

# PCR Primer

A LABORATORY MANUAL

SECOND EDITION

EDITED BY

Carl W. Dieffenbach

Gabriela S. Dveksler



COLD SPRING HARBOR LABORATORY PRESS

PCR Primer: A Laboratory Manual  
Second Edition

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Printed in the United States of America.

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**Library of Congress Cataloging-in-Publication Data**

PCR Primer : a laboratory manual / edited by Carl W. Dieffenbach, Gabriela S.

Dveksler.— 2nd ed.

p.cm.

Includes bibliographical references and index.

ISBN 0-87969-633-2 (cloth : alk. paper) — ISBN 0-87969-654-0 (pbk. : alk. paper)

1. Polymerase chain reaction—Laboratory manuals. I. Dieffenbach, Carl W. II.

Dveksler, Gabriela S.

QP606.D46P359 2003

572.43—dc21

2003053085

10 9 8 7 6 5 4 3 2 1

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This book is dedicated to

Sara,

Rebecca,

Elhan,

and Ari

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This is often due to a small contaminating fragment that was present in the original PCR product. Its mass may be low but, because it is small, the molarity is high. Generally the sequence beyond this point will be fine, and can be used to design a primer to obtain sequence data from the opposite strand of the region in question.

False terminations, in which the polymerase stalls but does not incorporate a dideoxynucleotide, are not a problem with dye terminators because these products are unlabeled. However, compression artifacts are sometimes seen. Compression refers to the sequence-dependent aberrant migration of some fragments with G and C residues at the 3' end, and is seen on both capillary and gel systems as abnormal spacing of the dye-terminated fragments. When there is compression, fragments of length  $n$  and  $n + 1$  have nearly identical mobilities. The traces will show two peaks close together, with an unusually large gap before the  $n + 2$  fragment. The ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit contains dITP, which eliminates most problems with compression of GC-rich regions. In addition, base-calling software is often sufficiently sophisticated to handle these artifacts. Usually visual inspection of the traces can resolve the sequence ambiguity, but the region should be sequenced on the opposite strand, as it is very rare to find compressions on both strands at identical locations.

Finally, sequence-independent compression is typically seen at one or more locations within 10–30 nucleotides from the beginning of the readable sequence. This is due to a salt front that distorts fragment migration but usually does not interfere significantly with base-calling in this region. However, if absolute accuracy is required and only one strand is being sequenced in this region, it is advisable to pick primers such that the critical sequence area is located well beyond the salt front.

## ACKNOWLEDGMENTS

I thank members of my laboratory for comments on the manuscript, and Mike Flora of the U.S. U.H.S. Biomedical Instrumentation Center for helpful discussions on sequencing.

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## WWW RESOURCE

<http://www.genome.wi.mit.edu/cgi-bin/primer/primer3 WWW.cgi> Primer3, web software, Whitehead Institute/MIT Center for Genome Research.

## SECTION

# 2

## SAMPLE PREPARATION

**S**ample preparation—the purification and concentration of nucleic acids from specimens collected for analysis—is the critical first step for successful PCR. Because of the variety of PCR applications, the starting materials for nucleic acid extraction may include such diverse specimens as 17- to 20-million-year-old fossilized magnolia leaves (Golenberg et al. 1990), mitochondrial DNA from dogs (Leonard et al. 2002), and human remains. Clearly, each specific sample type places unique constraints and requirements on the method of nucleic acid extraction.

## DNA ISOLATION

The chapter “DNA Purification” (p. 87), contains methods for isolation of DNA from blood; tissues, such as mouse tails, buccal smears, or plant tissues; and cultured cells. This chapter also provides methods for automating the DNA isolation process. All of the methods presented in this chapter are based on binding of nucleic acids to silica gel. This procedure allows the DNA to bind to the silica gel in the presence of strong protein denaturants and after washes to eliminate the presence of contaminants; the DNA is eluted in a low-salt buffer. This methodology reduces the number of steps in DNA purification by eliminating organic solvent extraction and alcohol precipitation. In addition, it reduces handling of the nucleic acid, which minimizes the risk of sample-to-sample contamination. The prevention of contamination of PCR by either sample-to-sample contamination or PCR product carry-over is discussed in Section 1.

Although the methods described here reproducibly liberate the DNA from the cells and tissues, prior handling and age of the specimen may affect the average length of the resulting DNA fragments. Therefore, consideration should be given to the size of the region to be amplified, based on the source of the DNA. It would be unreasonable to expect nucleic acid isolated from a formalin-fixed, paraffin-embedded tissue section to serve as template for products longer than 800 bp. However, with appropriate specimens, the isolated DNA can be used as template for PCR products in the 25- to 40-kb range.

This is possible because the method of nucleic acid isolation described in this chapter does not introduce single-strand nicks, but breaks the DNA to an average size of greater than 40 kb. As described in “Tips for Long and Accurate PCR” (p. 53) in Section 1, template DNA for this application requires additional special handling. Specifically, excess heating of the nucleic acid should be avoided. Temperatures of 95°C or higher for more than 5 minutes can result in the deamination of cytosine, giving rise to a number of deoxyuridine sites in the DNA. As described in “High-Fidelity PCR Enzymes” (p. 21), the archaeal ther-

mophilic DNA polymerases irreversibly bind to these sites and block amplification. Because of this problem, the judicious use of heat to denature the DNA template is recommended.

