INSTRUCTION MANUAL

Monarch® Total RNA Miniprep Kit

50 preps

NEB #T2010S

Version 5.0_05/24

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Kit Components

The materials provided are sufficient for 50 preps (NEB #T2010S). This kit should be stored at room temperature. Reconstituted Proteinase K and DNase I should be stored at -20°C (DNase I should be aliquoted before storage). Monarch RNA Lysis Buffer and Monarch RNA Priming Buffer contain chaotropic agents, and Monarch Priming Buffer also contains ethanol. For detailed information regarding the proper handling of these materials, please consult the Safety Data Sheets available on our website (www.neb.com). Proper laboratory safety practices should be employed, including the use of lab coats, gloves, and eye protection. Always keep buffer bottles tightly closed and keep columns sealed in the enclosed zip-lock bag.

NEB #		T2010S 50 preps	STORAGE TEMPERATURE
T2017	**Monarch gDNA Removal Columns**	50 columns	25°C
T2007	**Monarch RNA Purification Columns**	50 columns	25°C
T2118	**Monarch Spin Collection Tubes**	100 tubes	25°C
T2011	**Monarch DNA/RNA Protection Reagent (2X)**	28 ml	25°C
T2012	**Monarch RNA Lysis Buffer**	50 ml	25°C
T2001	**Monarch Proteinase K**	20.8 mg lyophilized	25°C (-20°C after reconstitution)
T2002	**Monarch Proteinase K Resuspension Buffer**	1.5 ml	25°C
T2003	**Monarch Proteinase K Reaction Buffer**	4 ml	25°C
T2004	**Monarch DNase I**	275 U lyophilized	25°C (aliquots at -20°C after reconstitution)
T2005	**Monarch DNase I Reaction Buffer**	4 ml	25°C
T2013	**Monarch RNA Priming Buffer**	28 ml	25°C
T2014	**Monarch RNA Wash Buffer (5X)**	25 ml	25°C
T2006	**Monarch Nuclease-free Water**	13 ml	25°C

Introduction

The Monarch Total RNA Miniprep Kit is a comprehensive solution for sample preservation, cell lysis, gDNA removal, and purification of total RNA from a wide variety of biological samples, including cultured cells, blood, and mammalian tissues. Additionally, bacteria, yeast, and plant samples can be processed with additional steps to enhance lysis in these tough-to-lyse samples. Cleanup of enzymatic reactions or purification of RNA from TRIzol® -extracted samples is also possible using this kit. Purified RNA has high quality metrics, including $A_{260/280}$ and $A_{260/230}$ ratios > 1.8, high RIN scores, and minimal residual gDNA. Captured RNA ranges in size from full-length rRNAs down to intact miRNAs. Additionally, differential binding conditions allow selective capture or exclusion of the sub-200 nucleotide RNA pool that includes miRNA, 5S rRNA, and tRNA. Purified RNA is suitable for downstream applications such as RT-qPCR, cDNA synthesis, RNA-seq, Northern blot analysis, etc.

Specifications

Input:	Cultured mammalian cells and blood cells: (PBMC's, WBC's): up to 1 x 10^6 Mammalian whole blood: up to 3 ml Tissue: up to 20 mg for high yield tissues up to 50 mg for low yield tissues Bacteria: up to 1 x 10^9 Yeast: up to 5 x 10^7 Plant: up to 100 mg
Binding Capacity:	100 µg RNA
Elution Volume:	30–100 µl
Yield:	Varies depending on sample type, see page 6 for details
RNA Size:	≥ 20 nt
Purity:	$A_{260/280}$ typically ≥ 2.0 $A_{260/230}$ typically ≥ 1.8

General Guidelines for Working with RNA:

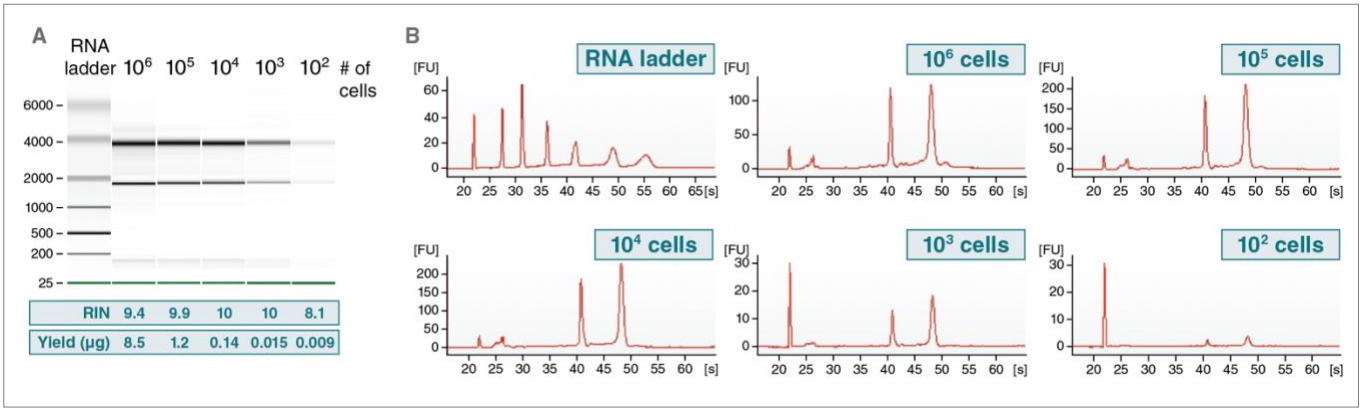
Successful RNA extraction and purification is greatly influenced by the type and physiological condition of the sample, as well as the user's ability to maintain RNA integrity during preservation, lysis, and purification. In general, samples should be flash frozen or rapidly processed after harvest to ensure RNA remains intact and accurately reflects the gene expression profile at the time of harvest. Sample disruption and homogenization should include RNase inactivation measures, and lysis methods should ensure complete cell disruption to enable maximal RNA recovery. RNA capture should be efficient for large and small RNAs and should include vigorous wash conditions to remove contaminants.

To maximize RNA yield, integrity and purity, please keep the following principles in mind:

1. RNases are stable and difficult to inactivate, and care must be taken when handling samples during and after preparation of RNA. Plasticware and glassware in direct contact with RNA-containing samples should be RNase-free. Gloves should be worn at all times when handling samples and kit components. Frequent glove changes are encouraged. Bench and equipment surfaces should be clean and can be decontaminated prior to work using commercially available cleaners such as RNaseZap®.
2. Quickly processing fresh samples avoids any concerns associated with sample storage.
3. Samples to be stored should be flash frozen and stored at -80°C. The use of cryoprotectants may also be beneficial. Storage at -20°C can be used for short periods of time (1 week), but RNA integrity will be better maintained at lower temperatures. Alternatively, sample preservation using lysis/stabilization reagents, such as Monarch DNA/RNA Protection Reagent or stabilization reagents such as RNAlater® should be employed for samples that will be maintained at 4°C or room temperature, or that will be prepared for transport. See supplier instructions for correct use.
4. Mechanical disruption and homogenization of tissue samples by use of a bead mill, homogenizer, etc. efficiently releases RNA and maximizes recovery.
5. After disruption and homogenization, perform all steps at room temperature.
6. Elution with nuclease-free water is standard, but for samples that will be stored for use later, EDTA can be added to 0.1–1.0 mM to limit degradation due to magnesium-requiring nucleases. Alternatively, elution with slightly alkaline TE can be employed.
7. Avoid unnecessary freeze-thaw cycles of purified RNA. Aliquots should be made, consistent with downstream needs.

