Disruption and homogenization of samples

   Efficient disruption and homogenization of the starting material is an absolute requirement for all total RNA purification procedures. Disruption and homogenization are two distinct steps.

   Disruption: Complete disruption of plasma membranes of cells and organelles is absolutely required to release all the RNA contained in the sample. Different samples require different methods to achieve complete disruption. Incomplete disruption results in significantly reduced RNA yields. Homogenization is necessary to reduce the viscosity of the lysates produced by disruption.

   Homogenization: Shears the high molecular-weight genomic DNA and other high-molecular-weight cellular components to create a homogeneous lysate. Incomplete homogenization results in inefficient binding of RNA to the RNase-free Spin Column CR1 membrane and therefore significantly reduces RNA yields. Homogenization could be operated by homogenizer, vortex, filtered column, syringe and pipette.

5. Carrier RNA

   The RNAprep Pure Micro Kit contains poly-A RNA for use as Carrier RNA. When added to lysates of very small samples, the Carrier RNA may in some cases improve the recovery of total RNA. Carrier RNA is not required when processing more than 5,000 cells or 10 μg tissue.

Important notes before starting

1. In samples that have not been fixed, RNA is vulnerable to degradation. For protection of RNA integrity, please avoid placing untreated samples at room temperature for long time and store samples in liquid nitrogen immediately.
2. The lysate in Buffer RL can be stored at (-90~-65°C) for later use or used directly in the procedure. 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.
3. Perform all steps at room temperature (15–30°C). During the procedure, work quickly.
4. Prepare ethanol solution with RNase-free ddH_2O before use.
5. β-Mercaptoethanol (β-ME) must be added to Buffer RL before use. The final concentration of β-ME is 1%. For example, add 10 μl β-ME per 1 ml Buffer RL. Buffer RL containing β-ME can be stored at 2-8°C for up to 1 month. Buffer RL may form a precipitate upon storage. If necessary, redissolve by warming, and then place at room temperature (15–30°C).
6. Add appropriate volumes of ethanol (96–100%) to Buffer RW before the first use as indicated on the bottle.

Solutions Preparation

- **Preparation of DNase I stock solution:** Dissolve the lyophilized DNase I (1500 U) in 550 μl of the RNase-free ddH_2O. Mix gently by inverting. Do not vortex.

  Divide it into single-use aliquots, and store at -30~-15°C for up to 9 months. Thawed aliquots can be stored at 2–8°C for up to 6 weeks. Do not refreeze the aliquots after thawing.

- **Preparation of Carrier RNA stock solution:** When processing <5000 cells, Carrier RNA may be added to the lysate before homogenization. Before using for the first time, dissolve the Carrier RNA (310 μg) in 1 ml RNase-free ddH_2O. Store this stock solution at -30~-15°C, and use it to make

fresh dilutions for each set of RNA preps. The concentration of this stock solution is 310 μg/ml (i.e. 310 ng/μl).

- **Preparation of Carrier RNA working solution:** To make a working solution (4 ng/μl) for 10 preps, add 5 μl stock solution to 34 μl Buffer RL and mix by pipetting. Add 6 μl of this diluted solution to 54 μl Buffer RL to give a working solution of 4 ng/μl. Add 5 μl of this working solution to the lysate.

Protocol Ⅰ: Purification of Total RNA from Microdissected Cryosections

Protocol I is suitable to isolate RNA from frozen microdissected tissue of animal. It is a challenge for isolating RNA from very small amounts of starting sample. In addition, fixation and staining steps may compromise the integrity of RNA.

1. Add appropriate volume of Buffer RL **(Ensure β-mercaptoethanol has been added to the Buffer RL at the final concentration of 1%)** to the sample. The volume depends on the collection vessel used for microdissection, but should not be greater than [A] 65 μl or [B] 300 μl.

   Note: (A) indicates the volume required by Leica AS LMD, (B) indicates the volume required by other instruments, the following (A) & (B) are consistent with this.