#### LACK OF AMPLIFICATION

There are occasions when samples cannot be successfully amplified. This could be due to a variety of problems with the template including high number of deoxyuridines, presence of contaminants, or degradation of the sample. Often the specimens for PCR analysis are not replaceable. The chapter "Strategies for Overcoming PCR Inhibition" (p. 149) provides a number of approaches for salvaging these specimens. This chapter covers a number of strategies to overcome lack of amplification, including changing the DNA polymerase to one that is less sensitive to the contaminants and the presence of deoxyuridine. When the DNA is too degraded, new primers designed to amplify shorter products, in the 130- to 150-bp range, are recommended.

A good method for handling and purification of PCR products is important for a number of applications, and there are several commercially available kits for this purpose. However, in some instances, it becomes important to minimize the chance of aerosol contamination of the laboratory with the product to be purified. The silica-based method is fast, simple, and effective, and contamination is less likely to occur than with other methods.

#### mRNA ISOLATION

Isolation of mRNA for reverse transcriptase (RT)-PCR presents a new set of challenges. The RNA must be released from within the cell, protected from endogenous nucleases, and recovered as quantitatively as possible. Additionally, the RNA must be free of salts and detergents, which inhibit or disrupt the enzymatic steps need for RT-PCR. "RNA Purification" (p. 117) describes the techniques used for specimen processing and storage as well as the steps of RNA purification. For RNA isolation, the protocols presented in this chapter detail the evolution of the technology.

The methods developed by Chirgwin and coworkers (1979) use strong chaotropic agents to simultaneously liberate the RNA and denature all the cellular proteins. Then the RNA is pelleted using cesium chloride equilibrium gradient ultracentrifugation, which keeps the cellular DNA in solution. One advantage of this method is that no organic solvents are used during the RNA extraction. This remains the method of choice when processing large amounts of tissue per sample or when the specimen contains large quantities of endogenous RNase. One disadvantage of this method is that it is very time-consuming.

The second method developed by Chomczynski and Sacchi (1987) uses a phenol-based extraction system combined with strong protein denaturants. The RNA partitions into the aqueous phase, and the denatured proteins and the bulk of the genomic DNA stay at the aqueous-organic interface. A limitation with this method is the variable amount of genomic DNA that is isolated with the RNA. If the RNA population is used directly in RT-PCR, then under certain circumstances the contaminating DNA could be misinterpreted as higher levels of gene expression. Treatment of all samples with RNase-free DNase removes this problem, but this adds a significant number of steps to the procedure.

The third method uses the selective binding and elution of the RNA from a silica gel membrane or matrix. A major advantage of the silica-based systems is the incorporation of the DNase treatment into the protocol so that the isolated RNA is ready for RT-PCR or other uses.

Equally important to the isolation procedure is the handling and storage of specimens. The standard method of sample processing requires the rapid processing of the specimens—from chilling of the sample, followed by the isolation of the cells and tissues, then either immediate lysis and RNA extraction, or rapid freezing of the specimen in liquid nitrogen or a dry ice-ethanol bath. If samples are flash-frozen, the specimens must be processed rapidly at the time of RNA extraction to prevent any thawing and RNA degradation prior to complete lysis. Ambion has developed a solution to this problem. By storing the cells and tissues in RNAlater, samples can be collected and stored at 4°C for short periods of time or at -20°C until all samples are ready to be batch processed. This greatly improves the reproducibility of RNA expression studies.

A major limitation of RNA expression studies is that the analysis reflects the average expression from all the cells in the tissue. "In Situ Localization of Gene Expression Using Laser Capture Microdissection" (p. 135) details a method for the visualization of the tissue and the selection and isolation of cells or groups of cells from the tissue section. This methodology extends the power of in situ analysis by providing a means to dissect tissues and perform gene expression studies of numerous genes using real-time PCR. Standard in situ methods are limited by the limit of detection and the single-use nature of a specimen. Collectively, these chapters provide isolation methods for RNA and DNA for nearly every possible PCR application.

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Craig Smith, Paul Otto, Rex Bitner, and Gary Shiels

*Promega Corporation, Madison, Wisconsin 53711-5399*

The amplification of nucleic acids from small amounts of starting material is a critical tool for basic research and diagnostics laboratories. Numerous methods have been developed, including self-sustaining sequence replication (SSR; Abrevaya et al. 1995), ligase chain reaction (LSR; Shimer and Backman 1995), and rolling circle amplification (Lizardi et al. 1998). However, PCR (Kleppie et al. 1971; Saiki et al. 1985) has become the standard.

Methods to isolate nucleic acids are a necessary part of the overall PCR process. They provide the means both to isolate source materials for amplification and to clean up, or purify, amplification products before their use in downstream applications. Genomic DNA is the most commonly used template for PCR amplification. DNA may be isolated from a wide range of starting materials, including whole blood, plant or animal tissue, and cultured cells. For some diagnostics and forensic applications, specialized techniques for isolation of DNA from processed food, buccal swabs, bodily fluids, and paraffin-embedded tissues are necessary. Post-PCR cleanup of amplified DNA fragments requires the removal of excess primers, salts, and nucleotides, as well as efficient recovery of the amplified product. In some cases, limitations in primer design can result in the amplification of multiple products of varying size. These contaminating fragments must also be removed before use in downstream applications.

