

Leukocyte RNA Purification 96-Well Kit Product # 37800

Product Insert

Norgen's Leukocyte RNA Purification 96-Well Kit provides a rapid method for the isolation and purification of total leukocyte (white blood cell) RNA from mammalian blood samples. RNA isolated from blood can be used in various expression studies including those focusing on diseases. However, a major problem with blood RNA isolation is that a large portion of the RNA present is globin mRNA, which is found primarily in red blood cells. In fact, up to 70% of the mass of mRNA in whole blood total RNA is globin transcripts. Therefore, it is desirable to be able to remove the red blood cells from the sample and isolate only the RNA associated with the leukocytes, which will result in improved expression profiling and other applications by removing the masking effects of this abundant globin mRNA. Norgen's Leukocyte RNA Purification Kit can be used to isolate and purify total leukocyte RNA, including all small RNAs, from mammalian blood samples.

Norgen's Purification Technology

Purification is based on 96-well column chromatography using Norgen's proprietary resin as the separation matrix. The RNA is preferentially purified from the other cellular components such as proteins, without the use of phenol or chloroform. For leukocyte RNA purification, whole blood samples are first collected with anticoagulants. The red blood cells are removed through differential red blood cell lysis, and the leukocytes are recovered by centrifugation (please see flow charts on pages 4 and 5). The recovered leukcoytes are then lysed. Ethanol is then added to the lysate, and the solution is loaded onto the 96-Well Filter Plate for purification. The purification could be performed on either a vacuum manifold or using centrifugation. Norgen's resin binds RNA in a manner that depends on ionic concentrations. Thus only the RNA will bind to the column, while the contaminating proteins will be removed in the flowthrough or retained on the top of the resin. The bound RNA is then washed three times with the provided wash solution in order to remove any remaining impurities, and the purified leukocyte RNA is eluted with the elution solution. Norgen's kit allows for the isolation of total leukocyte RNA, including all small RNA species. The purified RNA is of the highest guality and can be used in a number of downstream applications including real time PCR, reverse transcription PCR, northern blotting, RNase protection and primer extension, and expression array assays.

Specifications

Kit Specifications		
Binding Capacity Per Well	50 µg	
Maximum Loading Volume Per Well	500 μL	
Size of RNA Purified	All sizes, including small RNA (<200 nt)	
Maximum Blood Input*	1 mL or 1.5 x 10 ⁶ Leukocytes	
Standard Input	150 μL	
Minimum Blood Input	10 μL	
Time to Complete 10 Purifications	40 minutes	
Average Yield: 500 μL human blood	1.5 μg	

*Additional RBC Lysis Solution is required for input Volumes >150 µL

Storage Conditions and Product Stability

All solutions should be kept tightly sealed and stored at room temperature. The RBC Lysis Buffer should be stored at 4°C upon arrival. These reagents should remain stable for at least 1 year in their unopened containers.

Advantages

- Fast and easy processing using either a vacuum manifold or centrifugation
- No phenol or chloroform extractions
- Differential red blood cell lysis allows for the removal of a majority of globin mRNAs
- Isolate total leukocyte RNA, including all small RNA species
- High quality leukocyte RNA can be used in a number of downstream applications

Kit Components

Component	Product # 37800 (2 x 96 preps)
RBC Lysis Buffer	2 x 90 mL
Binding Solution	2 x 40 mL
Wash Solution	2 x 30 mL
Elution Solution	2 x 20 mL
RBC Lysis 96-Well Plate	2
96-Well Filter Plate	2
Adhesive Tape	4
96-Well Collection Plate	2
96-Well Elution Plate	2
Product Insert	1

Precautions and Disclaimers

User must determine the suitability of the product for their particular use. This kit is intended for research purposes only and not for human or drug use. This kit is not designed for diagnostic purposes. MSDS sheets are available upon request.

Blood of all human and animal subjects is considered potentially infectious. All necessary precautions recommended by the appropriate authorities in the country of use should be taken when working with whole blood. Ensure that a proper lab coat, disposable gloves and protective eyewear are worn when working with this kit.

The **Binding Solution** contains guanidine salts, and should be handled with care. Guanidine salt forms highly reactive compounds when combined with bleach, thus care must be taken to properly dispose of any of these solutions.

