PROTOCOL

ambion® | RNA
by life technologies™

LeukoLOCK™ Total RNA Isolation System

life technologies™

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Part Number 1923M Rev. D
01/2011

LeukoLOCK™ Total RNA Isolation System

(Part Number AM1923, AM1933, AM1934)

Protocol

I. Introduction

IMPORTANT

Before using this product, read and understand the "Safety Information" in the appendix in this document.

A. Background and Product Description

Globin mRNA interferes with expression profiling of whole blood

Blood is a dynamic storehouse of cellular information that can be used to diagnose and treat human disease. Molecular clues of infection, inflammation, and autoimmune disease are carried in circulating leukocytes, which include T- and B-lymphocytes, neutrophils, eosinophils, basophils, monocytes, and other, less abundant, cell types. Expression profiling tools, such as microarrays and real-time quantitative RT-PCR (qRT-PCR), provide a powerful means for identifying blood-based RNA biomarkers associated with pathological conditions (Burczynski et al. 2005, Cobb et al. 2005, Tsuang et al. 2005). However, the sensitivity of expression profiling methodology for detecting leukocyte-derived mRNAs of interest is diminished by the large proportion of reticulocyte-derived globin mRNA present in RNA prepared from whole blood.

The LeukoLOCK™ system enriches for leukocytes before RNA isolation

The LeukoLOCK™ Total RNA Isolation System is optimized for use with human whole blood. It is an innovative method for fractionation of whole blood, as well as stabilization and extraction of total RNA from the leukocyte population of whole blood. The LeukoLOCK System incorporates leukocyte depletion filter technology to isolate leukocytes from whole blood, and Ambion RNA*later*® Solution to stabilize the RNA in the cells captured on the filter. The RNA purified from the captured leukocytes is depleted of globin mRNA, improving its utility for expression profiling and other applications.

Purchase the entire system as a whole or as two separate modules

The LeukoLOCK System can be purchased as a single kit, and it is also available as two separate modules to accommodate researchers who plan to collect blood samples at one site, and process them for RNA isolation at a separate location.

Part Number	Product Name
P/N AM1923	LeukoLOCK Total RNA Isolation System
P/N AM1933	LeukoLOCK Fractionation & Stabilization Kit
P/N AM1934	LeukoLOCK Total RNA Isolation Kit

B. Procedure Overview

Isolation and stabilization of leukocytes

A 9–10 mL sample of anticoagulated blood is passed through a LeukoLOCK Filter that captures the total leukocyte (white blood cell; WBC) population, including both lymphoid and myeloid cells (Figure 1). Plasma, platelets, and red blood cells (RBCs), including reticulocytes, are eliminated.

The filter is flushed with phosphate-buffered saline (PBS) to remove residual RBCs and then with RNA*later* to stabilize leukocyte RNA. The RNA can be isolated immediately, or the stabilized cells can be maintained in the LeukoLOCK Filter at ambient temperature for several days, permitting sample transport to remote labs for RNA extraction (Figure 2 on page 4). The stabilized cells may also be stored on the filter at –20°C or –80°C for longer periods of time.

Purification of RNA from captured leukocytes

Captured cells are disrupted on the LeukoLOCK Filter in a guanidine thiocyanate-based solution that rapidly releases RNA while simultaneously inactivating nucleases. The cell lysate can be stored at –80°C after collection from the filter, or processing can continue immediately with a brief Proteinase K treatment to degrade cellular proteins. The RNA is then purified, using bead capture technology during washing, optional TURBO™ DNase treatment, and final cleanup. The entire procedure can be performed in under 1 hour when beads are recovered via centrifugation (alternatively, beads can be recovered at each step via magnetic capture). RNA recovery varies from donor to donor but is typically 10–20 µg per 9–10 mL blood sample from healthy donors.

Alternative procedure for isolation of total RNA, including microRNA, from cells captured on LeukoLOCK Filters

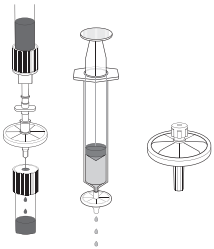
The LeukoLOCK Fractionation & Stabilization Kit can also be used for capture of leukocytes and stabilization of leukocyte RNA for isolation of total RNA using our alternative procedure. This alternative method uses glass-fiber filters rather than magnetic beads for RNA purification from captured leukocytes. If desired, total RNA that includes the small RNA fraction, including microRNA and other small RNAs can be recovered using this method. For more information, go to www.invitrogen.com/site/us/en/home/support/technical-support.html.

Purified RNA is depleted of globin mRNA

RNA recovered using the LeukoLOCK System contains less than 10% of the amount of reticulocyte-derived alpha and beta globin mRNAs present in typical mRNA samples from unfractionated whole blood (Figure 2). This level of globin mRNA removal is sufficient to rescue the detection of thousands of genes that would otherwise be called Absent on microarrays. Thus, the LeukoLOCK System improves the utility of RNA from blood for expression profiling and other applications.

Figure 1. LeukoLOCK™ Total RNA Isolation System Overview

Sample Collection and Capture of Leukocytes



1. Collect 9–10 mL whole blood samples in EDTA-containing tubes

2. Assemble the sample tube/LeukoLOCK Filter apparatus

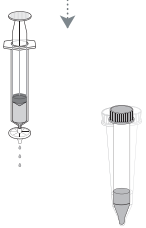
3. Pass blood through the LeukoLOCK Filter using an evacuated tube as vacuum source

4. Flush filter with 3 mL of PBS and 3 mL of RNAlater®

5. Seal the LeukoLOCK Filter ports

Potential stopping point

LeukoLOCK Filter Processing and Cell Lysis



1. Prepare pH-adjusted Lysis/Binding Solution

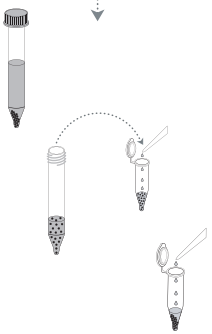
2. Remove residual RNAlater from LeukoLOCK Filter