The successful isolation of DNA requires methods that prevent nuclease degradation and eliminate PCR inhibitors, which can be problematic in some samples (Akane et al. 1994; Wilson 1997; Al-Soud et al. 2000). Methods that provide template in a concentrated form and in buffers that are compatible with downstream applications are also desirable. Numerous methods have evolved to isolate genomic DNA for amplification, as well as to purify PCR products post-amplification. Which method is the best depends on a number of factors, including the nucleic acid type and size, yield, purity, the presence or absence of contaminating fragments, throughput and the need for automation, and price per isolation.

Binding and elution from silica has become the method of choice for both template isolation and purification of amplification products. Silica-based methods have been developed to isolate all classes of nucleic acids from a range of sample types. Different formats are available to handle various throughput needs, ranging from individual samples to high-throughput, fully automated methods capable of processing thousands of samples per day. The high concentrations of chaotropes used to bind nucleic acids to silica surfaces are potent denaturants that, when used in combination with various detergents, serve to disrupt tissue, lyse cells, and inactivate endogenous nucleases. A hallmark of silica methods is

the elution of nucleic acids in nuclease-free water or other low-ion-c-strength buffer: such as TE (10 mM Tris HCl, pH 8.0; 0.1 mM EDTA), allowing use of isolated nucleic acids in downstream applications without further processing. Typically, DNA isolated by the following methods is stable for up to a year at 4°C, depending on the source material, and can be stored indefinitely at -20 to -70°C.

For large-scale isolation of milligram quantities of DNA, solution extraction and precipitation methods based on the differential solubility of DNA, RNA, and protein have been developed. These methods are best suited to the reagent volumes required for processing large amounts of starting material and can be readily scaled to isolate from 1 to 100 mg of nucleic acid.

## OVERVIEW OF METHODS

Here we outline a series of sample protocols for (1) purification of genomic DNA from blood, tissues, and cultured cells and (2) purification of PCR products from agarose gels and from solution—including the cleanup of dye terminator sequencing reactions. Solution extraction, silica membrane, and magnetic particle-based methods are included. Where applicable, more than one method is presented to provide users with a choice of methods to meet their individual throughput requirements.

### Genomic DNA Isolation

The purification of genomic DNA for downstream use in PCR can be achieved in a number of ways. With the advent of genomic-based DNA analyses, the requirements for easily processing large numbers of samples, particularly using automated or semiautomated methods, have led to greater use of solid-phase extraction methods. The common element with these methods is the reversible binding of DNA to a solid matrix, most commonly silica. The Wizard SV 96 technology, based on a high-density 96-well filter plate with a silica-impregnated membrane, and the Wizard MagneSil silica paramagnetic particle chemistry, are two technologies that satisfy these requirements.

Here we present a solution-based method for the isolation of genomic DNA from 10 ml of whole blood, a silica membrane-based method for isolation of genomic DNA from cultured cells and tissue, and magnetic silica paramagnetic particle-based methods for small-scale isolation of genomic DNA from whole blood, and for simultaneous isolation and quantitation of genomic DNA from buccal swabs.

## PROTOCOL 1 SOLUTION-BASED METHOD FOR GENOMIC DNA PURIFICATION FROM WHOLE BLOOD

Isolation of genomic DNA from whole blood (10 ml) using the Wizard Genomic DNA Purification Kit is based on a four-step process. The first step in the purification procedure involves lysis of the red blood cells and removal of the soluble fraction, followed by lysis of the white blood cells and their nuclei. An RNase digestion step may be included at this time; it is optional for some applications (see Table 1). Cellular proteins are selectively removed by a salt precipitation step, which leaves the high-molecular-weight genomic DNA in solution. Finally, the genomic DNA is concentrated and desalted by isopropanol precipitation (Fig. 1).

The Wizard Genomic DNA Purification Kit protocol is scalable and can be used to isolate genomic DNA from 20 µl to 10 ml of whole blood. A 96-well microwell plate format

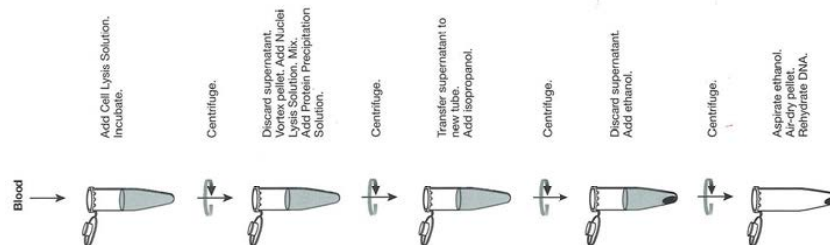


FIGURE 1. An overview of genomic DNA purification using the Wizard Genomic DNA Purification Kit. (Reprinted, with permission, from Wizard Genomic DNA Purification Kit protocol card #FB022, fig. 2818MAT1\_9A [© 2003 Promega Corporation].)

has been developed for processing volumes less than 50 µl. Purifications of equal quality and yield are achieved from whole-blood samples collected in EDTA-, heparin-, or citrate-coated tubes (Beutler et al. 1990).