Customer-Supplied Reagents and Equipment

You must have the following in order to use the Leukocyte RNA Purification 96-Well Kit:

- For Vacuum Format Purification:
 - Vacuum manifold with vacuum pump capable of generating a minimum pressure of -650 mbar or -25 in. Hg (such as Whatman UniVac 3 Vacuum to Collect Manifold)
 - Sealing tape or pads
- For 96-Well RBC Lysis and Centrifuge Format Purification:
 - Centrifuge with rotor for 96-well plate assembly (such as Thermo Fisher IEC Centra CL3 series or Beckman GS-15R)

- 95 100% ethanol
- β-mercaptoethanol (optional)
- Collection/Waste Tray for vacuum manifold or 96-well bottom plate (single or 96-well format) for centrifugation. Two 96-Well Collection Plates are provided with the kit.

Working with RNA

RNases are very stable and robust enzymes that degrade RNA. Autoclaving solutions and glassware is not always sufficient to actively remove these enzymes. The first step when preparing to work with RNA is to create an RNase-free environment. The following precautions are recommended as your best defense against these enzymes.

- The RNA area should be located away from microbiological work stations
- Clean, disposable gloves should be worn at all times when handling reagents, samples, pipettes, disposable tubes, etc. It is recommended that gloves are changed frequently to avoid contamination
- There should be designated solutions, tips, tubes, lab coats, pipettes, etc. for RNA only
- All RNA solutions should be prepared using at least 0.05% DEPC-treated autoclaved water or molecular biology grade nuclease-free water
- Clean all surfaces with commercially available RNase decontamination solutions
- When working with purified RNA samples, ensure that they remain on ice during downstream applications

Flow Chart 1 Procedure for Differential Red Blood Cell (RBC) Lysis



White Leukocyte Pellet

Flow Chart 2 Procedure for Total Leukocyte RNA Purification



Total Leukocyte RNA

Procedure

For *Vacuum Manifold*: All vacuum steps are performed at room temperature. The correct pressure can be calculated using the conversions:

For *Centrifugation*: All centrifugation steps are performed at room temperature. The correct rpm can be calculated using the formula:

$$RPM = \sqrt{\frac{RCF}{(1.118 \text{ x } 10^{-5}) (r)}}$$

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

Protocol for Total RNA Purification from Isolated Leukocytes

Notes Prior to Use

- Ensure that all solutions are at room temperature prior to use.
- Prepare a working concentration of the Wash Solution by adding 90 mL of 95% ethanol (provided by the user) to the supplied bottle containing the concentrated Wash Solution. This will give a final volume of 120 mL. The label on the bottle has a box that may be checked to indicate that the ethanol has been added.
- Prepare an appropriate amount of **Binding Solution** by adding 10 μL of β-mercaptoethanol (provided by the user) to each 1 mL of **Binding Solution** required.
 β-mercaptoethanol is toxic and should be dispensed in a fume hood.
- It is recommended that no more than 1 mL of blood be used in order to prevent possible clogging of the wells of the plate.
- Blood of all human and animal subjects is considered potentially infectious. All necessary precautions recommended by the appropriate authorities in the country of use should be taken when working with whole blood.
- Blood samples should be collected into a tube containing EDTA, such that the final concentration of the EDTA is ~ 4.8 mM.
- Only fresh blood can be used with this procedure. Frozen whole blood can not be used.
- For optimal results, blood samples should be processed within a few hours of collection.
- Two RBC Lysis 96-Well Plate with deep wells are provided for high throughput RBC Lysis of up to 150 μ L of blood input. For input blood volumes larger than 150 μ L, perform individual RBC Lysis in an appropriate vessel such as a 1.5 mL microcentrifuge tube or a 15 mL conical centrifuge tube.
- For input blood volume larger than 150 μL, additional volumes of RBC Lysis Solution (Part# 21201) are required.
- It is important to work quickly during this procedure.

Section 1. RBC Lysis and Lysate Preparation

1A. High Throughput Red Blood Cell Lysis in RBC Lysis 96-Well Plate

- a. Aliquot up to 150 µL of blood sample per well.
- b. Add 5 volumes of **RBC Lysis Buffer** to blood samples. (i.e.: Add 750 μ L of **RBC Lysis Buffer** to 150 μ L of blood).

c. Incubate at room temperature for 3 to 5 minutes, with gentle agitation during the incubation to mix.