2. If necessary, transfer the sample and buffer to a larger vessel (e.g., 1.5 ml or 2 ml RNase-free microcentrifuge tube, not supplied). Adjust the volume to (A) 75 μl and (B) 350 μl with Buffer RL.

   Note: If processing <5000 cells, 20 ng Carrier RNA (5 μl of a 4 ng/μl solution) may be added to the lysate before homogenization. Prepare the Carrier RNA as described before.

3. Vortex the sample for 30 sec.

4. Add 1 volume ((A) 75 μl and (B) 350 μl) of 70% ethanol, and mix well by pipetting. Proceed immediately to step 5.

   Note: The volume of 70% ethanol should be reduced accordingly if some lysate was lost during homogenization. Precipitates may be visible after addition of ethanol, which does not affect the procedure.

5. Transfer the sample, including any precipitate that may have formed, to an RNase-free Spin Column CR1 placed in a 2 ml Collection Tube (supplied). Close the lid gently, and centrifuge for 30 sec at 12,000 rpm (~13,400 × g). Discard the flow-through. Set the RNase-free Spin Column CR1 back into the Collection Tube.

6. Add 350 µl Buffer RW1 to the RNase-free Spin Column CR1. Close the lid gently, and centrifuge for 30-60 sec at 12,000 rpm (~13,400 × g) to wash the spin column membrane. Discard the flow-through. Set the Spin Column CR1 back into the Collection Tube.
7. Preparation of DNase I working solution: Add 10 µl DNase I stock solution (see Preparation of DNase I stock solution) to 70 µl Buffer RDD. Mix by gently inverting the tube.
8. Add 80 µl DNase I working solution directly to the center of RNase-free Spin Column CR1, and place at room temperature (15–30°C) for 15 min.
9. Add 350 µl Buffer RW1 to the RNase-free Spin Column CR1. Close the lid gently, and centrifuge for 30-60 sec at 12,000 rpm (~13,400 × g) to wash the spin column membrane. Discard the flow-through. Set the RNase-free Spin Column CR1 back into the Collection Tube.
10. Add 500 µl Buffer RW **(Ensure that ethanol has been added to Buffer RW before use)** to the RNase-free Spin Column CR1. Incubate at room temperature for 2 min, Close the lid gently, and centrifuge for 30-60 sec at 12,000 rpm (~13,400 × g). Discard the flow-through and set the RNase-free Spin Column CR1 back into the Collection Tube.
11. Repeat step 10.
12. Centrifuge for 2 min at 12,000 rpm (~13,400 × g). Discard the flow-through and place the RNase-free Spin Column CR1 in the air with lid open for several minutes to air dry the membrane.

    Note: This step is necessary, since residual ethanol in the eluate may affect downstream applications such as RT-PCR.
13. Place the RNase-free Spin Column CR1 in a clean 1.5 ml RNase-free microcentrifuge tube and apply 14 µl RNase-free ddH_2O to the center of the membrane. Close the lid gently and incubate at room temperature (15–30°C) for 2 min. Centrifuge at 12,000 rpm(~134,00×g) for 1 min.

    Note: Do not elute with less than 10 µl RNase-free water. As little as 10 µl RNase-free water can be used for elution if a higher RNA concentration is required, but the yield will be reduced.

Protocol:Purification of Total RNA from Formalin Fixed Microdissected Tissues

The protocol is suitable for purification of total RNA from Formalin Fixed Microdissected Tissues. In the Formalin Fixed Microdissected Tissues, RNA yield and purity is determined by the status of sample, procedure of fixing and storage condition. RNA may degrade into <300bp fragment because of above factors. Since the kit can be used for purification of total RNA (usually >200bp) only, it is possible to purify nothing from samples because of severe degradation.

1. Preheat the water bath or thermo bath at 55°C for digestion by Proteinase K (not supplied, Cat. no. GRT403-01/GRT403-02) from step 4.
2. Add appropriate volume of Buffer RL **(Ensure β-mercaptoethanol has been added to the Buffer RL at the final concentration of 1%)** to the sample . The volume depends on the collection vessel used for microdissection, but should not be greater than 140 µl.
3. If necessary, transfer the sample and buffer to a larger vessel (e.g., 1.5 or 2 ml tube, not supplied). Adjust the volume to 150 µl with Buffer RL.

