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sample size or type
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About The Cover:
 Acetylcholine
 (neurotransmitter)
 crystals dissolving.
 Photograph by
 Spike Walker.

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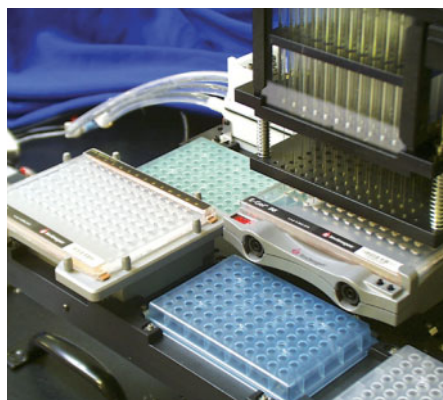


High-throughput electrophoresis using the Tango™ automated liquid handling system and the E-Gel® 96 system

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Abstract

Automating electrophoresis significantly reduces the time required for loading a large number of samples, increasing the speed and throughput of electrophoretic analysis. In addition, the reliability and consistency of automation maximizes resolving power and increases the precision of fragment separation. Here we demonstrate an automated, high-throughput method for simultaneously loading 96 samples onto an electrophoresis gel, using the Tango™ automated liquid handling system and the E-Gel® 96 system.



Loading an E-Gel® 96 gel using the Tango™ automated liquid handling system.

Introduction

Electrophoresis is a fundamental technique used to identify and separate DNA, RNA, and protein molecules. It is routinely used to quantify and determine the quality of samples prior to performing downstream applications. Here we demonstrate an automated, high-throughput method of simultaneously loading 96 samples onto an electrophoresis gel using the Tango™ system (Apogent Discoveries) and the Invitrogen E-Gel® 96 system. This procedure significantly reduces the time required for loading a large number of samples. Consequently, an increase in the overall speed of electrophoresis analysis is achieved. Furthermore, automation reduces sample-to-sample variability caused by errors in manually dispensing samples, resulting in an increase in the precision of electrophoresis analysis.

Materials and Methods

A Tango™ system (Apogent Discoveries, Sunnyvale, CA) equipped with 96, 100- μ l standard syringes with DuraFlex™ needles was used for liquid handling. The E-Gel® 96 2% agarose gel (Cat. no. G7008-02), E-Gel® 96 holder (Cat. no. G7300-01), E-Gel® 96 mother base (Cat. no. G7100-01), 10X BlueJuice™ Gel Loading Buffer (Cat. no. 10816-015), 0.24-9.5 Kb RNA Ladder (Cat. no. 15620-016) and E-Gel® 96 Low Range DNA Marker (Cat. no. 12369-013) were provided by Invitrogen Corporation, Carlsbad, CA. PCR core kit (Cat. no. 1 578 553) and human genomic DNA (Cat. no. 1 691 112) were purchased from Roche, Mannheim, Germany. A Human β -actin amplimer set (Cat. no. 5402-1) was purchased from Clontech

Laboratories, Palo Alto, CA. Samples were prepared in a skirted cycleplate-96 (Cat. no. 1047-20-0, Apogent Discoveries, Sunnyvale, CA) and loaded following the procedures outlined in the E-Gel® 96 High-Throughput Agarose Electrophoresis System manual.

Dispensing precision of the Tango™ liquid handling system. Prior to use of the Tango™ system, the uniformity and consistency of sample volumes dispensed across the 96 syringes used in this study were determined by measuring the coefficient of variance, C.V. (<http://www.robsci.com/hug.html>). A high uniformity for dispensing volumes equal to, and higher than, 100 nl was evident with C.V.s less than 5%.

Operating the Tango™ system for loading samples. The Tango™ system incorporates precision glass syringes (96 or 384) arrayed in standard SBS microplate spacing. The stage of the Tango™ system is composed of 12 nests. For this protocol, one nest was dedicated to the wash module, one to a reservoir containing 2% bleach, one to a reservoir containing deionized water, one to a skirted cycleplate-96 containing DNA or RNA samples (called the source plate), and one to an E-Gel® 96 gel (placed on the E-Gel® 96 holder). As many as four E-Gel® 96 holders can fit on the stage of the Tango™ system. In order to clean the syringes and prevent carry-over contamination, a Tango™ protocol was created that incorporated three water wash cycles and one wash cycle with 2% bleach, followed by an additional three water wash cycles, before and after loading the E-Gel® 96 gel

continued on page 4

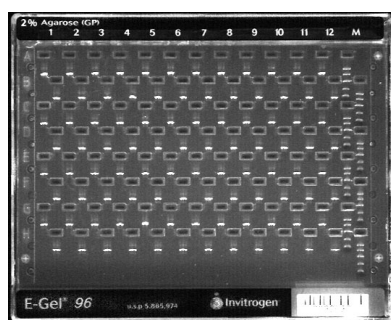
continued from page 3

(one “wash cycle” is defined as an aspiration and a dispense; in this instance, the wash volume was set at 20 μ l). To load the DNA/RNA samples, the Tango™ protocol called for pre-loading the 96 wells in the E-Gel® 96 gel with 10 μ l of water (a 5- μ l air gap, followed by 10 μ l of water was aspirated into the syringes and then emptied into the wells of the gel). Next, the samples (from the skirted cycleplate-96) were loaded onto the E-Gel® 96 gel (a 5- μ l air gap, followed by 10 μ l of sample was aspirated into the syringes and then emptied into the wells of the gel). Once the samples were loaded, the E-Gel® 96 gel was transferred onto the E-Gel® 96 mother base to begin electrophoresis. Electrophoresis was complete in 12 minutes. Gel results were visualized and photographed under ultraviolet light.

Results and Discussion

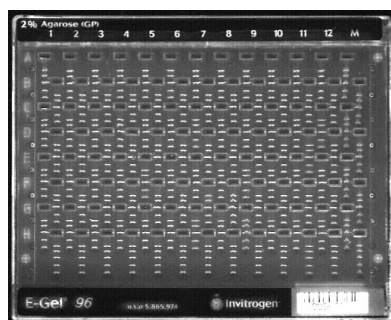
Using the Tango™ system, DNA and RNA samples were loaded onto E-Gel® 96 gels. The loading time, including the time required for the process of priming (trial dispensing required higher dispensing precision) was approximately 15 seconds. Figure 1A shows that DNA samples loaded resolved as a single, sharp, high-quality band. Figure 1B demonstrates the exceptional quality of separation between different lengths of DNA fragments. No electrophoresis flaws such as diffusion of the sample, smearing, or tailing were detected. In addition to the loading of DNA samples, polyA-tailed RNA samples* were also successfully loaded onto the E-Gel® 96 gel using the Tango™ system. As indicated in Figure 1C, no RNA degradation was observed when samples were loaded with the Tango™ system. These results demonstrate a simple,

Figure 1 – Electrophoresis results using the Tango™ automated liquid handling system and the Invitrogen™ E-Gel® 96 system



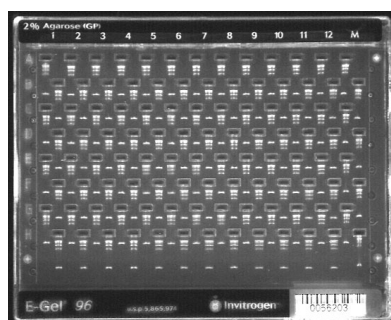
A: Sharp resolution

Sample: 1 μ l (50 ng) of β -actin PCR product (length: 838 bp), DNA marker (M): E-Gel® 96 Low Range DNA Marker.



B: Clear separation

Sample: E-Gel® 96 Low Range DNA Marker (90 ng).



C: RNA remains intact

Sample: 150 ng of 0.24-9.5 Kb RNA ladder.

fast, and precise method for automating electrophoresis using the E-Gel® 96 system on a robotic platform.

Conclusion

The Tango™ system and the Invitrogen™ E-Gel® 96 system work together to provide a fast, simple, precise, and automated

method for simultaneously loading and analyzing a large number of samples for high-throughput electrophoresis. ■

*E-Gels® are not guaranteed to be RNase-free.

E-Gel® is subject to Limited Use Label License No. 61. Please refer to the Invitrogen web site or catalog for the corresponding Limited Use Label License.

The ZOOM® IPGRunner™ System: simplified 2D gel electrophoresis

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Abstract

Two-dimensional (2D) gel electrophoresis is a technique that separates proteins based on two independent characteristics: charge and molecular weight. Proteins are first separated based on their intrinsic charge in the first dimension by isoelectric focusing (IEF) and then separated based on their mass in the second dimension by SDS-PAGE. The result of using these two independent separation techniques is a high degree of resolution within a protein population. The conventional 2D gel technique is costly, tedious, and time-consuming. The ZOOM® IPGRunner™ System provides a simple and fast method for performing first dimension isoelectric focusing without the use of mineral oil. Electrophoretic separation in both the first dimension using 7 cm ZOOM® immobilized pH gradient (IPG) strips and the second dimension using NuPAGE® Novex 4-12% Bis-Tris ZOOM® Gels can be completed in a total of three hours.

Introduction

Separation of proteins by two-dimensional (2D) gel electrophoresis is the gold standard whereby significant differences in biological samples can be analyzed with consequences for the diagnosis of disease, discovery of new drug targets, and analysis of underlying environmental and drug toxicities. Two-dimensional (2D) gel electrophoresis separation techniques take advantage of the electrophoretic mobilities of individual constituents of a complex mixture of proteins, fractionating by charge in one dimension and mass in the other. 2D gel electrophoresis coupled with mass spectrometry is the central technology of the emerging field of proteomics.

To date, the 2D electrophoresis technique has only been available on expensive, dedicated instrumentation requiring a large laboratory footprint and specialty training. To ensure reproducible results, extended periods of time and technical expertise were needed.

