GLOBINclear™ Kit

(Cat #AM1980, AM1981)

Instruction Manual

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I. Introduction

A. Product Description

The Ambion GLOBINclear™ Kit (patent pending) is designed to rapidly deplete alpha and beta globin mRNA from total RNA preparations derived from human or rodent whole blood. The kit employs a novel hybridization technology that takes advantage of the strength of biotin/streptavidin binding, the specificity of nucleic acid hybridization, and the convenience of magnetic bead separations to deplete globin mRNA from blood total RNA. RNA samples processed with the GLOBINclear Kit are a superior template for RNA expression profiling because the depletion procedure alleviates problems associated with the abundant globin mRNA found in whole blood RNA samples. GLOBINclear processed RNA provides more sensitive expression profile results than unprocessed whole blood RNA.

B. Background

Globin mRNA interferes with expression profiling of whole blood samples

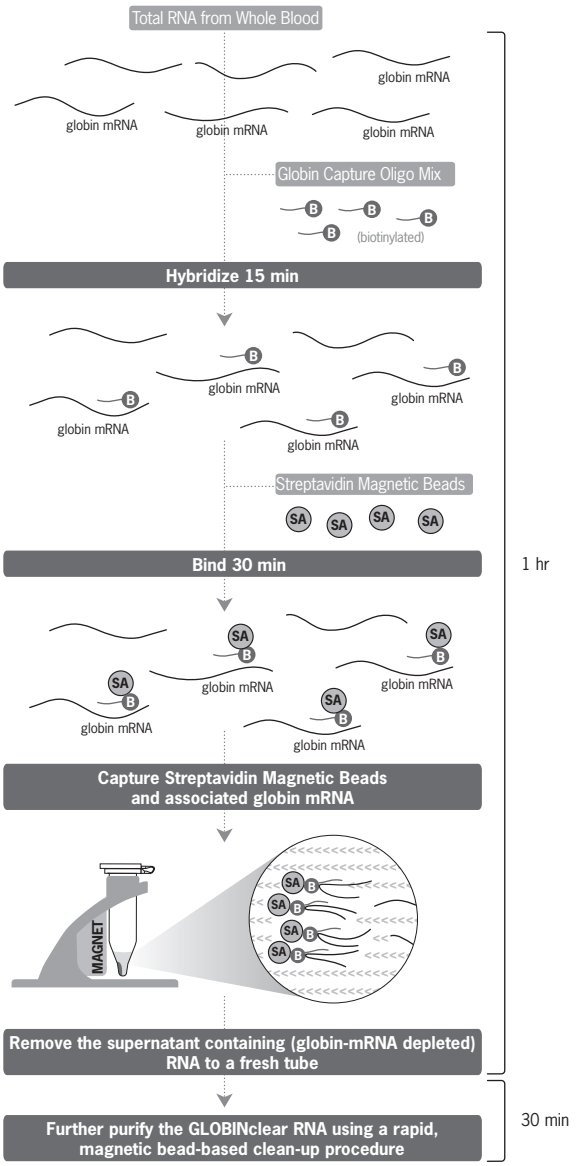
Expression profiling is widely used to study physiological, pathogenic, and therapeutic responses of the transcriptome. For clinical research, blood is the most commonly used tissue; unfortunately, blood samples present obstacles to gene expression analysis that are not encountered with most other tissues. Blood is made up of a heterogeneous population of erythrocytes, granulocytes, and other peripheral blood mononuclear cells (PBMC). The complex cellular nature of blood samples makes it difficult to detect differential gene expression that occurs in only a subset of cell types. Also, whole blood mRNA consists of a relatively large proportion of globin mRNA transcripts. Ambion has estimated that globin transcripts represent as much as 70% of the total mRNA population. These "unwanted" globin transcripts effectively dilute the mRNA population and decrease the sensitivity of detecting less abundant mRNAs using microarray technology.

The GLOBINclear advantage

Evidence of the dilution of mRNA by highly abundant globin mRNA has been observed on Affymetrix GeneChip arrays, where expression profiles from whole blood RNA show decreased Percent Present calls and increased variability among replicates, relative to RNA from the PBMC fraction of whole blood (which does not contain globin mRNA). The Ambion GLOBINclear Kit is a treatment for RNA derived from whole blood that eliminates the negative effects of globin mRNA during high density oligonucleotide expression profiling. GLOBINclear-processed RNA can be used in downstream applications such as RNA amplification reactions for microarray analysis, routine RT-PCR, and cDNA synthesis reactions.

C. GLOBINclear Procedure Overview

Figure 1. GLOBINclear Procedure Overview



The GLOBINclear Kit procedure is rapid and robust (Figure 1). First, globin mRNA is removed from whole blood RNA using a novel hybridization capture technology; then the remaining RNA is further purified with a rapid magnetic bead-based clean-up procedure.