## MATERIALS

## BUFFERS, SOLUTIONS, AND REAGENTS

Ethanol, 70% <1>, room temperature  
Isopropanol <1>, room temperature  
RNase A

Dissolve RNase A to 4 mg/ml in DNA Rehydration Solution, and boil for 10 minutes to remove contaminating DNase. Store in aliquots at -20°C.

## SPECIAL EQUIPMENT

Agarose gel electrophoresis equipment  
Centrifuge tubes, sterile 50-ml

## ADDITIONAL ITEMS

Wizard Genomic DNA Purification Kit (Promega; includes Cell Lysis Solution, Nuclei Lysis Solution, Protein Precipitation Solution, and DNA Rehydration Solution)  
Molecular-weight markers for use in agarose gel electrophoresis

## CELLS AND TISSUES

Whole blood <1> (10 ml)

## METHOD

## RED BLOOD CELL LYSIS

1. Add 30 ml of Cell Lysis Solution to a sterile 50-ml centrifuge tube.
2. Gently rock the blood sample tube until thoroughly mixed; then transfer blood (10 ml) to the tube containing the Cell Lysis Solution. Invert the tube 5 or 6 times to mix.
3. Incubate the mixture for 10 minutes at room temperature (invert 2 or 3 times once during the incubation) to lyse the red blood cells. Centrifuge at 2000g for 10 minutes at room temperature.
4. Remove and discard as much supernatant as possible without disturbing the visible white pellet. Approximately 1.4 ml of residual liquid will remain. If the blood sample has been frozen, add an additional 30 ml of Cell Lysis Solution, invert 5 or 6 times to mix, and repeat steps 3 and 4 until the pellet is nearly white. There may be some loss of DNA from frozen samples.

At step 4, some red blood cells or cell debris may be visible along with the white blood cells. If the pellet appears to contain only red blood cells, add an additional aliquot of Cell Lysis Solution after removing the supernatant above the cell pellet, and then repeat steps 3 and 4.

5. Vortex the tube vigorously until the white blood cells are resuspended (10–15 seconds).

## NUCLEI LYSIS AND PROTEIN PRECIPITATION

1. Add 10 ml of Nuclei Lysis Solution to the tube containing the resuspended cells. Pipette the solution 5 or 6 times to lyse the white blood cells. The solution should become very viscous. If clumps of cells are visible after mixing, incubate the solution at 37°C until the clumps are disrupted. If the clumps are still visible after 1 hour, add an additional 3 ml of Nuclei Lysis Solution and repeat the incubation.
2. *Optional:* Add RNase A, to a final concentration of 20 µg/ml, to the nuclear lysate and mix by inverting the tube 2–5 times. Incubate the mixture at 37°C for 15 minutes, then cool to room temperature.

3. Add 3.3 ml of Protein Precipitation Solution to the nuclear lysate and vortex vigorously for 10–20 seconds. Small protein clumps may be visible after vortexing.
4. Centrifuge at 2000g for 10 minutes at room temperature. A dark brown protein pellet should be visible.

## DNA PRECIPITATION AND REHYDRATION

1. Transfer the supernatant to a clean 50-ml centrifuge tube containing 10 ml of room-temperature isopropanol.
2. Gently mix the solution by inversion until the white, thread-like strands of DNA form a visible mass.
3. Centrifuge at 2000g for 1 minute at room temperature. The DNA will be visible as a small white pellet.
4. Decant the supernatant and add 10 ml of room-temperature 70% ethanol to the DNA. Gently invert the tube several times to wash the DNA pellet and the sides of the tube. Centrifuge as in step 3.
5. Carefully aspirate the ethanol. The DNA pellet is very loose at this point, and care must be used to avoid aspirating the pellet into the pipette. Invert the tube on clean, absorbent paper and air-dry the pellet for 10–15 minutes.
6. Add 800 µl of DNA Rehydration Solution, and rehydrate the DNA by incubating at 65°C for 1 hour. Periodically mix the solution by gently tapping the tube. Alternatively, rehydrate the DNA by incubating the solution overnight at room temperature or at 4°C.
7. Store the DNA at 2–8°C.

## CHARACTERIZATION OF ISOLATED DNA

DNA yield can be measured by absorbance at 260 nm or by agarose gel electrophoresis of purified samples relative to standards. This latter method is preferred over spectrophotometric determinations if precise concentrations are required, because absorbance readings can be artificially high due to the presence of low-molecular-weight, UV-absorbing material that may be present in the sample (Glaser 1995). A typical yield from 10 ml of whole blood is 250–500 µg (Table 1).

The quality of DNA isolated by this procedure can be assessed by length determination and functionality in PCR amplification (Saito et al. 1985). The majority of the DNA isolated using this method is 50–200 kb, as determined by pulsed-field gel electrophoresis (Fig. 2). Compatibility of the isolated DNA with PCR amplification was demonstrated by successful amplification of three polymorphic human loci. Samples (25 µg) from five individuals amplified efficiently, producing an identical pattern of alleles for each locus (Fig. 3).