3. Flush with 2.5 mL pH-adjusted Lysis/Binding Solution; collect lysate in 15 mL tube

Potential stopping point

4. Add 2.5 mL Nuclease-free Water and 25 µL Proteinase K, and shake for 5 min

RNA Isolation



1. Add 50 µL RNA Binding Beads and 2.5 mL 100% isopropanol, and incubate at room temp for 5 min

2. Recover the RNA Binding Beads and discard the supernatant

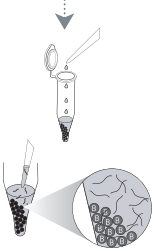
3. Wash with 1.2 mL Wash Solution 1 and transfer the RNA Binding Beads to a 1.5 mL Processing Tube

4. Recover the RNA Binding Beads and discard the supernatant

5. Wash RNA Binding Beads with 750 µL Wash Solution 2/3

(Optional) TURBO DNase Treatment

Final Wash and Elution



1. Wash the RNA Binding Beads with 750 µL Wash Solution 2/3 and air dry briefly

2. Elute the RNA with ≤150 µL Elution Solution

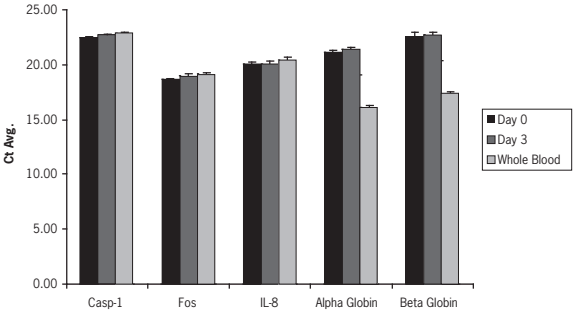


Figure 2. qRT-PCR of Total RNA Isolated with the LeukoLOCK™ Total RNA Isolation System

Total RNA was isolated from human blood samples using either the Ambion RiboPure™-Blood Kit (P/N AM1928; Whole Blood) technology, or using the LeukoLOCK Total RNA Isolation System and processing fractionated WBCs either immediately (Day 0) or after 3 days of storage on the LeukoLOCK Filter in RNA*later*® Solution at room temperature (Day 3). The RNA was analyzed in real-time one-step qRT-PCR using MessageSensor™ RT (P/N AM1745) conditions. Assays were performed with 3 different TaqMan® Gene Expression Assays (Casp-1, Fos, and IL-8; 10 ng input RNA) and TaqMan probes to alpha and beta globin (5 pg input RNA). C_T values are the average of triplicate reactions. Note the dramatic increase in C_T values for globin targets (indicating depletion of globin mRNA) in RNA prepared using the LeukoLOCK System compared to that from whole blood, and the stability of C_T values (indicating stable mRNA levels) for the remaining targets in Day 0 vs. Day 3 RNA.

Downstream applications

Quantitative and endpoint RT-PCR

RNA isolated from captured WBCs using the LeukoLOCK System is suitable for both qRT-PCR (Figure 2) and endpoint RT-PCR. The optional TURBO DNase treatment is recommended when using primers that do not cross intron-exon boundaries.

RNA amplification for array analysis

RNA isolated from biological replicates using the LeukoLOCK Total RNA Isolation System gives highly reproducible results after amplification and array analysis (Figure 3).

Other applications

High quality total RNA isolated from the LeukoLOCK Total RNA Isolation System is suitable for most other downstream applications, including Northern blot analysis and ribonuclease protection assay (RPA).

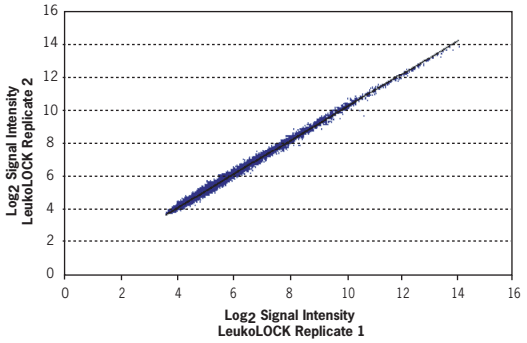


Figure 3. LeukoLOCK™ Total RNA Isolation System Performance on an Affymetrix® GeneChip® Array

RNA was prepared from biological replicates (duplicate isolations from the same donor) using the LeukoLOCK Total RNA Isolation System (including optional TURBO™ DNase treatment). Total RNA (0.5 µg) was amplified using the Ambion MessageAmp™ II-96 aRNA Amplification Kit (P/N AM1819), fragmented, and then hybridized to Human Focus arrays. Normalized signal intensity for Present calls in each replicate are plotted against the other to create a signal concordance graph. Pearson's Correlation Coefficient (r) = 0.997.

C. Components and Storage Conditions

The LeukoLOCK Total RNA Isolation System contains reagents to isolate RNA from 20 samples of 9–10 mL whole blood. It is optimized for use with human whole blood. To provide flexibility, we offer the LeukoLOCK System as a single kit or as two separate modules; this is outlined in the following tables.

Part Number	Product Name
P/N AM1923	LeukoLOCK Total RNA Isolation System
P/N AM1933	LeukoLOCK Fractionation & Stabilization Kit
P/N AM1934	LeukoLOCK Total RNA Isolation Kit

Part Number				
AM1923	AM1933	AM1934	Component	Storage
80 mL	80 mL		1X PBS pH 7.4	any temp*
80 mL	80 mL		RNA*later*® Solution	room temp
2	2		Rubber Septum Cap	room temp
20	20		White Slip Connector	room temp
22	22		Transfer Spike	room temp
20	20		LeukoLOCK™ Filter	room temp
5 mL		5 mL	Elution Solution†	room temp

Part Number			Component	Storage
AM1923	AM1933	AM1934		
60 mL		60 mL	LeukoLOCK™ Lysis/Binding Solution	room temp
1.5 mL		1.5 mL	LeukoLOCK™ pH Adjustment Solution	room temp
40		40	Processing Tubes	room temp
30 mL		30 mL	Wash Solution 1 Concentrate Add 10 mL 100% isopropanol before use	room temp
50 mL		50 mL	Wash Solution 2/3 Concentrate Add 40 mL 100% ethanol before use	room temp
50 mL		50 mL	Nuclease-free Water	any temp*
1.1 mL		1.1 mL	RNA Binding Beads	4°C‡
6.6 mL		6.6 mL	1X LeukoLOCK™ DNase Buffer	–20°C
550 µL		550 µL	Proteinase K	–20°C
110 µL		110 µL	TURBO™ DNase (20 U/µL)	–20°C

* Store PBS and Nuclease-free Water at –20°C, 4°C, or room temp.
† 1 mM KCl, 0.2 mM Na-citrate, pH 7
‡ Do not freeze RNA Binding Beads.