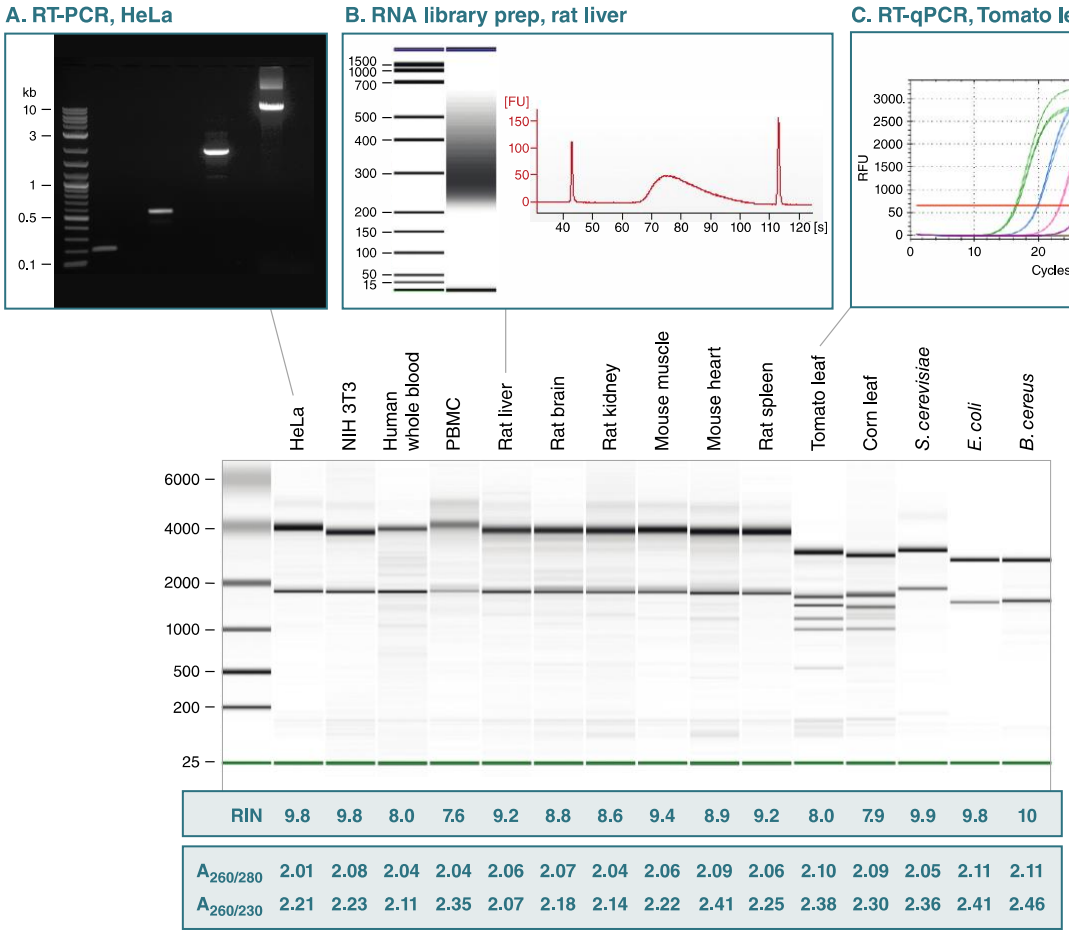
Performance Data:

Figure 1: The Monarch Total RNA Miniprep Kit can generate high quality RNA from as few as 100 HeLa cells.



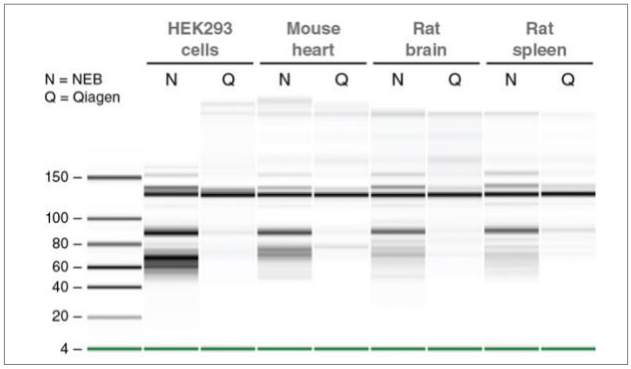
Total RNA was isolated using the Monarch Total RNA Miniprep Kit (NEB #T2010) from varying amounts of HeLa cells over 5 orders of magnitude and eluted in 100 µl of nuclease-free water. Samples were analyzed on a Bioanalyzer® Pico chip, with RIN values and total yields shown below the lanes (A). Electropherograms are included as a reference (B).

Figure 2: Monarch-purified RNA is high-quality and compatible with a wide variety of downstream applications.



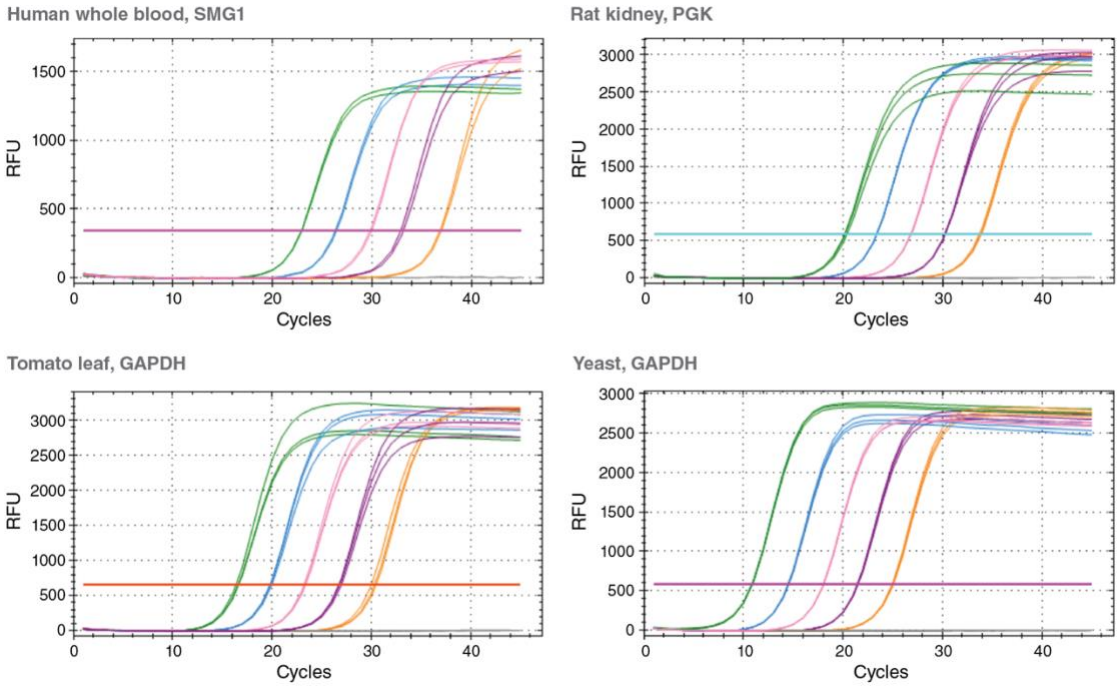
Total RNA from a broad array of sample types was purified using the Monarch Total RNA Miniprep Kit (NEB #T2010). Aliquots were run on an Agilent® Bioanalyzer 2100 using the Nano 6000 RNA chip (S. cerevisiae RNA was run using a plant Nano assay). RIN values and O.D. ratios confirm the overall integrity and purity of the RNA. To demonstrate compatibility with downstream applications, samples were subsequently used for RT-PCR (+/– RT) (A) for detection of 4 different RNA species using ProtoScript® II Reverse Transcriptase (NEB #M0368)/LongAmp® Taq DNA Polymerase (NEB #M0323), NGS library prep (B) using NEBNext® Ultra™ II RNA Library Prep Kit (NEB #E7760) and RT-qPCR (C) using Luna® One-Step RT-qPCR Reagents (NEB #E3005).

Figure 3: The Monarch Total RNA Miniprep Kit successfully purifies small RNAs below 200 nucleotides, enabling a more faithful representation of the total RNA pool.