   Note: If processing <5000 cells, 20 ng Carrier RNA (5 µl of a 4 ng/µl solution) may be added to the lysate before homogenization. Prepare the Carrier RNA referring to Preparation of solutions.
4. Add 295 µl RNase-free ddH_2O and then 5µl Proteinase K (20 mg/ml, not supplied), and mix well by pipetting. Incubate at 55°C for 10 min. Centrifuge for 3 min at 12,000 rpm (~13,400 × g).

   Note: some tissue particles or a layer of thin membrane may forms occasionally .
5. Transfer above layer of solutions (about 450 µl) into a clean RNase-free microcentrifuge tube (not supplied).

   Note: Do not pipet tissue debris and ensure the tip is below the layer of thin membrane to avoid transferring the thin membrane into the RNase-free microcentrifuge tube.
6. Add 0.5 volume of ethanol (>99%) (usually 225 µl), and mix well by pipetting.

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

7. Transfer the sample, including any precipitate that may have formed, to an RNase-free Spin Column CR1 placed in a 2 ml Collection Tube (supplied). Close the lid gently, and centrifuge for 30-60 sec at 12,000 rpm (~13,400 × g). Discard the flow-through. Set the RNase-free Spin Column CR1 back into the Collection Tube.
8. Add 350 µl Buffer RW1 to the RNase-free Spin Column CR1. Close the lid gently, and centrifuge for 30-60 sec at 12,000 rpm (~13,400 × g) to wash the spin column membrane. Discard the flow-through. Set the RNase-free Spin Column CR1 back into the Collection Tube.
9. Preparation of DNase I working solution: Add 10 µl DNase I stock solution (see Preparation of DNase I stock solution) to 70 µl Buffer RDD. Mix by gently inverting the tube.
10. Add 80 µl DNase I working solution directly to the center of the RNase-free Spin Column CR1, and place at room temperature (15-30°C) for 15 min.
11. Add 350 µl Buffer RW1 to the RNase-free Spin Column CR1. Close the lid gently, and centrifuge for 30-60 sec at 12,000 rpm (~13,400 × g) to wash the spin column membrane. Discard the flow-through. Set the RNase-free Spin Column CR1 into the Collection Tube.
12. Add 500 µl Buffer RW **(Ensure that ethanol has been added to Buffer RW before use)** to the RNase-free Spin Column CR1. Incubate at room temperature for 2 min, close the lid gently, and centrifuge for 30-60 sec at 12,000 rpm (~13,400 × g). Discard the flow-through and set the RNase-free Spin Column CR1 back into the Collection Tube.
13. Repeat step 12.
14. Centrifuge for 2 min at 12,000 rpm (~13,400 × g). Discard the flow-through and place the RNase-free Spin Column CR1 in the air with lid open for several minutes to air dry the membrane.

    Note: This step is necessary, since residual ethanol in the eluate may affect downstream applications such as RT-PCR.
15. Place the RNase-free Spin Column CR1 in a clean 1.5 ml RNase-free microcentrifuge and apply 14 µl RNase-free ddH_2O to the center of the membrane. Close the lid gently and incubate at room temperature (15–30°C) for 2 min. Centrifuge at 12,000 rpm (~13,400 × g) for 1 min.

    Note: Do not elute with less than 10 µl RNase-free water. As little as 10 µl RNase-free water can be used for elution if a higher RNA concentration is required, but the yield will be reduced.

Protocol |||: Purification of Total RNA from Animal Tissues

This protocol is suitable for the purification of total RNA from easy-to-lyse animal tissues. It is essential to use the correct amount of starting material in order to obtain optimal RNA yield and purity. A maximum amount of 5 mg fresh or frozen tissue can generally be processed. Some tissues such as spleen, parts of brain, lung, and thymus tend to form precipitates during the procedure. However, this does not affect RNA purification.