D. Materials Not Provided With the Kit

Reagents

- 100% isopropanol, ACS reagent grade or equivalent proof
- 100% ethanol, ACS reagent grade or equivalent proof

Supplies

- Blood collection tubes with anticoagulant; we recommend Greiner Bio-One 9 mL EDTA K3 Vacuette® #455036 or Terumo® Venosafe® EDTA K3 #VF-109SDK (www.terumo-europe.com) because these tubes have thinner septums that are more easily pierced than stopper-type closures.
- 10 mL evacuated blood collection tubes, no anti-coagulant, to provide a vacuum source and act as receiver tube for LeukoLOCK Filter flow-through; 10 mL Vacutainer® BD #366430 or Terumo Venoject® #VT-100SP recommended
- 25 G x 5/8 in. needles (e.g., Becton Dickinson #305122), to connect the receiver tube to the LeukoLOCK Filter
- 3 mL or 5 mL syringes, for flushing LeukoLOCK Filters with liquid reagents
- 1 mL or 3 mL syringes, for flushing residual RNA*later* from LeukoLOCK Filters
- Large-gauge needle (e.g., 16 G, Becton Dickinson #305197), for loading syringes with Lysis/Binding Solution
- 15 mL disposable conical plastic tubes with caps (e.g., Ambion P/N AM12500) for collecting cell lysate
- Pipettors and RNase-free tips

Equipment

- (Optional) Rotating shaker, capable of at least 250 rpm, with attachments for 15 mL conical tubes
- Vortex mixer
- Centrifuge capable of spinning 15 mL conical tubes at 2500 x g
- Microcentrifuge capable of ~16,000 x g
- (Optional) Vortex adapter for simultaneous hands-free mixing of several 1.5 mL Processing Tubes (e.g., P/N AM10024, AM10014)

E. Related Products

RNase*Zap*® P/N AM9780, AM9782, AM9784	RNase Decontamination Solution. RNase*Zap*® is simply sprayed, poured, or wiped onto surfaces to instantly inactivate RNases. Rinsing twice with distilled water will eliminate all traces of RNase and RNase*Zap*.
MessageAmp™ aRNA Amplification Kits see our web or print catalog	We offer a full line of MessageAmp Kits tailored for different array analysis applications. The MessageAmp II Kit offers maximum flexibility; samples can be amplified using either single- or double-round amplification, and the reagent cocktails are configured to accommodate modification. For arrays requiring biotin-labeled samples, We offer the MessageAmp Premier and MessageAmp III RNA Amplification Kit. For preparation of fluorescently-labeled samples, we recommend the Amino Allyl MessageAmp II Kits which are available with and without Cy™3 and Cy5. Bacterial RNA can be amplified using the MessageAmp II Bacteria RNA Amplification Kit. We also offer the MessageAmp II-96 and Amino Allyl MessageAmp II-96 aRNA Amplification Kits for high throughput applications.
MessageSensor™ RT Kit P/N AM1745	The MessageSensor RT Kit for one-step qRT-PCR includes an optimized set of reagents for exceptionally sensitive reverse transcription. The kit is designed to be used for single-tube amplification of mRNA using either real-time or end-point amplification strategies.
RNA 6000 Ladder P/N AM7152	The RNA 6000 Ladder is a set of 6 RNA transcripts designed for use as reference standards with the RNA 6000 Lab Chip Kits and the Agilent 2100 bioanalyzer.
DNA-*free*™ P/N AM1906	DNase treatment and removal reagents. This product contains our ultra-high quality RNase-free DNase I and reaction buffer for degrading DNA. It is ideal for removing contaminating DNA from RNA preparations. A novel reagent for removing the DNase without the hassles or hazards of phenol extraction or alcohol precipitation is also included.
TURBO™ DNA-*free*™ P/N AM1907	The TURBO DNA-*free* Kit employs our exclusive TURBO DNase; a specifically engineered hyperactive DNase that exhibits up to 350% greater catalytic efficiency than wild type DNase I. The kit also includes a novel reagent for removing the DNase without the hassles or hazards of phenol extraction or alcohol precipitation—and without heat inactivation, which can cause RNA degradation. TURBO DNA-*free* is ideal for removing contaminating DNA from RNA preparations.

II. LeukoLOCK Total RNA Isolation System Procedure

A. Equipment and Reagent Preparation

RNase precautions

Lab bench and pipettors

Before working with RNA, it is always a good idea to clean the lab bench and pipettors with an RNase decontamination solution (e.g., Ambion RNase*Zap*® Solution).

Gloves and RNase-free technique

Wear laboratory gloves for this procedure; they protect you from the reagents, and they protect the RNA from nucleases that are present on skin.

Use RNase-free pipette tips to handle the kit reagents, and avoid putting used tips into the reagent containers.

Prepare *Wash Solution 1* and *Wash Solution 2/3*

a. Add 10 mL 100% isopropanol to the bottle labeled Wash Solution 1 Concentrate.

b. Add 40 mL 100% ethanol to the bottle labeled Wash Solution 2/3 Concentrate.

c. Mix both solutions well, and mark the labels to indicate that the solutions are completed. Store at room temperature.
The completed solutions are referred to in the procedure as ***Wash Solution 1*** and ***Wash Solution 2/3***.

Replace the bottle caps for PBS and RNA*later* with Rubber Septum Caps and Transfer Spikes

a. Replace the cap on the bottle of 1X PBS with a Rubber Septum Cap, and insert a Transfer Spike through it.

b. Retain the screw cap on top of the Transfer Spike to prevent evaporation from the bottle.

c. Do the same for the bottle of RNA*later*.

Procedure notes

- Pass all solutions through the LeukoLOCK Filter in the same direction, i.e., from the inlet (flared) port to the outlet (tapered) port.
- The LeukoLOCK Total RNA Isolation System is designed for blood volumes of 9–10 mL; however, samples of ~3–30 mL have been used with a single LeukoLOCK Filter and the standard procedure.

B. Sample Collection, and Capture and Stabilization of Leukocytes

1. Collect 9–10 mL whole blood samples in EDTA-containing tubes

Collect 9–10 mL of whole blood samples according to standard procedures in tubes containing EDTA as an anticoagulant. Do not add any reagents to the blood prior to filtration.

