



Instruction Manual

PureLink™ Total RNA Blood Purification Kit

For isolating total RNA from whole blood

Catalog no. K1560-01

Version B

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Experienced Users Procedure

Introduction

This quick reference sheet is included for experienced users of the PureLink™ Total RNA Blood Purification Kit. If you are a first time user, follow detailed protocols provided in this manual.

The purification procedure is designed for purifying up to 500 µl fresh whole blood or $\sim 3.5 \times 10^6$ leukocytes using a microcentrifuge capable of centrifuging $>8,000 \times g$.

Step	Action
Leukocyte Enrichment	<ol style="list-style-type: none">1. To a sterile, RNase-free tube of appropriate size, add 5 volumes of Lysis Buffer (L5) to 1 volume of fresh whole blood sample (page 4 for blood sample requirements).2. Incubate for 10 minutes on ice. Vortex the tube briefly 2-3 times during the incubation. The solution turns translucent.3. Centrifuge the tube at 4°C for 10 minutes at 400 x g. Remove the supernatant completely and discard the supernatant. <i>Do not discard the pellet as the pellet contains leukocytes.</i>4. Resuspend the leukocyte pellet in 2 volumes of Lysis Buffer (L5). Mix well by vortexing briefly.5. Centrifuge the tube at 4°C for 5 minutes at 400 x g. Remove the supernatant completely and discard the supernatant. <i>Do not discard the pellet as the pellet contains leukocytes.</i> Proceed to Step 6 immediately. Do not store the pellet.6. Resuspend the leukocyte pellet in 350 µl Lysis Buffer (L3). Mix well by vortexing briefly to completely resuspend the pellet ensuring the absence of any cell clumps.7. Add 350 µl 70% ethanol to the tube and mix well by vortexing briefly. Proceed to the Purification Procedure, next page.

Continued on next page

Experienced Users Procedure, Continued

Step	Action
Purification Procedure	<ol style="list-style-type: none"> 1. Remove a Spin Cartridge in a Collection Tube from the package. Transfer the leukocyte lysate from Step 7, previous page, to the Spin Cartridge. 2. Centrifuge the Spin Cartridge at $8,000 \times g$ for 1 minute at room temperature. 3. Discard the flow through and return the Spin Cartridge to the collection tube. 4. Wash the Spin Cartridge with 700 μl of Wash Buffer (W4). Centrifuge at $8,000 \times g$ for 30 seconds at room temperature. 5. Perform Optional DNase Digestion Step (page 7) if needed to remove genomic DNA or proceed to Step 6. 6. Wash the Spin Cartridge with 500 μl of Wash Buffer (W4). If DNase I digestion was performed, incubate for 5 minutes at room temperature. Then centrifuge at $8,000 \times g$ for 30 seconds at room temperature. Discard the flow through. 7. Wash Spin Cartridge with 500 μl of Wash Buffer (W5) with ethanol (page 5). Centrifuge the cartridge at $8,000 \times g$ for 30 seconds at room temperature. 8. Repeat Step 7 one more time. 9. Discard the flow through and place the Spin Cartridge in the Wash Tube and centrifuge at $8,000 \times g$ for 1 minute at room temperature to remove any residual Wash Buffer (W5). 10. Place the Spin Cartridge into a clean 1.7-ml Elution Tube. 11. Elute with 30-100 μl of sterile, RNase-free water. Add the water to the center of the cartridge and incubate at room temperature for 1 minute. 12. Centrifuge the Spin Cartridge at $8,000 \times g$ for 1 minute at room temperature. <i>The elution tube contains your purified total RNA. Remove and discard the cartridge.</i> 13. Store the total RNA at -80°C or use total RNA for the desired downstream application.

Kit Contents and Storage

Shipping and Storage

All components of the PureLink™ Total RNA Blood Purification Kit are shipped at room temperature. Upon receipt, store all components at room temperature.

Contents

The components included in the PureLink™ Total RNA Blood Purification Kit are listed below. Sufficient reagents are included in the kit to perform 50 isolations.