Do not overload the RNase-free Spin Column CR1 as this will significantly reduce RNA yield and purity.

1. Weigh the amount of tissue. Do not use more than 5 mg. Proceed immediately to step 2.
2. Disruption and homogenization in the presence of Buffer RL

   (Please see step 2a & 2b)

   Notes: If processing <10 µg tissue, 20 ng Carrier RNA (5 µl of a 4 ng/µl solution) may be added to the lysate before homogenization. Prepare the Carrier RNA referring to preparation of solutions. Incomplete homogenization leads to significantly reduced RNA yields and can cause clogging of the RNase-free Spin Column CR1.

   2a Disruption and homogenization using the homogenizer

   Place the tissue in a suitably sized vessel. Add 350 µl Buffer RL and operate the homogenizer immediately at full speed until the lysate is homogeneous (usually 20-40 sec). Proceed to step 3.

   2b Disruption using a pestle (not supplied)

   Add 350 µl Buffer RL to a 1.5 ml RNase-free microcentrifuge tube (not supplied). Transfer tissue sample frozen at -80°C quickly to this microcentrifuge tube and grind to a fine powder without allowing the sample to thaw. Transfer the suspension into an RNase-free Filtration Column CS (not supplied), set the RNase-free Filtration Column CS into a Collection Tube (supplied), and centrifuge for 2 min at 12,000 rpm (~13,400 × g). Collect the flow-through and proceed to step 3.
3. Centrifuge the lysate (2a) and filtrate (2b) for 3 min at 12,000 rpm (~13,400 × g). Carefully pipette the supernatant and transfer it to a 1.5 ml RNase-free microcentrifuge tube (not supplied).
4. Add 1 volume of 70% ethanol (350 µl), and mix well by pipetting. Proceed immediately to step 5.

   Note: The volume of 70% ethanol should be reduced accordingly if some lysate was lost during homogenization and DNA removal. Precipitates may be visible after addition of ethanol, which does not affect the procedure.

5. Transfer the sample, including any precipitate that may have formed, to an RNase-free Spin Column CR1 placed in a 2 ml Collection Tube (supplied). Close the lid gently, and centrifuge for 30-60 sec at 12,000 rpm (~13,400 × g). Discard the flow-through. Set the Spin Column CR1 back into the Collection Tube.
6. Add 350 µl Buffer RW1 to the RNase-free Spin Column CR1. Close the lid gently, and centrifuge for 30-60 sec at 12,000 rpm (~13,400 × g) to wash the spin column membrane. Discard the flow-through. Set the RNase-free Spin Column CR1 into the Collection Tube.
7. Preparation of DNase I working solution: Add 10 µl DNase I stock solution (see Preparation of DNase I stock solution) to 70 µl Buffer RDD. Mix by gently inverting the tube.
8. Add 80 µl DNase I working solution directly to the center of the RNase-free Spin Column CR1, and place at room temperature (15-30°C) for 15 min.
9. Add 350 µl Buffer RW1 to the RNase-free Spin Column CR1. Close the lid gently, and centrifuge for 30-60 sec at 12,000 rpm (~13,400 × g) to wash the spin column membrane. Discard the flow-through and set the RNase-free Spin Column CR1 back into the Collection Tube.
10. Add 500 µl Buffer RW **(Ensure that ethanol has been added to Buffer RW before use)** to the RNase-free Spin Column CR1. Incubate at room temperature for 2 min, close the lid gently, and centrifuge for 30-60 sec at 12,000 rpm (~13,400 × g). Discard the flow-through and set the RNase-free Spin Column CR1 back into the Collection Tube.
11. Repeat step 10.
12. Centrifuge for 2 min at 12,000 rpm (~13,400 × g). Discard the flow-through and place the RNase-free Spin Column CR1 in the air with lid open for several minutes to air dry the membrane.

    Note: This step is necessary, since residual ethanol in the eluate may affect downstream applications such as RT-PCR.
13. Place the RNase-free Spin Column CR1 in a clean 1.5 ml RNase-free microcentrifuge tube and apply 14 µl RNase-free ddH_2O to the center of the membrane. Close the lid gently and incubate at room temperature (15–30°C) for 2 min. Centrifuge at 12,000 rpm (~13,400 × g) for 1 min.