To remove globin mRNA, total RNA from human, mouse, or rat whole blood is mixed with a biotinylated Capture Oligo Mix that is specific for either human or rodent (mouse and rat) globin mRNA. The mixture is incubated for 15 min to allow the biotinylated oligonucleotides to hybridize with the globin mRNA species. Streptavidin Magnetic Beads are then added, and the mixture is incubated for 30 min. During this incubation, streptavidin binds the biotinylated oligonucleotides, thereby capturing the globin mRNA on the magnetic beads. The Streptavidin Magnetic Beads are then pulled to the side of the tube with a magnet, and the RNA, depleted of the globin mRNA, is transferred to a fresh tube.

The treated RNA is further purified using a rapid magnetic bead-based purification method. This simple, ~30 min, purification consists of adding an RNA Binding Bead suspension to the samples, and using magnetic capture to wash and elute the GLOBINclear RNA.

Typically the GLOBINclear Kit procedure removes >95% of the alpha and beta globin mRNA in human, mouse or rat whole blood RNA. The precise amount of globin mRNA removed is influenced by factors such as the mass of input RNA, its globin mRNA content, and the integrity of the RNA.

D. Effects of GLOBINclear Processing on RNA Amplification

The GLOBINclear procedure was optimized primarily for RNA amplification using the MessageAmp™ II Kit. The goal was to develop a process to improve global expression screening of samples derived from human, mouse, or rat whole blood. Before GLOBINclear processing, aRNA amplified from whole blood samples shows a very distinct ~600 nt peak that represents amplified globin mRNA. We found that total RNA from whole blood that has been processed with the GLOBINclear procedure produces a very different aRNA profile, in which the distinctive peak disappears, and the aRNA produces a smooth curve (Figure 2). This is a result of the removal of globin mRNA transcripts from the amplification reaction.

GLOBINclear-processed RNA actually contains far less mRNA template for amplification in a given amount of total RNA than unprocessed whole blood RNA. This is because GLOBINclear depletes samples of >95% of their globin mRNA, which represents a substantial proportion, >70%, of the total mRNA present in whole blood RNA. The result is that aRNA yield from GLOBINclear-processed RNA is less than the yield from an equivalent mass of unprocessed RNA (Table 1 on page 13).

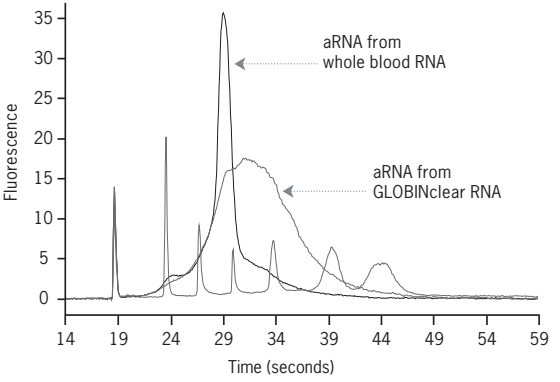


Figure 2. aRNA Amplified from GLOBINclear RNA and Untreated Whole Blood RNA.

Total RNA isolated from human whole blood using the RiboPure™-Blood Kit (Ambion Cat #AM1928) was processed using the GLOBINclear™-Human Kit. The MessageAmp™ II aRNA Amplification Kit (Cat #AM1751) was used to linearly amplify 1 µg total RNA or RNA enriched by the GLOBINclear Kit. The resulting aRNA was analyzed on an Agilent 2100 bioanalyzer using an RNA LabChip to compare aRNA from GLOBINclear-processed RNA with unprocessed RNA. Note the disappearance of the distinctive globin aRNA peak in the GLOBINclear-processed RNA compared to the aRNA from whole blood RNA.

E. Materials Provided with the Kit

The GLOBINclear Kit contains reagents for 20 globin mRNA depletion reactions—each starting with 1–10 µg of total RNA from human, mouse or rat whole blood.

Globin mRNA depletion reagents

GLOBINclear Kit			
Human Cat #AM1980	Mouse/Rat Cat #AM1981	Component	Storage
20 µL	20 µL	Capture Oligo Mix	–20°C
600 µL	600 µL	Streptavidin Magnetic Beads*	4°C
1.5 mL	1.5 mL	2X Hybridization Buffer	room temp
1.5 mL	1.5 mL	Streptavidin Bead Buffer	room temp
1.75 mL	1.75 mL	Nuclease-free Water	any temp†

* Do not freeze the Streptavidin Magnetic Beads.

† Store the Nuclease-free Water at –20°C, 4°C, or room temp.