The ZOOM® IPGRunner™ System provides a new method for conducting isoelectric focusing experiments that greatly simplifies and speeds up the process for performing 2D separations. Isoelectric focusing is performed on 7-cm immobilized pH gradient (IPG) strips in a disposable cassette. The ZOOM® IPGRunner™ Cassette accommodates up to six IPG strips and does not require a mineral oil overlay. The strips are rehydrated directly in the cassette and focusing takes place in a vertical format gel electrophoresis mini-cell using a specially designed electrophoresis core. The apparatus is easy to set up, requiring very

little time. Up to 12 strips can be run simultaneously in a mini-cell using two ZOOM® IPGRunner™ Cassettes. The apparatus is designed for optimal electrical efficiency, leading to focusing times of as little as 1.5 hours (Figure 1, page 6). The second dimension separation is performed in ZOOM® Gels that contain a well for a protein standard and a second well that accommodates the 7-cm IPG strip. The second dimension SDS-PAGE step takes only 40-45 minutes (Figure 1, page 6) when using the NuPAGE® Novex Bis-Tris ZOOM® Gels. These gels provide the same highly reproducible performance and excellent resolution as Novex® minigels. Dozens of 2D gels can be run in a single day. The simplicity of the system makes 2D gel electrophoresis accessible to novice users.




Methods

First Dimension IEF. Rehydration of ZOOM® Strips, pH 4-7 (Invitrogen, Cat. no. ZM0012) was carried out in the ZOOM® IPGRunner™ Cassette (Invitrogen, Cat. no. ZM0003). The ZOOM® IPGRunner™ Cassette was placed on the bench top with the windows in the cover film facing upwards. Rehydration buffer (155 µl) containing 2 M Thiourea, 7 M urea, 0.5% ZOOM® Carrier ampholytes (Invitrogen, Cat. no. ZM0022), 2.0% CHAPS, 20 mM DTT and an *E. coli* lysate was pipetted into the IPGRunner™ cassette window. The acidic (+) end of the strip was inserted into the slot with the gel side facing toward the film or cover side of the cassette. Sample wells were sealed with sealing tape (included in the kit) to provide an airtight environment for rehydration. Strips were rehydrated

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Figure 1 – ZOOM® IPGRunner™ System

Step	Procedure	Time
1	Apply sample, insert IPG strips, and seal loading wells 	10 minutes
2	Rehydrate strips	incubate overnight
3	Remove wells, apply wicks, and assemble the IPGRunner™ Mini-Cell	5 minutes
4	Perform isoelectric focusing 	run 90 minutes
5	Reduce, alkylate, and insert strips into a ZOOM® gel	35 minutes
6	Perform SDS-PAGE 	run 40 minutes
7	Stain gel using the SilverQuest™ Silver Staining Kit or SimplyBlue™ SafeStain	90 or 45 minutes

for 8-16 hours at room temperature. Six IPG strips were run in each IPGRunner™ Cassette. Following rehydration, the tape and sample wells were removed, an electrode wick was placed at each end of the IPGRunner™ Cassette, and the wick was wetted with 750 µl of deionized water. Excess water was removed by blotting with filter paper so that the wicks were damp but not wet. The cassettes and buffer core were assembled and inserted into the ZOOM® IPGRunner™ Mini-Cell (Invitrogen, Cat. no. ZM0001). The mini-cell outer chamber was filled with 650 ml of water. Isoelectric focusing was performed using a power supply with a 50 µA/strip current limit and a 0.1 W/strip power limit and the following voltage steps:

1. 175 V for 15 minutes
2. 175-2000 V ramp for 45 minutes
3. 2000 V for 30 minutes

Following isoelectric focusing, the ZOOM® IPGRunner™ Mini-Cell was disassembled. The film cover on the cassette was removed and the ZOOM® Strips equilibrated for second dimension analysis.

Second Dimension SDS-PAGE. Second dimension electrophoresis was performed using NuPAGE® Novex 4-12% Bis-Tris ZOOM® Gels. Equilibration for the second dimension was carried out in NuPAGE® LDS Sample Buffer in the presence of reducing and alkylating agents. Alkylation of the sulfhydryl groups of the proteins reduces vertical streaking due to reformation of disulfide bonds and the presence of residual DTT during SDS electrophoresis. The ZOOM® Strips were incubated for 15 minutes in 5 ml

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1X NuPAGE® LDS Sample Buffer containing 50 mM DTT followed by incubation for 15 minutes in 1X NuPAGE® LDS Sample Buffer containing 125 mM iodoacetamide. Each IPG strip was then placed into a ZOOM® Gel well and sealed in the well with 400 µl of 0.5% agarose solution prepared in the appropriate running buffer. Molecular weight standards (Mark12™ Unstained Protein Standard, Invitrogen, Cat. no. LC5677) were loaded in the molecular weight marker well. The strips were run on NuPAGE® Novex 4-12% Bis-Tris ZOOM® Gels (Invitrogen, Cat. no. NP0330) in the XCell SureLock™ Mini-Cell (Invitrogen, Cat. no. EI001) according to standard protocol. Electrophoresis was performed at 200 V for 45 minutes.

Staining. 2D gels were stained using the SilverQuest™ Silver Staining kit (Invitrogen, Cat. no. LC6070) or SimplyBlue™

SafeStain (Invitrogen, Cat. no. LC6060) following standard procedures.

Results and Discussion

The electrophoresed gels in Figure 2 are *E. coli* lysate run as described in the flow chart in Figure 1. The total time for 2D separation and staining was 3 hr 35 min for the gel stained with SimplyBlue™ SafeStain and 4 hr 20 min for the gel stained using the SilverQuest™ Kit. For both gels, total run time for first and second dimension separation was 2 hrs 50 min. The isoelectric focusing time is shortened to 1.5 hrs with a total of ~1300 volt-hours compared to the 2-8 hours with a total of 6,000-20,000 volt-hours needed to focus proteins in conventional oil-immersion systems. The ZOOM® IPGRunner™ apparatus eliminates sources of resistance in the circuit so that the voltage applied across the strip is closer to the actual voltage output from the

power supply. The efficiency of this electrical system allows complete focusing using a voltage of 2000 volts or less.

The time-saving benefit of the ZOOM® IPGRunner™ System, in combination with the convenience of a disposable cassette, offers the user the ability to run numerous samples per day in the first dimension. Focused strips can be batch analyzed or stored for a later second dimension run by keeping them in the cassette at -80°C, without removing the film cover.

Because of its small footprint, the IPGRunner™ Mini-Cell can be stored and run on any bench top, eliminating the need for a dedicated IEF station. The simplicity of the ZOOM® IPGRunner™ process makes 2D electrophoresis technology accessible to everyone. Transfer of skills and training is simple, making any laboratory function more effective and efficient.

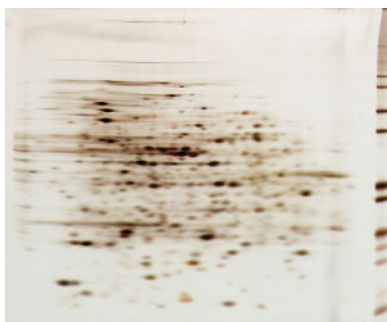
The ZOOM® IPGRunner™ System fulfills the need for day-to-day reproducibility and technical simplicity in 2D gel electrophoresis. This permits every researcher to have access to this powerful technique on their bench top. ■

Figure 2 – Stained 2D gels run with the ZOOM® System

A: Coomassie®-stained gel



B: Silver-stained gel



The total run time for both dimensions of 2D electrophoresis is 2 hr 50 min. Staining time for SimplyBlue SafeStain™ Coomassie stain is 45 min and for SilverQuest™ Silver stain is 1 hr 30 min.

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MagicMark™ and SeeBlue® Protein Standards can be run together in the same lane by following a few simple guidelines

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James Frazier*

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Introduction

Researchers running and blotting protein gels currently face the problem of having to choose between running a pre-stained protein standard to monitor the gel run and running a different standard for detection on a western blot. While these two standards can be run in adjacent lanes, this wastes valuable gel space that is better utilized by samples. In this paper we discuss a method for running a pre-stained protein standard (SeeBlue® Protein Standard) and western blot standard (MagicMark™ Western Protein Standard) in the same lane while maintaining the integrity and resolution of both markers.

Methods and Materials

All components used are from Invitrogen, with the exception of the ECL™ detection system from Amersham Biosciences.

Protein standards. The MagicMark™ Western Protein Standard (Cat. no. LC5600) and SeeBlue® Pre-Stained Protein Standard (Cat. no. LC5625) were used in this study. The MagicMark™ Western Standard proteins are expressed from a construct containing repetitive units of a fusion protein forming the size variation and an IgG binding site. This IgG binding site allows the MagicMark™ standard bands to bind antibodies from a wide variety of species*. The SeeBlue® Pre-Stained Standard contains nine blue-stained proteins that can be seen during an electrophoresis run and after transferring to a membrane.

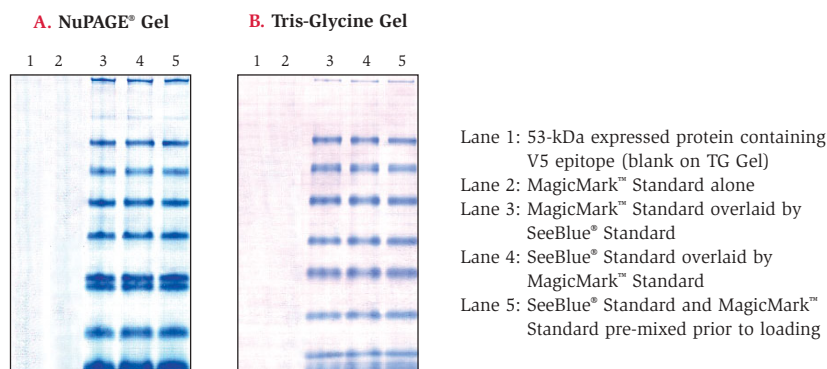
Loading the standards. In all cases except where noted, 5 µl of SeeBlue® Standard and 5 µl of MagicMark™ Standard were loaded. Lanes containing both standards were loaded in three different ways. In the first, MagicMark™ Standard was loaded and then SeeBlue® Standard was added on top in the same well. In the second, the SeeBlue® Standard was loaded first and then the MagicMark™ Standard was loaded on top. In the third, the MagicMark™ and SeeBlue® Standards were mixed together and then loaded onto the gel. The amount of time that the standards were mixed showed no effect. Samples were run on both NuPAGE® 4-12% Bis-Tris gels (MES) and Novex® 4-20% Tris-Glycine gels. For comparison, the MagicMark™ Standard was loaded into a separate lane along with a 53-kDa expressed protein containing the V5 epitope.

