app*GENE* Total RNA Kit Manual

Fast, Efficient, and Flexible

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Ver1.0

appleton®
For the Life Scientist

KIT COMPONENTS

Product Codes	ARN015	ARN016
Pack size	50 preps	250 preps
ALY buffer ɸ (volume of 100% b-mercaptoethanol to add) ⊕	33ml (330µl) ⊕	165ml (1.65ml) ⊕
AW1 buffer ɸ	35ml	175ml
AW2 buffer (volume of 96-100% ethanol to add)*	16.5ml (66ml)*	82ml (328ml)*
AEB buffer (for RNA elution)	5ml	5x 5ml
EM reagent (anti-foam)	1 ml	5 ml
Nuclease-free water	510µl	2.55ml
DNase I (RNase-free, lyophilised)	2 tubes	10 tubes
10X DNase I reaction buffer	4.5ml	22.5ml
app*GENE* RNA spin columns	50 columns	5x 50 columns
Collection tubes (2ml)	50 tubes	5x 50 tubes

BEFORE STARTING:

1. Mix each buffer well, but do not mix the ALY buffer vigorously as it may foam due to its non-ionic detergent content. (To prevent foaming, add EM Reagent to the ALY buffer during the protocol to a final concentration 3.3% in the lysis mixture – see sample preparation section below).

2. Examine the ALY and AW1 buffers. If sedimentation has occurred, incubate the ALY buffer at 50°C, and the AW1 buffer at 37°C, mixing occasionally until the sediment has dissolved. Cool to room temperature.

3. ⊕ - Add 100% β-mercaptoethanol to the ALY buffer, to a final concentration of 1%. The combined ALY buffer and β-mercaptoethanol will remain stable at 2-8°C for four weeks. When isolating in parts, transfer enough of the ALY buffer for one isolation to a separate RNase-free bottle/tube and add β-mercaptoethanol. Marking the bottle after adding β-mercaptoethanol is recommended.

4. ɸ - Protect ALY and AW1 buffers from sunlight.

5. * - Add appropriate amount of 96-100% ethanol to the AW2 buffer; for details, see the instructions on the bottle label and in the table above. Marking the bottle after adding the alcohol is recommended.

6. DNase I is shipped lyophilized. Each vial contains sufficient amount enzyme for 25 preps. Before using for the first time, reconstitute the DNase I lyophilizate in 255µl of nuclease-free water. Incubate 1 minute at room temperature. Mix carefully by inverting the tube several times. DNase I is sensitive to physical denaturation. Therefore, do not vortex DNase I solution. Divide it into aliquots to avoid excessive freeze-thawing. Do not freeze/thaw more than three times. After reconstitution the DNase I should be kept at -20°C and it is stable for 6 months.

7. Prepare a freezing rack for storage of the eluted RNA.

8. Pre-chill a sterile mortar and pestle.

9. Read the entire protocol bearing in mind the Considerations section to read tips about how to avoid issues like poor RNA integrity due to incorrect storage or the use of inappropriately treated consumables, inefficient elution due to lack of washing, and how to amend the elution volume depending on the concentration of RNA required.

STORAGE

Store the kit at room temperature (18 - 25°C). Avoid evaporation from buffer bottles by ensuring they are tightly closed before storing. Under proper storage the kit will remain stable for at least 12 months.

DESCRIPTION

The **app*GENE* Total RNA kit** provides an easy-to-use and rapid method for the isolation of up to 230µg of from total RNA from cultured eukaryotic cells and from fresh or frozen tissues. Multiple samples of up to 1 x 10^7 eukaryotic cells or up to 30 mg of tissue can be processed in parallel in fewer than 20 minutes. Purified RNA can be used in many downstream applications such as RT-PCR, RT-qPCR, Northern blotting, nuclease protection assays, and in vitro translation. The product is intended for research use only.

SAMPLE PREPARATION

TISSUE

1.1 Homogenisation via Mortar and Pestle

Put ≤ 30mg tissue previously frozen in liquid nitrogen or dry ice into a pre-chilled, sterile mortar. Using a chilled pestle, carefully, but firmly crush the tissue into smaller pieces and then, into a pulp. Transfer the powder thus obtained into a sterile RNase-free 2ml microcentrifuge tube containing 600µl ALY buffer and 20µl EM reagent and go to step 2 of the protocol. After pulping, a thin, sticky layer may be formed, rather than a powder. If this occurs, add 600µl ALY buffer containing 20µl EM reagent-to the mortar and reconstitute the tissue by pipetting and then transfer the lysate into a sterile RNase-free 2ml microcentrifuge tube. Retrieve the tissue remains from the pestle as well.