## PROTOCOL 2

## SILICA MEMBRANE-BASED METHOD FOR ISOLATION OF GENOMIC DNA FROM TISSUES AND CULTURED CELLS IN A 96-Well Format

Silica chemistry has become the method of choice for the purification of high-quality genomic DNA from samples such as mouse tail clippings, animal tissue, and tissue culture cells. The Wizard SV Genomic DNA Purification System uses a solid-phase silica-impregnated filter membrane for the purification of genomic DNA. The procedure consists of tissue disruption (if necessary), cell lysis, binding of DNA to the SV membrane, washing to remove residual contaminants, and elution of DNA in nuclease-free water. Sample and reagents can be driven through the membrane by either centrifugation or vacuum filtration.

TABLE 1. DNA yield from various starting materials using the Wizard Genomic DNA Purification Kit

| Species and material                                                                              | Amount of starting material                                      | Typical DNA yield                                              | RNase treatment                              |
|---------------------------------------------------------------------------------------------------|------------------------------------------------------------------|----------------------------------------------------------------|----------------------------------------------|
| Human whole blood<br>(Yield depends on the quantity of white blood cells present)                 | 300 $\mu$ l<br>1.0 ml<br>10.0 ml                                 | 5–15 $\mu$ g<br>25–50 $\mu$ g<br>250–500 $\mu$ g               | optional<br>optional<br>optional             |
| Mouse whole blood<br>96-well plate<br>EDTA (4%) treated<br>Heparin (4%) treated<br>96-well plates | 50 $\mu$ l/well<br>300 $\mu$ l<br>300 $\mu$ l<br>50 $\mu$ l/well | 0.2–0.7 $\mu$ g<br>6 $\mu$ g<br>6–7 $\mu$ g<br>0.2–0.7 $\mu$ g | optional<br>optional<br>optional<br>optional |
| Cell lines                                                                                        |                                                                  |                                                                |                                              |
| K562                                                                                              | 3 $\times$ 10 <sup>6</sup> cells                                 | 15–30 $\mu$ g                                                  | required                                     |
| COS                                                                                               | 1.5 $\times$ 10 <sup>6</sup> cells                               | 10 $\mu$ g                                                     | required                                     |
| NH-3T3                                                                                            | 2.25 $\times$ 10 <sup>6</sup> cells                              | 9.5–12.5 $\mu$ g                                               | required                                     |
| PC12                                                                                              | 8.25 $\times$ 10 <sup>6</sup> cells                              | 6 $\mu$ g                                                      | required                                     |
| CHO                                                                                               | 1–2 $\times$ 10 <sup>6</sup> cells                               | 6–7 $\mu$ g                                                    | required                                     |
| Animal tissue                                                                                     |                                                                  |                                                                |                                              |
| Mouse liver                                                                                       | 11 mg                                                            | 15–20 $\mu$ g                                                  | required                                     |
| Mouse tail                                                                                        | 0.5–1 cm of tail                                                 | 10–30 $\mu$ g                                                  | optional                                     |
| Insect                                                                                            |                                                                  |                                                                |                                              |
| Sf9 cells                                                                                         | 5 $\times$ 10 <sup>6</sup> cells                                 | 16 $\mu$ g                                                     | required                                     |
| Plant tissue                                                                                      |                                                                  |                                                                |                                              |
| Tomato leaf                                                                                       | 40 mg                                                            | 7–12 $\mu$ g                                                   | required                                     |
| Gram-negative bacteria                                                                            |                                                                  |                                                                |                                              |
| <i>Escherichia coli</i> JM109                                                                     | 1 ml                                                             | 20 $\mu$ g                                                     | required                                     |
| overnight culture,                                                                                | 5 ml                                                             | 75–100 $\mu$ g                                                 | required                                     |
| ~2 $\times$ 10 <sup>8</sup> cells/ml                                                              |                                                                  |                                                                |                                              |
| <i>Enterobacter cloacae</i> ,                                                                     | 1 ml                                                             | 20 $\mu$ g                                                     | required                                     |
| overnight culture,                                                                                | 5 ml                                                             | 75–100 $\mu$ g                                                 | required                                     |
| ~6 $\times$ 10 <sup>8</sup> cells/ml                                                              |                                                                  |                                                                |                                              |
| Gram-positive bacteria                                                                            |                                                                  |                                                                |                                              |
| <i>Staphylococcus epidermidis</i> ,                                                               | 1 ml                                                             | 5–13 $\mu$ g                                                   | required                                     |
| overnight culture,                                                                                |                                                                  |                                                                |                                              |
| ~3.5 $\times$ 10 <sup>8</sup> cells/ml                                                            |                                                                  |                                                                |                                              |
| Yeast                                                                                             |                                                                  |                                                                |                                              |
| <i>Saccharomyces cerevisiae</i> ,                                                                 | 1 ml                                                             | 4.5–6.5 $\mu$ g                                                | required                                     |
| overnight culture,                                                                                |                                                                  |                                                                |                                              |
| ~1.9 $\times$ 10 <sup>8</sup> cells/ml                                                            |                                                                  |                                                                |                                              |

The procedure can be performed manually or in liquid-handling robots such as the Biomek FX and Biomek 2000 Laboratory Automation Workstations (Beckman Coulter). Individual minicolumns can be used for: manual processing of low numbers of samples. Amplifiable DNA can be isolated from up to 5  $\times$  10<sup>6</sup> cells, 20 mg of tissue, or from up to 1.2 cm of mouse tail without centrifugation of the lysates. Isolation from tissue or mouse tails requires an overnight digestion with proteinase K. The genomic DNA isolated with this system is of high quality and serves as an excellent template for PCR.

