

Product Information

GenElute™ Direct mRNA Miniprep Kit

Catalog Numbers **DMN10, DMN 70**

TECHNICAL BULLETIN

Product Description

The GenElute™ Direct mRNA Miniprep kit provides a convenient format to isolate polyadenylated mRNA directly from mammalian cells and tissues. The direct mRNA isolation procedure is based on that of Badley.¹ Up to 10⁷ mammalian cells or 40 mg tissue are lysed and homogenized, either with the filtration columns provided or with a mechanical homogenizer. RNase is eliminated during a 10 minute proteinase K digestion. Sodium chloride is added, and polyadenylated RNA is captured on oligo(dT) polystyrene beads during a 10 minute incubation. For further enrichment, RNA may be released from the beads into fresh lysis solution and recaptured with the original beads. After 3 washes in a spin column, purified mRNA is eluted in 100 µl of 10 mM Tris-HCl, pH 7.4. Note that if all traces of DNA contamination must be eliminated for RT-PCR, further treatment with Amplification Grade DNase I is recommended, Catalog No. AMPD1.

Storage

Store at room temperature. If any reagent forms a precipitate, see Preparation Instructions that follow.

Precautions and Disclaimer

The GenElute Direct mRNA Miniprep Kit is for laboratory use only. Not for drug, household or other uses. Wear gloves, safety glasses, and suitable protective clothing when handling any reagent provided with the kit. See the Material Safety Data Sheet (MSDS).

RNases are ubiquitous and very stable proteins, which are concerns for any researcher attempting to isolate RNA. Conditions during lysis inhibit RNase activity, and the proteinase K digestion eliminates RNases present in the original cell or tissue sample. **Care must be taken not to introduce RNase, especially during the wash and elution steps.** Use RNase-free pipette tips, preferably those having an aerosol barrier. Wear latex or vinyl gloves and change them frequently. Keep bottles and tubes closed when not adding or removing their contents. References 2-4 given at the end of this bulletin are good sources of additional information on working with RNA.

Reagents Provided	Catalog No.	DMN10 10 Preps	DMN70 70 Preps
Lysis Solution	L2163	20 ml	120 ml
Proteinase K	P2308	5 mg	25 mg
40% Glycerol	P0484	0.6 ml	3 ml
5 M NaCl Solution	S5150	1.5 ml	8 ml
Oligo(dT) Polystyrene Beads	O8512	0.3 ml	2 ml
Wash Solution	W2140	4 ml	30 ml
Low Salt Wash Solution	W2265	8 ml	60 ml
Elution Solution (10 mM Tris-HCl, pH 7.4)	E8026	1.5 ml	10 ml
GenElute Filtration Columns with Tubes	C9346	10 each	70 each
Spin Filters with Tubes	C9596	10 each	70 each
Dolphin Collection Tubes	D7688	10 each	70 each

Equipment and Reagents Required But Not Provided

- Heating block set at 65 °C
- Tissue homogenizer and vessels for tissue samples
- Microcentrifuge capable of 16,000 x *g* (maximum speed in rotors that hold 18 tubes)

Preparation Instructions

Before beginning the procedure, do the following:

1. Thoroughly mix reagents. Examine for precipitation. If any reagent forms a precipitate, warm at 55-65 °C until the precipitate dissolves and allow to cool to room temperature before use.
2. Dissolve the proteinase K in the 40% glycerol. Use 0.5 ml of 40% glycerol for 5 mg proteinase K (10 prep package) or 2.5 ml of 40% glycerol for 25 mg proteinase K (70 prep package). Let sit at room temperature for a few minutes, and mix thoroughly before use. This product is stable at room temperature in its dry form, but **store the proteinase K solution at 2-8 °C**.
3. Add the proteinase K solution to a sufficient volume of Lysis Solution for that day's use. The amount of Lysis Solution containing proteinase K required per preparation is 0.5 ml for cultured cells and 1.0 ml for tissue. Add 20 µl of proteinase K solution per ml of Lysis Solution and mix thoroughly.
4. Transfer approximately 120 µl of Elution Solution per preparation into a microcentrifuge tube and heat to 65 °C in a heating block.
5. Ensure that the oligo(dT) beads are at room temperature and vortex thoroughly before use.