RNA purification

GLOBINclear Kit			
Human Cat #AM1980	Mouse/Rat Cat #AM1981	Component	Storage
200 µL	200 µL	RNA Binding Beads*	4°C
5 mL	5 mL	RNA Wash Solution Concentrate Add 4 mL of 100% ethanol before use	4°C or room temp
1 mL	1 mL	Elution Buffer	4°C or room temp
80 µL	80 µL	RNA Bead Buffer	room temp
4 mL	4 mL	RNA Binding Buffer Concentrate Add 2 mL of 100% isopropanol before use	room temp
100	100	1.5 mL Non-stick Tubes	room temp

* Do not freeze the RNA Binding Beads.

F. Materials Not Provided with the Kit

Lab equipment and supplies

- Spectrophotometer or RiboGreen RNA Quantitation Reagent (Molecular Probes, Inc.) to quantitate RNA before starting the procedure

  For fast, easy, and accurate UV/Vis analyses, Ambion recommends the NanoDrop ND-1000A Spectrophotometer. The NanoDrop works with 1.5–2 µL samples and does not require dilutions, cuvettes, or capillaries. See www.nanoambion.com for more information.

- General laboratory equipment including vortex mixer, microcentrifuge, pipettors, and RNase-free tips
- 50°C hybridization oven or fixed-temperature incubator
- 58°C heat block
- Magnetic stand for 1.5 mL tubes, e.g. the Ambion 6 Tube Magnetic Stand (Cat #AM10055)

Reagents

- Total RNA isolated from human, mouse, or rat whole blood (see section *II.A. Input RNA Requirements* on page 6 for more information)
- 100% ethanol, ACS grade or higher quality
- 100% isopropanol, ACS grade or higher quality
- Reagents for precipitation of input RNA. If your RNA sample concentration is ≤70 ng/µL, you will need the following reagents to concentrate it by precipitation:
  - 5 M ammonium acetate or 3 M sodium acetate
  - Glycogen
  - TE (10 mM Tris-HCl pH 8, 1 mM EDTA) or Nuclease-free Water (e.g., Cat #AM9937)

G. Related Products Available from Ambion

RiboPure™-Blood Cat #AM1928	The RiboPure-Blood Kit is designed for purification of total RNA directly from whole blood—without isolating white blood cells. It employs a fast simple procedure that yields RNA from whole blood samples in about 30 minutes.
6 Tube Magnetic Stand Cat #AM10055	Designed to hold up to six standard microcentrifuge tubes, the 6 Tube Magnetic Stand houses a strong magnet for fast, efficient separation of magnetic beads from solutions.
MessageAmp™ aRNA Amplification Kits see our web or print catalog	Ambion offers 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, Ambion offers 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.

II. GLOBINclear™ Protocol

A. Input RNA Requirements

Use total RNA isolated from whole blood

Total RNA obtained from whole blood samples using any high quality whole blood RNA isolation procedure is compatible with the GLOBINclear Kit. The Ambion RiboPure-Blood™ Kit was used extensively during GLOBINclear Kit development; the RiboPure-Blood Kit purifies total RNA directly from whole blood without prior recovery of white blood cells (WBCs) in less than 30 min.

Use 1–10 µg of human, mouse, or rat whole blood total RNA; accurate quantitation is important

It is important to accurately quantitate input RNA so as not to overload the globin mRNA hybridization reaction. The GLOBINclear Kit has been extensively optimized for globin mRNA removal from 1–10 µg of input whole blood total RNA from human, mouse, or rat. If more than the recommended 10 µg of RNA is used in the procedure, globin mRNA capture will be incomplete. Ambion recommends using a high quality, calibrated spectrophotometer or RiboGreen RNA Quantitation Reagent to accurately quantitate RNA for use in this procedure.

Use only high quality RNA

RNA integrity is critically important for the success of the GLOBINclear procedure, as it is for many downstream applications. Even moderate levels of RNA degradation can lead to inefficient removal of globin mRNA by the capture reagents. This will result in recovery of mRNA that is not as highly enriched as it could be. Several factors, such as blood storage, RNA isolation method, and residual RNase activity can all have an effect on RNA quality. Evaluate the integrity of the RNA you want to use in the procedure by Agilent bioanalyzer or by electrophoresis.

Precipitate RNA, if necessary, to concentrate it

The input RNA volume should be ≤14 µL

The input RNA concentration must be ≥70 ng/µL for use in the GLOBINclear procedure: the recommended amount of RNA per reaction is 1–10 µg and the recommended maximum volume is 14 µL. Determine how much RNA to process (up to 10 µg) based on RNA availability and the requirements of your downstream applications. If the RNA volume is >14 µL, reduce the volume to 14 µL or less for use in the GLOBINclear procedure. If the RNA is dissolved in water, you can reduce its volume by vacuum drying; RNA in other solutions must be precipitated to concentrate it—e.g., using the precipitation protocol below.