RNA preps were performed on HEK293 cells, mouse heart, rat brain, or rat spleen using the Monarch Total RNA Miniprep Kit (N) (NEB #T2010) and the RNeasy® Mini Kit from Qiagen® (Q). Equivalent amounts were resolved on a Bioanalyzer 2100 using the Small RNA chip. Monarch-purified RNA contains significantly more RNA in the sub-200 nucleotide pool.

Figure 4: RT-qPCR using Monarch-purified RNA generates high-quality qPCR curves demonstrating accurate detection and quantitation of targets across a wide variety of RNA sources.



Monarch-purified RNA from human whole blood, rat kidney, tomato leaf, and the yeast S.cerevisiae was diluted to produce a five-log range of input template concentrations. Primers targeting GAPDH variants (tomato, yeast), PGK (rat), or SMG1 (human blood) were used for RT-qPCR assays, assembled as directed using Luna One-Step RT-qPCR reagents (NEB #E3005) and cycled on a Bio-Rad® CFX384.

Choosing Input Amounts

Using the right amount of starting material ensures sufficient RNA yield without exceeding the binding capacity of the column. **To ensure the column is not overloaded, we do not recommend exceeding the maximum input.** Large sample input amounts reduce lysis efficiency, introduce excessive amounts of cellular components other than RNA (DNA, polysaccharides, proteins, lipids, etc), and compromise RNA binding to the silica-based capture column.

SAMPLE TYPE*		RECOMMENDED INPUT AMOUNT	TYPICAL YIELD (µg)	OBSERVED RIN	MAXIMUM INPUT AMOUNT
CULTURED CELLS					
HeLa		1 x 10^6 cells	12–15	9–10	1 x 10^6 cells
HEK 293		1 x 10^6 cells	12–14	9–10	1 x 10^6 cells
NIH3T3		1 x 10^6 cells	8–12	9–10	1 x 10^6 cells
MAMMALIAN BLOOD					
Human	Fresh	200 µl	0.5–1.0	7–8	3 ml
	Frozen	200 µl	0.5–1.0	7–8	3 ml
	Stabilized	200 µl	0.5–1.0	7–8	3 ml
Rat	Frozen	100 µl	5.6	9	1 ml**
BLOOD CELLS					
PBMC (isolated from 5 ml whole blood)		5 ml	3	7	1 x 10^6 cells
TISSUE					
Rat liver	Frozen pulverized	10 mg	25	8–9	20 mg
	Stabilized solid	10 mg	50–60	8–9	20 mg
Rat spleen (stabilized solid with bead homogenizer)		10 mg	40–50	9	20 mg
Rat kidney (frozen pulverized)		10 mg	7–10	9	50 mg
Rat Brain	Frozen pulverized	10 mg	2–3	8–9	50 mg
	Stabilized solid	10 mg	0.5–1.5	8–9	50 mg
	Stabilized solid with bead homogenizer	10 mg	5–8	8–9	50 mg
Rat muscle (frozen pulverized)		10 mg	2–3	8–9	50 mg
Mouse muscle	Frozen pulverized	10 mg	3	8–9	50 mg
	Powder with bead homogenizer	10 mg	5	7–8	50 mg
	Stabilized solid with bead homogenizer	10 mg	8–10	9	50 mg
Mouse heart (stabilized solid w/bead homogenizer)		10 mg	5–6	8–9	50 mg
YEAST					
S. cerevisiae	Frozen with bead homogenizer	1 x 10^7 cells	50	9–10***	5 x 10^7 cells
	Frozen with Zymolyase	1 x 10^7 cells	60	9***	5 x 10^7 cells
BACTERIA					
E. coli	Frozen	1 x 10^9 cells	5	10	1 x 10^9 cells
	Frozen with bead homogenizer	1 x 10^9 cells	10	10	1 x 10^9 cells
	Frozen with lysozyme	1 x 10^9 cells	70	10	1 x 10^9 cells
B. cereus	Frozen with lysozyme	1 x 10^8 cells	20–30	9	1 x 10^9 cells
	Frozen with bead homogenizer	1 x 10^8 cells	8	9–10	1 x 10^9 cells
PLANT					
Corn leaf (frozen pulverized with bead homogenizer)		100 mg	45	8	100 mg
Tomato leaf (frozen pulverized with bead homogenizer)		100 mg	30	8	100 mg

* Additional sample types have been validated; protocols are available in Supplemental Protocols Section.

** Mouse blood also has a maximum input of 1 ml.

*** *S. cerevisiae* total RNA was run on an Agilent Nano 6000 Chip using plant assay.

Considerations for Sample Disruption and Homogenization:

The Sample disruption and homogenization are critical parts of every RNA extraction. Maximal recovery will only be achieved by releasing all available cellular RNA. Lysis of cell walls and plasma membranes, as well as reduction of the viscosity of the lysate, ensures quantitative binding to the capture matrix. Each sample type has different requirements for lysis, so the method used should take this into consideration. **Incomplete disruption and homogenization will reduce the yield of recovered RNA.**

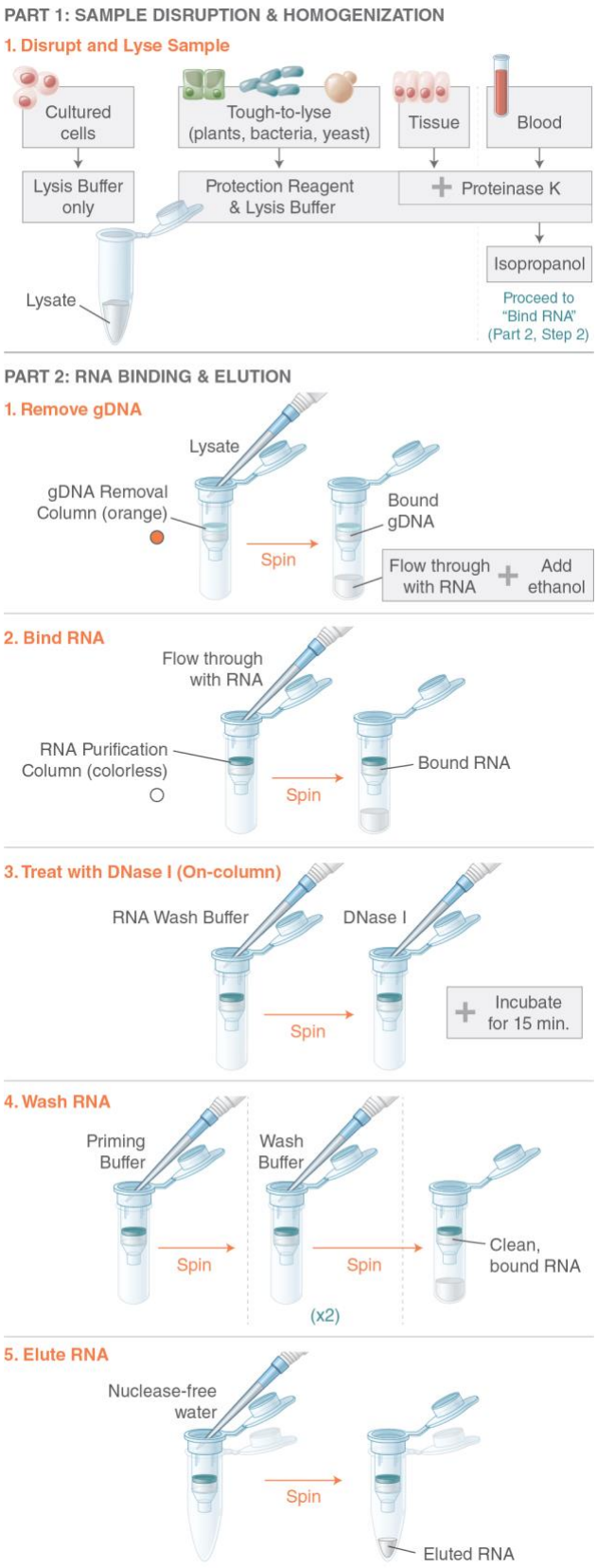
Cultured mammalian cells: Cells grown in suspension, in a monolayer, or as adherent cells should be pelleted prior to use of this kit to ensure the cell culture medium is removed. Alternatively, adherent cells can be lysed directly in plate wells (see page 16). The plasma membrane of these samples is easily lysed by the detergents in the lysis buffer and no additional disruption is necessary.