Procedure

Note: All centrifugation speeds are given in units of *g*. Please refer to Table 1 for information on converting *g*-force to rpm. If centrifuges/rotors for the required *g*-forces are not available, use the maximum *g*-force possible and increase the spin time proportionally. Spin until all liquid passes through the column.

Table 1. Conversion of Centrifugal Force (in units of *g*) to RPM for Common Rotors

Centrifuge	Rotor	Tubes (max)	Radius (cm)	RPM at 300 x <i>g</i>	RPM at 16,000 x <i>g</i>
Eppendorf					
5410	-	12	5.8	2,143	15,652
5415C	F45-18-11	18	7.3	1,917	14,000
5415D&R	F45-24-11	24	8.3	1,801	13,155
5417C,D,&R	F45-30-11	30	9.5	1,681	12,279

See table above for spin speeds in rpm for selected common centrifuges and rotors. The correct rpm for unlisted rotors can be calculated using the formula:

$$RPM = \sqrt{RCF / 1.118 \times 10^{-5} r}$$

where *RCF* = required gravitational acceleration (relative centrifugal force) in units of *g*; *r* = radius of the rotor in cm; *RPM* = the number of revolutions per minute required to achieve the necessary *g*-force.

All steps are carried out at room temperature except as noted. All centrifugation (spin) steps should be performed at maximum speed (16,000 x g).

1. **Harvest cells or prepare tissue.** For best yields of intact RNA, use only rapidly growing cells before they reach their maximum density or harvest tissue immediately from a freshly sacrificed animal.
 - a. **Suspension cell cultures:** Count cells. Pellet up to 10^7 cells for 5 minutes at 300 x g. Remove the culture medium completely and discard. Continue with step 2.
 - b. **Attached cell cultures:** Release attached cells with trypsin and pellet before lysis. To release cells with trypsin, see the protocol under Trypsin in the technical information within the Tissue Culture section of the Sigma Catalog. Count cells. Pellet up to 10^7 cells and proceed as for suspension cultures.
 - c. **Mammalian tissue:** Quickly slice and weigh a piece of fresh or frozen tissue. Use 10 to 40 mg per preparation. Transfer to an appropriate vessel for homogenization and continue with step 2.
2. **Lyse and homogenize cells/tissue.** This is a critical step that must be accomplished quickly and thoroughly.
 - a. **Pelleted cells:** Vortex the pellet to loosen the cells. Add 0.5 ml Lysis Solution containing proteinase K. Vortex or pipette thoroughly until all clumps disappear. Transfer the lysed cells into a GenElute filtration column (blue insert with a 2 ml receiving tube) and spin for 2 minutes. Discard the blue filtration column, and continue to step 3 with the homogenized lysate (flow-through liquid).
 - b. **Mammalian tissue:** Add 1 ml of Lysis Solution containing proteinase K and homogenize immediately until no visible pieces remain. Continue with step 3.

Note: Approximately 30 to 60 seconds of homogenization with a Brinkman Polytron PT 1200 is sufficient for most tissues. However, with fibrous tissues, such as muscle, several 30 second bursts are required to completely disrupt the tissue.

3. **Digest with proteinase K.** Incubate homogenized lysate at 65 °C for 10 minutes to degrade nucleases and other proteins. After incubation, remove the tube(s) from the heating block, and proceed with steps 4 and 5.
4. **Prepare mRNA for binding.** Add 32 μ l of the 5 M NaCl solution to the digested cell lysate, or 64 μ l of 5 M NaCl to the digested tissue lysate.

Note that the sodium chloride and oligo(dT) beads (step 5) may be added while the lysate is still warm.