Electrophoresis and blotting conditions. The gels were run in the XCell SureLock™ Mini-Cell. Samples were transferred onto 0.45 µm nitrocellulose membrane using the XCell II™ Blot Module following standard protocols from the product manuals. The blots were then blocked and probed with a 1° Anti-V5 antibody (Invitrogen, Cat. no. R960-25) at a 1:5000 dilution. Blotted bands were detected using either the WesternBreeze® kit (anti-mouse) (Invitrogen, Cat. no. WB7104) or ECL™ kit. All detection experiments were performed according to the manufacturer's protocol.

Results

The results of these experiments illustrate that both the MagicMark™ Standard and the SeeBlue® Standard can be loaded into the same gel lane and still resolve bands clearly. The general progression of the

Figure 1 – The transferred blots, as they appear post-transfer

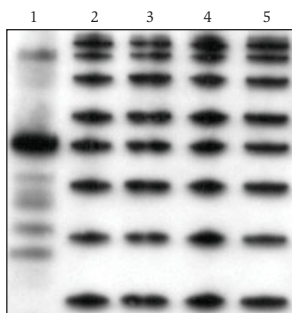


Standards and an expressed protein containing a V5 epitope were loaded onto a NuPAGE® 4-12% Bis-Tris Gel (A) and a 4-20% Tris-Glycine Gel (B) and then transferred onto a 0.45 µm nitrocellulose membrane according to Invitrogen's product instruction manuals.

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continued from page 8

Figure 2 – The transferred NuPAGE® blot immunodetected with the WesternBreeze® Chemiluminescent Kit



Immunodetection results of blot A in Figure 1 using chemiluminescence according to manufacturer's protocol.

experiments is shown in Figures 1 through 3. Figure 1 (page 8) shows the transfer from both the NuPAGE® and Tris-Glycine gels prior to immunodetection. The blue bands correspond to the SeeBlue® Pre-Stained Protein Standard. In Figures 2 and 3, the same membranes are shown after immunodetection with the WesternBreeze® Chemiluminescent Kit. Blots developed using the ECL™ System are not shown.

Band shape. An interesting effect regarding band shape is observed. In lane 3 of both Figures 2 and 3, where the MagicMark™ Standard was loaded first and the SeeBlue® Standard layered on top, there is a slight pinching-in that runs down the center of the MagicMark™ bands. Likewise, when the SeeBlue® Standard was added first and the MagicMark™ Standard layered on top (lane 4), the MagicMark™ Standard bands balled up into a shape somewhat resembling a

football. This effect was seen in both the NuPAGE® gels and the Tris-Glycine gels. Interestingly, when the standards are pre-mixed (lane 5), the MagicMark™ bands remain sharp and resemble those of lane 2 (MagicMark™ Standard alone). This effect was also observed in blots detected using the ECL™ System.

Chromogenic detection. The same NuPAGE® blot used in Figure 2 was also developed with the substrate in the WesternBreeze® Chromogenic Kit—Anti-Mouse (Invitrogen, Cat. no. WB7103) (Figure 4, page 10) following the chemiluminescent detection. This blot shows that although the SeeBlue® Standard is not affected either in shape or intensity (lanes 3 through 5), the MagicMark™ bands are distorted in the overlaid (non-premixed) lanes (lanes 3 and 4).

Band intensity. A significant difference in the band intensities appeared on the Tris-Glycine gel only. In the NuPAGE® gel (Figures 2 and 4), there is no decrease in signal intensity between the MagicMark™ bands in lane 2 and the pre-mixed MagicMark™ and SeeBlue® bands in lane 5. However, in the Tris-Glycine gel (Figure 3), lane 2 (MagicMark™ Standard alone) is noticeably more intense than lane 5 (pre-mixed). As the amount of SeeBlue® Standard is increased from 5 μ l to 10 μ l, this band intensity effect becomes more pronounced. In fact, at the 10- μ l level, slightly less intense MagicMark™ bands are apparent in the pre-mixed lanes in NuPAGE® gels as well. To isolate the specific part of the SeeBlue® Standard that is contributing to this effect, we loaded the SeeBlue® loading buffer (without the

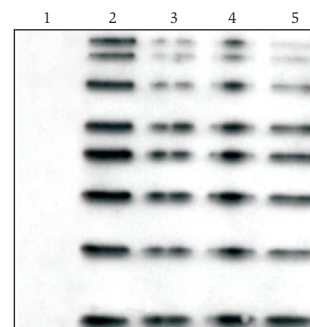
SeeBlue® peptides) pre-mixed with MagicMark™ Standard onto a NuPAGE® 4-12% Bis-Tris and a Novex® 4-20% Tris-Glycine Gel. It is observed that the buffer alone lanes do not show significant reduction in signal intensity on the MagicMark™ bands. This effect appeared in blots detected with both the WesternBreeze® Kit and ECL™ detection system (data not shown).

Band intensity recovery. Figure 5 (page 10) shows the recovery of MagicMark™ band intensity by adding increasing amounts of MagicMark™ Standard on a 4-20% Tris Glycine gel. For most applications, adding 2X more MagicMark™ Standard will recover sufficient band intensity (See lanes 1 and 4 of Figure 5).

Discussion

The results of this study indicate that a pre-stained standard such as the SeeBlue® Standard can be utilized in the same well

Figure 3 – The transferred Tris-Glycine blot immunodetected with the WesternBreeze® Chemiluminescent Kit



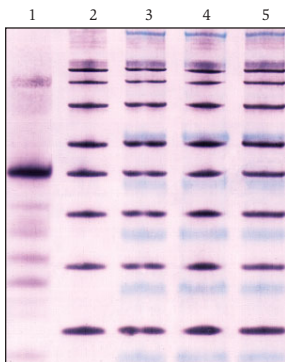
Immunodetection results of blot B in Figure 1 using chemiluminescence according to manufacturer's protocol.

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as the MagicMark™ Western Protein Standard so long as certain parameters are followed. Limiting the SeeBlue® loading volume to 5 µl, pre-mixing the SeeBlue® and MagicMark™ Standards and running on NuPAGE® gels gave the best results. If Tris-Glycine gels are to be utilized, then additional MagicMark™ Standard needs to be used to yield the equivalent signal that one would achieve with the MagicMark™ Standard alone. The data in Figure 5 indicate that the signal can be recovered to starting levels for the majority of the MagicMark™ bands in Tris-Glycine gels by loading 2X the volume of

Figure 4 – The transferred NuPAGE® blot detected with WesternBreeze® Chromogenic Kit



Immunodetection results using chromogenic substrate according to the WesternBreeze® kit manual.

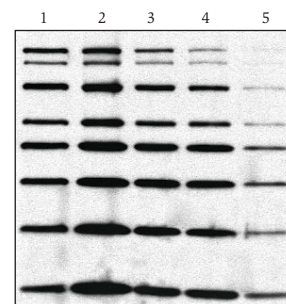
- Lane 1: 53-kDa expressed protein containing V5 epitope
- Lane 2: MagicMark™ Standard alone
- Lane 3: MagicMark™ Standard overlaid by SeeBlue® Standard
- Lane 4: SeeBlue® Standard overlaid by MagicMark™ Standard
- Lane 5: SeeBlue® Standard and MagicMark™ Standard pre-mixed prior to loading

MagicMark™ Standard (10 µl MagicMark™ + 5 µl SeeBlue®).

The reason for the anomalous band shapes in the lanes where one standard was simply layered upon the other likely stems from the differing buffer densities and composition in which the two standards are provided. In the lanes where the MagicMark™ signal is somewhat pinched in the middle (Figures 2 and 3, lane 3), the SeeBlue® buffer pushed down into the middle of the pre-loaded MagicMark™ band. Likewise, in lane 4 of these same gels (and in lane 4 of Figure 4) where the balling effect on the MagicMark™ bands is observed, the MagicMark™ bands can be actually be seen to fall into the pre-loaded SeeBlue® buffer and form a ball in the center. Interestingly, the SeeBlue® Standard itself does not seem similarly affected. When the standards are pre-mixed, this band distortion effect disappears.

The second effect is the diminishment in signal intensity observed in Tris-Glycine gels with the SeeBlue® Standard, but not in NuPAGE® gels. This observed drop in signal when the two standards are loaded together led to the question: what factors contribute to this diminished signal? At the lower volumes of SeeBlue® Standard normally used in western blots (5 µl or less), the MagicMark™ bands show no loss of signal intensity in NuPAGE® gels and a noticeable loss of band intensity in Tris-Glycine gels. Thus, the intensity drop is not a quenching of the chemiluminescent detection. Our first inclination was to believe that the sample buffer in the SeeBlue® Standard was the contributing factor.

Figure 5 – Recovery of MagicMark™ band intensity on a 4-20% Tris-Glycine Gel



Transferred Tris-Glycine gel. Immunodetection performed with WesternBreeze® Anti-Mouse Chemiluminescent Kit. All Lanes contain 5 µl of SeeBlue® Standard pre-mixed with differing volumes of MagicMark™ Standard.

- Lane 1: MagicMark™ Standard alone
- Lane 2: 5 µl SeeBlue® Standard pre-mixed with 20 µl MagicMark™ Standard
- Lane 3: 5 µl SeeBlue® Standard pre-mixed with 15 µl MagicMark™ Standard
- Lane 4: 5 µl SeeBlue® Standard pre-mixed with 10 µl MagicMark™ Standard
- Lane 5: 5 µl SeeBlue® Standard pre-mixed with 5 µl MagicMark™ Standard

To answer this question, we mixed MagicMark™ with SeeBlue® and the SeeBlue® buffer. The answer was surprising in that the SeeBlue® buffer alone lanes showed a minimal effect on MagicMark™ band intensity. However, the complete SeeBlue® Standard caused the significant band decreases previously observed. Although, the NuPAGE® gel also showed some effect from the SeeBlue® Standard, this was only evident at the 10 µl level, and was much less significant than what was observed on the Tris-Glycine gel. ■

* For more information on the MagicMark™ Western Protein Standard, visit www.invitrogen.com.

High specificity and robustness of AccuPrime™ *Taq* provides the ideal tool for demanding miniaturized, multiplex, and high-throughput PCR

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Introduction

In the post-sequenced genomic era, Polymerase Chain Reaction (PCR) is one of the most utilized techniques for analysis of the information encoded in the human genome. To accurately study particular stretches of genomic DNA or open reading frames in genomic DNA, sequence specificity must be maintained.