    Note: Do not elute with less than 10 µl RNase-free water. As little as 10 µl RNase-free water can be used for elution if a higher RNA concentration is required, but the yield will be reduced.

Protocol IV: Purification of Total RNA from Fibrous Tissues

Fibrous tissues contain a large amount of contractile proteins, conjunctive tissues, collagens such as bones, heart, and skin. Digestion of Proteinase K is introduced since removal of these proteins is critical to purification of total RNA from fibrous tissues.

This protocol is suitable for purification of total RNA from heart, muscles, skin and other fibrous tissues. In the process, the buffer does not inhibit activity of RNase for long time, so it is not for the tissue rich in RNase such as spleen, intestines, etc.

Note: We recommend starting with no more than 5 mg tissue. Do not overload the RNase-free Spin Column CR1, as this will significantly reduce RNA yield and purity.

1. Preheat the water bath or thermo bath at 55°C for digestion by Proteinase K (not supplied) from step 4.
2. Weigh the amount of tissue (<5 mg), proceed to step 3 immediately.
3. Disruption and homogenization in the presence of Buffer RL **(Ensure β-mercaptoethanol has been added to the Buffer RL at the final concentration of 1%)** (Please see step 3a & 3b)

   Notes: If processing <10 μg tissue, 20 ng Carrier RNA (5 μl of 4 ng/μl solution) may be added to the lysate before homogenization. Prepare the Carrier RNA referring to preparation of solutions on the page 4. Incomplete homogenization leads to significantly reduced RNA yields and can cause clogging of the RNase-free Spin Column CR1. Homogenization using homogenizer or a filtered column results in more RNA yield.

   3a Disruption and homogenization using the homogenizer

   Weigh the tissue and place in a suitably sized vessel. Add 150 μl Buffer RL. Operate the homogenizer immediately at full speed until the lysate is homogeneous (usually 20-40 sec). Proceed to step 4.

   3b Disruption using a pestle (not supplied)

   Add 150 μl Buffer RL to a 1.5 ml RNase-free microcentrifuge tube (not supplied). Transfer tissue sample frozen at -80°C quickly to this microcentrifuge tube and grind thoroughly without allowing the sample to thaw. Proceed to step 4.

   Note: Ensure the tissue sample is disrupted thoroughly when using pestle.

4. Add 295 µl RNase-free ddH_2O and then 5 µl Proteinase K (20 mg/ml, not supplied, cat.no.GRT403-01/GRT403-02), and mix well by pipetting. Incubate at 55°C for 10min. Centrifuge for 3 min at 12,000 rpm (~13,400 ×g).

   Note: some tissue particles or a layer of thin membrane forms occasionally .

5. Transfer top layer of solutions (about 450 µl) into a clean 1.5 ml RNase-free microcentrifuge tube (not supplied).

   Note: Do not pipet tissue particles and ensure the tip is below the layer of thin membrane to avoid transferring the thin membrane into the RNase-free microcentrifuge tube.