Item	Amount
Lysis Buffer (L3)	17.5 ml
Lysis Buffer (L5)	175 ml
Wash Buffer (W4)	60 ml
Wash Buffer (W5)	10 ml
RNase-free water	5 ml
Spin Cartridges with Collection Tubes	50
Wash Tubes (2.0 ml)	50
Elution Tubes (1.7 ml)	50

Product Qualification

The PureLink™ Total RNA Blood Purification Kit is functionally qualified by isolating total RNA from 200 µl human whole blood as described in this manual and must produce the following results:

- $OD_{260/280}$ between 1.6-2.2
- Intact RNA as determined by visual inspection on an agarose gel

In addition, each kit component is free of ribonuclease contamination and is lot qualified for optimal performance.

Accessory Products

Additional Products

The following products are also available from Invitrogen. For more details on these products, visit our Web site at www.invitrogen.com or contact Technical Service (page 13).

Product	Quantity	Catalog no.
Reagents for RT-PCR		
SuperScript™ One-Step RT-PCR System with Platinum® <i>Taq</i> DNA Polymerase	100 reactions	10928-042
Platinum® Quantitative RT-PCR ThermoScript™ One-Step System	100 reactions	11731-015
LUX™ Fluorogenic Primer Set, FAM-labeled	50 nmol or 200 nmol	Design and order LUX™ Primer Sets at www.invitrogen.com/lux
LUX™ Fluorogenic Primer Set, JOE-labeled	50 nmol or 200 nmol	
Reagents		
RNase AWAY®	250 ml	10328-011
DNase I	20,000 units	18047-019
DNase I, Amplification Grade	100 units	18068-015
0.16-1.77 Kb RNA Ladder	75 µg	15623-010
0.24-9.5 Kb RNA Ladder	75 µg	15620-016
UltraPure™ DEPC-treated Water	1 L	750023
UltraPure™ DNase/RNase-Free Distilled Water	500 ml	10977-015
Quant-iT™ RNA Assay Kit	1000 assays	Q-33140

Introduction

Overview

The PureLink™ Total RNA Blood Purification Kit allows purification of high-quality total RNA from 50-500 µl fresh, whole mammalian blood.

The kit uses a leukocyte enrichment step (see below) and a non-phenol based purification system resulting in the isolation of high-quality total RNA that is suitable for use in any downstream application of choice (see next page).

Whole Blood RNA Purification

Whole blood consists of two major cell types, erythrocytes (red blood cells) and leukocytes (white blood cells).

Erythrocytes (red blood cells) are the most numerous blood cells with a concentration of 4–6 million/mm³ and are not nucleated in mammals (do not contain any nucleic acids).

Leukocytes (white blood cells) are less abundant than erythrocytes with a concentration of 5000–7000/mm³ and are nucleated (contain nucleic acids), unlike erythrocytes.

Thus, the blood total RNA purification is actually the purification of total RNA from leukocytes only.

Since whole blood contains a high number of non-nucleated erythrocytes, purifying total RNA from whole blood without the removal of erythrocytes results in low RNA yields and clogging of purification columns.

The depletion of abundant erythrocytes is therefore a key step in the purification of whole blood total RNA and is performed by selective lysis of erythrocytes using hypotonic shock. The erythrocytes are resuspended in a hypotonic buffer that causes an influx of water into erythrocytes and ruptures the erythrocyte cell membranes. Leukocytes are not affected by the hypotonic shock and are easily separated from lysed erythrocytes by centrifugation.

The enriched leukocytes are used for RNA purification as described on the next page.

Continued on next page

Introduction, Continued

System Overview

Leukocytes from whole blood are enriched as described in the previous page and then lysed using Lysis Buffer (L3) containing guanidine isothiocyanate, a chaotrope capable of protecting the RNA from endogenous RNases. Ethanol is added to the lysate and the lysate is processed through the spin cartridge. The RNA binds to the silica-based membrane in the spin cartridge and impurities are removed by thorough washing with Wash Buffers. The RNA is eluted in RNase free water.