Note: Ensure that the solution changes from a milky, opaque pink to clear red before proceeding to the next step.

- d. Centrifuge at 650 x g (~800 1,000 RPM) for 3 minutes and remove the supernatant by pipetting.
- Add 1 additional volumes of RBC Lysis Buffer to pelleted white blood cells and mix by gentle vortexing for 10 seconds. (i.e. Add 150 μL of RBC Lysis Buffer to every 150 μL of input blood volume)
- f. Centrifuge at 650 x g (~800 1,000 RPM) for 3 minutes and remove the supernatant by pipetting. A few μ L of media may be left behind with the pellet in order to ensure that the pellet is not dislodged.

Note: The leukocyte pellet should be white. If the pellet is red, then the red blood cell lysis procedure was incomplete. Please refer to the troubleshooting guide at the back of the manual if this occurs.

1B. Higher Volume Red Blood Cell Lysis in Microcentrifuge or Centrifuge Tubes

Notes Prior to Use

- For input blood volumes larger than 150 μL, additional volumes of RBC Lysis Solution (Part# 21201) are required.
- a. Aliquot up to 300 μ L of blood sample to a 1.5 mL microcentrifuge tube or up to 1 mL of blood sample to a 15 mL conical centrifuge tube.
- b. Add 5 volumes of **RBC Lysis Buffer** to blood samples. (i.e.: Add 500 μ L of **RBC Lysis Buffer** to every 100 μ L of blood).
- c. Incubate at room temperature for 3 to 5 minutes, with gentle agitation during the incubation to mix.

Note: Ensure that the solution changes from a milky, opaque pink to clear red before proceeding to the next step.

- d. Centrifuge at 650 x g (~2,000 3,000 RPM in a microcentrifuge or ~800 1,000 RPM in a swing bucket centrifuge) for 3 minutes and remove the supernatant by decanting or by pipetting.
- e. Add 1 additional volumes of **RBC Lysis Buffer** to pelleted white blood cells and mix by gentle vortexing for 10 seconds. (i.e. Add 100 μL of **RBC Lysis Buffer** to every 100 μL of input blood volume)
- f. Centrifuge at 650 x g (~2,000 3,000 RPM in a microcentrifuge or ~800 1,000 RPM in a swing bucket centrifuge) for 3 minutes and and remove the supernatant by decanting or by pipetting. A few μ L of media may be left behind with the pellet in order to ensure that the pellet is not dislodged.
 - **Note:** The leukocyte pellet should be white. If the pellet is red, then the red blood cell lysis procedure was incomplete. Please refer to the troubleshooting guide at the back of the manual if this occurs.

2. Cell Lysate Preparation

- a. Add 300 µL of **Binding Solution** directly to pelleted leukocytes.
- b. Lyse cells by gentle vortexing or pipetting up and down until homogeneity is reached.

- c. Add 140 μ L of 95 100% ethanol (provided by the user) to the mixture and mix by vortexing or pipetting up and down for 10 seconds.
- **Note:** For input amounts greater than 500 μ L of blood or 10⁶ leukocytes, it is recommended that the lysate is passed through a 25 gauge needle attached to a syringe 5-10 times at this point, in order to shear the genomic DNA prior to loading onto the column.

Section 2. Total RNA Purification from All Types of Lysate

Note: The purification of total RNA from the lysate prepared in Section 1 could be performed using either a vacuum manifold or centrifugation. For purification using vacuum, please follow the procedure outlined in Section 2A. For purification using centrifugation, please follow the procedure outlined in Section 2B

A. Leukocyte RNA Purification Using Vacuum Manifold

3. Binding RNA to 96-Well Filter Plate

a. Assemble the 96-Well Filter Plate and the vacuum manifold according to manufacturer's recommendations.

Note: The provided 96-Well Collection Plate can be used as the collection/waste tray if desired.

b. Apply the lysate with the ethanol (from Step 2) into each well of the 96-Well Filter Plate. Tape the plate or any unused wells using sealing tape or pads (provided by the user) according to the vacuum manifold manufacturer's recommendations. Apply vacuum for 2 minutes.