Solid tissues, cells from blood (PBMCs, buffy coat): Solid tissues and white blood cells (leukocytes) can be effectively lysed after resuspension in Monarch DNA/RNA Protection Reagent and incubated with Proteinase K. Lysis time is reduced when tissues are mechanically disrupted with a mortar and pestle. Maximal disruption can be achieved using a bead homogenizer, leading to maximal RNA release.

Whole blood: Mammalian whole blood contains little RNA and an abundance of protein (hemoglobin). Effective lysis can be achieved with Monarch DNA/RNA Protection Reagent. Proteinase K treatment is necessary to lower hemoglobin levels prior to loading the column.

Tough-to-lyse samples (bacteria, yeast, plants, etc.): Cells grown in liquid culture should be pelleted and all culture medium removed prior to sample disruption and homogenization. These samples benefit from mechanical disruption and homogenization to effectively release RNA due to carbohydrate-containing cell walls. Alternatively, samples may be lysed enzymatically by the addition of glycanases (i.e. lysozyme, Zymolyase).

Figure 5: Workflow for RNA Purification using the Monarch Total RNA MiniPrep Kit



Monarch Total RNA Miniprep Kit Protocol

- Required equipment: microcentrifuge, water bath or heat block (55°C)
- Reagents supplied by user: ≥ 95% ethanol, RNase-free microfuge tubes.
- Additional equipment/reagents that may be required: nuclease-free water, additional collection tubes, homogenizer (bead mill or other), isopropanol for whole blood samples, glycanases for bacteria and yeast, Xylene or similar for deparaffinization of FFPE tissue.

IMPORTANT NOTES BEFORE YOU BEGIN

- Monarch DNA/RNA Protection Reagent is supplied as a 2X concentrate. Dilute only as needed, as some sample types require resuspension in the 2X concentrate, while others require a 1X solution. If purifying samples stored in Monarch DNA/RNA Protection Reagent, please review the related guidance in the Appendix.
- For the 50-prep kit, add 275 µl nuclease-free water to the lyophilized DNase I vial and resuspend by gentle inversion. We suggest making aliquots of DNase I, sized to your processing needs, and storing at -20°C to minimize freeze-thaw cycles (3 F/T cycles maximum).
- For the 50-prep kit, add 1,040 µl Proteinase K Resuspension Buffer to the lyophilized Proteinase K (Prot K) vial and vortex to resuspend. Store at -20°C.
- For the 50-prep kit, add 100 ml ethanol ≥ 95% (not included) to the 25 ml RNA Wash Buffer concentrate and store at room temperature.
- Addition of RNA Lysis Buffer and all subsequent steps should be performed at room temperature (this will prevent precipitation of detergent in the lysis buffer). If samples are accidentally placed on ice and precipitate forms, allow the samples to return to room temperature to resolubilize before loading onto the column.

RNA Purification Consists of Two Stages:

PART 1: Sample Disruption and Homogenization

PART 2: RNA Binding and Elution

PART 1 SAMPLE DISRUPTION AND HOMOGENIZATION

Please follow the protocol specific to your starting material: For sample types not listed, please see Supplemental Protocols.

❑ Cultured Mammalian Cells

1. **Pellet cells by centrifugation (500 x *g*) for 1 minute. Discard supernatant.**
   Alternatively, cells in suspension can be directly processed by adding 4 volumes of RNA Lysis Buffer, mixing, and proceeding to Step 1 of PART 2: RNA Binding and Elution, page 12. The buffers provided in this kit are optimized for pelleted cells. Lysing cells in suspension often requires large volumes of lysis buffer. Adherent cells can be lysed in-well; please see page 16 for instructions.

2. **Resuspend pellet in 300 µl of RNA Lysis Buffer by pipetting gently to avoid foaming. Do not place samples on ice**.
   For frozen pellets, thaw briefly before resuspension. For cells previously mixed with DNA/RNA Protection Reagent, add an equal volume of RNA Lysis Buffer.

3. **Proceed to** **Step 1 of PART 2: RNA Binding and Elution, page 12.**

❑ Mammalian Whole Blood (fresh or frozen)

1. **Add an equal volume (up to 200 µl) of DNA/RNA Protection Reagent (2X concentrate) to an aliquot of whole blood and vortex briefly. Do not place samples on ice.** For frozen samples, quickly thaw in the presence of 2X DNA/RNA Protection Reagent while vortexing or shaking. Blood cells are lysed during this step, releasing the RNA.
2. **For every 400 µl of DNA/RNA Protection Reagent/blood mixture, add 10 µl of Proteinase K (Prot K). Vortex briefly and incubate at room temperature for 30 minutes.**
3. **Add an equal volume of isopropanol (not included) and vortex briefly. Proceed to Step 4 of Part 2: RNA Binding and Elution, page 12.** Addition of isopropanol is necessary to create favorable binding conditions before application onto the RNA Purification Column. Up to 3 ml blood may be processed on a single column with reloading by repeating Steps 1-3 with 200 µl of blood at a time.

❑ Tissue or Leukocytes

1. **Determine the amount of 1X DNA/RNA Protection Reagent that you will need according to the table below. Prepare the required amount of 1X DNA/RNA Protection Reagent by diluting the 2X concentrate with nuclease-free water (not included for this step).**

SAMPLE INPUT AMOUNT	VOLUME 1X DNA/RNA PROTECTION REAGENT
Tissue (up to 10 mg)	300 µl
Tissue (10-30 mg)	300-600 µl
Tissue (30-50 mg)	≥ 600 µl
Leukocytes (up to 3 x 10^6)	300 µl

2. **Add the 1X DNA/RNA Protection Reagent to sample.** Solid tissue samples should be submerged in DNA/RNA Protection Reagent, not to exceed 10% (w/v). For maximal RNA recovery, tissues can be mechanically homogenized using a bead mill or similar device.
3. **For every 300 µl of DNA/RNA Protection Reagent/Sample mixture, add 30 µl Prot K Reaction Buffer + 15 µl Prot K*. Vortex briefly and incubate at 55°C according to the chart below:**

SAMPLE TYPE	INCUBATION TIME AT 55°C**
Homogenized Tissues	5 minutes
Solid Tissues	5-30 minutes
Leukocytes	30 minutes

* Doubling the amount of Prot K may increase RNA yield for some tissues.
** Longer incubation times may result in decreased RNA integrity. Optimal time should be determined empirically as yield and integrity vary with incubation time.

4. **Vortex sample briefly and spin for 2 minutes (16,000 x *g*) to pellet debris. Transfer supernatant to an RNase-free microfuge tube (not included).**
5. **Add an equal volume of RNA Lysis Buffer and vortex briefly. Proceed to Step 1 of Part 2: RNA Binding and Elution, page 12.**

❑ Tough-to-Lyse Samples (bacteria, yeast, plant, etc.):

Mechanical lysis/homogenization is recommended for tough-to-lyse samples. Alternatively, bacteria and yeast may be lysed enzymatically. Some gram-negative bacteria (e.g., *E. coli*) do not require mechanical or enzymatic lysis and can by lysed directly by resuspension in DNA/RNA Protection Reagent, however, yields may be lower. For enzymatic lysis of bacteria or yeast, see Enzymatic Lysis below.