RNA precipitation instructions

NOTE

*The reagents for this RNA precipitation are **not** supplied with the kit.*

a. Add the following to each RNA sample and mix thoroughly:
   - 0.1 volume 5 M ammonium acetate or 3 M sodium acetate
   - (Optional) 5 µg glycogen
   - 2.5–3 volumes 100% ethanol

   Glycogen acts as a carrier to improve precipitation efficiency from dilute RNA solutions; it is unnecessary for solutions with >200 µg RNA/mL.

b. Place the mixture at –20°C overnight, or quick-freeze it in ethanol and dry ice or in a –70°C freezer for 30 min.

c. Centrifuge at >12,000 x g for 30 min at 4°C to recover the RNA.

d. Carefully remove and discard the supernatant.

e. Add 1 mL ice cold 70% ethanol, and vortex the tube.

f. Re-pellet the RNA by centrifugation for 10 min at 4°C. Remove the supernatant carefully as in step d above.

g. Repeat steps e and f to wash the pellet a second time.

h. Dissolve the RNA in <14 µL TE (10 mM Tris-HCl pH 8, 1 mM EDTA) or in Nuclease-free Water.

i. Measure the RNA concentration again after precipitation.

B. Reagent Preparation

IMPORTANT

Prepare the following reagents before starting the procedure.

1. Add 2 mL of 100% isopropanol to the RNA Binding Buffer Concentrate

Add 2 mL of 100% isopropanol to the bottle labeled RNA Binding Buffer Concentrate. Mix well and mark the label to indicate that the isopropanol was added.

Store at room temperature.

The resulting mixture is referred to as ***RNA Binding Buffer*** in these instructions.

2. Add 4 mL of 100% ethanol to the RNA Wash Solution Concentrate

Add 4 mL of 100% ethanol to the RNA Wash Solution Concentrate bottle. Mix well and indicate on the label that the ethanol was added.

Store at room temperature.

The resulting mixture is referred to as ***RNA Wash Solution*** in these instructions.

3. Dilute the RNA Binding Beads in RNA Bead Buffer, and add isopropanol

IMPORTANT

The prepared Bead Resuspension Mix can be stored at room temp for up to 1 month. If necessary, scale down the reagent volumes proportionally to prepare the amount of mix needed for 1 month.

In a 1.5 mL tube, combine RNA Bead Buffer with RNA Binding Beads according to the table below and mix briefly. Then add the 100% isopropanol and mix thoroughly by vortexing.

Store at room temperature.

This mixture is referred to as ***Bead Resuspension Mix*** in these instructions.

a. Combine the following:	1 Rxn	20 Rxn*
RNA Binding Beads†	10 µL	200 µL
RNA Bead Buffer	4 µL	80 µL
b. Mix briefly, then add:		
100% Isopropanol	6 µL	120 µL
c. Mix well by vortexing.		

* When preparing Bead Resuspension Mix for more than 2 samples, include 5–10% overage to compensate for pipetting error.

† Mix the RNA Binding Beads thoroughly by vortexing before dispensing.

C. Preparation of Streptavidin Magnetic Beads

NOTE

Follow the instructions in this section to equilibrate and preheat the Streptavidin Magnetic Beads needed for the samples to be processed that day before proceeding to section *II.D. Hybridization of Globin mRNA and Globin Capture Oligonucleotides starting on page 9.*

Before you start:

- Set a dry incubator to 50°C; we recommend using a hybridization oven or other fixed-temperature air incubator.
- Warm the 2X Hybridization Buffer and the Streptavidin Bead Buffer to 50°C for at least 15 min before starting the procedure. Vortex well before use.

1. Place 30 µL of Streptavidin Magnetic Beads per sample into a 1.5 mL tube

a. You will need 30 µL of Streptavidin Magnetic Beads for each sample; calculate the volume of beads needed for the samples being processed that day. When there are more than 2 samples, it is prudent to include 5–10% overage to cover pipetting error.

b. Vortex the tube of the Streptavidin Magnetic Beads to suspend the settled beads, and transfer the volume needed into a 1.5 mL Non-stick Tube provided with the kit. Up to 20 reactions can be processed in a single tube.

c. Briefly centrifuge (<2 sec) at low speed (<1000 x g) to collect the mixture at the bottom of the tube.

2. Magnetically capture the beads and carefully remove and discard the supernatant

a. Place the tube on a magnetic stand to capture the Streptavidin Magnetic Beads. Leave the tube on the magnetic stand until the mixture becomes transparent (~3–5 min), indicating that capture is complete. The capture time will depend on the magnetic stand used.

b. Carefully aspirate the supernatant using a pipet without disturbing the Streptavidin Magnetic Beads. Discard the supernatant, and remove the tube from the magnetic stand

3. Equilibrate the beads with an equal volume of Streptavidin Bead Buffer and place at 50°C

a. Add Streptavidin Bead Buffer to the Streptavidin Magnetic Beads; use a volume equal to the original volume of Streptavidin Magnetic Beads (volume transferred in step 1.b). Vortex vigorously until the beads are resuspended.

b. Place the prepared Streptavidin Magnetic Beads beads at 50°C (hybridization oven recommended) and immediately proceed to the next step. The beads should remain at 50°C for at least 15 min before they are used in step E.1 on page 10.