NOTE

Potassium or sodium EDTA are the recommended anticoagulants. Blood collection tubes containing other anticoagulants, such as citrate and heparin, have not been tested.

NOTE

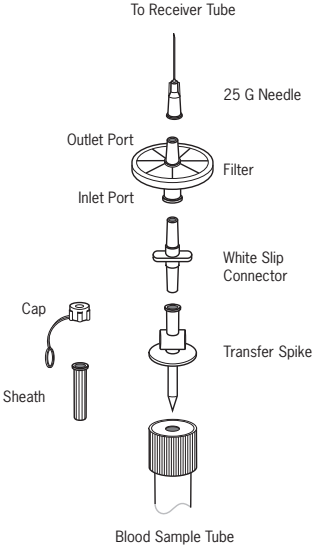
The LeukoLOCK Total RNA Isolation System is optimized for use with human whole blood.

An evacuated blood collection tube provides the vacuum source for drawing the blood sample through the LeukoLOCK Filter in step 3 on page 10. To achieve complete filtration of the sample volume, we suggest collecting a slightly lower volume of blood than the evacuated receiver tube capacity. For instance, if you will be using a 10 mL evacuated tube in step 3, collect a 9 mL blood sample.

Process blood samples through step 5 on page 10 as soon as possible after collection. Levels of some mRNAs in blood have been shown to change dramatically during ex vivo storage (Rainen et al. 2002).

2. Assemble the sample tube/LeukoLOCK Filter apparatus

Assemble the sample tube/LeukoLOCK Filter apparatus as shown in the illustration to the left.



a. Pierce the rubber septum of the blood sample tube with a Transfer Spike. Use a twisting motion and push the Transfer Spike through the cap or stopper of the sample tube.

NOTE

Save the Transfer Spike sheath and screw cap; they will be used to seal the LeukoLOCK Filter for storage or shipping (step 5 on page 10).

b. Insert a White Slip Connector into the top of the Transfer Spike.

c. Connect the inlet port (flared port) of the LeukoLOCK Filter to the White Slip Connector.

d. Connect a 25 gauge needle to the outlet port (tapered port) of the LeukoLOCK Filter. Remove the sheath from the needle.

3. Pass blood through the LeukoLOCK Filter using an evacuated tube as vacuum source

The LeukoLOCK Filter captures the total leukocyte population, while plasma, platelets, and red blood cells (RBCs) are eliminated.

a. Invert the sample tube/LeukoLOCK Filter assembly and stab the needle into an empty 10 mL evacuated blood collection tube (receiver tube).
The evacuated tube is used as a vacuum source to draw the blood through the LeukoLOCK filter.

b. Allow all of the blood to filter through the LeukoLOCK Filter until the wedge-shaped sectors of the filter have cleared of blood.
The filtration process typically takes ~2 min. The assembly can be placed in a rack during filtration.

c. Remove the LeukoLOCK Filter from the assembly.
The needle may be left in the tube containing the filtrate.
The leukocyte-depleted blood filtrate is typically not hemolyzed and can be saved for other tests.

4. Flush filter with 3 mL of PBS and 3 mL of RNA*later*®

The filter is flushed with phosphate-buffered saline (PBS) to remove residual RBCs and then with RNA*later* to stabilize leukocyte RNA.

a. Attach a 3 mL or 5 mL syringe to the Transfer Spike on the bottle of 1X PBS, invert the bottle, and withdraw 3 mL.


NOTE

Pass all solutions through the LeukoLOCK Filter in the same direction, i.e., from the inlet (flared) port to the outlet (tapered) port.

b. Attach the PBS-loaded syringe to the LeukoLOCK Filter and flush the filter with the PBS at a rate of ~3–5 drops/second. Collect the PBS in a biological waste container.

c. Using a new 3 mL or 5 mL syringe, withdraw 3 mL of RNA*later* using the method described in step a.

d. Attach the syringe to the LeukoLOCK Filter and flush the filter with RNA*later* at a rate of ~3–5 drops/second.


IMPORTANT

Leave the filter saturated in RNAlater for storage.

e. Detach the syringe from the filter, leaving the filter saturated with RNA*later*: do not expel all of the RNA*later* from the LeukoLOCK Filter, and do not retract the plunger of the syringe before detaching the syringe.

5. Seal the LeukoLOCK Filter ports

Seal the LeukoLOCK Filter ports with the sheath and screw cap from the Transfer Spike.


STOPPING POINT

Store the LeukoLOCK Filter with stabilized leukocytes at room temperature for up to 3 days, or at –20°C or –80°C for long term storage (several months).

C. LeukoLOCK Filter Processing and Cell Lysis

1. Prepare pH-adjusted Lysis/Binding Solution

Add pH Adjustment Solution to LeukoLOCK Lysis/Binding Solution according to the table below, and mix briefly by vortexing. Prepare only the amount needed for immediate use.

Do not add pH Adjustment Solution to the entire bottle of LeukoLOCK Lysis/Binding Solution; you will need some of the unadjusted Lysis/Binding Solution in step E.4 on page 14.

1 Rxn	20 Rxn	Component
2.5 mL	50 mL	Lysis/Binding Solution Concentrate
70 µL	1.4 mL	pH Adjustment Buffer

2. Remove residual RNA*later* from LeukoLOCK Filter

a. If the LeukoLOCK filter with captured cells was stored frozen, allow it to thaw at room temperature for ~5 min before proceeding.

b. Remove the closures from the LeukoLOCK Filter.

c. Attach a 1 mL or 3 mL syringe, with the plunger retracted, to the inlet port of the LeukoLOCK Filter, and then depress the plunger to expel residual RNA*later* from the filter and ports.
Typically, ~8–10 drops of RNA*later* are expelled from the LeukoLOCK Filter.