Note: Depending on the starting material, a small quantity of precipitates may appear in the lysate-ethanol mix. No additional step is required to remove these precipitates prior to application of the mixture to the wells.

c. Turn off vacuum and ventilate the manifold. Discard the flowthrough. Reassemble the 96-Well Filter Plate and the vacuum manifold.

Note: Ensure that all of the lysate from each well has passed through into the collection/waste tray. If the entire lysate volume has not passed, apply vacuum for an additional 2 minutes.

Optional Step:

Norgen's Total RNA Purification 96-Well Kit isolates total RNA with minimal amounts of genomic DNA contamination. However, an optional **On-Plate DNA Removal Protocol** is provided in Appendix A for maximum removal of residual DNA that may affect sensitive downstream applications. This step should be performed at this point in the protocol.

4. RNA Wash

- a. Apply 400 μL of Wash Solution to each well of the 96-Well Filter Plate. Tape the plate or any unused wells using sealing tape or pads (provided by the user) according to the vacuum manifold manufacturer's recommendations. Apply vacuum for 2 minutes.
 - **Note:** Ensure the entire wash solution has passed through into the collection/waste tray by inspecting the 96-Well Filter Plate. If the entire wash volume has not passed, apply vacuum for an additional 2 minutes.

- b. Turn off vacuum and ventilate the manifold. Discard the flowthrough.
- c. Reassemble the 96-Well Filter Plate and the vacuum manifold. Repeat steps **4a** and **4b** to wash column for a second time.
- d. Reassemble the 96-Well Filter Plate and the vacuum manifold. Repeat steps **4a** and **4b** to wash column for a third time.
- e. Pat the bottom of the 96-Well Filter Plate dry. Reassemble the 96-Well Filter Plate and the vacuum manifold. Apply vacuum for an additional 5 minutes in order to completely dry the plate.
- f. Turn off vacuum and ventilate the manifold.

5. RNA Elution

- a. Replace the collection/waste tray in the vacuum manifold with the provided 96-Well Elution Plate. Complete the vacuum manifold assembly with the 96-Well Filter Plate.
- b. Add 75 μ L of **Elution Solution** to each well of the plate.
- c. Apply vacuum for 2 minutes.

6. Storage of RNA

Use the provided adhesive tape to seal the 96-Well Elution Plate. The purified RNA samples may be stored at -20 °C for a few days. It is recommended that samples be placed at -70 °C for long term storage.

B. Leukocyte RNA Purification Using Centrifugation

Note: The remaining steps of the procedure for the purification of total RNA using centrifugation are the same from this point forward for all the different types of lysate.

3. Binding RNA to 96-Well Filter Plate

- a. Place the 96-Well Filter Plate on top of a provided 96-Well Collection Plate.
- b. Apply up to 500 μ L of the lysate with the ethanol (from **Step 2**) into each well of the 96-Well Filter Plate. Centrifuge the assembly at maximum speed or 3,000 *x g* (~3,000 RPM) for 2 minutes.

Note: Depending on the starting material, a small quantity of precipitates may appear in the lysate-ethanol mix. No additional step is required to remove these precipitates prior to application to the wells

c. Discard the flowthrough. Reassemble the the 96-Well Filter Plate and the bottom plate.

Note: Ensure that all of the lysate from each well has passed through into the bottom plate. If the entire lysate volume has not passed, centrifuge for an additional 2 minutes.

Optional Step:

Norgen's Total RNA Purification 96-Well Kit isolates total RNA with minimal amounts of genomic DNA contamination. However, an optional **On-Plate DNA Removal Protocol** is provided in Appendix A for maximum removal of residual DNA that may affect sensitive downstream applications. This step should be performed at this point in the protocol

4. RNA Wash

a. Apply 400 μ L of **Wash Solution** to each well of the 96-Well Filter Plate. Centrifuge the assembly at maximum speed or 3,000 *x g* (~3,000 RPM) for 2 minutes.