D. Hybridization of Globin mRNA and Globin Capture Oligonucleotides

1. Combine 1–10 µg RNA and 1 µL Capture Oligo Mix

Combine the following in a 1.5 mL Non-stick Tube provided with the kit:

- 1–10 µg human, mouse, or rat whole blood total RNA (in a maximum volume of 14 µL, i.e. the RNA concentration must ≥70 ng/µL)
  Use human RNA for GLOBINclear-Human (Cat #AM1980); use mouse or rat RNA for GLOBINclear-Mouse/Rat (Cat #AM1981).
- 1 µL of Capture Oligo Mix

2. Add Nuclease-free Water for a final volume of 15 µL

If necessary, add Nuclease-free Water to the sample mixture from step 1 to a final volume of 15 µL.

3. Add 15 µL 2X Hybridization Buffer

a. Add 15 µL of 50°C 2X Hybridization Buffer to the sample.

b. Vortex briefly to mix and centrifuge briefly at low speed to collect the contents in the bottom of the tube.

4. Hybridize at 50°C for 15 min

Place the sample in a prewarmed 50°C incubator and allow the Globin Capture Oligo Mix to hybridize to the globin mRNA for 15 min.


NOTE

Both the time and temperature of this incubation are important for efficient globin mRNA depletion.

E. Removal of Globin mRNA

1. Add 30 µL prepared Streptavidin Magnetic Beads to each sample

a. Remove the prepared Steptavidin Magnetic Beads (from step C.3.b on page 9) from the 50°C incubator, and resuspend them by gentle vortexing. Briefly centrifuge (<2 sec) at low speed (<1000 x g) to collect the mixture at the bottom of the tube.

b. Add 30 µL of prepared Streptavidin Magnetic Beads to each RNA sample (from step D.4).

c. Vortex to mix well and centrifuge briefly at low speed as in the previous steps to collect the contents in the bottom of the tube.

d. Flick the tube very gently to resuspend the beads, being careful to keep the contents at the bottom of the tube.

2. Incubate 30 min at 50°C

Place the RNA bead mixture at 50°C (hybridization oven or other fixed temperature air incubator recommended) and incubate for 30 min.

3. Magnetically capture the beads

a. Remove sample from the incubator, and vortex briefly to mix. Then centrifuge briefly at low speed as in the previous steps to collect the contents in the bottom of the tube.

b. Capture the Streptavidin Magnetic Beads on a magnetic stand. Leave the tube on the magnetic stand until the mixture becomes transparent (~3–5 min), indicating that capture is complete.

4. Transfer the supernatant containing the RNA to a new tube

Carefully draw up the supernatant, which contains the globin mRNA-depleted RNA, using a pipet without disturbing the Streptavidin Magnetic Beads. Transfer the RNA to a new 1.5 mL Non-stick Tube supplied with the kit, and place on ice. ***The supernatant contains the*** GLOBINclear ***RNA; do not discard the supernatant.***

(You can discard the tube with the Streptavidin Magnetic Beads.)

F. Purify the GLOBINclear RNA

Before you start:
Warm the Elution Buffer to 58°C for use in step 6.

1. Add 100 µL RNA Binding Buffer to each sample

Add 100 µL prepared RNA Binding Buffer (prepared in step B.1 on page 7) to each enriched RNA sample.

2. Add 20 µL *Bead Resuspension Mix* to each sample; mix for 10 sec

a. Vortex the ***Bead Resuspension Mix*** (prepared in step B.3), then immediately dispense 20 µL to each sample. It is important to resuspend the beads thoroughly before adding them to the samples.

b. Vigorously vortex the sample for 10 sec to fully mix the reagents, and to allow the RNA Binding Beads to bind the RNA.

c. Briefly centrifuge (<2 sec) at low speed (<1000 x g) to collect the mixture at the bottom of the tube.

3. Magnetically capture the RNA Binding Beads and discard the supernatant

a. Capture the RNA Binding Beads by placing the tube on a magnetic stand. Leave the tube on the magnetic stand until the mixture becomes transparent (~3–5 min), indicating that capture is complete.

b. Carefully aspirate the supernatant using a pipet without disturbing the RNA Binding Beads. Discard the supernatant.

IMPORTANT

It is important to remove as much of the supernatant as possible at this step.

c. Remove the tube from the magnetic stand. It is critical for effective washing to remove the tube from the magnetic stand before adding the RNA Wash Solution (next step).