3. Flush with 2.5 mL pH-adjusted Lysis/Binding Solution; collect lysate in 15 mL tube

In this step, the leukocytes that are trapped on the LeukoLOCK Filter are lysed, and the lysate is flushed off the filter and collected in a 15 mL conical tube.

a. Load a 3 mL syringe with 2.5 mL of ***pH-adjusted*** Lysis/Binding Solution.
Use a large-gauge needle to withdraw the solution; remove the needle before proceeding to step b.

b. Attach the syringe with Lysis/Binding Solution to the inlet (flared) port of the filter, and depress the plunger of the syringe to flush the Lysis/Binding Solution through the filter, collecting the lysate in a 15 mL conical tube. The process should take 5–10 seconds.

c. Disconnect the syringe, retract the plunger, and then reconnect it to the LeukoLOCK Filter. Depress the plunger to expel residual drops of cell lysate into the same tube.

d. (Optional) If desired, repeat step c several times, collecting the foamy material that is expelled.

In our experience, ~90% of the RNA is recovered from the lysate collected as described in steps b and c.

STOPPING POINT

The lysate may be stored at –80°C at this point. To continue, thaw the lysate at room temperature and proceed immediately to step 4.

4. Add 2.5 mL Nuclease-free Water and 25 µL Proteinase K, and shake for 5 min

This brief Proteinase K treatment degrades cellular proteins.

a. Add 2.5 mL of Nuclease-free Water to the collected lysate and mix thoroughly by vortexing or inversion.

b. Add 25 µL of Proteinase K.

c. Shake the tube at moderate speed (~250 rpm) on a shaker for 5 min at room temperature.
Alternatively, the sample can be mixed manually by intermittent inversion of the tube.

D. RNA Isolation

In these steps, the RNA is captured from the Proteinase K-treated lysate on RNA Binding Beads and purified.

1. Add 50 µL RNA Binding Beads and 2.5 mL 100% isopropanol, and incubate at room temp for 5 min

a. Resuspend the RNA Binding Beads by vortexing, then remove 50 µL of RNA Binding Beads and add to the Proteinase K-treated lysate. Vortex briefly to mix.

b. Add 2.5 mL of 100% isopropanol to the lysate and vortex briefly to mix.

c. Incubate with mixing for 5 min at room temperature to allow the RNA to bind to the beads.
Incubate on a rocking platform at medium speed, or with intermittent manual mixing.

2. Recover the RNA Binding Beads and discard the supernatant

a. Pellet the beads by centrifugation at 2000 x g (~3,200 rpm in an IEC tabletop centrifuge) for 3 min.
Alternatively, place the tube on a magnetic stand for 15 mL conical tubes (e.g., Dynal MPC-L #120.21) for 5–10 min, until the mixture becomes transparent, indicating that capture is complete. Aspirate and discard the supernatant before removing the tube from the magnetic stand.

IMPORTANT

Avoid losing RNA Binding Beads when removing the supernatant—the RNA is bound to the beads.

b. Carefully decant or aspirate the supernatant without disturbing the RNA Binding Beads. Discard the supernatant.
See *Loss of RNA Binding Beads* on page 18 for helpful techniques.

3. Wash with 1.2 mL Wash Solution 1 and transfer the RNA Binding Beads to a 1.5 mL Processing Tube

a. Add 600 µL of Wash Solution 1 to the RNA Binding Beads, vortexing or pipetting up and down to disperse the beads sufficiently to transfer the mixture to the Processing Tube in step b.
The beads may not fully disperse during this step; this will not affect RNA purity or yield.

b. Transfer the RNA Binding Beads/Wash Solution 1 mixture to a 1.5 mL Processing Tube.

c. Rinse the 15 mL tube with a second 600 µL aliquot of Wash Solution 1, and transfer to the same Processing Tube.

4. Recover the RNA Binding Beads and discard the supernatant

a. Spin the sample for 15–30 sec in a microcentrifuge set at ~16,000 x g to pellet the beads.
The centrifuge may not attain 16,000 x g in 15 sec. The goal is to create a pellet that will not be dislodged when the supernatant is removed.
In this and following steps, the RNA Binding Beads can be recovered by magnetic capture instead of by centrifugation. Move the Processing Tube to a magnetic stand for 1.5 mL tubes (e.g., P/N AM10055). Leave the tube on the magnetic stand until the mixture becomes transparent, indicating that capture is complete. The capture time depends on the magnetic stand used; using the Ambion Magnetic Stand for 1.5 mL tubes, the capture time is ~3–5 min. Aspirate the supernatant before removing the tube from the stand.

b. Carefully decant or aspirate and discard the supernatant without disturbing the beads.

5. Wash RNA Binding Beads with 750 µL Wash Solution 2/3

a. Add 750 µL Wash Solution 2/3 and vortex vigorously for 15–30 sec to dislodge the pellet.

b. Recover the RNA Binding Beads by centrifugation for 15–30 sec at ~16,000 x g.

c. Carefully aspirate and discard the supernatant without disturbing the RNA Binding Beads.

d. Proceed to section *E. (Optional) TURBO DNase Treatment* starting on page 14 or section *F. Final Purification and Elution* starting on page 15 for final wash and elution steps.

E. (Optional) TURBO DNase Treatment

TURBO DNase treatment is recommended when the RNA will be used for RT-PCR with primers that do not cross intron-exon boundaries.

1. Briefly air dry the RNA Binding Beads

Leave the tube open at room temperature for 2 min to allow any remaining alcohol from Wash Solution 2/3 to evaporate.

IMPORTANT

It is important to remove residual Wash Solution 2/3 from the samples, because the ethanol may affect TURBO DNase digestion efficiency.

2. Prepare TURBO DNase master mix

Using the following table, prepare enough TURBO DNase master mix to treat all of the RNA samples in your experiment, plus 5–10% overage. The master mix can be kept at room temperature up to 15 minutes.

Table 1. TURBO™ DNase master mix

Amount (per sample)	Component
296 µL	1X LeukoLOCK DNase Buffer
4 µL	TURBO™ DNase (20 U/µL)

3. Add 300 µL TURBO DNase master mix and incubate for 10 min with agitation

When the TURBO DNase master mix is added to the sample, nucleic acids are released from the RNA Binding Beads, and genomic DNA is removed.

a. Add 300 µL of the prepared TURBO DNase master mix to each sample.

b. Vortex briefly or pipet up and down to disperse the bead pellet, then agitate gently (1000 rpm) for 10 min at room temperature.
Use a hands-free adapter for a vortex mixer, or gently vortex the sample several times during the digestion.