AccuPrime™ *Taq* DNA polymerase is a powerful and efficient tool proven to consistently provide the highest levels of sensitivity and specificity in demanding PCR applications. In this paper, we describe the use of AccuPrime™ *Taq* DNA polymerase to amplify DNA sequences accurately in various applications, including PCR miniaturization, multiplex PCR, and high-throughput colony screening.

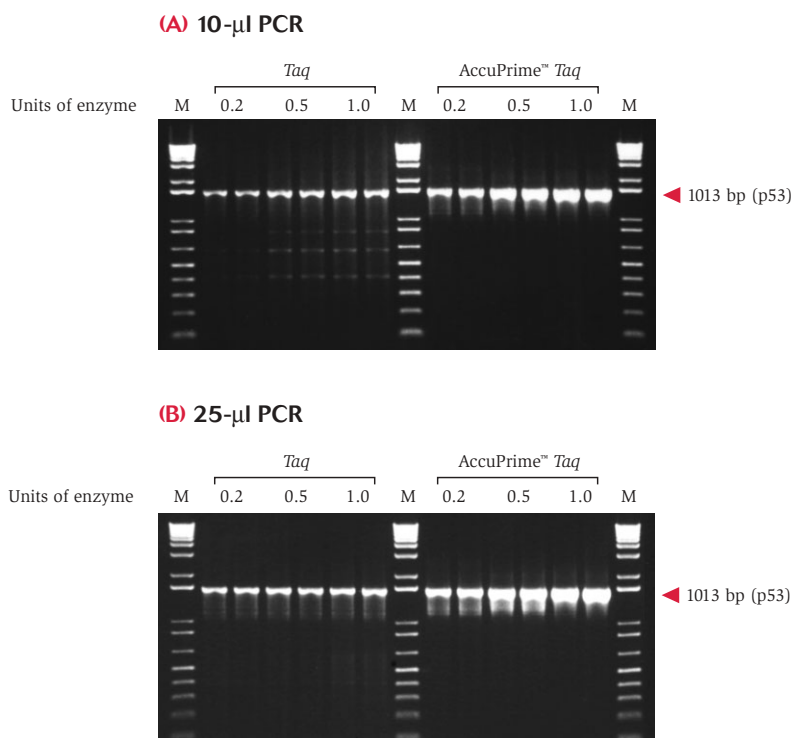
Methods

PCR miniaturization. PCR amplification reactions were performed in 10- μ l or 25- μ l volumes using 0.2, 0.5, and 1 unit of *Taq* DNA polymerase or AccuPrime™ *Taq* DNA polymerase (Invitrogen, Cat. no. 12339-016). Components were proportionally reduced in volume for each reaction, with concentrations of 0.2 μ M primers, 200 mM each dNTP, 1X PCR buffer, and 1.5 mM MgCl₂. Cycling conditions were 94°C, 2 min pre-incubation followed by 35 cycles of 94°C, 15 s; 55-60°C, 30 s; and 68°C, for 1 min/kb. Twenty percent of each amplification reaction was analyzed by electrophoresis with 0.5X TBE 1.2% agarose containing 0.5 μ g/ml ethidium bromide.

Multiplex PCR. PCR amplifications were prepared using 2, 5, or 10 units of hot-start *Taq* or AccuPrime™ *Taq* DNA Polymerase under standard PCR conditions in a 50- μ l reaction. A mix of 20 different primer pairs targeting amplicons ranging in length from 44 to 469 base pairs were prepared in a single reaction

tube and amplified. Cycling conditions were 94°C, 2 min pre-incubation followed by 35 cycles of 94°C, 15 s; 55-60°C, 30 s; and 68°C, for 1 min/kb. Twenty percent of each amplification reaction was analyzed by electrophoresis with a 0.5X TBE-3% agarose containing 0.5 μ g/ml ethidium bromide.

Figure 1 – PCR miniaturization using AccuPrime™ *Taq* DNA polymerase



Unlike *Taq* DNA polymerase alone, AccuPrime™ *Taq* DNA polymerase functions efficiently regardless of the reaction volume and the amount of the enzyme. AccuPrime™ *Taq* enzyme amounts can be lowered proportionally to the reaction volume without losing the robustness or specificity of the reaction. M: 1 Kb Plus DNA Ladder.

continued on page 12

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High-throughput PCR. AccuPrime™ *Taq* DNA polymerase was compared to hot-start *Taq* DNA polymerase in a high-throughput colony PCR screening application. pUC19 was selected as the target DNA sequence. PCR was performed in a 96-well microtiter plate for 18 cycles using either 2 units of AccuPrime™ *Taq* DNA polymerase or 2 units of hot-start *Taq* DNA polymerase. PCR cycling parameters were 94°C for 2 min, followed by 18 cycles of 94°C for 15 s; 55°C for 30 s; and 68°C for 3 min. The PCR products were

analyzed by electrophoresis with 0.5X TBE 0.8% agarose containing 0.5 µg/ml ethidium bromide.

Results and Discussion

PCR miniaturization. A typical result from PCR miniaturization with AccuPrime™ *Taq* is shown in Figure 1 (page 11). The target was a 1013 base pair sequence of the p53 gene. As a direct comparison, equal amounts of *Taq* polymerase were used in similar reactions. In a 10-µl reaction, AccuPrime™ *Taq* polymerase produced

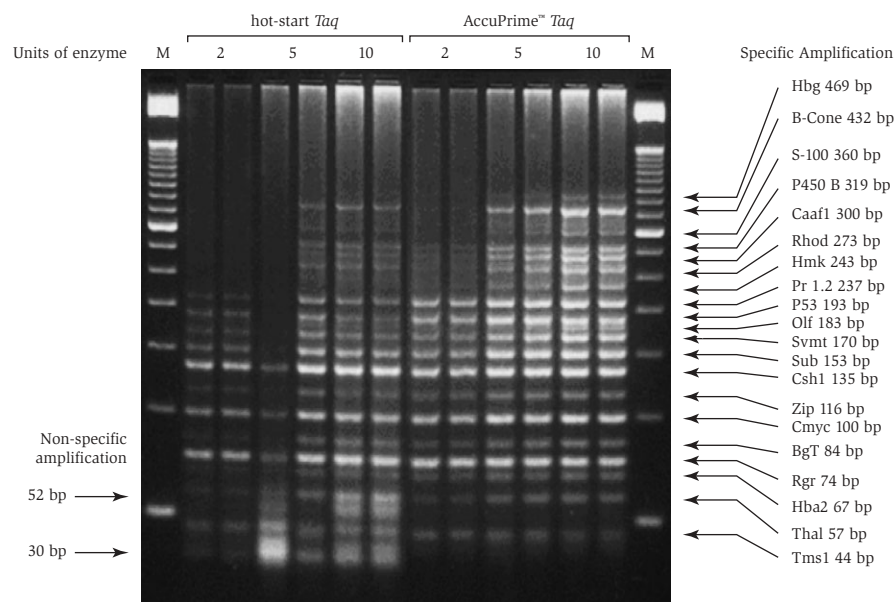
more product than *Taq* polymerase, using as little as 0.2 units, or approximately 2.5 times less enzyme than *Taq* polymerase, (Figure 1A). Even as enzyme units were gradually increased, specificity was maintained with AccuPrime™ *Taq*. However, non-specific priming was observed with increasing units of *Taq* polymerase, (Figure 1A).

The specificity of *Taq* polymerase improved with an increase in reaction volume (Figure 1, panels A and B), regardless of reaction volume. Even with this increase, AccuPrime™ *Taq* maintained its superiority over *Taq* regarding yield and specificity. Unlike *Taq*, the amount of the specific product increased proportionally to the amount of AccuPrime™ *Taq* added, indicating that the enzyme had not yet reached its performance plateau under the conditions tested.

Multiplex PCR. Multiplex PCR is a highly desirable tool for detecting several genes within a single reaction. However, due to the high degree of complexity, extensive optimization is usually required. Using standard PCR conditions, we compared the amplification of 20 different target sequences using either hot-start *Taq* or AccuPrime™ *Taq* at 2, 5, or 10 units of enzyme (Figure 2). AccuPrime™ *Taq* was able to amplify all 20 distinct targets, while hot-start *Taq* failed to amplify some of the targets and also produced non-specific bands.

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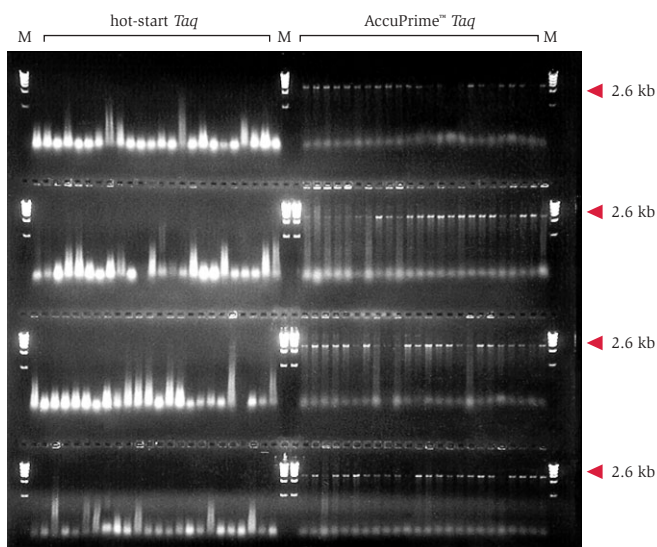
Figure 2 – Multiplex PCR results using hot-start *Taq* and AccuPrime™ *Taq* DNA polymerase



Comparison of multiplex PCR performance between hot-start *Taq* and AccuPrime™ *Taq* for 20 sets of primers with varying amounts of polymerase. Only AccuPrime™ *Taq* amplified all 20 amplicons. M: 50 bp DNA Ladder.

continued from page 12

Figure 3 – Comparison of high-throughput screening between hot-start *Taq* and AccuPrime™ *Taq*



Comparison of high-throughput PCR screening with hot-start *Taq* and AccuPrime™ *Taq*. Colonies from an agar plate were sampled with pipette tips and mixed with PCR amplification mixes. An 18 cycle PCR was used. Only AccuPrime™ *Taq* DNA polymerase successfully amplified the specific target in greater than 90% of the 96 colonies. The high specificity of AccuPrime™ *Taq* DNA polymerase promoted the high success rate of this high-throughput application. M: High DNA Mass™ Ladder.