4. Wash the RNA Binding Beads with 200 µL RNA Wash Solution

a. Add 200 µL RNA Wash Solution (prepared in step B.2) to each sample and vortex for 10 sec. The RNA Binding Beads may not fully disperse during this step; this is expected, and it will not affect RNA purity or yield.

b. Briefly centrifuge (<2 sec) at low speed (<1000 x g) to collect the mixture at the bottom of the tube.

c. Capture the RNA Binding Beads on a magnetic stand as in the previous magnetic bead capture steps.

d. Carefully aspirate and discard the supernatant, and remove the tube from the magnetic stand.

5. Remove any remaining supernatant and leave the tube open for 5 min

a. Briefly centrifuge the tube as in previous steps and place it back on the magnetic stand.

b. Remove any liquid in the tube with a small-bore pipet tip (e.g. P10 or P20).

c. Remove the tube from the magnetic stand and allow the beads to air-dry for 5 min with the caps left open.


NOTE

Do not air-dry the beads for >5 min.

6. Add 30 µL Elution Buffer and incubate at 58°C for 5 min to elute the enriched RNA

a. Add 30 µL warm (58°C) Elution Buffer to each sample, and vortex vigorously for ~10 sec to thoroughly resuspend the RNA Binding Beads.

b. Incubate the mixture at 58°C for 5 min.


IMPORTANT

This incubation is critical for optimal RNA recovery.

c. Vortex the sample vigorously for ~10 sec to thoroughly resuspend the RNA Binding Beads and centrifuge briefly at low speed as in previous steps to collect the mixture at the bottom of the tube.

7. Magnetically capture the RNA Binding Beads and transfer the GLOBINclear RNA to a new tube

a. Capture the RNA Binding Beads on a magnetic stand as in the previous magnetic bead capture steps. Be especially careful at this step to avoid disturbing the RNA Binding Beads when collecting the supernatant. ***The purified RNA will be in the supernatant.***

b. Transfer the supernatant containing the RNA to a 1.5 mL Non-stick Tube (supplied). Store the purified RNA at –20°C.


NOTE

Frequently some of the RNA Binding Beads are carried over to the eluate, tinting it brownish. The RNA Binding Beads have been shown to have no effect on absorbance readings or downstream enzymatic applications. If desired, however, magnetic bead contamination can usually be reduced by recapturing the beads on a magnetic stand for 3–5 min, and transferring the RNA solution to a new tube.

III. Assessing the Yield and Quality of Enriched RNA

A. Expected RNA Yield After GLOBINclear Processing and MessageAmp RNA Amplification

Percent RNA recovery from the GLOBINclear procedure will depend on RNA quality, the amount of globin mRNA in the sample, and most importantly on the amount of input RNA used in the procedure; data from Ambion are shown in Table 1. Also shown are aRNA yield data from GLOBINclear-processed RNA. Note that aRNA yield drops after GLOBINclear processing, this is a direct result of removing a large proportion of the mRNA (globin mRNA) in the sample.

Table 1. Empirical GLOBINclear™ RNA and aRNA Yield Data for Human Samples

Input RNA	GLOBINclear RNA Recovered (+/– SD)	Input for amplification	aRNA yield (+/– SD)
0.5 µg	0.45 µg (.01)	0.3 µg	19.62 µg (2.93)
1 µg	0.81 µg (.11)	0.6 µg	30.03 µg (2.85)
3 µg	2.24 µg (.03)	1 µg	61.59 µg (.63)
5 µg	3.54 µg (.08)	1 µg	79.89 µg (.48)
7 µg	4.81 µg (.03)	1 µg	64.77 µg (1.29)
10 µg	10.78 µg (.74)	1 µg	56.98 µg (1.52)
Control: no GLOBINclear	na	1 µg	148.92 µg (1.82)

Triplicate GLOBINclear-Human reactions and MessageAmp II-96 RNA amplification reactions were performed with the indicated input levels of human whole blood total RNA or GLOBINclear RNA respectively. RNA and aRNA yield were determined with a NanoDrop spectrophotometer. Values in parentheses represent 1 standard deviation. In the control reaction a whole blood RNA sample was amplified directly without GLOBINclear processing.

B. Quantitation of RNA

Assessing RNA yield by UV absorbance

The concentration of an RNA solution can be determined by measuring its absorbance at 260 nm. Ambion scientists recommend using the NanoDrop 1000A Spectrophotometer (www.nanoambion.com) because it is extremely quick and easy to use. No dilutions and no cuvettes are needed; just measure 1.5 µL of the aRNA sample directly.

Alternatively, the RNA concentration can be determined by diluting an aliquot of the preparation (usually a 1:50 to 1:100 dilution) in 10 mM Tris-HCl pH 8, 1 mM EDTA (TE), and reading the absorbance in a traditional spectrophotometer at 260 nm. Be sure to zero the spectrophotometer with the TE used for sample dilution. Calculate the concentration in µg/mL by multiplying the A_{260} by the dilution factor and the extinction coefficient. (1 A_{260} = 40 µg RNA/mL)

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

The typical yield, after GLOBINclear processing, from 5 µg of high quality total RNA is ~3–5 µg. The RNA is eluted in 30 µL, thus the concentration will be ~100–167 ng/µL.