4. Add 300 µL Lysis/Binding Solution and 300 µL 100% isopropanol, and incubate for 3 min

The RNA is rebound to the RNA Binding Beads in this step.

a. Add 300 µL of Lysis/Binding Solution ***that has not been pH-adjusted*** to each sample.

b. Add 300 µL of 100% isopropanol, mix, and briefly centrifuge (~2 sec) at low speed (<1000 x g) to remove any liquid from the lid of the tube.

c. Incubate at room temperature for 3 min.

5. Recover the RNA Binding Beads and discard the supernatant

a. Recover the RNA Binding Beads by centrifugation for 15–30 sec at ~16,000 x g.

b. Carefully aspirate and discard the supernatant without disturbing the beads.

6. Wash the RNA Binding Beads with 750 µL Wash Solution 2/3

a. Add 750 µL Wash Solution 2/3 and vortex vigorously for 15–30 sec.

b. Recover the RNA Binding Beads by centrifugation for 15–30 sec at ~16,000 x g.

c. Carefully aspirate and discard the supernatant without disturbing the beads. Proceed to *F. Final Purification and Elution*, below.

F. Final Purification and Elution

In these steps, the RNA bound to the RNA Binding Beads is washed a final time and eluted.

1. Wash the RNA Binding Beads with 750 µL Wash Solution 2/3 and air dry briefly

a. Add 750 µL Wash Solution 2/3 and vortex vigorously for 15–30 sec.

b. Recover the RNA Binding Beads by centrifugation for ***1 min*** at ~16,000 x g.

c. Carefully aspirate and discard the supernatant without disturbing the beads.

d. Centrifuge the tube briefly and remove any remaining liquid from the tube, then leave the tube open at room temperature for ~3 min to allow any residual liquid to evaporate.
To achieve maximum recovery of RNA, do not overdry the RNA Binding Beads; they should still have a glossy sheen. If they lose the sheen, or cracks appear in the bead pellet, they may be overdry.

2. Elute the RNA with ≤150 µL Elution Solution

a. Add ≤150 µL Elution Solution and vortex vigorously for 30 seconds. RNA can be eluted in 50–150 µL Elution Solution. Using a larger volume will increase the RNA recovery, but will result in a lower final concentration.

b. Thoroughly pellet the RNA Binding Beads by centrifugation for ***2 min*** at ~16,000 x g.


NOTE

The purified RNA will be in the supernatant.

c. Transfer the RNA-containing supernatant to a new Processing Tube or other nuclease-free container appropriate for your application.

d. Store the purified RNA at –20°C.

III. Assessing RNA Yield and Integrity

A. RNA Yield

Expected RNA yield

RNA yield is typically 10–20 µg from 9 mL human blood.

Spectrophotometry

The concentration of an RNA solution can be determined by measuring its absorbance at 260 nm. The NanoDrop 1000A Spectrophotometer is extremely quick and easy to use; measure 1–2 µL of the RNA sample directly, following the manufacturer's instructions.

Alternatively, the RNA concentration can be determined by diluting an aliquot of the preparation in TE (10 mM Tris-HCl, pH 8, 1 mM EDTA) and reading the absorbance in a traditional spectrophotometer at 260 nm. To determine the RNA concentration in µg/mL, multiply the A_{260} by the dilution factor and the extinction coefficient (1 A_{260} = 40 µg RNA/mL).

$$A_{260} \text{ X dilution factor X } 40 = \text{µg RNA/mL}$$

Note that any contaminating DNA in the RNA prep will lead to an overestimation of yield, since all nucleic acids absorb at 260 nm.

Fluorometry

If a fluorometer or a fluorescence microplate reader is available, Molecular Probes' RiboGreen® fluorescence-based assay for RNA quantitation is a convenient and sensitive way to measure RNA concentration. Follow the manufacturer's instructions for using RiboGreen.

The RiboGreen signal intensity from double stranded DNA is roughly 2-fold more than that for the same mass of RNA; if significant amounts of DNA contaminate an RNA sample, it will affect the RiboGreen quantitation.

B. RNA Quality

Microfluidics analysis

The Agilent® 2100 bioanalyzer with Caliper's RNA LabChip® Kits provides better qualitative data than conventional gel analysis for characterizing RNA. When used with Ambion RNA 6000 Ladder (P/N AM7152), this system can quickly provide an accurate size distribution profile of RNA samples (see Figure 4 on page 17). Follow the manufacturer's instructions for performing the assay.

The 28S to 18S rRNA ratio is often used as an indicator of RNA integrity. Total RNA isolated from freshly collected whole blood using this kit usually has a 28S to 18S rRNA ratio of >1.0.

Using a bioanalyzer, the RIN (RNA Integrity Number) can be calculated to further evaluate RNA integrity. A new metric developed by Agilent, the RIN algorithm analyzes information from both rRNA bands,

as well as information contained outside the rRNA peaks (i.e., potential degradation products) to provide a more complete picture of RNA degradation states. Search for "RIN" at Agilent's website for information:

www.chem.agilent.com

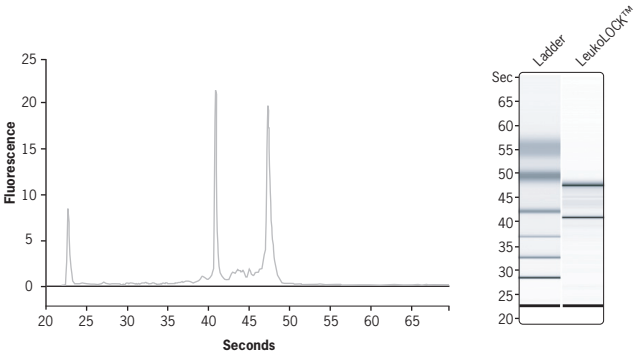


Figure 4. Bioanalyzer Profile of RNA Prepared With the LeukoLOCK™ Total RNA Isolation System

Using the LeukoLOCK Total RNA Isolation System, including optional TURBO™ DNase treatment, RNA was purified from a freshly drawn 9 mL blood sample from a healthy male donor. RNA quality was determined on an Agilent® 2100 bioanalyzer using an RNA LabChip® Kit and 2100 expert software. The 28S to 18S rRNA ratio for this sample was 1.5, and the RIN was 9.0 (the RIN algorithm generates a number from 1 to 10; higher numbers indicate more intact RNA).