- **Note:** Ensure the entire wash solution has passed through into the bottom plate by inspecting the 96-Well Filter Plate. If the entire wash volume has not passed, centrifuge for an additional 2 minutes.
- b. Discard the flowthrough. Reassemble the 96-Well Filter Plate and the bottom plate.
- c. Repeat steps **4a** and **4b** to wash column for a second time.
- d. Repeat steps 4a and 4b to wash column for a third time.
- e. Pat the bottom of the 96-Well Filter Plate dry. Reassemble the 96-Well Filter Plate and the bottom plate. Centrifuge the assembly at maximum speed or $3,000 \times g$ (~ $3,000 \times g$ (~ $3,000 \times g$) RPM) for 5 minutes in order to completely dry the plate.

5. RNA Elution

- a. Stack the 96-Well Filter Plate on top of the 96-Well Elution Plate.
- b. Add 75 μ L of **Elution Solution** to each well of the 96-Well Filter Plate.
- c. Centrifuge the assembly at maximum speed or 3,000 x g (~3,000 RPM) for 2 minutes.

6. Storage of RNA

Use the provided adhesive tape to seal the 96-Well Elution Plate. The purified RNA sample may be stored at -20 °C for a few days. It is recommended that samples be placed at -70 °C for long term storage.

Appendix A

Protocol for Optional On-Column DNA Removal

Norgen's Leukocyte RNA Purification 96-Well Kit isolates total RNA with minimal amounts of genomic DNA contamination. However, an optional protocol is provided below for maximum removal of residual DNA that may affect sensitive downstream applications. It is recommended that Norgen's RNase-Free DNase I Kit (Product # 25710) be used for this step.

- 1. For every on-column reaction to be performed, prepare a mix of 10 μL of **DNase I** and 65 μL of **Enzyme Incubation Buffer** using Norgen's RNase-Free DNase I Kit (Product # 25710). Mix gently by inverting the tube a few times. **DO NOT VORTEX**.
 - **Note:** If using an alternative DNase I, prepare a working stock of 0.25 Kunitz unit/ μ L RNase-free DNase I solution according to the manufacturer's instructions. A 75 μ L aliquot is required for each column to be treated.
- 2. Perform the appropriate Leukocyte RNA Isolation Procedure up to and including "**Binding RNA to 96-Well Filter Plate**" (Steps 1 to 3 of all protocols)
- 3. *For Vacuum Manifold*: Apply 400 μL of **Wash Solution** to each well of the 96-Well Filter Plate. Tape the plate or any unused wells using sealing tape or a pad (provided by the user) according to the vacuum manifold manufacturer's recommendations. Apply vacuum for 2 minutes.

For Centrifugation: Apply 400 μ L of **Wash Solution** to each well of the 96-Well Filter Plate. Centrifuge the assembly at maximum speed or 3,000 *x g* (~3,000 RPM) for 2 minutes.

- 4. Discard the flowthrough. Reassemble the 96-Well Filter Plate with the vacuum manifold or the bottom plate.
- 5. Apply 75 μ L of the RNase-free DNase I solution prepared in Step 1 to each well of the 96-Well Filter Plate.

For Vacuum Manifold: Apply vacuum for 30 seconds.

For Centrifugation: Centrifuge the assembly at maximum speed or $3,000 \times g$ (~3,000 RPM) for 30 seconds.

- 6. After the centrifugation or vacuum in Step 5, pipette the flowthrough that is present in the collection plate back onto the top of the column.
 - **Note:** Ensure Step 6 is performed in order to ensure maximum DNase activity and to obtain maximum yields of RNA, in particular for small RNA species
- 7. Incubate the assembly at 25 30°C for 15 minutes.
- 8. Without any further centrifugation, proceed directly to "**RNA Wash**" Section 2A, Step 4c for **Vacuum Manifold** procedure or Section 2B, Step 4c for **Centrifugation** procedure.