The ratio of A_{260} to A_{280} values, a measure of RNA purity, should fall in the range of ~1.9 to 2.2.

Assessing RNA yield with RiboGreen

If you have a fluorometer, or a fluorescence microplate reader, 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.

C. Agilent 2100 Bioanalyzer Analysis of Processed RNA

The Agilent 2100 bioanalyzer with the RNA 6000 Nano LabChip Kit provides a particularly effective method for evaluating RNA quality. Follow the instructions for RNA analysis provided with the RNA 6000 Nano LabChip Kit. At Ambion, we have found that this system performs best by loading 1 µL of a 50–250 ng/µL RNA solution.

It is a good idea to compare the total RNA used in the GLOBINclear procedure with the RNA recovered to be sure that RNA degradation did not occur during the GLOBINclear procedure. The following article from Ambion's TechNotes newsletter discusses interpretation of bioanalyzer results and RNA quality:

www.ambion.com/techlib/tn/111/8.html

IV. Troubleshooting

A. Low Final Yield of RNA

NOTE

See section III.A on page 13 for a discussion of expected yield from the GLOBINclear Kit.

Wrong quantity of input RNA or incorrectly quantitated RNA

For optimal results, it is very important to use 1–10 µg of total RNA from whole blood in the GLOBINclear Kit procedure (instructions for quantitation of RNA are provided in section III.B on page 13). Accurate quantitation is important for many downstream procedures as well.

RNA is degraded

See section *IV.C. RNA Degradation* on page 16.

RNA Binding Beads were not fully resuspended/ dispersed

In general, the RNA Binding Beads will disperse more easily when the temperature of the mixture is warmer than 20°C.

a. Make sure the ***Bead Resuspension Mix*** is fully resuspended before adding it to the RNA sample in step II.F.2 on page 11.

b. Avoid over-drying the RNA Binding Beads in step II.F.5 before eluting the RNA because this may make the beads more difficult to resuspend.

c. Make sure that the RNA Binding Beads are fully resuspended in Elution Buffer to efficiently elute the RNA from the beads in step II.F.6 on page 12.

RNA Binding Beads were unintentionally lost

Any loss of RNA Binding Beads will result in loss of GLOBINclear processed RNA. Avoid aspirating the RNA Binding Beads when removing supernatant from the captured beads. To determine whether RNA Binding Beads have been inadvertently aspirated with supernatant, it may be helpful to collect all supernatants (except the final RNA-containing supernatant) in a single container. Observe the color of the collected supernatant: if RNA Binding Beads are in the supernatant, they will color it light brown.

To prevent aspiration of RNA Binding Beads in subsequent experiments:

- Increase the magnetic capture time.
- Remove the supernatant from the captured beads more slowly.
- Avoid touching the captured beads when removing supernatant.

B. Inefficient Removal of Globin mRNA

To maximize removal of globin mRNA, follow the procedure exactly. Following is a list of some of the most crucial parts of the procedure:

More than 10 µg of input total RNA was used in the procedure

The GLOBINclear Kit procedure is optimized for depletion of globin mRNA from 10 µg or less total RNA from human, mouse, or rat whole blood. Using more than 10 µg RNA in the procedure will compromise removal of globin mRNA.

Hybridization conditions are suboptimal

Be sure to follow the temperature and time recommendations for the hybridizations in steps II.D.4 on page 9 and II.E.2 on page 10 for optimal depletion of globin mRNA.

C. RNA Degradation

The total RNA was degraded even before starting the procedure

RNA integrity can be evaluated by microfluidics analysis using the Agilent 2100 bioanalyzer and an RNA LabChip Kit. Primarily full-length RNA will exhibit a ratio of 28S to 18S rRNA bands that approaches 2:1. Using a bioanalyzer, the RIN (RNA Integrity Number) can be calculated to further evaluate RNA integrity. A new metric developed by Agilent, the RIN analyzes information from both rRNA bands, as well as information contained outside the rRNA peaks (potential degradation products) to provide a fuller picture of RNA degradation states. Search for "RIN" at the following web address for more information:

http:\\www.chem.agilent.com

Denaturing agarose gel electrophoresis and nucleic acid staining can also be used to separate and visualize the major rRNA species. When the RNA resolves into discrete rRNA bands (i.e. no significant smearing below each band), with the 28S rRNA band appearing 1.5–2 fold brighter than the 18S rRNA band, then the mRNA in the sample is likely to be mostly full-length. The primary drawback to gel electrophoresis is that microgram amounts of RNA must be sacrificed.