Spectrophotometry

An effective measure of RNA purity is the ratio of absorbance readings at 260 and 280 nm. The total RNA isolated with this kit should have an A_{260}/A_{280} ratio of 1.8–2.1. Lower ratios can indicate protein or other contamination in the sample. However, RNA with absorbance ratios outside of this range may still function well for downstream applications.

IV. Troubleshooting

A. Poor RNA Yield

Variation among donors

The yield of RNA may vary due to donor to donor variation. The total RNA yield is typically 10–20 µg per 9 mL of human blood from healthy donors.

LeukoLOCK Filter was not stored in RNA*later* prior to RNA purification step

Leave the LeukoLOCK Filter saturated with RNA*later* during storage (step II.B.4 on page 10).

- Ensure that the residual RNA*later* in the filter is not flushed out by an air bubble in the syringe.
- Do not retract the plunger of the syringe before detaching the syringe from the filter.
- Avoid trapping air bubbles on the LeukoLOCK Filter before storage in RNA*later*.

RNase contamination

- Blood contains a high level of RNase activity, which can lead to RNA degradation.
  - The procedure has been optimized to inactivate endogenous RNases immediately upon cell lysis and to minimize carryover of any remaining RNase activity; follow the protocol's recommended incubation times and stop only at the suggested steps to maximize the integrity of your recovered RNA.
  - Avoid blood contamination of your work area, which can lead to external RNase contamination at later stages of the isolation.
- To avoid external contamination with RNase, follow the best practices described in the section *II.A. RNase precautions* on page 8. Use RNase-free tubes, tips, and reagents.
- For more information, go to www.invitrogen.com/site/us/en/home/support/technical-support.html.

Loss of RNA Binding Beads

Since the basis of the RNA purification procedure is to immobilize RNA on the RNA Binding Beads, any loss of beads during the procedure will result in loss of RNA.

The following techniques may be helpful to avoid aspirating the RNA Binding Beads when removing the supernatant.

- Use the side of the tube opposite the captured beads as a guide when removing the supernatant.
- Aspirate or decant the supernatant slowly.
- Leave 100–200 µL of supernatant on top of the pellet in the first bead recovery step, II.D.2 on page 12.

- Ensure that beads are not left in the pipet tip used to transfer the sample to the Processing Tube in step II.D.3 on page 13.

Poor elution of RNA from the RNA Binding Beads

- Resuspend the RNA Binding Beads fully in Elution Solution. See *RNA Binding Beads were not fully resuspended/dispersed,* below.
- Performing two successive elutions of 75 µL each, instead of a single elution in 150 µL, may improve RNA yield by 15–20%.
- Increasing the volume in which the RNA is eluted in step II.F.2 will enhance recovery, but the RNA will be more dilute in downstream applications. Sufficient Elution Solution is supplied for 250 µL elution volume per sample.
- Avoid overdrying the RNA Binding Beads before eluting the RNA because this may make the beads more difficult to resuspend for elution. Beads that are overdry lose their glossy sheen, and cracks may appear in the bead pellets. If the beads are inadvertently overdried, increase the resuspension time during the elution step II.F.2 on page 15 by vortexing for 30 sec 1–2 more times, or by pipetting up and down, to allow the beads to rehydrate. There should be no clumps of beads in the RNA Binding Beads/Elution Solution mixture.

RNA Binding Beads were not fully resuspended/dispersed

- In general, the stock tube of RNA Binding Beads will disperse more easily when the temperature of the mixture is warmer than 20°C.
- Fully resuspend the stock of RNA Binding Beads before adding them to the Proteinase K-treated lysate in step II.D.1 on page 12.
- Avoid overdrying the RNA Binding Beads before eluting the RNA because this may make the beads more difficult to resuspend. See *Poor elution of RNA from the RNA Binding Beads* above.

B. Poor RNA Quality

Too much time elapsed between sample collection and LeukoLOCK processing

It is best to use blood that has been drawn into an EDTA-containing collection tube and processed as soon as possible. Studies have shown that blood expression profiles can change during storage in EDTA-containing collection tubes at room temperature for 4 hr (Rainen et al., 2002) and, for some mRNAs, in as little as 20 min (Life Technologies unpublished data). Storage in EDTA for longer than 1 day may result in degradation of RNA in the sample (Rainen et al., 2002).

Suboptimal Proteinase K digestion

The pH of the Lysis/Binding Solution must be adjusted (see step II.C.1 on page 11) for optimal Proteinase K digestion in step II.C.4 on page 12. The unadjusted pH of Lysis/Binding Solution is optimal for binding RNA to the RNA Binding Beads in step II.E.4 on page 14.

RNase contamination

Follow the best practices described in the section *II.A. RNase precautions* on page 8. Use RNase-free tubes, tips, and reagents.

See also *RNase contamination* on page 18 for additional tips.

DNA contamination

The RNA can be subjected to a rigorous post-elution DNase digestion:

a. Use the Ambion DNA-*free*™ Kit (P/N AM1906), which contains a novel DNase Removal Reagent.

b. If the sample was not treated with TURBO DNase in section II.E starting on page 14, an alternative is to treat with the TURBO DNase provided with this kit, followed by phenol extraction to inactivate the DNase and ethanol precipitation to purify the RNA from the reaction.

   i. Add an equal volume of 1X LeukoLOCK™ DNase Buffer and 1 µL of TURBO DNase (20 U/µL).

   ii. Incubate at room temperature for 10 min with agitation.

   iii. Add a volume equal to the final DNase reaction volume of phenol/chloroform/isoamyl alcohol (50:49:1; P/N AM9730) and vortex to mix.
   If the original elution volume was 150 µL, use 150 µL of 1X DNase Buffer and 300 µL of phenol/chloroform/isoamyl alcohol.

   iv. Centrifuge at maximum speed in a microcentrifuge for ~5 min to separate the organic and aqueous phases. Remove the top, aqueous phase to a new RNase-free tube.

   v. Add 0.1 volume (relative to the volume of aqueous phase) of 3 M sodium acetate (P/N AM9740), or 5 M ammonium acetate (P/N AM9071), and 2.5 volume of 100% ethanol, vortex to mix, and chill for ≥1 hr at –20°C.

   vi. Centrifuge at 4°C for 5–15 min and remove the supernatant.

   vii. Wash the pellet with ~1 mL 70% ethanol, and let it air dry for 10–15 min.

   viii. Resuspend in Elution Solution or other buffer.