5. **Bind mRNA to oligo(dT) beads.** Mix the oligo (dT) beads thoroughly by vortexing and inverting until resuspended and homogenous. Add 25 μ l of the resuspended oligo(dT) beads to the lysate-NaCl mixture, cap tube, and mix thoroughly by vortexing. Incubate the lysate/bead mixture at room temperature for 10 minutes. No mixing or rocking is necessary. During this incubation, the poly(A) tails of mRNA will hybridize with the oligo(dT) on the beads.
6. **Collect bead:mRNA complex.** Pellet the oligo(dT) beads:mRNA complex by centrifuging for 5 minutes at maximum speed. Carefully remove and discard the supernatant, leaving behind approximately 50 μ l to avoid disturbing the pellet.
7. **Release and rebind mRNA (optional).** For a more highly enriched mRNA preparation, bound material may be released from the beads into fresh lysis solution; mRNA is then rebound to the same beads. Add 0.5 ml of Lysis Solution and 32 μ l of 5 M NaCl solution. Vortex thoroughly to resuspend the pellet. A series of short bursts on a vortex mixer are usually more effective than one long burst. Invert tube and inspect to verify complete release of pellet.

Incubate suspension at 65 °C for 5 minutes.

Remove from heat and incubate at room temperature for 5 minutes.

Repellet the bead:mRNA complex for 2 minutes. Remove and discard all but ~50 μ l of the supernatant as above.

Note: This step may be omitted if rRNA contamination is not a concern, or if purification will be repeated as described in the note at the end of the procedure.

8. **First wash.** Resuspend the pellet in 350 μ l of Wash Solution by vortexing or pipetting. A series of short bursts on a vortex mixer will help release the pellet. Transfer the suspension into a GenElute spin filter-collection tube assembly by pipetting. Make sure that all suspension is expelled from the pipette tip and that the pellet is completely removed from tube. Spin for 1-2 minutes at maximum speed. Discard the flow-through liquid, but retain the collection tube.
9. **Second wash.** Pipette 350 μ l of Low Salt Wash Solution into the column. Spin for 1-2 minutes. Discard the flow-through liquid, but retain the collection tube.
10. **Third wash.** Pipette another 350 μ l of Low Salt Wash Solution into the column. Spin for 1-2 minutes.
11. **First elution.** Transfer the spin filter into a fresh collection tube. Discard the flow-through liquid and the original collection tube. Pipette 50 μ l of preheated Elution Solution (65 °C) onto the spin filter ensuring that it contacts the bead:mRNA complex. Incubate for 2-5 minutes at 65 °C. Spin for 1-2 minutes. Save the flow-through liquid; it contains most of the purified mRNA.
12. **Second elution.** To maximize recovery of mRNA, pipette an additional 50 μ l of preheated Elution Solution (65 °C) onto the bead:mRNA complex. Incubate for 2-5 minutes at 65 °C. Spin for 1-2 minutes.

The poly(A)⁺ mRNA is now in the flow-through eluate (90-100 μ l total), and is ready for immediate use or storage at -70 °C. Keep the mRNA on ice whenever it is thawed for use.

Note: If a highly enriched preparation is desired, repurify the mRNA by adding 400 μ l of Lysis Solution, 32 μ l of 5 M NaCl solution, and a fresh 25 μ l aliquot of oligo(dT) beads to the 100 μ l eluted mRNA. Vortex and incubate 10 minutes at room temperature. Pellet the beads for 2 minutes, then wash and elute as in steps 8 through 12 above.

Results

Determining yield

Due to the low levels of mRNA present in cells and in tissues (only 1-5% of total RNA), estimating the concentration and quality of mRNA from mini-preparations spectrophotometrically by measuring absorbance at 260 and 280 nm is not recommended. Typically, the absorbance must be read using the neat sample or with little dilution. As a consequence, most or all of the mRNA isolated will be used. The sample may be recovered for downstream applications if RNase-free cuvettes are used. However, fluorometric determination with the RiboGreen[®] RNA Quantitation Reagent from Molecular Probes is a more sensitive and practical method, and is recommended.