Mechanical Lysis:

1. Determine the amount of 1X DNA/RNA Protection Reagent that you will need according to the table below. Prepare the aliquot of 1X DNA/RNA Protection Reagent by diluting the 2X concentrate with nuclease-free water (not provided for this step).

SAMPLE INPUT AMOUNT			VOLUME 1X DNA/RNA PROTECTION REAGENT
BACTERIA	**YEAST**	**PLANT**	
$< 5 \times 10^7$	$< 5 \times 10^6$		400 µl
$5 \times 10^7 – 1 \times 10^9$	$5 \times 10^6 – 5 \times 10^7$	≤ 100 mg	800 µl

2. Add 1X DNA/RNA Protection Reagent to sample.
3. Disrupt sample mechanically using a bead mill or similar device.
4. Transfer lysate/homogenate to an RNase-free microfuge tube (not included).
5. Spin for 2 minutes (16,000 x g) to pellet debris. Transfer supernatant to an RNase-free microfuge tube (not included).
6. Add an equal volume of RNA Lysis Buffer and vortex briefly. Proceed to **Step 1 of Part 2: RNA Binding and Elution**, page 12.

Enzymatic Lysis of Bacteria or Yeast:

1. Add appropriate reaction buffer and glycanase (not included), and incubate at appropriate temperature according to the chart below:

SAMPLE TYPE	ENZYME	INCUBATION TIME	DIGESTION TEMP.
Bacteria (gram neg.)	Lysozyme (1 mg/ml)	5 minutes	25°C
Bacteria (gram pos.)	Lysozyme (3 mg/ml)	10 minutes	25°C
Yeast	Zymolyase (25 U)	30-40 minutes	30°C

2. Add 2 volumes RNA Lysis Buffer and vortex vigorously for ~10 sec.
3. Centrifuge for 2 minutes at 16,000 x *g* to pellet cellular debris.
4. Transfer supernatant to an RNase-free microfuge tube (not included). Proceed to **Step 1 of Part 2: RNA Binding and Elution**, page 12.

❑ Reaction Cleanup (IVT, Labeling, DNase Treatment etc.):

The Monarch Total RNA Miniprep Kit can also be used to clean up RNA after enzymatic reactions including IVT, labeling reactions, or DNase I treatment. NEB also supplies Monarch RNA Cleanup Kits for faster, more streamlined cleanup of RNA (NEB #T2030, #T2040, and #T2050).

1. For every 50 µl of reaction volume, add 100 µl RNA Lysis Buffer and mix thoroughly.
2. Add an equal volume of ethanol (≥ 95%) (not included) and mix thoroughly. For example, if sample is now 150 µl, add 150 µl ethanol. Proceed to **Step 4 of Part 2: RNA Binding and Elution**, page 12.

❑ Samples Stored in Monarch DNA/RNA Protection Reagent

1. Allow samples to equilibrate to room temperature
2. Proceed to the **Part 1** protocol specific to your starting material.

❑ RNAlater Samples:

Samples stored in RNAlater can be processed according to sample type after the RNAlater is removed by pipetting. If RNAlater is not removed prior to processing (for example, liquid samples), please follow the protocol below.

1. Add an equal volume of nuclease-free water (not provided for this step) to the sample.

2. Add four volumes RNA Lysis Buffer to the sample and mix thoroughly. Proceed to **Step 1 of Part 2: RNA Binding and Elution**, page 12.

❑ **TRIzol Extracted Samples:**

1. Transfer the aqueous phase of a TRIzol extracted sample to an RNase-free tube (not included).
2. Add an equal volume of ethanol (≥ 95%) (not included) and mix thoroughly. Proceed to **Step 4 of Part 2: RNA Binding and Elution**, page 12.

PART 2 RNA BINDING AND ELUTION

All centrifugation steps should be performed at 16,000 x *g*.

For sample volumes > 800 µl (column reservoir capacity), columns may be reloaded.

1. **Transfer up to 800 µl of the sample from Part 1 to a gDNA Removal Column (orange) fitted with a collection tube.** For sample identification, **label collection tubes**, as gDNA removal columns will be discarded after spinning.
2. **Spin for 30 seconds to remove most of the gDNA. SAVE THE FLOW-THROUGH (RNA partitions here).** Discard the gDNA removal column.
3. **Add an equal volume of ethanol (≥ 95%) (not included) to the flow-through and mix thoroughly by pipetting. Do not vortex**. To exclude RNA ≤ 200 nt, add only 1/2 volume ethanol to flow-through. The addition of ethanol creates favorable conditions for RNA to bind to the RNA Purification column.
4. **Transfer mixture to an RNA Purification Column (colorless) fitted with a collection tube. Spin for 30 seconds. Discard flow-through.** If further gDNA removal is essential for downstream applications, proceed to on-column DNase I treatment, Step 4A–4C (recommended). If not, proceed to Step 5.

   Optional (but recommended): On-column DNase I treatment for enzymatic removal of residual gDNA

   4A. **Add 500 µl RNA Wash Buffer and spin for 30 seconds. Discard flow-through.** This ensures all salts are removed prior to the addition of DNase I.

   If using a vacuum manifold, add 500 µl of RNA Wash Buffer and switch the vacuum on. Allow the solution to pass through the column, then switch the vacuum source off.

   4B. **In an RNase-free microfuge tube (not included), combine 5 µl DNase I with 75 µl DNase I Reaction Buffer and pipet mixture directly to the top of the matrix.**

   4C. **Incubate for 15 minutes at room temperature.** Proceed to Step 5.

5. **Add 500 µl RNA Priming Buffer and spin for 30 seconds.** Discard flow-through.

   If using a vacuum manifold, add 500 µl of RNA Priming Buffer and switch the vacuum on. Allow the solution to pass through the column, then switch the vacuum source off.

6. **Add 500 µl RNA Wash Buffer and spin for 30 seconds.** Discard flow-through.

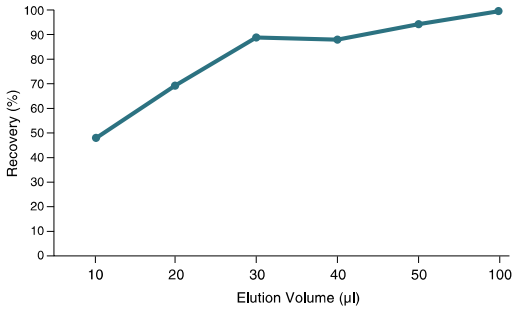
   If using a vacuum manifold, add 500 µl of RNA Wash Buffer and switch the vacuum on. Allow the solution to pass through the column, then switch the vacuum source off.

7. **Add another 500 µl RNA Wash Buffer and spin for 2 MINUTES. Transfer column to an RNase-free microfuge tube (not included).** Use care to ensure the tip of the column does not contact the flow-through. If in doubt, re-spin for 1 minute to ensure no ethanol is carried over.

   If using a vacuum manifold, add 500 µl of RNA Wash Buffer and switch the vacuum on. Allow the solution to pass through the column, then switch the vacuum source off.

8. **Add 30-100 µl Nuclease-free Water directly to the center of column matrix and spin for 30 seconds.** For best results, elute with at least 50 µl, which is the minimum volume needed to wet the membrane. Lower elution volumes can be used but will result in lower recovery (elution with 30 µl results in > 80% recovery and 100 µl provides maximum recovery). For spectrophotometric analysis of eluted RNA, it may be necessary to re-spin eluted samples and pipet aliquot from top of the liquid to ensure that the $A_{260/230}$ is unaffected by possible elution of silica particles.

Figure 6: Percent Recovery as a Function of Elution Volume for the Monarch Total RNA Miniprep Kit (NEB #T2010)



Total RNA (2 µg, 16S and 23S Ribosomal Standard from E. coli, Sigma) was loaded onto a Monarch RNA Purification Column (NEB #T2007) following the "Reaction cleanup" protocol with optional DNase I treatment for the Monarch Total RNA Miniprep Kit (NEB #T2010). Elutions using the volumes listed were performed on triplicate preps and percent recovery was calculated using concentration values measured on a Nanondrop spectrophotometer and conversion to a percentage of the input amount.