Advantages

Using the PureLink™ Total RNA Blood Purification Kit offers:

- High quality total RNA purification from whole blood using non-phenol based purification
 - Designed to isolate total RNA from fresh whole blood samples from various mammalian species in <1 hour
 - Minimal genomic DNA contamination of the purified RNA
 - Reliable performance of the high-quality purified total RNA in downstream applications (see below)
-

Downstream Applications

Total RNA isolated using the PureLink™ Total RNA Blood Purification Kit is suitable for:

Direct Use	Use after reverse transcription
Northern blotting	RT-PCR
Nuclease protection assays	Real time quantitative PCR (qPCR)
Reverse transcription	

Specifications

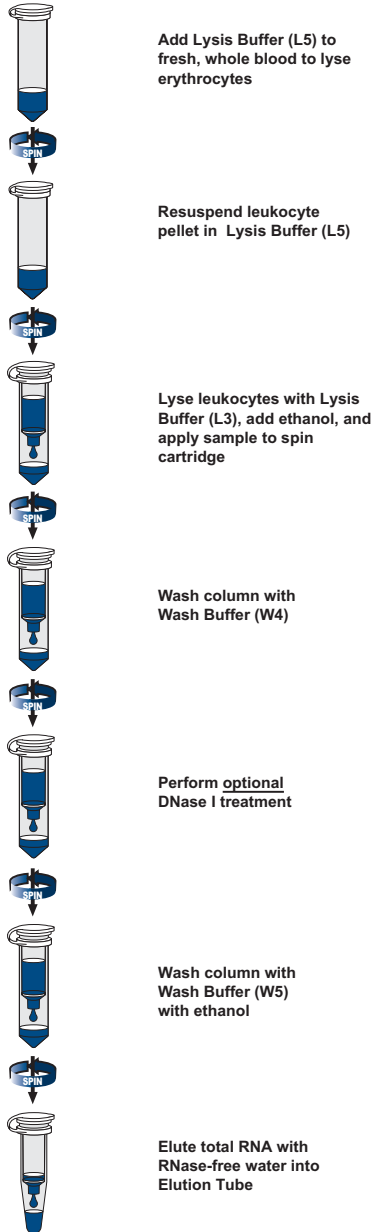
Starting Material:	50-500 µl whole blood (up to 3.5 x 10 ⁶ leukocytes)
Binding Capacity:	~1 mg nucleic acid
Column Reservoir Capacity:	700 µl
Wash Tube Capacity:	2.0 ml
Elution Tube Capacity:	1.7 ml
Centrifuge Compatibility:	Capable of centrifuging >8,000 x g

The RNA yield is dependent on the age of blood sample and conditions (certain disease states reduce the leukocyte count resulting in lower RNA yield).

Experimental Overview

Introduction

The flow chart describes the steps for isolating total RNA.



Isolating Total RNA

Introduction

Instructions are provided below to isolate total RNA from mammalian whole blood.



The PureLink™ Total RNA Blood Purification Kit buffers contain guanidine isothiocyanate. Always wear a laboratory coat, disposable gloves, and eye protection when handling solutions containing this chemical.

Do not add bleach or acidic solutions directly to solutions containing guanidine isothiocyanate or sample preparation waste. Guanidine isothiocyanate forms reactive compounds and toxic gases when mixed with bleach or acids.

Be sure to take the appropriate precautions (wear a laboratory coat, disposable gloves, and eye protection) when handling whole blood samples. Dispose of blood samples and washes during the purification procedure as biohazardous waste.

General Handling of RNA

Observe the following guidelines to prevent RNase contamination:

- Use disposable, individually wrapped, sterile plasticware
 - Use only sterile, new pipette tips and microcentrifuge tubes
 - Wear latex gloves while handling reagents and RNA samples to prevent RNase contamination from the surface of the skin
 - Always use proper microbiological aseptic techniques when working with RNA
 - Use RNase AWAY® Reagent (page viii) to remove RNase contamination from surfaces
-

Whole Blood Sample

The PureLink™ Total RNA Blood Purification Kit is designed to purify total RNA from **50-500 µl** of the following whole blood samples:

- Fresh whole blood from any mammalian species
- Fresh whole blood collected in the presence of anti-coagulants such as EDTA, heparin, or citrate

The kit is not designed for use with frozen whole blood samples.