Acknowledgements

We thank Sharon Cates, Lori Lebruska, and Patrick Gilles for their critical reading of the manuscript and helpful advice.

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High-throughput PCR. AccuPrime™ *Taq* can also be used in high-throughput colony PCR screening applications (Figure 3). We performed colony PCR screening with either hot-start *Taq* DNA or AccuPrime™ *Taq* polymerase with bacteria cells grown on a solid agar plate. The target was a 2.6 kilobase pair sequence of the pUC19 plasmid. The bacterial cells were touched with pipette tips, mixed directly with reaction mixtures, and amplified in an 18-cycle PCR. The high specificity of AccuPrime™ *Taq* poly-

merase allowed for more than 90% of the 96 reactions to be successfully amplified.

Conclusion

AccuPrime™ *Taq* DNA polymerase consistently outperforms hot-start *Taq* in all of the tested applications. Providing for unparalleled performance in sensitivity, specificity, and yield, AccuPrime™ *Taq* is the most suitable enzyme for delivering accurate and reliable performance in highly demanding applications as miniaturized PCR, multiplex PCR, or high-throughput PCR. ■

Optimized RT-PCR with Invitrogen™ Cloned AMV Reverse Transcriptase

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Introduction

Avian Myeloblastosis Virus reverse transcriptase (AMV RT) has been used to synthesize cDNA from mRNA since 1972 (1). The enzyme is widely used, particularly in RT-PCR applications, because of its relatively high thermostability and processivity (2). Expression of active, cloned retroviral avian RT has lagged behind that of murine RT, primarily due to the complex subunit structure of the avian enzyme. AMV RT has been recently cloned and over-expressed in a fully active form in both baculovirus-infected insect cells and yeast (2-4). In this article, we describe the use of Invitrogen™ Cloned AMV RT under optimized reaction conditions in RT-PCR applications.

Methods

Reverse transcriptases. Cloned AMV RT was over-expressed in, and purified from, insect cells infected with recombinant baculovirus or *Saccharomyces cerevisiae* in a subunit structural form identical to native AMV RT (2). Other native AMV RT preparations were obtained from commercial suppliers.

RNA. Total RNA was isolated from HeLa cells using TRIzol® Reagent (5).

RT-PCR. cDNA synthesis reactions were performed at 45-50°C for 60 min. (except where noted otherwise) and consisted of 2.5 µM oligo(dT)₂₀, 1 pg to 1 µg of HeLa RNA, 50 mM Tris-acetate (pH 8.4), 75 mM K-acetate, 8 mM Mg-acetate, 1 mM of each dNTP, 5 mM DTT, 40 units of RNaseOUT™ Recombinant Ribonuclease

Inhibitor, and 10 or 15 units of Cloned AMV RT. Amplification reactions consisted of 0.2 µM primers, 200 µM of each dNTP, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2 µl of cDNA reaction, and 2 units of Platinum® Taq DNA Polymerase

(Invitrogen, Cat. no. 10966-018) for fragments up to 4 kb or 1 unit of Platinum® Taq DNA Polymerase High Fidelity (Invitrogen, Cat. no. 11304-011) for fragments up to 9 kb. Magnesium con-

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Figure 1 – Sensitivity of RT-PCR with Invitrogen™ Cloned AMV RT

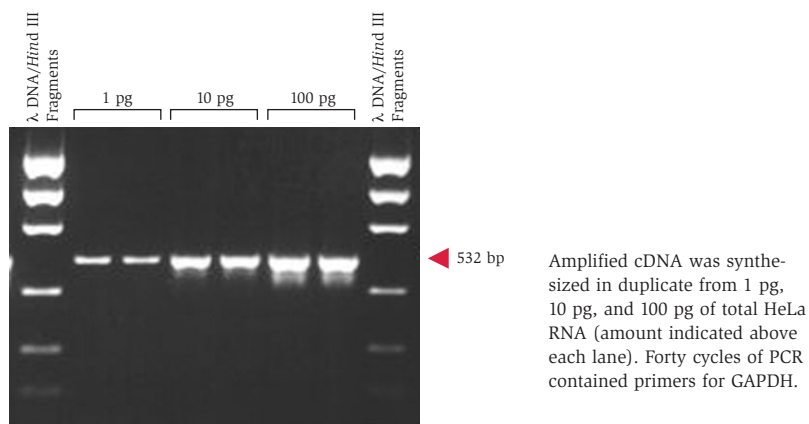
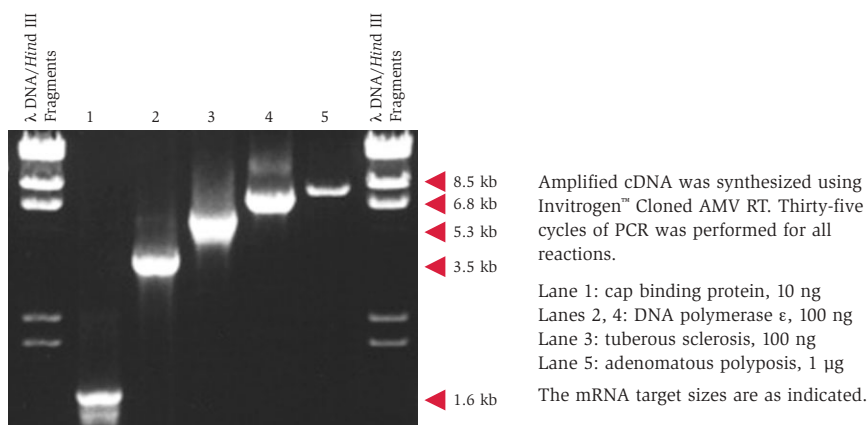


Figure 2 – Range of RT-PCR product size with Invitrogen™ Cloned AMV RT



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centration was 1.5 mM MgCl₂ for Platinum® *Taq* DNA Polymerase and 2 mM MgSO₄ for Platinum® *Taq* DNA Polymerase High Fidelity. After incubation at 94°C for 2 min., amplification followed with 35 to 40 cycles at 94°C for 15 s, 55-60°C for 30 s., and 68°C for 1 min./kb.

Gel electrophoresis. RT-PCR products (10 µl) were analyzed by electrophoresis on 0.8% to 1.5% (w/v) agarose gels in 0.5X TBE with 0.4 µg/ml ethidium bromide.

Results and Discussion

First-strand cDNA synthesis reaction conditions using Invitrogen™ Cloned AMV RT were developed to generate high sensitivity and yield during RT-PCR. Multiple factors were examined, including the Tris buffer counter ion used, Mg²⁺ concentration, dNTP concentration, and

the effect of the addition of RNase inhibitor. By employing the set of optimized RT reaction conditions, Invitrogen™ Cloned AMV RT was able to detect a 532 bp human GAPDH mRNA with as little as 1 pg of total HeLa RNA (Figure 1, page 14). Similar results were also seen with RT-PCR products from β-actin (data not shown).

The ability of Invitrogen™ Cloned AMV RT to produce cDNA from different RNA targets was demonstrated with cap binding protein (1.6 kb), DNA polymerase ε (3.5 kb), tuberous sclerosis (5.3 kb), DNA polymerase ε (6.8 kb), and adenomatous polyposis (8.5 kb). RT-PCR catalyzed by Invitrogen™ Cloned AMV RT and Platinum® *Taq* or Platinum® *Taq* DNA Polymerase High Fidelity produced amplicon DNA from these targets in good yield and with good reproducibility (Figure 2, page 14). The versatility of

Invitrogen™ Cloned AMV RT was shown by its ability to successfully synthesize cDNA at higher temperatures. With cap binding protein (1.6 kb), the yield of PCR products remained high up to 60°C (Figure 3).

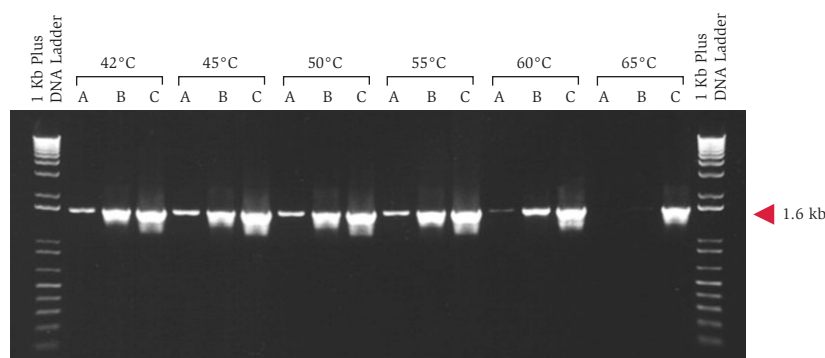
In summary, these results show that Invitrogen™ Cloned AMV RT demonstrates high sensitivity, the ability to detect a wide variety of targets, and the capability for efficient RT-PCR at elevated temperatures. ■

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Figure 3 – Effect of increased RT reaction temperatures on RT-PCR



Amplified cDNA was synthesized from 1 ng (A), 10 ng (B), or 100 ng (C) of total HeLa RNA with Invitrogen™ Cloned AMV RT. cDNA synthesis was performed at temperatures from 42°C to 65°C. Thirty-five cycles of PCR contained primers for cap binding protein.

SuperScript™ II RNase H⁻ Reverse Transcriptase: Focus on stability and performance at various storage temperatures

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Introduction

The Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) is an RNA-directed DNA polymerase that uses single-stranded RNA or DNA in the presence of a primer to synthesize a complementary DNA strand (1). SuperScript™ II RT contains a series of point mutations in the RNase H domain of the enzyme, making it an RNase H⁻ version of M-MLV RT. This RNase H mutation makes SuperScript™ II RT unable to cleave RNA in a DNA:RNA hybrid, resulting in intact RNA template, higher yields, a greater proportion of full-length products, consistent high quality, and efficient synthesis of cDNA from ssRNA or mRNA. The objective of this study is to evaluate the short-term stability of the SuperScript™ II RT enzyme at various temperatures. In this article, we demonstrate that SuperScript™ II RT is able to maintain the above-mentioned properties over a wide range of storage temperatures for a finite time period.

Methods

In order to assess the durability of reverse transcription activity of the enzyme, triplicate samples of SuperScript™ II RT (Invitrogen, Cat. no. 18064-022) were stored at temperatures ranging from -80°C to +8°C over a 48-hour time period. The enzymatic and functional activity of SuperScript™ II RT were determined using the unit assay and the functional assay, respectively.