Troubleshooting Guide

Problem	Possible Cause	Solution and Explanation	
Poor RNA Recovery	Incomplete lysis of leukocytes	Ensure that the appropriate amount of Binding Solution was used to lyse the leukocyte pellet.	
	Lysis of red blood cells was incomplete	Ensure that the blood sample is collected with the appropriate amount of EDTA, which will prevent coagulation of the red blood cells and allow for proper lysis. Also check that the appropriate amount of RBC Lysis Buffer is added to the blood sample, and that it is mixed and incubated properly.	
	Ethanol was not added to the lysate	Ensure that 140 μL of 95-100% ethanol is added to the lysate before binding to the column.	
	Ethanol was not added to the Wash Solution	Ensure that 90 mL of 95% ethanol is added to the supplied Wash Solution prior to use.	
	An alternative elution solution was used	It is recommended that the Elution Solution supplied with this kit be used for maximum RNA recovery.	
	The column has become clogged	Do not exceed 1mL of blood or 1.5×10^{6} leukocytes per well. The amount of blood used may need to be decreased if the wells show clogging below the recommended level. See also "Clogged Wells" below.	
Leukocyte pellet is red	Incomplete red blood cell lysis	The leukocyte pellet should be white, with only residual traces of red blood cells. If red blood cell lysis is incomplete, the pellet will be red. In this case resuspend the leukocyte pellet in another 5 volumes of RBC Lysis Solution and incubate at room temperature for another 5 minutes.	

Problem	Possible Cause	Solution and Explanation
Clogged Wells	Incomplete lysis of leukocytes	Ensure that the appropriate amount of Binding Solution was used to lyse the leukocyte pellet.
	Insufficient Vacuum	Ensure that a vacuum pressure of at least -650 mbar or -25 in. Hg is developed
	Lysis of red blood cells was incomplete	Ensure that the blood sample is collected with the appropriate amount of EDTA, which will prevent coagulation of the red blood cells and allow for proper lysis. Improperly lysed red blood cells will clog the column.
	Amount of blood used exceeds kit specifications	It is recommended that no more than 1 mL of blood or 1.5×10^{6} leukocytes be used in order to prevent possible clogging of the column.
	Centrifuge temperature too low	Ensure that the centrifuge remains at room temperature throughout the procedure. Temperatures below 15°C may cause precipitates to form that can cause the columns to clog.
Cloudy Pink Solution Does Not Become Clear Red During RBC Lysis	Incomplete red blood cell lysis	The solution should become a translucent red colour after RBC Lysis Solution has been added and incubated with the blood. If not, pellet the leukocytes and remove as much of the supernatant as possible. Add another 5 volumes of RBC Lysis solution and incubate again.
RNA is Degraded	RNase contamination	RNases may be introduced during the use of the kit. Ensure proper procedures are followed when working with RNA. Please refer to " <i>Working with RNA</i> " at the beginning of this user guide.
	Procedure not performed quickly enough	In order to maintain the integrity of the RNA, it is important that the procedure be performed quickly.
	Improper storage of the purified RNA	For short term storage RNA samples may be stored at -20 °C for a few days. It is recommended that samples be stored at -70 °C for longer term storage.
	Leukocyte pellets were too old	Leukocyte pellets generated at the end of Step 1 may be stored for up to 2 weeks at -70 °C and used in this procedure. It is not recommended that samples be frozen for longer than 2 weeks, as the integrity of the RNA may be compromised.

Problem	Possible Cause	Solution and Explanation
RNA does not perform well in downstream applications	RNA was not washed 3 times with the provided Wash Solution	Traces of salt from the binding step may remain in the sample if the wells are not washed 3 times with Wash Solution. Salt may interfere with downstream applications, and thus must be washed from the wells of the plate.
	Ethanol carryover	Ensure that the dry spin under Column Wash in the centrifugation protocol or the extended vacuum in the vacuum protocol is performed in order to remove traces of ethanol prior to elution. Ethanol is known to interfere with many downstream applications.
Residual genomic DNA contamination	Large amounts of genomic DNA in starting material	Perform RNase-free DNasel digestion on the RNA sample after elution to remove genomic DNA contamination. It is recommended that Norgen's RNase-Free DNase I Kit (Product # 25710) be used for this step.

Related Products	Product #
RBC Lysis Solution	21210
RNase-Free DNase I Kit	25710
Total RNA Purification Kit	17200
Total RNA Purification 96-Well Kit	24300
Leukocyte RNA Purification Kit	21200
Cytoplasmic & Nuclear RNA Purification Kit	21000
microRNA Purification Kit	21300
100b RNA Ladder	15002
1kb RNA Ladder	15003

Technical Support

Contact our Technical Support Team between the hours of 8:30 and 5:30 (Eastern Standard Time) at (905) 227-8848 or Toll Free at 1-866-667-4362.

Technical support can also be obtained from our website (www.norgenbiotek.com) or through email at <u>techsupport@norgenbiotek.com</u>.

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