Continued on next page

Isolating Total RNA, Continued



Follow the recommendations below to obtain the best results:

- Recommended whole blood volume is 50-500 μ l
 - Use Lysis Buffer (L5) for erythrocyte lysis and Lysis Buffer (L3) for leukocyte lysis
 - Pipet the RNase-free water in the center of the cartridge and perform a 1 minute incubation
 - Perform the DNase I digestion on the cartridge before eluting the RNA as performing the DNase I digestion after the purification will affect RNA integrity
-

Materials Needed

You will need the following items:

- Whole Blood Sample (see previous page)
 - Lysis Buffer (L5) for lysis of erythrocytes (supplied with the kit)
 - Lysis Buffer (L3) for lysis of leukocytes (supplied with the kit)
 - Wash Buffer (W4) (supplied with the kit)
 - Wash Buffer (W5) with ethanol (see below)
 - RNase-free water (supplied with the kit)
 - Spin Cartridges with collection tubes, Wash Tubes, and Elution Tubes (supplied with the kit)
 - Sterile, RNase-free tube (~15 ml) for performing erythrocyte lysis (next page)
 - 70% ethanol
 - Ice bucket
 - 96-100% ethanol
 - Centrifuge capable of centrifuging 15 ml tubes at 4°C
 - Microcentrifuge capable of centrifuging >8,000 x g
-

Before Starting

Add 40 ml 96–100% ethanol to 10 ml Wash Buffer (W5) included with the kit.

Store the Wash Buffer (W5) with ethanol at room temperature.

Continued on next page

Isolating Total RNA, Continued

Leukocyte Enrichment

1. To a sterile, RNase-free tube of appropriate size, add 5 volumes of Lysis Buffer (L5) to 1 volume of fresh whole blood sample (page 4 for blood sample).
For example, add 500 μl of Lysis Buffer (L5) to 100 μl fresh whole blood sample.
 2. Incubate for 10 minutes on ice. Vortex the tube briefly 2-3 times during the incubation step to allow complete lysis of erythrocytes. The solution turns translucent.
 3. Centrifuge the tube at 4°C at 400 \times g for 10 minutes. Remove the supernatant completely and discard the supernatant.
Do not discard the pellet as the pellet contains leukocytes.
 4. Resuspend the leukocyte pellet in 2 volumes of Lysis Buffer (L5). Mix well by vortexing briefly.
For example, use 200 μl Lysis Buffer (L5)/100 μl of whole blood from Step 1.
 5. Centrifuge the tube at 4°C at 400 \times g for 5 minutes. Remove the supernatant completely and discard the supernatant.
Do not discard the pellet as the pellet contains leukocytes. The leukocyte pellet should be white with no traces of red. If the pellet is significantly red, wash the pellet with Lysis Buffer (L5).
 6. Resuspend the leukocyte pellet in 350 μl Lysis Buffer (L3). Mix well by vortexing briefly to completely resuspend the pellet ensuring the absence of any cell clumps.
 7. Add 350 μl 70% ethanol to the tube and mix well by vortexing briefly.
 8. Proceed to **Binding RNA**, below.
-

Binding RNA

1. Remove a Spin Cartridge in a Collection Tube from the package. Transfer the leukocyte lysate from Step 7, above, to the Spin Cartridge.
 2. Centrifuge the Spin Cartridge at 8,000 \times g for 1 minute at room temperature.
 3. Discard the flow through and place the Spin Cartridge into the collection tube.
 4. Proceed to **Washing RNA**, next page.
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Isolating Total RNA, Continued

Washing RNA

1. Add 700 μl of Wash Buffer (W4) supplied in the kit to the Spin Cartridge.
 2. Centrifuge Spin Cartridge at $8,000 \times g$ for 30 seconds at room temperature.
 3. Proceed to DNase I digestion if you need to remove genomic DNA or proceed directly to Step 4.
Optional (DNase Digestion): To remove genomic DNA from the samples, add 80 μl of DNase I solution (page 12 for a recipe) to the Spin Cartridge. Incubate at room temperature for 15 minutes.
 4. Add 500 μl of Wash Buffer (W4) to the Spin Cartridge. If DNase I digestion is performed, incubate for 5 minutes at room temperature.
 5. Centrifuge the Spin Cartridge at $8,000 \times g$ for 30 seconds at room temperature. Discard the flow through.
 6. Add 500 μl of **Wash Buffer (W5) with ethanol** (page 5) to the Spin Cartridge.
 7. Centrifuge the Spin Cartridge at $8,000 \times g$ for 30 seconds at room temperature. **Repeat Step 7 once.**
 8. Discard the flow through and place the Spin Cartridge into the Wash Tube supplied with the kit and centrifuge the Spin Cartridge at $8,000 \times g$ for 1 minute at room temperature to remove any residual Wash Buffer (W5).
 9. Proceed to **Eluting RNA**, below.
-