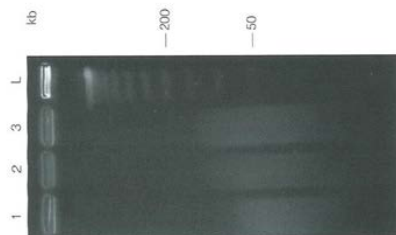


FIGURE 2. Pulsed-field gel electrophoresis of genomic DNA isolated from whole blood using the Wizard Genomic DNA Purification Kit. DNA samples were resolved by electrophoresis through a 1% agarose gel using a CHEF Mapper apparatus (BioRad, Hercules, CA). The initial and final switch intervals were varied with a time ramp of 2 seconds (initial) to 30 seconds (final). Electrophoresis was performed in 0.5X TBE buffer at 14°C for 20 hours. (Lane L) Lambda Ladders Molecular Weight Markers. DNA was purified from whole blood collected in a tube coated with EDTA (lane 1), heparin (lane 2), or citrate (lane 3). (Reprinted, with permission, from Micka et al. 1996 Promega Notes 56, p. 2, fig. 0896GA12 [©2003 Promega Corporation].)

## MATERIALS

### BUFFERS, SOLUTIONS, AND REAGENTS

1X PBS (for tissue culture cells)

Dissolve the following in 1 liter of sterile, deionized H<sub>2</sub>O. Autoclave. The pH of 1X PBS should be 7.4.

Na<sub>2</sub>HPO<sub>4</sub> <1> 1.15 g

KH<sub>2</sub>PO<sub>4</sub> <1> 0.2 g

NaCl 8 g

KCl <1> 0.2 g

### ENZYMES AND ENZYME BUFFERS

Digestion Solution Master Mix (for tissues and mouse tails):

For each tissue sample, combine the following:

Nuclei Lysis Solution 200  $\mu$ l

EDTA (0.5 M) 50  $\mu$ l

Proteinase K <1> (20 mg/ml) 20  $\mu$ l

RNase A solution (4 mg/ml) 5  $\mu$ l

Proteinase K <1> (20 mg/ml solution; for tissues and mouse tails)

Resuspend proteinase K with nuclease-free H<sub>2</sub>O to a concentration of 20 mg/ml. Dispense into working volumes determined by the average number of preps done at a time. Store at -20°C and thaw on ice. Avoid multiple freeze-thaw cycles.

### CAUTION

See Appendix for appropriate handling of materials marked with <1>.

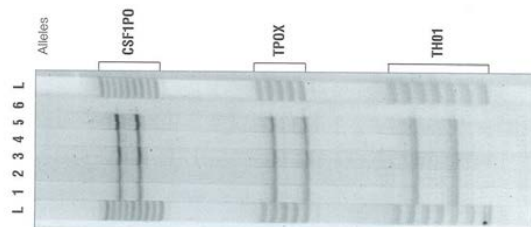


FIGURE 3. PCR amplification of polymorphic short tandem repeat (STR) sequences from genomic DNA isolated using the Wizard Genomic DNA Purification Kit. Amplification was performed using the *GeneAmp* CSF1PO, TPOX, TH01 Multiplex (Promega, DG6001). Products were separated by electrophoresis in a 4% denaturing polyacrylamide gel and detected by silver staining. (Lane 1) STR multiplex CSF1PO, TPOX, TH01 Ladder Mix. (Lanes 1–5) Replicate amplification reactions from samples purified from the same blood sample. (Lane 6) Negative (no template) control. (Reprinted, with permission, from Micka et al. 1996, Promega Notes 56, p. 2, fig. 0897GA12 [©2003 Promega Corporation].)

#### SPECIAL EQUIPMENT

- 3 single-position labware ALP (Beckman Coulter)
- 4 P250 tip rack assemblies (Beckman Coulter)
- 4 × 4 position labware ALP (Beckman Coulter)
- 96-well Tip Wash ALP (Beckman Coulter)
- Adhesive plate sealers (foil)
- Heating and Cooling ALP (Beckman Coulter)
- Liquid-handling robot (for automated procedure) such as the Biomek FX or Biomek 2000 Laboratory Automation Workstation (Beckman Coulter)
- Orbital Shaker ALP (Beckman Coulter)
- Tip Loader ALP (Beckman Coulter)
- Vac-Man 96 Vacuum Manifold (Promega)
- Vacuum pump capable of 15–20 inches of Hg
- Vacuum trap for waste collection (1-liter size)
- Vacuum tubing
- Water bath, 55°C

#### ADDITIONAL ITEMS

Wizard SV 96 Genomic DNA Purification System (Promega; includes Binding Plate, 96-well deep-well plate, DNA elution plate, Nuclei Lysis Solution, 0.5 M EDTA, Wizard SV Lysis Buffer, Wizard SV Wash Solution, RNase A solution, and nuclease-free H<sub>2</sub>O)

#### CELLS AND TISSUES

Samples for genomic DNA purification (mouse tail clippings, animal tissue, or tissue culture cells)

#### METHOD

##### PREPARATION OF MOUSE TAIL AND TISSUE LYSATES FOR AUTOMATED PURIFICATION

1. Place a 0.5- to 1.2-cm mouse tail clipping or up to 20 mg of other tissue into each well of a 96-well, deep-well plate.
2. Add 275 µl of the Digestion Solution Master Mix per sample. Cover the plate with an adhesive seal.
3. Place the plate in a 55°C water bath and incubate overnight (16–18 hours).