Individual vials of SuperScript™ II RT (200 units/μl), vialled from the same lot, were subjected to different temperature conditions. Triplicate samples of SuperScript™ II RT were incubated at each

of the following temperatures: -80°C freezer, -20°C freezer (non-frost free), 0°C (wet ice was packed in a cooler and the entire package was placed at +4°C), and +8°C (refrigerator). After 48 h, all samples were placed on ice and tested for unit and functional activity using the protocols described below.

Unit assay. Each enzyme sample to be tested and a positive control enzyme were diluted 1:600 in RT dilution buffer (20 mM Tris-HCl pH 7.5, 0.1 mM EDTA, 100 mM NaCl, 5% Glycerol, 0.01% NP-40, 1 mM DTT). Dilution buffer without enzyme served as the negative control to test for background activity. A unit assay reaction mix was prepared containing a final concentration of the following components: 5X First-Strand Buffer (50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM MgCl₂), 3 mM MgCl₂, 10 mM DTT, 0.5 mM dTTP, 1 mM poly(A)-0.6 mM d(T)₂₅, and 20 μCi/ml [α -³²P]dTTP.

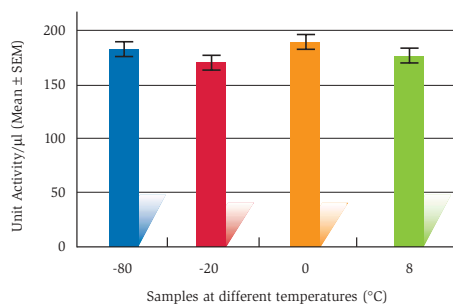
The Multi-Probe IIEX robotic liquid handling system (Packard, Meriden, CT, USA) was used to dispense 45 μl of unit assay reaction mix in triplicate into each

well of a 96-well thin-walled PCR plate maintained at 37°C. The reaction was initiated by adding 5 μl of each diluted enzyme in a staggered fashion (*i.e.*, every 30 seconds) to the reaction mix. After ten minutes, each reaction was stopped by adding 10 μl EDTA (0.5 M, pH 8.0) in the same 30-second interval. Next, 10 μl of each reaction was spotted onto a 96-well DEAE filter plate. The filter plate was washed three times with 400 mM Sodium Phosphate (pH 7.0). In addition, 5 μl of the reaction mix was spotted into three unused wells for determination of the total counts. The filter plate was dried, filled with EcoLite (ICN, CA, USA), and the cpm incorporated determined using a microplate scintillation and luminescence counter (Top Count.NXT, Packard, Meriden, CT, USA). The data was analyzed and the unit activity/μl calculated as follows:

$$\text{Units}/\mu\text{l} = \frac{(\text{cpm test} - \text{cpm background}) \times (250 \text{ U}/\mu\text{l})}{(\text{cpm control} - \text{cpm background})}$$

Functional assay. The following reaction mixture was prepared in an autoclaved

Figure 1 – Unit activity of SuperScript™ II RT at various temperatures



SuperScript™ II RT samples were maintained for 48 h at various temperatures: -80°C, -20°C, 0°C and +8°C. Unit activity was determined using the Unit assay. Each value represents the Mean ± SEM of samples in triplicates maintained at each temperature.

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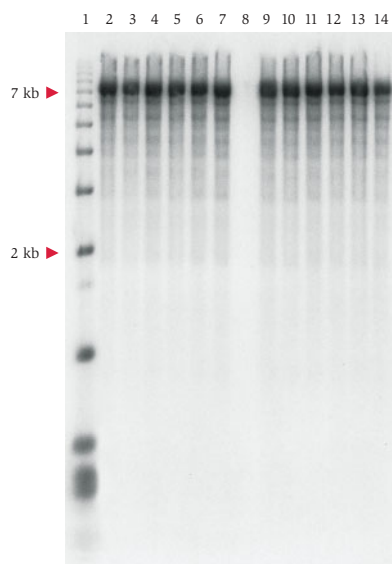
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microcentrifuge tube kept on ice: 220 μ l distilled water (Gibco™), 200 μ l 5X First-Strand Buffer, 100 μ l 0.1 M DTT, 50 μ l 10 mM dNTP mix, 25 μ l 0.1 mg/ml oligo(dT)₁₂₋₁₈, 125 μ l 1 mg/ml 7.5-kb poly(A) RNA, and 5 μ l [α -³²P]dATP (10 mCi/ml).

Nineteen microliters of the reaction mix was dispensed into labeled tubes and the tubes placed in a 45°C water bath for 5 min. to equilibrate to temperature. The reverse transcription reaction was initiated by adding 1 μ l of each test or control enzyme to each tube. A tube with reaction mix alone (no enzyme) served as the negative control. The tubes were vortexed gently and incubated for 60 min. at 45°C. The reaction was stopped by adding 5 μ l of 0.5 M EDTA to each tube and vortexing gently. Five microliters of this mixture was spotted onto GF/C filters that were then TCA precipitated to determine the yields of the first-strand reaction.

Alkaline gel electrophoresis. The remaining 20 μ l of the reverse transcription reaction mixture was mixed with an equal volume of alkaline agarose solution. Then, 20 μ l of each sample was analyzed by 1.4% alkaline agarose gel electrophoresis (2,3). Diluted ³²P-labeled 1-Kb DNA Ladder (~500,000 cpm; protocol described below) was run in the first lane. After electrophoresis in 1X alkaline agarose electrophoresis solution, the gel was removed and placed on filter paper, covered with plastic wrap, dried under vacuum, and exposed to X-ray film. A phosphor imager screen was also exposed (1 h) to the dried gel for quantification studies (% full-length cDNA yield) using the Cyclone™ Storage Phosphor System

Figure 2 – 7.5-kb cDNA synthesis autoradiograph



An autoradiograph showing the ³²P-labeled cDNA synthesis from a 7.5-kb RNA template with SuperScript™ II RT samples maintained at various temperatures for 48 h. Triplicate samples at each temperature except 20°C (duplicate samples), were loaded on the gel for the study.

Lane 1: ³²P-Labeled 1-Kb DNA Ladder
Lanes 2,3,4: SuperScript™ II RT samples at +8°C
Lanes 5,6,7: SuperScript™ II RT samples at 0°C
Lane 8: Blank control (No RT)
Lanes 9,10: SuperScript™ II RT samples at -20°C
Lanes 11,12,13: SuperScript™ II RT samples at -80°C
Lane 14: QC SuperScript™ II RT Gold Standard Control

(Packard, Meriden, CT, USA) and the image obtained was analyzed using the Optiquant Image Analysis Software (Packard, Meriden, CT, USA).

Measuring the level of incorporation. For the TCA precipitation, the spotted filters were washed with cold 10% (w/v) TCA containing 1% (w/v) sodium pyrophosphate for 10 min. followed by three washes with 5% TCA for 5 min. each. The filters were then washed with ethanol for 5 min., dried under a heat lamp, and counted (Beckman LS 6000IC, CA, USA) using the EcoLite (ICN, CA, USA). To determine the specific activity of the dATP in the reaction, 2 μ l of the reaction mix containing no enzyme was spotted onto a filter, dried (without any washes) and then counted.

The following equation was used to determine the specific activity (SA) of the dATP in the first-strand reaction from the counts obtained from the unwashed filter (total counts).

$$SA = \frac{\text{Average Total Counts} \times (25 \mu\text{l}/2 \mu\text{l})}{1.0 \times 10^4 \text{ pmol dATP}} = X \text{ cpm/pmol dATP}$$

The following equation was used to determine the yield of cDNA from the counts obtained from the washed filter and the SA.

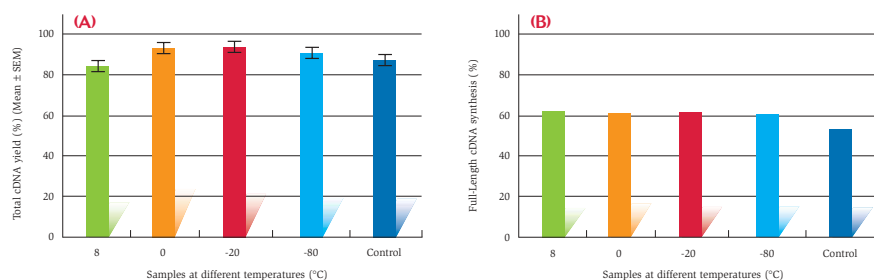
$$\text{Yield \%} = \frac{(\text{cpm} - \text{background}) \times 20 \times 100\%}{SA \times 3030}$$

[³²P] 5'-end labeling of the 1-Kb DNA Ladder. The following reaction was set up in a 1.5-ml microcentrifuge tube: 5 μ l

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continued from page 17

Figure 3 – First-strand cDNA synthesis of SuperScript™ II RT



Two hundred units of each SuperScript™ II RT sample was used in the cDNA synthesis reaction using a 7.5-kb RNA template. **(A)** Total yield of first-strand cDNA was calculated from TCA precipitation analysis. Each bar in the graph represents the Mean ± SEM of three samples maintained at the same temperature. **(B)** Percent full-length cDNA synthesis was determined following alkaline agarose gel electrophoresis and quantification using phosphor imaging. The counts of each full-length band (> 6-kb) were divided by the total counts in the lanes and then were multiplied by 100 to give the percent of full-length cDNA synthesis.

5X Exchange buffer, 5 mg 1-Kb DNA ladder (Invitrogen, Cat. no. 15615-016), 5 µl [γ -³²P]ATP, 1 µl T4 kinase (10 units/µl), and autoclaved water to 25 µl.

After incubation at 37°C for 30 min., 12.5 µl (one-half the original reaction volume) of 7.5 M ammonium acetate was added to stop the reaction and 75 µl of absolute ethanol was then added to this mix. The mix was placed at -20°C for > 1 h and then centrifuged at +4°C for 15 min. The pellet was washed with ethanol and dissolved in 25 µl 0.1 mM EDTA and 25 µl ethanol. One microliter was spotted onto a GF/C filter that was then TCA precipitated, dried, and counted to determine the activity incorporated. The labeled DNA was stored (shielded) at -20°C.

Results

Activity was retained at all temperatures. The results of the unit assay demonstrate

that the SuperScript™ II RT maintains enzymatic (unit) activity independent of the temperature used to store the enzyme (Figure 1, page 16). There was no significant difference in the unit activity among samples at each temperature over a 48-h period.