1.2 Homogenisation via Mechanical Homogenizer

Place ≤ 30mg tissue in a sterile RNase-free 2ml microcentrifuge tube, add 100µl ALY buffer and 3.3µl EM reagent and carefully homogenize with a sterile homogenizer tip. After homogenization, retrieve the tissue remains from the knife tip by washing it with 500µl ALY buffer. Combine the fractions thus obtained and transfer the entire volume to a new sterile, RNase-free 2ml microcentrifuge tube. Go to step 1 of the below protocol.

CULTURED CELLS

Thaw 10^4-10^7 cell suspension or adherent cells, fresh or frozen at 37°C. Centrifuge the cells suspended in growth medium or PBS buffer in a 15ml falcon tube or a 1.5-2ml sterile, RNase-free microcentrifuge tube at 400 x g. If a compact cell pellet is not formed, wash the cells twice with 1ml cold PBS buffer. Add 600µl ALY buffer containing 20µl EM reagent. Mix thoroughly by vortexing for 30 sec and subsequent pipetting. In some cases when cells tend to form syncytia (myoblasts) or tight connections (epithelial cells) or they are high in number (approx. 10^7 cells), it may be difficult to re-suspend them in ALY buffer. In such cases, pipette carefully, using a ≥1000µl pipette tip or a sterile syringe. Do not use filter tips. Transfer everything to a new 2ml sterile, RNase-free microcentrifuge tube. Go to step 1 of the below protocol.

TOTAL RNA EXTRACTION PROTOCOL

1. Vortex either fragmented tissue / cells (with pre-added 600µl ALY buffer containing 20µl EM reagent) for 1 minute.

2. If more isolations are carried out ALY buffer may be prepared for all samples adding EM Reagent to the final concentration 3.3%. E.g. For 10 tissue preps, transfer 6ml of ALY Buffer to an RNase and DNase free tube and add 200µl EM Reagent and use this buffer for lysis.

3. Centrifuge for 2 minutes at 15,000 x g.

4. Transfer the supernatant into an RNase, DNase free 1.5-2ml sterile, RNase-free microcentrifuge tube.

5. Add 600µl 70% ethanol to the transferred supernatant.

6. Mix well by pipetting or vortexing.

7. Transfer 700µl of the resulting mixture into an app*GENE* RNA spin column placed in a collection tube.

8. Centrifuge for 15 sec at ≥ 8000 x g.

9. Discard the flow-through and re-use the column, together with the collection tube.

10. Transfer the remaining mixture into the same app*GENE* RNA spin column and centrifuge at ≥ 8000 x g for 15 sec.

11. Discard the flow-through and place the app*GENE* RNA spin column in a new collection tube.

DNA Removal (OPTIONAL):

a. Prewash the app*GENE* RNA spin column with 500µl AW2 buffer and centrifuge for 1 min at 15,000 x g.

b. For each isolation mix 90µl 10x DNase I reaction buffer and 10µl reconstituted DNase I. Mix by inverting the tube.

c. Apply 95µl of the above DNase mixture onto the centre of the app*GENE* RNA spin column.

d. Incubate for 15 minutes at room temperature.

e. Add 600µl AW1 buffer and centrifuge for 15 sec at ≥ 8000 x g.

f. Discard the flow-through and re-use the collection tube. **Proceed to step 14.**

12. Add 700µl AW1 buffer and centrifuge for 15 sec at ≥ 8000 x g.

13. Discard the flow-through and reuse the collection tube.

14. Add 500µl AW2 buffer and centrifuge for 30 sec at ≥ 8000 x g.

15. Discard the flow-through and reuse the collection tube.

16. Repeat steps 14 & 15.

17. Centrifuge for 90 sec at 15,000 x g (read Considerations, Section 1.3 on washing).

18. Discard the collection tube and flow-through and carefully transfer the app*GENE* RNA spin column to a sterile, 1.5ml sterile, RNase-free microcentrifuge (read Considerations, Section 1.4 on elution).

19. Add 50-100µl elution buffer AEB precisely, onto the centre of the app*GENE* RNA spin column membrane.

20. Centrifuge at ≥ 8000 x g for 1 minute.

21. Remove the app*GENE* RNA spin column and place the tube with the eluted RNA in a freezing rack. The isolated RNA is ready for use in downstream applications or for storage at -80°C.

CONSIDERATIONS

1.1 Tissue Storage Prior to RNA Extraction

Proper sampling and storing of the biological material prior to RNA isolation is crucial to obtaining a high purity RNA sample. After sampling, the material should either be preserved by deep freezing at -80°C or in liquid nitrogen or stored in RNase inactivating buffers at -20°C. Most tissues must be preserved within 30 minutes of sampling. Tissues rich in RNases (pancreas, liver) must be preserved immediately. When isolating from cell cultures, the best results are achieved with fresh material. If storage is unavoidable, discard the supernatant after centrifugation and freeze the cell pellet at -80°C or in liquid nitrogen.