If genomic DNA will not be purified directly after overnight proteinase K digestion, the sample lysate may be stored frozen at –70°C. Before proceeding with automated genomic DNA purification, the frozen lysate must be warmed to 55°C for 1 hour.

*Optional:* If undigested hair and cartilage remain after overnight proteinase K digestion, spin the plate in a centrifuge at 200g to pellet the undigested sample. Transfer the supernatant to a new 96-well, deep-well plate.

##### AUTOMATED PURIFICATION OF GENOMIC DNA FROM PREPARED TISSUE LYSATES

After proteinase K digestion of sample tissue, all subsequent genomic DNA purification steps can be automated on a liquid-handling workstation without further user intervention. After the required tools, reagents, and the warm tissue lysate plate are placed on the deck of the automated liquid-handler, perform the following steps automatically.

1. Add 250 µl of Wizard SV Lysis Buffer to each sample well.
2. Mix the contents of each well by pipetting several times.
3. Transfer the tissue lysates to the wells of the Binding Plate sitting in the vacuum manifold. Bind genomic DNA to the plate by applying a vacuum until all of the lysate has passed through the Binding Plate.
4. Add 1 ml of Wizard SV Wash Solution to each well of the Binding Plate.
5. Apply the vacuum until the wash solution passes through the Binding Plate.
6. Repeat steps 4 and 5 for a total of 3 washes.
7. After the wells have emptied, continue to apply vacuum for an additional 6 minutes to allow the binding matrix to dry.
8. Prepare the vacuum manifold assembly for elution. Move the Binding Plate and position a deep-well elution plate in the vacuum manifold. Then position the Binding Plate on top of the elution plate in the same orientation so that the Binding Plate tips are centered on the deep-well elution plate.

9. Add 200  $\mu$ l of room-temperature, nuclease-free  $H_2O$  to each well of the Binding Plate, and incubate for 2 minutes at room temperature.

10. Apply the vacuum for 1 minute to elute purified genomic DNA from the SV 96 Binding Plate into the 96-well deep-well plate.

11. Repeat steps 9–10 for a total elution volume of 400  $\mu$ l.

12. Purification of genomic DNA is complete, and the purified sample is contained in the 96-well deep-well plate. Purified samples can be stored at  $-20^\circ C$  or  $-70^\circ C$  by covering the plate tightly with a plate seal.

#### AUTOMATED PURIFICATION OF GENOMIC DNA FROM TISSUE CULTURE CELLS

The following procedure is for automated purification of genomic DNA from tissue culture cells in 96-well culture plates. A maximum of  $5 \times 10^6$  cells per well can be purified. Exceeding this number of cells per well may cause clogging of the SV 96 Binding Plate during purification. The number of cells may need to be adjusted depending on cell type and function.

1. Wash the cells once with sterile 1X PBS before automated purification. All subsequent steps can be automated on a liquid-handling workstation without further user intervention. After the required tools, reagents, and cells (without media or PBS) are placed on the deck of the automated liquid-handler, perform the following steps automatically.

2. Add 150  $\mu$ l of Wizard SV Lysis Buffer to the washed cells. Mix by pipetting.

If genomic DNA will not be purified immediately, the method may be stopped after the addition of the Wizard SV Lysis Buffer and the samples can be stored at  $-70^\circ C$ . With thawed sample lysates, purification of genomic DNA may resume at step 3.

3. Transfer the cell lysates to the Binding Plate sitting in a vacuum manifold assembly. Bind the genomic DNA to the binding matrix by applying a vacuum until the lysate has passed through the Binding Plate.

4. Add 1 ml of Wash Solution to each well of the Binding Plate. Apply the vacuum until the Wash Solution passes through the Binding Plate.

5. Repeat step 4 two more times for a total of 3 washes with the Wash Solution.

6. After the wells have emptied, continue to apply the vacuum for an additional 6 minutes to dry the binding matrix.

7. Prepare the vacuum manifold assembly for elution. Move the Binding Plate, and position a deep-well elution plate in the vacuum manifold. Then position the Binding Plate on top of the elution plate in the same orientation so that the Binding Plate tips are centered on the deep-well elution plate.

8. Add 200  $\mu$ l of room-temperature, nuclease-free  $H_2O$  to each well of the Binding Plate and incubate for 2 minutes at room temperature.

9. Apply a vacuum for 1 minute to elute purified genomic DNA from the SV 96 Binding Plate to the 96-well deep-well plate.

10. Purification of genomic DNA is complete, and the purified sample is contained in the 96-well, deep-well plate. Purified samples can be stored at  $-20^\circ C$  or  $-70^\circ C$  by covering the plate tightly with a plate seal.