Eluting RNA

1. Place the Spin Cartridge in a clean 1.7-ml Elution Tube supplied with the kit.
 2. Add 30-100 μl of sterile, RNase-free water (supplied with the kit) to the center of the cartridge.
 3. Incubate at room temperature for 1 minute. Centrifuge the Spin Cartridge at $8,000 \times g$ for 1 minute at room temperature.
 4. The elution tube contains your purified total RNA. Remove and discard the cartridge.
Based on the volume of elution buffer used for elution, the recovery of the elution volume will vary and is usually 95% of the elution buffer volume used.
 5. Store the total RNA at -80°C or use total RNA for the desired downstream application.
-

Determining the RNA Quality and Quantity

Introduction

Once you have isolated total RNA, you may determine the quantity and quality of the purified RNA as described below.

Estimating RNA Quantity

The quantity of the purified total RNA is easily quantitated using UV absorbance at 260 nm or Quant-iT™ RNA Assay Kit.

UV Absorbance

1. Dilute an aliquot of the total RNA sample in 10 mM Tris-HCl, pH 7.0. Mix well. Transfer to a cuvette (1-cm path length).

Note: The RNA must be in a neutral pH buffer to accurately measure the UV absorbance.

2. Determine the OD₂₆₀ of the sample using a spectrophotometer blanked against 10 mM Tris-HCl, pH 7.0.
3. Calculate the amount of total RNA using the following formula:

$$\text{Total RNA } (\mu\text{g}) = \text{OD}_{260} \times 40 \mu\text{g} / (1 \text{ OD}_{260} \times 1 \text{ ml}) \times \text{dilution factor} \times \text{total sample volume (ml)}$$

Example:

Total RNA was eluted in water in a total volume of 150 μl . A 40 μl aliquot of total RNA was diluted to 500 μl in 10 mM Tris-HCl, pH 7.5. An OD₂₆₀ of 0.188 was obtained. The amount of RNA in the sample is determined as shown below:

$$\begin{aligned} \text{Total RNA } (\mu\text{g}) &= 0.188 \times 40 \mu\text{g} / (1 \text{ OD}_{260} \times 1 \text{ ml}) \times 12.5 \times 0.15 \\ &= 14.1 \mu\text{g of total RNA} \end{aligned}$$

Quant-iT™ RNA Assay Kits

The Quant-iT™ RNA Assay Kit (page viii for ordering information) provides a rapid, sensitive, and specific method for RNA quantitation with minimal interference from DNA, protein, or other common contaminants that affect UV absorbance readings.

The kit contains a state-of-the-art quantitation reagent, pre-diluted standards for standard curve, and a ready-to-use buffer. The assay is performed in a microtiter plate format and is designed for reading in standard fluorescent microplate readers. Follow manufacturer's recommendations to perform the assay.

Continued on next page

Determining the RNA Quality and Quantity, Continued

Analyzing RNA Quality

Typically, RNA isolated using the PureLink™ Total RNA Blood Purification Kit has an $OD_{260/280}$ of >1.6 when samples are diluted in Tris-HCl (pH 7.5). An $OD_{260/280}$ of >1.6 indicates that RNA is reasonably clean of proteins and other UV chromophores (heme, chlorophyll, etc.) that could either interfere with downstream applications or negatively affect the stability of the stored RNA.

Agarose gel electrophoresis of RNA isolated using the PureLink™ Total RNA Blood Purification Kit shows the 28S to 18S band ratio to be >1.5 . RNA is judged to be intact if discreet 28S and 18S ribosomal RNA bands are observed. See next page for an example of expected results.

Gel analysis reveals if contaminating DNA is present either as a band at the well or between the well and 28S band or as some background smearing. Contaminating DNA is easily removed by treating the RNA samples with DNase I during purification.

The human ribosomal RNA sizes are 1.9 kb (18S) and 5.0 kb (28S).

The Next Step

Total RNA isolated using the PureLink™ Total RNA Blood Purification Kit is suitable for use in any downstream application of choice (RT-PCR, reverse transcription, and qPCR reactions) using kits available from Invitrogen (page viii) without the need to perform any additional steps.