C. Bead Carryover in the Recovered RNA

If RNA Binding Beads are carried over into the eluate containing the RNA, they will cause the solution to be light brown in color. A small quantity of beads in the sample does not inhibit reverse transcriptase reactions or RT-PCR. However, significant bead carryover can affect the absorbance reading at 260 nm.

Pellet beads that are carried over

Centrifuge the tube for 2 min at ~16,000 x g, and carefully transfer the supernatant to a new RNase-free tube.

Techniques to avoid bead carryover

- Aspirate slowly and carefully when transferring the RNA.
- Avoid touching the bead pellet with the pipette tip when removing the eluted RNA in step II.F.2 on page 15.
- If necessary, leave a few microliters of Elution Solution on the beads when removing the eluted RNA in step II.F.2 on page 15.

Use a glass-fiber filter method for purification

Users who prefer a glass-fiber filter based method for RNA purification can contact our Technical Services Department for an alternative protocol.

V. Appendix

A. References

Burczynski ME, Twine NC, et al. (2005) Transcriptional profiles in peripheral blood mononuclear cells prognostic of clinical outcomes in patients with advanced renal cell carcinoma. *Clin Cancer Res.* **11**(3):1181–1189.

Cobb JP, Mindrinos MN, et al. (2005) Application of genome-wide expression analysis to human health and disease. *Proc Natl Acad Sci U S A.* **102**(13):4801–4806.

Rainen L, Oelmueller U, et al. (2002) Stabilization of mRNA expression in whole blood samples. *Clin Chem.* **48**:1883–1890.

Tsuang MT, Nossova N, et al. (2005) Assessing the validity of blood-based gene expression profiles for the classification of schizophrenia and bipolar disorder: a preliminary report. *Am J Med Genet B Neuropsychiatr Genet.* **133**(1):1–5.

B. Quality Control

Functional testing

All kit components are tested functionally by isolating RNA from whole blood using the procedure described in this protocol. RNA recovery is assessed by absorbance measurements using the NanoDrop Spectrophotometer and by qRT-PCR.

Nuclease testing

Relevant kit components are tested in the following nuclease assays:

RNase activity
Meets or exceeds specification when a sample is incubated with labeled RNA and analyzed by PAGE.

Nonspecific endonuclease activity
Meets or exceeds specification when a sample is incubated with supercoiled plasmid DNA and analyzed by agarose gel electrophoresis.

Exonuclease activity
Meets or exceeds specification when a sample is incubated with labeled double-stranded DNA, followed by PAGE analysis.

Protease testing

Meets or exceeds specification when a sample is incubated with protease substrate and analyzed by fluorescence.

C. Safety Information

WARNING

GENERAL SAFETY. *Using this product in a manner not specified in the user documentation may result in personal injury or damage to the instrument or device. Ensure that anyone using this product has received instructions in general safety practices for laboratories and the safety information provided in this document.*

- *Before using an instrument or device, read and understand the safety information provided in the user documentation provided by the manufacturer of the instrument or device.*
- *Before handling chemicals, read and understand all applicable Safety Data Sheets (SDSs) and use appropriate personal protective equipment (gloves, gowns, eye protection, etc). To obtain SDSs, see the "Documentation and Support" section in this document.*

1. Chemical safety

WARNING

GENERAL CHEMICAL HANDLING. *To minimize hazards, ensure laboratory personnel read and practice the general safety guidelines for chemical usage, storage, and waste provided below, and consult the relevant SDS for specific precautions and instructions:*

- *Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, see the "Documentation and Support" section in this document.*
- *Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).*
- *Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood).*
- *Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended in the SDS.*
- *Handle chemical wastes in a fume hood.*
- *Ensure use of primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)*
- *After emptying a waste container, seal it with the cap provided.*
- *Characterize (by analysis if necessary) the waste generated by the particular applications, reagents, and substrates used in your laboratory.*
- *Ensure that the waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.*
- ***IMPORTANT!*** *Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.*

2. Biological hazard safety

WARNING

Potential Biohazard. *Depending on the samples used on the instrument, the surface may be considered a biohazard. Use appropriate decontamination methods when working with biohazards.*

WARNING

BIOHAZARD. *Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. Follow all applicable local, state/provincial, and/or national regulations. Wear appropriate protective equipment, which includes but is not limited to: protective eyewear, face shield, clothing/lab coat, and gloves. All work should be conducted in properly equipped facilities using the appropriate safety equipment (for example, physical containment devices). Individuals should be trained according to applicable regulatory and company/institution requirements before working with potentially infectious materials. Read and follow the applicable guidelines and/or regulatory requirements in the following:*

In the U.S.:

- *U.S. Department of Health and Human Services guidelines published in Biosafety in Microbiological and Biomedical Laboratories found at: www.cdc.gov/biosafety*
- *Occupational Safety and Health Standards, Bloodborne Pathogens (29 CFR§1910.1030), found at: www.access.gpo.gov/nara/cfr/waisidx_01/29cfr1910a_01.html*
- *Your company's/institution's Biosafety Program protocols for working with/handling potentially infectious materials.*
- *Additional information about biohazard guidelines is available at: www.cdc.gov*

In the EU:

Check local guidelines and legislation on biohazard and biosafety precaution and refer to the best practices published in the World Health Organization (WHO) Laboratory Biosafety Manual, third edition, found at: www.who.int/csr/resources/publications/biosafety/WHO_CDS_CSR_LYO_2004_11/en/

VI. Documentation and Support

A. Obtaining SDSs

Safety Data Sheets (SDSs) are available from:

www.invitrogen.com/sds

or

www.appliedbiosystems.com/sds

Note: For the SDSs of chemicals not distributed by Life Technologies, **contact the chemical manufacturer.**

B. Obtaining support

For the latest services and support information for all locations, go to:

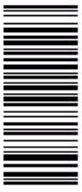
www.invitrogen.com

or

www.appliedbiosystems.com

At the website, you can:

- Access worldwide telephone and fax numbers to contact Technical Support and Sales facilities
- Search through frequently asked questions (FAQs)
- Submit a question directly to Technical Support
- Search for user documents, SDSs, vector maps and sequences, application notes, formulations, handbooks, certificates of analysis, citations, and other product support documents
- Obtain information about customer training
- Download software updates and patches



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