9. **Place RNA on ice if being used for downstream steps, at -20°C for short-term storage (less than 1 week), or at -80°C for long-term storage.** Addition of EDTA to 0.1–1.0 mM may reduce the activity of any contaminating RNases.

Supplemental Protocols

In-tube DNase I Treatment

Sensitive applications such as RT-qPCR require that RNA input samples contain little to no genomic DNA. To further reduce any residual genomic DNA, you can treat your eluted sample with DNAse I in a separate reaction and clean up the sample using a second RNA Purification Column from this kit or by using a separate cleanup kit.

1. For each sample to be treated, prepare a DNase I reaction mix in an RNase-free tube (not included) according to the table below. Mix well by gentle pipetting.

MIXTURE COMPONENT	VOLUME
RNA Sample (≤ 10 µg) Volume Adjusted with Water or TE Buffer	40 µl
Monarch DNase I (reconstituted)	5 µl
Monarch DNase I Reaction Buffer	5 µl
Total Volume	**50 µl**

2. Incubate at room temperature (20–30°C) for 15 minutes.
3. Proceed to Reaction Cleanup, page 11.

Fractionation of Small and Large RNA

Modification of the protocol used for cultured cells can allow for selective depletion or enrichment of the sub-200 nucleotide RNA pool. This modification may also be used on previously purified total RNA to enable fractionation. The procedure is compatible with up to 10^6 animal cells, or previously isolated RNA (with a sample volume adjusted to ≥ 50 µl).

1. Prepare an equal mixture of RNA Lysis Buffer and ethanol (≥ 95%) (not included).

   Choose the amount needed based on the number and type of samples. You will need 300 µl of the mixture for each cell pellet, or 2 volumes of the mixture for each RNA aliquot.

2. Add 300 µl of the mixture to each cell pellet, or 2 volumes of the mixture to each purified RNA aliquot. Mix samples by pipetting.
3. Transfer the sample into an RNA Purification Column (colorless) fitted with a collection tube and spin for 30 seconds at 16,000 x *g*. **SAVE THE FLOW-THROUGH.** RNAs > 200 nt bind to the column, whereas RNAs < 200 nt partition into the flow-through.

4a. To purify RNAs > 200 nt that are bound to the column, proceed to either Step 4A (for DNase I treatment) or Step 5 of Part 2: RNA Binding and Elution, page 12.

4b. To enrich for RNAs < 200 nt, add an equal volume of ethanol (≥ 95%) (not included) to the flow-through from Step 3 and transfer to a new RNA Purification Column fitted with a collection tube. Spin for 30 seconds at 16,000 x *g*, discard flow-through, and proceed to either Step 4A (for DNase I treatment) or Step 5 of Part 2: RNA Binding and Elution, page 12.

FFPE Tissue Deparaffinization

Paraffin is removed from FFPE samples by treating with xylene or a commercially available FFPE deparaffinization solution (see manufacturer's recommendations for details).

Rapid deparaffinization with xylene (tissue sections)

1. Remove (trim) as much excess paraffin from the sample as possible and transfer to 1.5 ml tube (not included).
2. Add 1 ml xylene (not included) and vortex vigorously 30 seconds.
3. Centrifuge for 1 minute at 16,000 x *g* and remove xylene.
4. Wash sample with 1 ml ethanol (≥ 95%) (not included) and vortex vigorously 30 seconds.
5. Centrifuge for 1 minute at 16,000 x *g* and remove ethanol. Repeat Steps 4 and 5.
6. Dry samples by vacuum centrifugation (e.g. Speed-Vac) or by incubating uncapped tubes at ≤ 37°C for up to 40 minutes.
7. Proceed to Tissue Digestion.

Tissue Digestion

1. Prepare a mixture of the following:

DNase/RNase-Free Water	170 µl
Proteinase K Reaction Buffer	20 µl
Proteinase K	10 µl

2. Add the mixture to the deparaffinized tissue sample (≤ 25 mg).
3. Incubate at 55°C for 15 minutes.
4. Transfer the tube to a preheated block at 65°C and incubate for 15 minutes. Note: If only one heat block is available, place sample at room temperature after the 55°C incubation until heating block has reached 65°C.
5. Add 600 µl RNA Lysis Buffer and mix thoroughly. Centrifuge for 1 min at 16,000 x *g* to pellet debris. Transfer supernatant to an RNase-free microfuge tube (not included). Proceed to Step 1 of Part 2: RNA Binding and Elution, page 12.

Purification of Total RNA from Saliva

We recommend stabilizing saliva samples in Monarch DNA/RNA Protection Reagent and processing samples as described below. It is important to note that RNA purified from saliva is typically degraded with RIN values between 2.0-3.0. Prepare all reagents as indicated in the Buffer Preparation section.

1. Immediately stabilize 200 µl of the saliva sample after collection by mixing with an equal volume of 2X Monarch DNA/RNA Protection Reagent. If unable to add the Protection reagent immediately, briefly place saliva sample on ice until Protection Reagent can be added.
2. For every 400 µl of DNA/RNA Protection Reagent/Sample mixture add 20 µl Monarch Proteinase K.
3. Vortex briefly and incubate at room temperature for 30 minutes.
4. Add an equal volume of Monarch RNA Lysis Buffer, vortex briefly and proceed to **Step 1 of Part 2: RNA Binding and Elution.**

RNA Purification from Buccal (Cheek) Swabs and Nasopharyngeal (NP) Swabs

We recommend collecting samples into Monarch DNA/RNA Protection reagent and processing samples as described below. Prepare all reagents as indicated in the Buffer Preparation section.

Note: *This protocol has been validated with buccal swabs and saliva samples. We have also confirmed the compatibility of this workflow with viral transport medium using reference samples that do not require cell and/or viral envelope lysis. At this time, we have not yet internally validated this kit with nasopharyngeal samples*.

Samples stored in transport medium

1. Add an equal volume (up to 200 µl) of 2X Monarch DNA/RNA Protection Reagent to an aliquot of transport medium and vortex briefly.
2. For every 400 µl of DNA/RNA Protection Reagent/Sample mixture add 20 µl Monarch Proteinase K.
3. Vortex briefly and incubate at room temperature for 30 minutes.
4. Add an equal volume of Monarch RNA Lysis Buffer and vortex briefly. **Proceed to Step 1 of Part 2: RNA Binding and Elution**, page 12.

Samples not stored in transport medium

1. Place swab into a tube containing 300 ul 1X Monarch DNA/RNA Protection Reagent. Vigorously swirl the swab to resuspend the sample material in Protection Reagent.
2. For every 300 µl of DNA/RNA Protection Reagent/Sample mixture, add 15 µl Monarch Proteinase K. Vortex briefly and incubate at room temperature for 30 minutes.
3. Vortex sample briefly and spin for 2 min (16,000 x g) to pellet debris. Transfer supernatant to an RNase-free microfuge tube (not included).
4. Add an equal volume of Monarch RNA Lysis Buffer and vortex briefly. Proceed to **Step 1 of Part 2: RNA Binding and Elution**, page 12.

Purification of Total RNA from Nucleated Blood (e.g. bird, fish, reptile):

We recommend stabilizing nucleated blood in Monarch DNA/RNA Protection Reagent and processing samples as described below.