Expected yield

The yield of mRNA varies according to the organism and developmental stage. In general, younger and more rapidly growing cultures or animals will contain more RNA. Furthermore, RNA levels vary over a wide range in different tissues. Brain, heart, and skeletal muscle generally have low levels of RNA, while kidney, pancreas, spleen, and liver can contain up to 10-fold more RNA. Typical yields from 40 mg of rat liver or 10⁷ HEK 293 cells are 5-10 μ g of RNA by the RiboGreen assay method.

Ethanol precipitation

Some applications will require a more concentrated mRNA preparation than that prepared with this kit. The mRNA may be concentrated by adding 20 μ g of glycogen (Product Code G 1767), 0.1 volume of 3 M sodium acetate buffer, pH 5.2 (Product Code S 7899), and 3 volumes of ice cold absolute ethanol (Product Code E 7023), and precipitating overnight at -20 °C. The mRNA can be stored indefinitely as an ethanol precipitate.

Before use, pellet the precipitated mRNA at 16,000 x g at 2-6 °C for 15 minutes. Carefully remove the supernatant, wash the pellet with 70% ethanol, and spin at the maximum speed for 3-5 minutes. Carefully remove the supernatant and allow the tube to air dry while lying flat with the lid open for 30 minutes. Once dry, the pellet can be resuspended in RNase-free molecular biology reagent water (Product Code W 4502).

Agarose gel electrophoresis

The mRNA may be fractionated on a denaturing formaldehyde agarose gel as described by Farrell.³ Staining with SYBR[®] Green II (Product Code S 9305) is recommended, because formaldehyde gels stained with SYBR Green II do not require destaining. The mRNA should appear as a series of very closely spaced bands or smear at 0.5 kb or greater. More intense bands at approximately 5.3 and 2.0 kb are residual 18S and 28S rRNA.

Northern analysis

The integrity of the mRNA can be determined by transferring the gel pattern to a nylon membrane and hybridizing with a probe specific for an mRNA present in the population. See Related Products list for products and procedures to perform Northern blots, label probes and perform hybridization. Intact mRNA should result in a discrete band or bands, with no smearing downwards.

RT-PCR

While most DNA is eliminated during mRNA isolation, no single procedure removes 100% of the DNA. Because PCR can detect even a single molecule of DNA, RNA samples should be digested with Amplification Grade DNase I (Product Code AMP-D1) before RT-PCR, and parallel samples should be assayed without adding reverse transcriptase. These precautions are especially recommended if PCR primers do not span an intron, or if pseudogenes that lack the intron may be present in the target cells or tissue.

References

1. Badley, J.E., *et al.*, A simple, rapid method for the purification of poly(A)⁺ RNA. *BioTechniques* **6**(2), 114-116 (1988)
2. Ausubel, F.M., *et al.*, *Current Protocols in Molecular Biology* (John Wiley & Sons, NY, 1995) sections 4.1-4.10
3. Farrell, Robert E., Jr. *RNA Methodologies*, 2nd Edition (Academic Press, NY, 1998) pp. 37-53 and 153-156 (Product Code Z35,035-4)
4. Sambrook, J., *et al.*, *Molecular Cloning: A Laboratory Manual*, 2nd ed., (Cold Spring Harbor Laboratory Press, Plainview, NY, 1989) pp. 7.3-7.8