Full-length cDNA synthesis by SuperScript™ II RT. SuperScript™ II RT yields more full-length cDNA product than any other reverse transcriptase (1). In order to determine whether this ability was retained along with the unit activity over the same temperature spectrum, a 7.5-kb RNA functional assay was performed to assess the reverse transcription activity of the enzyme. Radiolabeled cDNA products generated in the functional assay were analyzed on an alkaline agarose gel (Figure 2, page 17). The gel was then exposed to a phosphor imager screen

and the cDNA product was quantified (Figure 3). The results of the functional assay demonstrate that there was no loss in the ability of SuperScript™ II RT to synthesize full-length 7.5-kb products when maintained at the various temperatures. There was no significant difference in the percentage of total cDNA yield and full-length cDNA synthesis among the samples at each temperature over the 48-h period.

Discussion

In the present study, the stability and subsequent full-length cDNA yield of SuperScript™ II RT is achieved consistently at temperatures ranging from -80°C to +8°C for up to 48 hours. This, in turn, demonstrates the robustness of the SuperScript™ II RT over short-term storage at temperatures other than -20°C. For long-term storage, it is recommended to store SuperScript™ II RT at -20°C. At this temperature, the enzyme is stable for at least one year. ■

Acknowledgements

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Preparation of Gateway™ destination vectors for optimal recombination efficiencies

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Introduction

Historically, Gateway™ destination vectors for expression and analysis have been linearized with a specific restriction endonuclease prior to recombination with the entry clone of choice. Linearization of the destination vector was thought to improve the efficiencies of Gateway™ LR recombination reactions. Recent studies have shown that linearization of the destination vector does not significantly increase the number of colony forming units (CFU). In a panel of destination vectors, differences no greater than ~2.5X fold difference, with an average variability of 1.3X, were demonstrated. Consequently, Gateway™ destination vectors do not require linearization, saving the time and resources required for this additional step.

Methods

Recombination. All reagents and enzymes were obtained from Invitrogen. For each LR reaction, an entry clone (pENTR™-tet) was recombined with a linearized or supercoiled version of the Gateway™ destination vector. The LR recombination reaction was set up by mixing 100 ng entry clone, 300 ng destination vector, 1X LR Clonase™ reaction buffer, and TE buffer to a final volume of 16 µl. To each sample, 4 µl LR Clonase™ enzyme mix was added. The reactions were incubated at 25°C for 1 hour. After the incubation, the reaction mix was treated with 2 µl of Proteinase K solution for 10 minutes at 37°C.

Transformation. Fifty microliters of Library Efficiency® DH5α™ competent

cells were transformed with one microliter of each LR recombination reaction. The mixture was incubated on ice for 30 minutes, heat shocked at 42°C for 30 seconds, and transferred to ice. Four hundred-fifty microliters of SOC medium was added to the transformation and the cells were allowed to recover at 37°C for 1 hour. A dilution (10⁻²) was plated on LB-amp plates. In addition, a positive control (pUC19) and negative control (no entry clone) were performed to determine the transformation efficiency of the competent cells and the background of the LR recombination reaction.

Results

The efficiency of the LR recombination was determined by comparing the ratio of CFUs using supercoiled and linearized destination vectors. As shown in Table 1, the range was 0.93-2.4X and the median

was 1.66X at the 10⁻² dilution. Most destination vectors did not show a significant difference in the number of colony forming units when they were not linearized. The one exception was pBAD-DEST49, which showed higher LR recombination efficiency using a linearized vector. However, using a supercoiled destination vector produced ~80 colonies, a satisfactory number to analyze given the high cloning efficiency of the Gateway™ Technology.

Controls. A background control was performed for each LR recombination reaction. For each reaction, an undiluted aliquot was plated, and no colonies were obtained for any of the supercoiled vector samples. A minimal number of colonies (0-3) grew for the linearized vector samples. The pUC19 positive control produced satisfactory results.

Table 1 – Compares the LR recombination efficiencies of linearized or supercoiled Gateway™ destination vectors

Destination Vector	Supercoiled (no. of CFU)	Linear (no. of CFU)	Ratio of Linear (CFU): Supercoiled (CFU)
pBAD-DEST49	79.5	192	2.4
pcDNA-DEST47	97.5	179.5	1.8
pcDNA-DEST53	124	183	1.4
pDEST*17	122.5	142.5	1.1
pEF5/FRT/V5-DEST	278.5	491.5	1.76
pET104-DEST	392	324	0.8
pMT/Biotag-DEST	258	398	1.5
pMT-DEST48	231	224.5	0.96
pT-REx-DEST31	452.5	521	1.15
pXInsect-DEST39	299	519	1.7
pYES-DEST52	320	300	0.93
Total Average	241.3	315.9	1.3

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Conclusion

For most applications, we recommend performing the LR recombination reaction using a supercoiled *attL*-containing Gateway™ entry clone and supercoiled *attR*-containing destination vector. However, for some vectors, linearization may increase the LR recombination efficiency. To linearize the destination vector, choose a unique restriction site that cuts within the *attR* cassette but does not disrupt the *attR* sites. Alternatively, you may use topoisomerase I to relax the destination vector if suitable restriction sites are unavailable. Refer to the Gateway™ Technical manual for additional information. ■

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Site-specific recombination in Gateway™ Technology

Gateway™ Technology is a novel universal system for cloning and subcloning DNA sequences, facilitating functional gene analysis, and protein expression. Once in this versatile operating system, DNA segments are transferred between vectors using site-specific recombination.

Gateway™ Technology is based on the well-characterized lambda phage site-specific recombination system ($attB \times attP \rightarrow attL \times attR$). Two reactions, BP and LR, constitute the Gateway™ Technology (Table 1 and Figure 1). The BP reaction uses a recombination reaction between an *attB* DNA segment or expression clone and an *attP* donor vector to create an entry clone. The LR reaction is a recombination between an *attL* entry clone and an *attR* destination vector. The LR reaction is used to move the sequence of interest to one or more destination vectors in parallel reactions. ■

Figure 1 – Gateway™ Technology overview

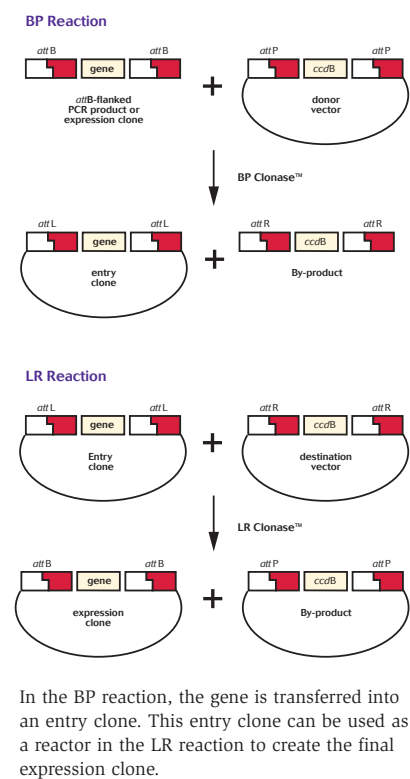


Table 1 – Summary of reactions and nomenclature

Reaction	Reacting Sites	Product	Structure of Product
BP Reaction	<i>attB</i> x <i>attP</i>	entry clone	<i>attL</i> 1-gene- <i>attL</i> 2
LR Reaction	<i>attL</i> x <i>attR</i>	expression clone	<i>attB</i> 1-gene- <i>attB</i> 2

Micro-to-Midi Total RNA Purification System: A system for a wide range of sample types and amounts

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Introduction

Non-phenolic silica-based cartridge systems allow fast sample processing without generation of hazardous waste and they isolate high-quality RNA. Unlike other cartridge systems, the Micro-to-Midi Total RNA Purification System is designed to isolate RNA from a variety of biological materials and quantities. This article provides examples of yields and RNA quality from a variety of samples.

Methods

Total RNA was isolated using the Micro-to-Midi Total RNA Purification System (Invitrogen, Cat. no. 12183-018) according to the product instructions (Figure 1). cDNA libraries were generated using poly(A)⁺-selected total RNA isolated from 2 µg of rat liver and rat brain and the SuperScript™ Plasmid System for cDNA Synthesis and Cloning.

Results

RNA yields from a variety of samples are shown in Table 1 (page 23). As expected, yields vary with sample type. Yields compare favorably with yields of RNA using a standard acidic phenol/guanidine method. For example, with TRIzol® Reagent, yields are 8 to 15 µg of RNA/10⁶ HeLa cells and 6 to 10 µg of RNA/mg liver or spleen (1).

RNA quality was examined several ways. The A_{260/280} ratio was determined to be 1.8 or higher (data not shown). Gel analysis showed the prominent 28S and 18S ribosomal RNA bands; had unde-

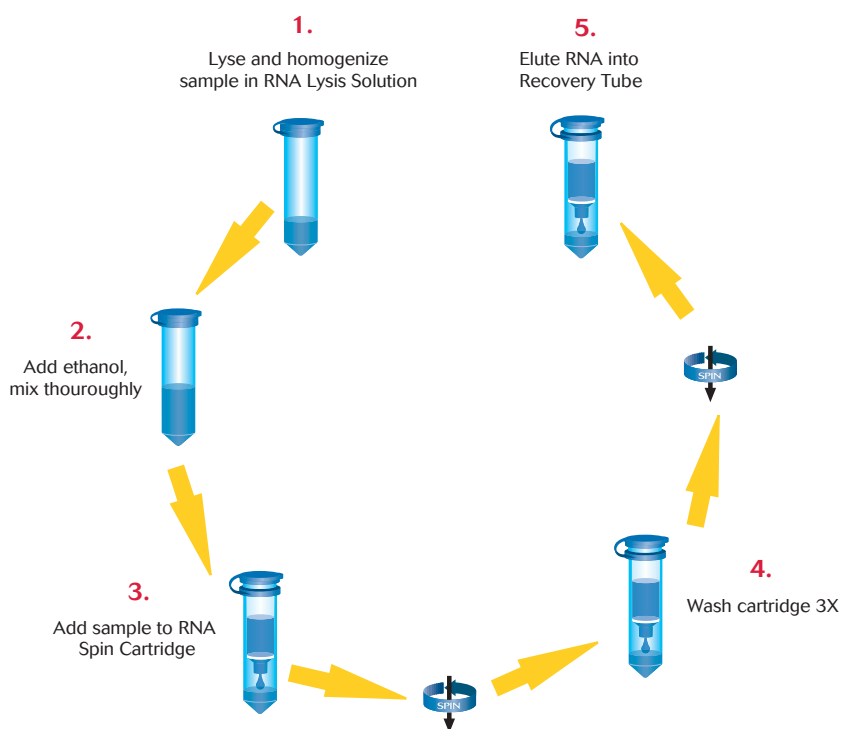
tectable background smearing; and had reduced signal from tRNA, 5S rRNA, and 5.8S rRNA. In addition, in plants, 23S, 16S, 14.5S, and 13S bands are visible due to RNA found in chloroplasts (Figure 2, page 22).