When isolating from more than the recommended quantity of starting material (>30 mg, >10^7 cells), divide the material into several isolations so that each 30mg (or 10^7 cells) of sample material is isolated with a separate buffer and spin column set. If this quantity is exceeded, the homogenizing column may become clogged and/or the isolated RNA may be of low purity.

1.2 RNases

RNases are very active enzymes which do not require any cofactors and are resistant to autoclaving at 121°C for 15 minutes. In order to avoid the degrading effect of the enzymes on the RNA, the following recommendations should be followed:

- Use disposable latex, vinyl or nitrile gloves at all times when working with the RNA. Do not touch any items not designed specifically for RNA work.
- If possible, keep the samples at 2-8°C at all stages of the procedure, including centrifugation. Use decontaminated freezing racks instead of ice in order to avoid RNase contamination. Keeping RNA after elution in the freezing racks is mandatory.
- Disposable plastic ware (tips, tubes) should be RNase-free or autoclaved at 134°C for 18-20 minutes.
- Re-useable plastic ware, glass and porcelain should be soaked overnight in 0.1 N NaOH/0.1% DEPC water (or RNase-free water) and then washed with 0.1% DEPC water (or RNase-free water). When applicable, glass and porcelain (mortars) should be parched at 150-140°C for 2-4 hrs and cooled to room temperature.
- Wipe surfaces, pipettes, centrifuge (wipe the rotor separately) and tube racks with 3% hydrogen peroxide or <0.5% sodium hypochlorite (or any other commercially available RNase inactivating fluid). Prior to decontamination, test the decontaminant on a small area of the material for possible undesired reactions.

1.3 Washing

The wash buffer contains alcohol, which may interfere with some enzymatic reactions and also decrease the elution efficiency. It is therefore vital to remove the alcohol completely from the app*GENE* RNA spin column prior to elution.

1.4 Elution

The optimal volume of the AEB elution buffer used should be chosen in line with the amount of the sample material and the final RNA concentration expected. The use of 30-50µl AEB is recommended when extracting from 2-10mg of tissue or <10^4 cells, as is increasing the elution buffer volume to 100µl when isolating from 10-30mg of tissue or 10^4-10^7 cells. If a high RNA concentration is desired, the elution volume may be reduced to 50µl. It should be noted that this may reduce the efficiency of the RNA retrieval. It is essential to apply the elution buffer precisely to the centre of the membrane. When more sample material is to be used for isolation (not recommended as the column can then easily become clogged), full RNA retrieval can be obtained by performing a second elution (100µl). For the second elution, repeat steps 19 to 21 of the above protocol, placing the app*GENE* RNA spin column in a new, sterile 1.5 ml microcentrifuge tube. AEB buffer does not contain EDTA, which may interfere with some enzymatic reactions.

1.5 DNA contamination

All the biological material used for RNA isolation also contains DNA. There is no RNA isolation method which guarantees complete DNA removal unless the RNA sample is treated with DNase after isolation. Even slight DNA contamination (several gDNA copies per reaction) may give an additional signal in a quantitative PCR analysis after reverse transcription. The app*GENE* Total RNA kit provides efficient on-column digestion of the DNA during RNA purification. The DNase I is removed by wash buffer AW1.

1.6 Average DNA Yields from Different Sample Types

Sample Material	Amount	DNA Yield
293 HEK cell line	10^6	5 µg
HeLa cell line	10^6	7 µg
Liver	10mg	75 µg
Heart	5 mg	10 µg
Brain	5 mg	5 µg
Colon	10mg	16 µg

TROUBLE SHOOTING / TECHNICAL SUPPORT

For troubleshooting please visit www.appletonwoods.co.uk/app*GENE*-Total-RNA-kit-troubleshooting.pdf for a trouble shooting guide on RT.

ASSOCIATED PRODUCTS

Product	Pack Size	Product Code
app*SCRIPT* cDNA synthesis kit	25 reactions	ARP601
app*GREEN* 1-Step Extreme Low ROX Kit	200 reactions	ARP742
app*GREEN* 1-Step Extreme High ROX Kit	200 reactions	ARP752
app*GREEN* 1-Step Opti Low ROX Kit	200 reactions	ARP792
app*GREEN* 1-Step Opti High ROX Kit	200 reactions	ARP802
app*TAQ* Polymerase	500 units	ARP001
app*TAQ* RedMix (2X)	200 reactions	ARP062
app*TAQ* Hot Start Polymerase	250 units	ARP021
app*HiFi* Polymerase	200 units	ARP041
Molecular biology grade water	100mL	BMW001

More pack sizes available at www.appletonwoods.co.uk