1. Add 200 µl 1X DNA/RNA Protection Reagent to 10 µl nucleated blood. Mix by pipetting.
2. Add 160 µl Proteinase K Reaction Buffer and 4 µl Proteinase K. Mix well.
3. Incubate at 55°C for 30 minutes (note: optimal incubation times may vary).
4. Add an equal volume of isopropanol and mix by vortexing.
5. Transfer the sample to a gDNA Removal Column fitted with a collection tube and spin for 30 seconds; discard the flow-through. Transfer column to a new collection tube.
6. Add 200 µl RNA Lysis Buffer to the matrix and let stand for 5 minutes. Spin for 30 seconds. SAVE THE FLOW-THROUGH and discard the gDNA Removal Column.
7. Add an equal volume of ethanol to the flow-through and mix thoroughly by pipetting.
8. Proceed to **Step 4 of Part 2: RNA Binding and Elution (Loading sample onto the RNA Purification Column)**, page 12.

Purification of Total RNA from Drosophila:

For insects, we recommend mechanical lysis and homogenization in Monarch RNA Lysis Buffer, followed by RNA Binding and Elution as described below.

1. Transfer drosophila (up to 10 mg) into a tube containing beads for homogenization and add 800 µl RNA Lysis Buffer.
2. Disrupt sample mechanically using a bead homogenizer.
3. Transfer lysate/homogenate to an RNase-free microfuge tube.
4. Spin for 2 minutes (16,000 x *g*) to pellet debris. Transfer supernatant to an RNase-free microfuge tube.
5. Proceed to **Step 1 of Part 2: RNA Binding and Elution.**

Purification of Total RNA from Zebrafish Embryos and Larvae:

We recommend stabilizing embryos or larvae in Monarch DNA/RNA Protection Reagent, followed by mechanical lysis/homogenization and processing as described below.

1. Add up to 20 embryos or larvae to 300 µl of 1X DNA/RNA Protection Reagent, vortex briefly, and transfer sample to a tube containing beads for homogenization.
2. Disrupt sample mechanically using a bead homogenizer.
3. Transfer lysate to an RNase-free microfuge tube.
4. Add 30 µl Proteinase K Reaction buffer and 15 µl Proteinase K, mix briefly, and incubate at 55°C for 5 minutes.

5. Vortex briefly and spin for 2 minutes (16,000 x *g*) to pellet debris. Transfer supernatant to an RNase-free microfuge tube.
6. Add an equal volume of RNA Lysis Buffer and vortex briefly. Proceed to **Step 1 of Part 2: RNA Binding and Elution**, page 12.

Purification of Total RNA from Plasma and Serum:

We recommend stabilizing 200 µl to 1 ml plasma or serum by mixing with an equal volume of 2X Monarch DNA/RNA Protection Reagent and following the Mammalian Whole Blood Protocol. It is important to note that RNA yields from plasma and serum are typically low, ranging from 1-100 ng/ml.

In-well Lysis of Adherent Cells:

Adherent cells can be lysed directly in the wells of a multi-well plate by following the suggested protocol below.

For Immediate Processing:

1. Remove the media from the well and rinse with PBS.
2. Add Monarch RNA Lysis Buffer (NEB #T2012) directly to the well according to the table below.

MULTI-WELL PLATE SIZE	VOLUME OF MONARCH RNA LYSIS BUFFER
6 well	≥ 600 µl
12 well	300-600 µl
24 well	300 µl
48 well	300 µl

3. Mix thoroughly and proceed to **Step 1 of Part 2: RNA Binding and Elution.**

For Storage:

1. Remove the media from the wells and rinse with PBS.
2. Add 1X Monarch DNA/RNA Protection Reagent (NEB #T2011) (be sure to dilute the 2X concentrate) directly to the well according to the table below:

MULTI-WELL PLATE SIZE	VOLUME OF 1X MONARCH DNA/RNA PROTECTION REAGENT
6 well	≥ 600 µl
12 well	300-600 µl
24 well	300 µl
48 well	300 µl

3. Mix thoroughly and store samples until ready for processing. Samples may be frozen for long-term storage or may be refrigerated or maintained at room temperature for medium-term or short-term storage (see Sample Stabilization and Storage Instructions on
page 17).
4. When ready to process, add an equal volume of Monarch RNA Lysis Buffer (NEB #T2012) and proceed to **Step 1 of Part 2: RNA Binding and Elution**, page 12.

Appendices

Monarch DNA/RNA Protection Reagent Guidance:

Monarch DNA/RNA Protection Reagent preserves nucleic acid integrity in a wide variety of sample types. DNA and RNA from cells, blood, and tissues are protected from degradation across a broad range of storage temperatures, simplifying handling, storage and transport. Compatibility with the Monarch Total RNA Miniprep kit enables seamless sample storage and RNA purification without the need to remove the reagent prior to cell lysis.

Sample Stabilization & Storage Instructions:

These recommendations are based on observed RIN values across a variety of samples. Certain individual sample types may be successfully preserved for longer times and/or at higher temperatures and researchers are encouraged to empirically determine optimal conditions for their sample of interest.

Sample Stabilization:

- **Cells, tissues, and other solid samples:** Submerge in 1X Monarch DNA/RNA Protection Reagent with the sample comprising no more than 10% of the final volume.
- **Blood and other liquid samples:** Mix with an equal volume of the 2X Protection Reagent concentrate.

Sample Homogenization Prior to Storage:

For cultured cells, blood, and small tissue pieces (less than 20 mg), simply adding/mixing sample with Protection Reagent is sufficient for subsequent storage. For larger tissue pieces, samples should be homogenized in Protection Reagent prior to storage.

Sample Storage:

Long-term storage (> 30 days): Use of freezers at –20 or –80°C is recommended

Medium-term storage (1-4 weeks): Refrigeration can be used

Short-term storage (< 7 days): Samples may be stored at 20-25°C

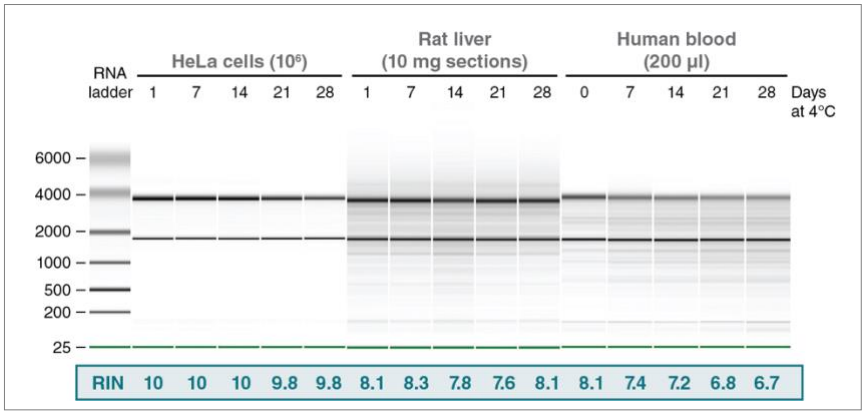
Usage Instructions for the Monarch Total RNA Miniprep Kit:

Samples not previously preserved in Monarch DNA/RNA Protection Reagent– samples are typically mixed with DNA/RNA Protection Reagent prior to Proteinase K digestion or bead homogenization. For cell culture pellets, DNA/RNA Protection Reagent is not required.

Samples preserved in Monarch DNA/RNA Protection Reagent– samples can be directly processed for RNA purification as long as the volume of DNA/RNA Protection Reagent used is consistent with protocol recommendations. For cultured cells in DNA/RNA Protection Reagent, an equal volume of RNA Lysis buffer should be added prior to transfer to the gDNA Removal column.

Additional DNA/RNA Protection Reagent should be added when the total volume of DNA/RNA Protection Reagent is below the suggested volume. If the volume of DNA/RNA Protection Reagent exceeds the suggested volume, additional lysis buffer and ethanol are required to maintain the proper ratio of reagents for processing.

Figure 7: Monarch DNA/RNA Protection Reagent preserves RNA integrity for up to 1 month at 4°C.