The RNA isolated with the Micro-to-Midi System was tested in several applications, including Northern blots, cDNA library construction, and RT-PCR. Northern blot analysis of RNA isolated from rat brain, liver, and spleen detected

the 6-kb clathrin mRNA (data not shown). cDNA libraries were constructed from poly(A)⁺-selected rat brain and rat liver RNA. The rat brain cDNA library had 4.4 x 10⁷ primary clones, with an average insert size of 1.5 kb. The rat liver cDNA library had 9.4 x 10⁷ primary clones, with an average insert size of 1.4 kb.

Examples of RT-PCR results with RNA isolated using the Micro-to-Midi Total RNA Purification System are shown in Figures 3 and 4 (page 22). One hundred

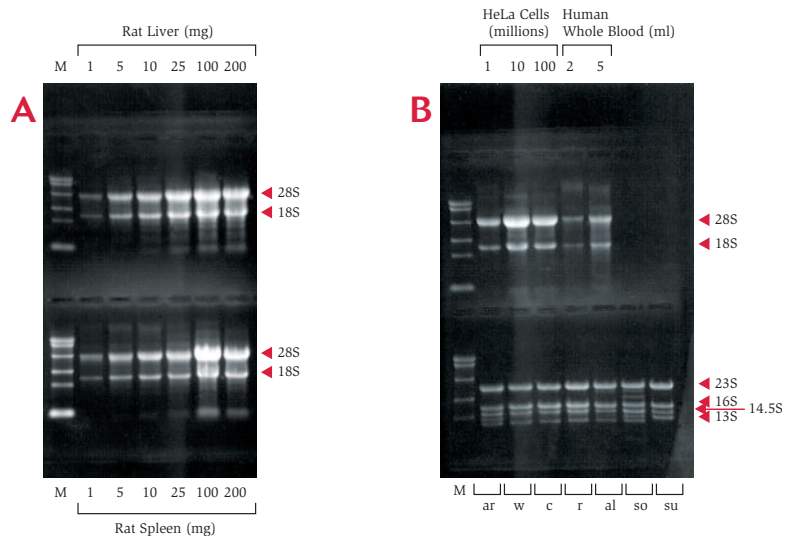
Figure 1 – Schematic of Micro-to-Midi Total RNA Purification System protocol



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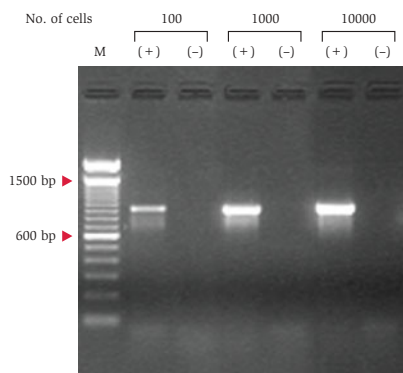
Figure 2 – Electrophoretic analysis of RNA



RNA was electrophoresed on a 1.2% agarose gel containing 50 mM MOPS (pH 7.0), 10 mM EDTA, and stained with ethidium bromide. The Invitrogen™ 0.24-9.5 Kb RNA Ladder was used for the molecular weight marker (M).

Panel A: RNA from 1-200 mg of rat liver (top) and rat spleen (bottom).
Panel B: RNA from 10⁶-10⁸ HeLa cells and 2-5 ml whole blood (top), and 100 mg leaf tissue of *Arabidopsis* (ar), wheat (w), corn (c), rice (r), alfalfa (al), soybean (so) and sugarbeet (su) (bottom).

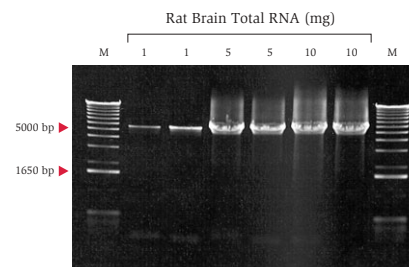
Figure 3 – RT-PCR from HeLa cell RNA



cDNA was synthesized in duplicate in 20- μ l reactions from one fifth of the isolated RNA after DNase I treatment, with (+) or without (-) Superscript™ II RT. One tenth of the reaction was amplified with primers for human elongation factor 1 α (911-bp fragment) using Platinum® *Taq* DNA Polymerase with 1.5 mM MgCl₂. PCR parameters were 35 cycles of 94°C for 30 s, 55°C for 60 s, and 72° C for 90 s. One fifth of the reaction was analyzed on an agarose/TAE gel stained with ethidium bromide. The Invitrogen™ 100 bp DNA Ladder was used for the molecular weight marker (M).

HeLa cells provided more than enough RNA for RT-PCR. A dose-response was visible with increasing amounts of HeLa cells. The negative controls (cDNA synthesis without RT) showed there was no contribution to the PCR signal from DNA (Figure 3). The RNA was high quality, as shown by long RT-PCR analysis for rat brain RNA (Figure 4).

Figure 4 – Long RT-PCR from various RNA samples



cDNA was synthesized from rat brain total RNA (1, 5, and 10 mg) in 20 μ l with SuperScript™ II RT after DNase I treatment, and 0.5 to 2 μ l was amplified with primers for clathrin (5.7 kb fragment) using eLONGase® Enzyme Mix with 1.8 mM MgSO₄. PCR was 40 cycles of 94°C for 20 s, 60°C for 30 s, and 68°C for 7 min. One fifth of the reaction was analyzed on an agarose/TAE gel stained with ethidium bromide. The Invitrogen™ 1 Kb Plus DNA Ladder was used for the molecular weight marker (M).

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Table 1 – Samples processed with the Micro-to-Midi Total RNA Purification System

Source	Amount of Sample	RNA Yield Exp. 1 (µg)	RNA Yield Exp. 2 (µg)
Cells			
HeLa Cells	10 ⁵	1.6	1.4
	10 ⁶	20	19
	10 ⁷	144	179
	10 ⁸	1,123	900
Yeast Cells	3 x 10 ⁷	4.3	6.4
Bacteria Cells	5 x 10 ⁸	6.3	5.3
Animal Tissues			
Rat Liver (mg)	1	4.7	5.7
	10	57	63
	100	280	340
Rat Brain (mg)	1	0.6	0.6
	10	6.3	5.9
	100	102	74
Rat Spleen (mg)	1	4.4	7.0
	10	55	61
	100	380	260
Plant tissues			
<i>Arabidopsis</i> Leaf (mg)	100	36	16
Wheat Leaf (mg)	100	28	34
Corn Leaf (mg)	100	35	36
Rice Leaf (mg)	100	35	39
Alfalfa Leaf (mg)	100	32	32
Soybean Leaf (mg)	100	43	34
Sugarbeet Leaf (mg)	100	17	31
Blood			
Human Whole Blood (ml)	0.5	1.3	1.4
	5	18	14

Discussion

In summary, the Micro-to-Midi Total RNA Purification System is a versatile tool for RNA isolation from a wide variety of sources and quantities, yielding high-quality RNA suitable for use in many applications.

Acknowledgements

We thank Dr. Jun Lee and Dr. Donna Fox for providing primers, PCR conditions, and valuable suggestions.

Reference

1. Simms, D., Cizdziel, P.E., and Chomczynski, P. (1993) *Focus*® **15(4)**: 99-102.

Help Box – Tips for RNA Isolation

Q. Why use the Homogenizer?

The Homogenizer reduces the viscosity of the lysate by simply placing the sample in the unit and centrifuging. The lysate is simultaneously homogenized and clarified. Traditional homogenization methods process the lysate through a small-bore needle attached to a syringe to reduce the viscosity. This method is laborious, inconsistent, and unsafe due to the risk of puncturing oneself. An alternative method is to disrupt samples using rotor-stator homogenizers (motor-driven devices that usually have stainless steel blades in a variety of styles). Rotor-stator homogenizers are efficient and are the method of choice for larger samples (>100 mg of animal tissue).

Q. Do I need to DNase I digest my RNA for RT-PCR?

DNA contamination of RNA varies with sample type. For example, RNA isolated from rat spleen tends to have higher DNA contamination than that from rat liver. Spleen has greater DNA contamination regardless of the method used for RNA isolation. Therefore, some RNA samples may require DNase I digestion. To test for DNA contamination, use a no-RT control in RT-PCR. Alternatively, primers can be designed to give a different-sized product for genomic DNA compared to product from cDNA.

Q. The A_{260/280} ratio of my RNA is not >1.8. Why is that?

The A_{260/280} ratio of the same RNA preparation can differ depending on what was used to dilute the sample (1). Dilute the RNA in TE (not water) before measuring A_{260/280}.

Reference:

1. Wilfinger, W.W., Mackey, K., and Chomczynski, P. (1997) *BioTechniques* **22**: 474.



The Invitrogen Grant Program is working. We're giving money away!

In January 2002, Invitrogen launched an international US \$5 million funding opportunity for researchers: the Invitrogen Research Tools Development Grant Program. Monetary awards of up to US \$100,000 per year were provided for research projects directed at developing new products or procedures to use in cell and molecular biology research. Investigators working in biotech, academic, and not-for-profit institutions were encouraged to apply.

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tors were asked to submit full proposals. To date, about 25% of these projects will be receiving funding, with topics from amplification to purification and distribution from California to the Netherlands.

Submit an idea. Invitrogen is driven by the mission to provide innovative products and services that accelerate biological discovery and understanding. If you have an idea or you are working on a project that can complement this mission, submit your preproposal today. What are you waiting for? The Research Tools Development Grant Program still has funds available for research. Take some of our money, please. For more information, visit www.invitrogen.com, e-mail grants@invitrogen.com, or call 800 955 6288, ext. 66140 today.



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