6. Add 0.5 volume of ethanol (>99%) (usually 225 µl), and mix well by pipetting.

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

7. Transfer the sample, including any precipitate that may have formed, to an RNase-free Spin Column CR1 placed in a 2 ml Collection Tube (supplied). Close the lid gently, and centrifuge for 30-60 sec at 12,000 rpm (~13,400 × g). Discard the flow-through. Set the RNase-free Spin Column CR1 back into the Collection Tube.
8. Add 350 µl Buffer RW1 to the RNase-free Spin Column CR1. Close the lid gently, and centrifuge for 30-60 sec at 12,000 rpm (~13,400 × g) to wash the spin column membrane. Discard the flow-through. Set the RNase-free Spin Column CR1 back into the Collection Tube.
9. Preparation of DNase I working solution: Add 10 µl DNase I stock solution (see Preparation of DNase I stock solution) to 70 µl Buffer RDD. Mix by gently inverting the tube.
10. Add 80 µl DNase I working solution directly to the center of the RNase-free Spin Column CR1, and place at room temperature (15–30°C) for 15 min.
11. Add 350 µl Buffer RW1 to the RNase-free Spin Column CR1. Close the lid gently, and centrifuge for 30-60 sec at 12,000 rpm (~13,400 × g) to wash the spin column membrane. Discard the flow-through. Set the RNase-free Spin Column CR1 back into the Collection Tube.
12. Add 500 µl Buffer RW **(Ensure that ethanol has been added to Buffer RW before use)** to the RNase-free Spin Column CR1. Incubate at room temperature for 2 min, close the lid gently, and centrifuge for 30-60 sec at 12,000 rpm (~13,400 × g). Discard the flow-through and set the RNase-free Spin Column CR1 back into the Collection Tube.

13. Repeat step 12.
14. Centrifuge for 2 min at 12,000 rpm (~13,400 × g). Discard the flow-through and place the RNase-free Spin Column CR1 in the air with lid open for several minutes to air dry the membrane.

    Note: This step is necessary, since residual ethanol in the eluate may affect downstream applications such as RT-PCR.
15. Place the RNase-free Spin Column CR1 in a clean 1.5 ml RNase-free microcentrifuge tube and apply 14 µl RNase-free ddH_2O to the center of the membrane. Close the lid gently and incubate at room temperature (15–30°C) for 2 min. Centrifuge at 12,000 rpm (~13,400 × g) for 1 min.

    Note: Do not elute with less than 10 µl RNase-free water. As little as 10 µl RNase-free water can be used for elution if a higher RNA concentration is required, but the yield will be reduced.

Protocol V: Purification of Total RNA from Animal Cells

It is essential to use the correct amount of starting material in order to obtain optimal RNA yield and purity. The RNA binding capacity of the RNase-free Spin Column CR1 is 45 µg RNA. We recommend starting with no more than 5 × 10^5 cells. Do not overload the RNase-free Spin Column CR1, as this will significantly reduce RNA yield and purity.

1. Treat cells according to step 1a, 1b or 1c.

1a Cells precipitation: Flick the bottom of tube to separate the cells and add 350 µl Buffer RL **(Ensure β-mercaptoethanol has been added to the Buffer RL at the final concentration of 1%)**, Mix by vortex or pipetting and proceed to step 2.

1b Cells grown in suspension: Calculate the number of cells (please see Table 2). Add the appropriate number of cells in a RNase-free microcentrifuge tube (not supplied), and centrifuge for 5 min at 300 × g. Carefully remove all supernatant by aspiration, and add 350 µl Buffer RL **(Ensure β-mercaptoethanol has been added to the Buffer RL at the final concentration of 1%)**. Proceed to step 2.

1c Cells grown in a monolayer: Cells grown in a monolayer in cell-culture vessels can be either lysed directly[*1] in the vessel (up to 10 cm diameter) or trypsinization and collected as a cell pellet prior to lysis[*2]. Cells grown in a monolayer in cell-culture flasks should always be trypsinized.

[*1] **To lyse cells directly**

Calculate the number of cells. Aspirate the medium completely and add 350 µl Buffer RL **(Ensure β-mercaptoethanol has been added to the Buffer RL at the final concentration of 1%)** to lyse cells. Collect lysate and transfer

it into 1.5 ml RNase-free microcentrifuge tube (not supplied). Mix by vortex or pipetting until the cluster of cells is not visible. Proceed to step 2.

[*2] **To trypsinize and collect cells (usually in cell-culture flask)**

Calculate 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 detaching from the dish or flask, add medium containing serum to inactivate the trypsin, transfer the cells to an RNase-free microcentrifuge tube (not supplied), and centrifuge at 300 × g for 5 min. Completely aspirate the supernatant, add 350 µl Buffer RL **(Ensure β-mercaptoethanol has been added to the Buffer RL at the final concentration of 1%)** to lyse cells and proceed to step 2.

Note 1: 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 yield and purity.