Endogenous RNase contamination of RNA

If the total RNA used in the procedure is contaminated with small amounts of endogenous RNase, significant RNA degradation could occur during the GLOBINclear procedure. To test for endogenous RNase contamination, incubate a sample of the RNA overnight at room temp or 37°C. Then, using denaturing agarose gel electrophoresis or microfluidics analysis, compare the integrity of the RNA incubated at room temperature or 37°C with a sample that was kept frozen. If the overnight incubation leads to appreciable RNA degradation, it indicates that the sample may contain endogenous RNase. If so, consider cleaning up your RNA by treating it with 100–200 µg/mL proteinase K, and

0.5% SDS at 50°C for 30 min. Follow this with phenol/chloroform extraction (using an equal volume) and ethanol precipitation to remove any RNase from the sample.

RNase contamination was introduced during the GLOBINclear procedure

Observe all of the typical precautions against RNase contamination while using the GLOBINclear Kit. Wear gloves at all times and change them frequently to avoid the introduction of RNases. Keep bags of 1.5 mL tubes and the kit reagent tubes and bottles closed when they are not in use to avoid contamination with dust. Any plasticware or reagents not supplied with the kit, which will contact the RNA, should be bought or prepared so that they are free from RNases.

For more information on avoiding RNase contamination see Ambion Technical Bulletins 159 and 180; they are available on our website at the following addresses:

www.ambion.com/techlib/tb/tb_159.html

www.ambion.com/techlib/tb/tb_180.html

D. Processed RNA Performs Poorly in Downstream Applications

Residual salt remaining in RNA preparation

Residual salt in the GLOBINclear RNA sample may negatively affect downstream applications. To avoid this, be sure to remove as much of the supernatant as possible from the RNA Binding Beads in section II.F.3.b on page 11.

RNA degradation

See section *IV.C. RNA Degradation* on page 16.

Low A_{260}:A_{280} Ratio

An A_{260}:A_{280} ratio of the enriched mRNA of <1.7 can indicate a sample with excess protein or other contaminating substances. Excessive protein contamination of total RNA preparations is not uncommon, especially when using a protocol based on organic extraction, and some of this protein may be carried over to the enriched RNA.

V. Appendix

A. GLOBINclear Kit Specifications

Kit contents and storage conditions

GLOBINclear Kit			
Human Cat #AM1980	Mouse/Rat Cat #AM1981	Component	Storage
20 µL	20 µL	Capture Oligo Mix	–20°C
600 µL	600 µL	Streptavidin Magnetic Beads*	4°C
200 µL	200 µL	RNA Binding Beads*	4°C
5 mL	5 mL	RNA Wash Solution Concentrate Add 4 mL of 100% ethanol before use	4°C or room temp
1 mL	1 mL	Elution Buffer	4°C or room temp
80 µL	80 µL	RNA Bead Buffer	room temp
4 mL	4 mL	RNA Binding Buffer Concentrate Add 2 mL of 100% isopropanol before use	room temp
1.5 mL	1.5 mL	2X Hybridization Buffer	room temp
1.5 mL	1.5 mL	Streptavidin Bead Buffer	room temp
2 x 50	2 x 50	1.5 mL Non-stick Tubes	room temp
1.75 mL	1.75 mL	Nuclease-Free Water	any temp†

* Do not freeze these reagents.

† Store the Nuclease-free Water at –20°C, 4°C, or room temp.

To obtain Material Safety Data Sheets

- Material Safety Data Sheets (MSDSs) can be printed or downloaded from product-specific links on our website at the following address:
  www.ambion.com/techlib/msds
- Alternatively, e-mail your request to MSDS_Inquiry_CCRM@appliedbiosystems.com. Specify the catalog or part number(s) of the product(s), and we will e-mail the associated MSDSs unless you specify a preference for fax delivery.
- For customers without access to the internet or fax, our technical service department can fulfill MSDS requests placed by telephone or postal mail. (Requests for postal delivery require 1–2 weeks for processing.)

B. Quality Control

Functional testing

RNA is processed with GLOBINclear following the procedure described in section II starting on page 6. The RNA recovered after processing is evaluated for yield, globin mRNA reduction, and integrity.

Nuclease testing

Relevant kit components are tested in the following nuclease assays:

RNase activity
Meets or exceeds specification when a sample is incubated with 25 ng labeled RNA and analyzed by PAGE.

Nonspecific endonuclease activity
Meets or exceeds specification when a sample is incubated with 300 ng supercoiled plasmid DNA and analyzed by agarose gel electrophoresis.

Exonuclease activity
Meets or exceeds specification when a sample is incubated with 40 ng labeled *Sau*3A fragments of pUC19 and analyzed by PAGE.

Protease testing

Meets or exceeds specification when a sample is incubated with 1 µg protease substrate and analyzed by fluorescence.