Troubleshooting Guide

Problem	Cause	Solution
Clogged spin filter	Sample size was too large	For future preparations, use fewer cells or smaller tissue samples. Alternatively, undigested material may be pelleted for 2-5 minutes after the proteinase K digestion in step 3, and the supernatant transferred to a new tube before adding the 5 M NaCl solution and oligo(dT) beads. To salvage the current preparation, spin longer than 1-2 minutes until solutions pass through the spin filter. The yield and purity of mRNA will likely be reduced.
	Homogenization was incomplete	Cell lysates must be spun through filtration columns to shear the DNA. Tissues must be thoroughly homogenized until no visible particles remain.
	Digestion was incomplete	Store proteinase K at 2-8 °C after it is dissolved in glycerol. Add proteinase K solution to the Lysis Solution immediately before use. The enzyme is not stable in the Lysis Solution for extended storage. Verify that the homogenized cells or tissues were incubated at 65 °C for 10 minutes before the 5 M NaCl solution was added.
	Centrifugal force was low	If the microcentrifuge used cannot attain 16,000 x g, longer spin times may be required.
Low yield of mRNA	Cells or tissue had low mRNA levels	Yields will vary greatly among different types of cells and tissues. See "Expected Yield" in the Results section.
	Elution Solution was not pre-heated or samples were not incubated at 65 °C	Transfer ~120 µl of Elution Solution per preparation into a microcentrifuge tube and heat to 65 °C in a heating block before starting the procedure. Incubate the bead:mRNA complex with Elution Solution for 2-5 minutes at 65 °C before spinning.
Degraded mRNA	Tissue or culture was too old	Use cultures before they reach maximum density or become fully confluent, and harvest tissues as rapidly as possible from freshly sacrificed animals.
	Cells or tissues were stored improperly	If immediate preparation of mRNA is not possible, flash-freeze cell pellets or small pieces of tissue in liquid nitrogen and store at -70 °C. Do not allow material to thaw before it is disrupted in Lysis Solution.
	Cells or tissue contained high levels of RNase	Cells such as monocytes and macrophages, and tissues such as pancreas, spleen, and thymus, are rich in RNases and require immediate and thorough disruption in Lysis Solution to prevent degradation of RNA.
	Cells or tissues were not disrupted sufficiently	Vortex or pipet cell lysates until no clumps remain. Homogenize tissues in Lysis Solution until no visible particles remain.
	Proteinase K digestion was incomplete	See "Digestion is incomplete" above.
	RNase was introduced during the procedure	Pay special attention to precautions for handling RNA samples and related lab equipment listed at the beginning of this bulletin and in references 2-4.

Troubleshooting Guide (cont.)

Problem	Cause	Solution
Excessive rRNA contamination	Abundance of rRNA is high; sequence of rRNA contains poly(A) regions	Detectable amounts of rRNA are expected. Non-specific binding to oligo(dT) can occur due to the vast excess of rRNA over mRNA. Also, poly(A) regions in rRNA can bind specifically to the beads. If a more enriched preparation is desired, repurify the mRNA as described in the note under Step 12 of the Procedure.
	Miniprep capacity was exceeded	Repurify as described in the note under Step 12 of the Procedure. For future preparations, use smaller amounts of starting cells or tissue.
	Releasing and re-binding procedures were omitted	Repurify as described in the note under Step 12 of the Procedure.
Poor results in downstream procedures	Salt was carried over into eluate	Spin beads dry before adding Elution Solution.
	Improper storage or handling of mRNA	Store eluted mRNA in Elution Solution at -70°C or as an ethanol precipitate at -70°C until needed. Keep the mRNA on ice whenever it is thawed for use.

Related Products	Catalog No.	Related Products	Catalog No.
GenElute™ mRNA Miniprep Kits	MRN10 and MRN70	Deoxyribonuclease I, Amplification Grade	AMPD1
Ethanol, Absolute, Molecular Biology Reagent	E7023	Enhanced Avian RT-PCR Kits	RTPCR20 RTPCR100
Glycogen, Molecular Biology Grade	G1767	Enhanced Avian Reverse Transcriptase	A4464
3 M Sodium Acetate Buffer, pH 5.2, Molecular Biology Grade	S7899	Deoxynucleotide (dNTP) Mix	D7295
Agarose, Molecular Biology Grade	A9539	JumpStart™ REDTaq® Readymix™	P0982
Formaldehyde, Molecular Biology Grade	F8775	Taq DNA Polymerase	D1806 D4545
MOPS-EDTA-Sodium Acetate Buffer	M5755	RNA/cDNA Inspector Kit	INSP1
RNA Sample Loading Buffer	R1386	PerfectHyb™ Plus Hybridization Buffer	H7033
RNA markers, 0.2-10 kb	R7020	Uniscript™ T7 Transcription Kit	UST7
SYBR Green II, 10,000X concentrate	S9305	CDP-Star® Universal Detection kit	UALK

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