Note 2: When processing ≤1 × 10^5 cells, the volume of Buffer RL could be reduced to 75 µl and a smaller microcentrifuge tube can be adopted. Homogenize by vortex for 1 min and proceed to step 3.

Note 3: If processing <5000 cells, 20 ng Carrier RNA (5 µl of 4 ng/µl solution) may be added to the lysate before homogenization. Prepare the Carrier RNA as described on page 4.

Note 4: If cells are not suspended thoroughly, it will result in incomplete lysis and reduce the yield of RNA.

2. Sample homogenization (using either 2a or 2b)

Note: Incomplete homogenization leads to significantly reduced RNA yields and can cause clogging of the RNase-free Spin Column CR1. Homogenization using homogenizer or a filtered column results in higher RNA yield.

2a Transfer the cells in Buffer RL into RNase-free Filtration Column CS (in a 2ml Collection Tube) (not supplied). Centrifuge at 12,000 rpm (13,400 × g) for 2 min and collect the filtrate. Proceed to step 3.

2b Homogenize for 30 sec by homogenizer.

3. Add 1 volume of 70% ethanol (usually 350 µl), and mix well by pipetting. Proceed immediately to step 4.

Note: The volume of 70% ethanol should be reduced accordingly if some lysate was lost during homogenization and DNA removal. Precipitates may be visible after addition of ethanol, which does not affect the procedure.

If 75 µl Buffer RL is added in the step2, add only 75 µl 70% ethanol in this step too. Precipitates may be visible after addition of ethanol, which does not affect the procedure.

4. Transfer the sample, including any precipitate that may have formed, to an RNase-free Spin Column CR1 placed in a 2 ml Collection Tube (supplied). Close the lid gently, and centrifuge for 30-60 sec at 12,000 rpm (~13,400 × g). Discard the flow-through. Set the RNase-free Spin Column CR1 into the Collection Tube.
5. Add 350 µl Buffer RW1 to the RNase-free Spin Column CR1. Close the lid gently, and centrifuge for 30-60 sec at 12,000 rpm (~13,400 × g) to wash the spin column membrane. Discard the flow-through. Set the RNase-free Spin Column CR1 back into the Collection Tube.
6. Preparation of DNase I working solution: Add 10 µl DNase I stock solution (see Preparation of DNase I stock solution) to 70 µl Buffer RDD. Mix by gently inverting the tube.
7. Add 80 µl DNase I working solution directly to the center of the RNase-free Spin Column CR1, and place at room temperature (15–30°C) for 15 min.
8. Add 350 µl Buffer RW1 to the RNase-free Spin Column CR1. Close the lid gently, and centrifuge for 30-60 sec at 12,000 rpm (~13,400 × g) to wash the spin column membrane. Discard the flow-through. Set the RNase-free Spin Column CR1 back into the Collection Tube.
9. Add 500 µl Buffer RW **(Ensure that ethanol has been added to Buffer RW before use)** to the RNase-free Spin Column CR1. Incubate at room temperature for 2 min, close the lid gently, and centrifuge for 30-60 sec at 12,000 rpm (~13,400 × g). Discard the flow-through and set the RNase-free Spin Column CR1 into the Collection Tube.
10. Repeat step 9.
11. Centrifuge for 2 min at 12,000 rpm (~13,400 × g). Discard the flow-through and place the RNase-free Spin Column CR1 in the air with lid open for several minutes to air dry the membrane.

    Note: This step is necessary, since residual ethanol in the eluate may affect downstream applications such as RT-PCR.
12. Place the RNase-free Spin Column CR1 in a clean 1.5 ml RNase-free microcentrifuge tube (supplied) and apply 14 µl RNA-free ddH_2O to the center of the membrane. Close the lid gently and incubate at room temperature (15–30°C) for 2 min. Centrifuge at 12,000 rpm (~13,400 × g) for 1 min to elute the RNA.

    Note: Do not elute with less than 10 µl RNase-free water. As little as 10 µl RNase-free water can be used for elution if a higher RNA concentration is required, but the yield will be reduced.