Samples were incubated in Monarch DNA/RNA Protection Reagent (NEB #T2011) at 4°C for the indicated time and subsequently processed using the Monarch Total RNA Miniprep Kit (NEB #T2010). Samples were eluted in 100 µl nuclease-free water. Rat liver and blood samples were diluted 5-fold (HeLa cells were not diluted), and 1 µl of each was loaded onto a Bioanalyzer Nano chip (HeLa and rat liver) or Pico chip (blood). The RIN values demonstrate the ability of the Protection Reagent to preserve RNA integrity over time.

RNA Quantification

- Quantitation of RNA can be performed using direct spectrophotometric measurement (NanoDrop™, Trinean), RNA-specific dye-assisted fluorometric measurements (Qubit®, RiboGreen®), or by RT-qPCR. Each method has advantages and disadvantages relating to accuracy, time requirements, equipment requirements, and expense.
- Direct spectrophotometric analysis of samples with a micro-volume spectrophotometer (NanoDrop) is easy, rapid, and appropriate for routine measurements where absolute concentrations are not required. These devices do not perform well on dilute samples (below 20 ng/µl), nor do they allow de-convolution of the contribution of genomic DNA to the absorbance at 260 nm. Additionally, the contribution of other macromolecules to the absorbance spectra is not always appreciated with these devices. Use of a spectrophotometer with content profiling (Trinean DropSense) can be helpful.
- Fluorescent dyes that specifically bind to RNA can provide a more accurate way to determine concentration but require additional effort because of the need to generate standard curves with samples of known concentration. Many kits exist for this approach, and the overall workflows have been optimized for efficiency, providing a reasonable balance between accuracy and effort/cost. NEB routinely utilized these methods during RNA kit development and sample manipulations.
- RT-qPCR remains the gold standard for absolute quantitation of RNA and provides unrivaled limits of detection. Care must be taken to design appropriate primer sets to detect RNA only and appropriate controls must be utilized to ensure amplification is RNA specific and not from residual host DNA. We recommend NEB's Luna RT-qPCR products.

RNA Purity & Integrity

- Purity of eluted RNA samples can be quickly assessed by reviewing OD ratios collected during routine spectrophotometry. Pure RNA typically has an $A_{260/280}$ of 1.9–2.1, and an $A_{260/230}$ of 2.0–2.2. Many factors can influence these values such as the use of a proper reference blank solution, the buffer pH, and contaminants such as protein, buffer salts, ethanol, etc.
- RNA integrity can be assessed using an Agilent Bioanalyzer or similar device to resolve the sample and determine the ratio of 28S to 18S rRNA, as well as the amount of lower molecular weight species. This approach produces an RNA Integrity Number (RIN) which encapsulates these generally agreed upon metrics. RIN values greater than 7 are often referred to as "good," though the specific downstream application of the purified RNA determines the true needs of RNA quality. Researchers are encouraged to establish their own criteria.

Troubleshooting

Clogged Column

- Insufficient disruption or homogenization
  - increase time of digestion or homogenization
  - centrifuge sample to pellet debris and use only supernatant for next steps
  - use larger volume of buffer for lysis and homogenization
- Too much sample
  - reduce amount of starting material to match kit specifications to ensure buffer amounts are sufficient and column is not overloaded

Low Yield

- Incomplete elution
  - increase incubation time after addition of elution buffer to 5–10 minutes
  - Increase elution volume or perform second elution step (note: this will dilute sample)
- Sample is degraded
  - store sample at -80°C prior to use
  - use Monarch DNA/RNA Protection Reagent (NEB #T2011) to maintain RNA integrity during storage
- Insufficient disruption or homogenization
  - increase time of digestion or homogenization
  - centrifuge sample to pellet debris and use only supernatant for next steps
  - use larger volume of buffer for lysis and homogenization
  - for Proteinase K treated samples, doubling Proteinase K (from 5% to 10%) may lead to an increase in RNA yield
- Too much sample
  - reduce amount of starting material to match kit specifications to ensure buffer amounts are sufficient and column is not overloaded

RNA Degradation

- Starting material not handled/stored properly. Degradation of RNA may occur if sample is not flash frozen or protected by a preservation reagent.
- Deviation from the stated protocol may expose RNA to unwanted RNase activities. Refer to the General Guidelines for working with RNA, Step 1, page 3.
- RNase contamination of eluted materials or kit buffers may have occurred. See guidance (page 3) for advice on reducing risks of contamination.

Low OD Ratios

- Low $A_{260/280}$ values indicate residual protein in the purified sample. Ensure the Proteinase K step was utilized for the recommended time. Ensure samples have no debris prior to addition of ethanol and loading onto RNA purification column.
- Low $A_{260/230}$ values indicate residual guanidine salts have been carried over during elution. Ensure wash steps are carried out prior to eluting sample. Use care to ensure the tip of the column does not contact the flow-through. If unsure, please repeat centrifugation. When reusing collection tubes, blot rim of tube on a Kimwipe™ prior to reattachment to the column to remove any residual wash buffer.

DNA Contamination

- Perform optional on-column DNase I treatment to remove unwanted gDNA from lysed sample
- Too much sample
  - reduce amount of starting material to match kit specifications to ensure buffer amounts are sufficient and column is not overloaded
- Perform in-tube/off-column DNase I treatment to remove gDNA (see protocol in Supplemental Protocols, page 13).

Low Performance of RNA in Downstream Steps

- Salt and/or ethanol carry-over has occurred.
  - use care to ensure the tip of the column does not contact the flow-through after the final wash. If unsure, please repeat centrifugation.
  - be sure to spin column for 2 minutes following final wash with RNA Wash Buffer
  - when reusing collection tubes, blot rim of tube on a Kimwipe prior to reattachment to the column to remove any residual wash buffer
- Add additional wash step and/or extend spin time for final wash.

Ordering Information

PRODUCT	NEB #	SIZE
Monarch Total RNA Miniprep Kit	T2010S/L	50 preps
Monarch RNA Purification Columns	T2007L	100 columns + collection tubes
Monarch gDNA Removal Columns	T2017L	100 columns + collection tubes
Monarch Spin Collection Tubes	T2118L	100 collection tubes
Monarch DNA/RNA Protection Reagent	T2011L	56 ml
Monarch RNA Lysis Buffer	T2012L	100 ml
Monarch Total RNA Miniprep Enzyme Pack	T2019L	1 pack
Monarch RNA Priming Buffer	T2013L	56 ml
Monarch RNA Wash Buffer	T2014L	50 ml
Nuclease-free Water	B1500S/L	25/100 ml

Revision History

REVISION #	DESCRIPTION	DATE
1.0	New Document	9/17
2.0		4/18
3.0	New format applied; new protocol for nasopharyngeal samples	4/20
4.0	Updated the correct max input amount for cultured cells to $1x10^7$ cells on page 6. Also updated legal footnote and table styling.	7/23
5.0	Updated the max input amount for cultured cells on page 6. Updated column color text description in Figure 5, page 8. Also updated header and footer.	5/24

How to Recycle Monarch Kit Components*

Component	Recycling Notes**
Kit Box (paper)	For the greatest environmental benefit, please reuse this box. It is fully recyclable in paper recycling. The small magnets do not prohibit recycling.
Columns and Collection Tubes (hard plastic)	Columns and collection tubes are made from polypropylene 5 and are recyclable. After use, please refer to your institutional policies for proper disposal, especially when working with biohazardous materials.
Plastic Bottles (hard plastic)	Bottles are made from high-density polyethylene 2, and caps are polypropylene 5. Please rinse before recycling.
Plastic Bags (plastic film)	Bags are made from low-density polyethylene 4 and can be recycled with other plastic bags and films.
Protocol Card (paper)	Recycle with mixed paper, or keep in your lab notebook for reference. The finish on this card does not prohibit recycling.

* Information as of November 2015. Please visit NEBMonarchPackaging.com for updates.
** Please defer to your institutional policies for proper disposal of this kit and its components.
Consult with your local and institutional authorities to learn how to maximize your landfill diversion and materials recovery.

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