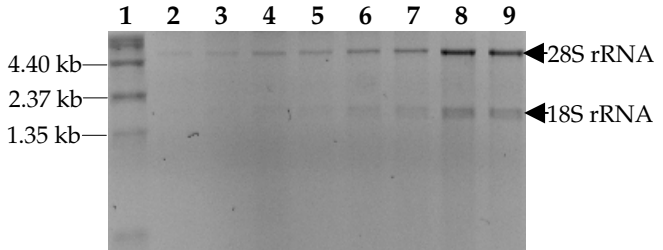
Expected Results

Results

The quality of total RNA obtained using the PureLink™ Total RNA Blood Purification Kit is shown below.

Total RNA (10 µl eluate) isolated from 50 µl (lanes 2, 3), 100 µl (lanes 4, 5), 200 µl (lanes 6, 7), and 500 µl (lanes 8, 9) of fresh human whole blood was analyzed by denaturing formaldehyde gel electrophoresis. The gel shows 28S and 18S bands in a ratio >1.5 with minimal DNA contamination.

Lane 1: 1 µl 0.24-9.5 Kb RNA Ladder



Troubleshooting

Introduction

Review the information below to troubleshoot your experiments with the PureLink™ Total RNA Blood Purification Kit.

Problem	Cause	Solution
Low RNA yield	Incomplete lysis or too much sample has clogged the filter	Avoid loading more than 500 µl whole blood or >3.5 x 10 ⁶ leukocytes on the Spin Cartridge. Follow the leukocyte enrichment protocol on page 6 to obtain the best results. Decrease the sample volume used, if cartridge is clogged or load the sample on 2 spin cartridges.
	RNA quantitation performed with water	Be sure the RNA quantitation using UV absorbance is performed with 10 mM Tris-HCl, pH 7.0 (page 8) to accurately measure the UV absorbance.
RNA degraded	RNA contaminated with RNase	Follow the guidelines on page 4 to prevent RNase contamination.
	Poor quality blood sample	Always use fresh whole blood samples for purification. Do not use frozen blood samples. Perform the leukocyte enrichment protocol at 4°C to prevent RNA degradation.
Genomic DNA contamination	--	Use optional DNase I digestion step included in the protocol to remove genomic DNA contamination. Avoid DNase I digestion after the RNA elution step.
Poor performance of RNA in downstream applications	Presence of ethanol in RNA	Traces of ethanol from the Wash Buffer (W5) may interfere with downstream applications. To remove Wash Buffer (W5), discard Wash Buffer (W5) flow through from the collection tube. Place the spin cartridge into a new Wash tube and centrifuge for 1 minute to completely dry the spin cartridge.

Recipes

DNase I Solution

DNase I is available from Invitrogen (catalog no. 18047-019). DNase I, Amplification Grade (catalog no. 18068-015) is also available from Invitrogen (see **Note** below).

Use freshly prepared DNase I solution.

You will need 80 μ l DNase I solution for each Spin Cartridge. Prepare 100 μ l DNase I solution as follows:

1. Prepare 10X DNase I Buffer using RNase-free water to yield the following final concentration:

200 mM Tris-HCl, pH 8.4

20 mM MgCl₂

500 mM KCl

2. To a sterile RNase-free tube, add:

10X DNase I Buffer (from Step 1)	10 μ l
DNase I (catalog no. 18047-019)	10 units
RNase-free water	to 100 μ l

3. Mix the contents and use this solution for DNase I digestion.
-



Note

DNase I, Amplification Grade (catalog no. 18068-015) is available from Invitrogen (page viii) and is supplied with a vial of 10X DNase I reaction buffer (200 mM Tris-HCl, pH 8.4, 20 mM MgCl₂, 500 mM KCl). If you are using this enzyme, use 10 units of enzyme for each reaction and there is no need to prepare the 10X DNase I buffer as described above.

Technical Service

World Wide Web



Visit the Invitrogen Web Resource using your World Wide Web browser. At the site, you can:

- Get the scoop on our hot new products and special product offers
- View and download vector maps and sequences
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Once connected to the Internet, launch your Web browser (Internet Explorer 5.0 or newer or Netscape 4.0 or newer), then enter the following location (or URL):

<http://www.invitrogen.com>

...and the program will connect directly. Click on underlined text or outlined graphics to explore. Don't forget to put a bookmark at our site for easy reference!

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Technical Service, Continued

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