RNA may be copurified with genomic DNA. To remove copurified RNA, add 2  $\mu$ l of RNase A Solution per 250  $\mu$ l of nuclease-free  $H_2O$  before elution of genomic DNA from the column. After elution, incubate the purified genomic DNA at room temperature for 10 minutes. Alternatively, the RNase A Solution (2  $\mu$ l) may be added after elution.

#### CHARACTERIZATION OF ISOLATED DNA

Yield and quality of Wizard SV-purified samples can be determined by spectrophotometric analysis at  $A_{260}$  and  $A_{280}$ . Typical yield of high-molecular-weight genomic DNA is 5–20  $\mu$ g, depending on sample source (Table 2), with  $A_{260}/A_{280}$  ratios of  $1.7 \pm 0.08$ . Yield and quality of individual mouse tail samples processed by a Biomek 2000 robotic workstation are given in Figure 4. The utility of purified genomic DNA as a template in PCR amplification was also assessed. One  $\mu$ l of genomic DNA purified from either mouse tail or tissue culture samples was amplified by PCR. Mouse-tail samples were evaluated by amplifying with interleukin-1 $\beta$  (IL-1 $\beta$ )-specific primers. Genomic DNA isolated from HeLa cells was evaluated by amplifying with primers specific for Factor V DNA (Fig. 5).

### PROTOCOL 3 MAGNETIC PARTICLE-BASED METHODS FOR HIGH-THROUGHPUT GENOMIC DNA ISOLATION FROM WHOLE BLOOD AND BUCCAL SWABS

The Wizard MagneSil Genomic DNA Purification System uses a solid-phase silica paramagnetic particle for the purification of genomic DNA. This technology was developed as an alternative to vacuum filtration and centrifugation-based purification formats to allow cost-efficient, high-throughput sample processing. MagneSil technology is readily adaptable to robotic platforms, allowing complete automation of the purification process in either 96- or 384-well microwell plate formats.

A significant advantage of using magnetic particles for bioseparation applications is their utility with variously sized starting samples. With most nucleic acid purification systems, the amount of starting material is limited by the fixed size of the product's purification matrix. With MagneSil particles, the amount of magnetic solid phase can be scaled to the amount of starting material. Thus, a single system provides increased flexibility with protocols covering small- to large-scale purifications. Magnetic particles can also be used with most off-the-shelf plasticware, making them a cost-effective alternative to membrane-based purification methods.

Magnetic DNA purification systems based on MagneSil particles can be used to isolate genomic DNA from sample types such as whole blood, cultured cells, a variety of foods and foodstuffs, and plant leaf or seed tissue. Two systems have been developed for the isolation of genomic DNA from human whole blood: The first processes 200  $\mu$ l of whole blood, generating 4–9  $\mu$ g of high-quality genomic DNA. This method is scalable down to 50  $\mu$ l, providing added flexibility for downstream DNA testing.

For users who prefer to avoid the need for post-purification DNA quantitation, the DNA IQ System provides a magnetic-based method for the purification of a predetermined

TABLE 2. Typical yields of genomic DNA isolated from a variety of sources using the Wizard SV Genomic DNA Purification System

| Sample              | Amount                | Average yield |
|---------------------|-----------------------|---------------|
| Mouse tail clipping | 20 mg                 | 20 $\mu$ g    |
| Mouse liver         | 20 mg                 | 15 $\mu$ g    |
| Mouse heart         | 20 mg                 | 10 $\mu$ g    |
| Mouse brain         | 20 mg                 | 6 $\mu$ g     |
| CHO cells           | $1 \times 10^6$ cells | 5 $\mu$ g     |
| NIH-3T3 cells       | $1 \times 10^6$ cells | 9 $\mu$ g     |
| 293 cells           | $1 \times 10^6$ cells | 8 $\mu$ g     |

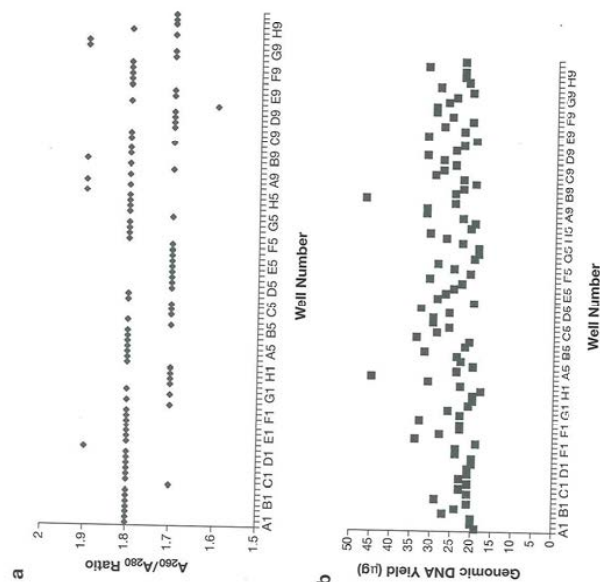


FIGURE 4. Purity (a) and yield (b) measurements of genomic DNA from 96 separate 20-mg mouse tail clippings purified with the Wizard SV 96 Genomic DNA Purification System. Purity was measured by  $A_{260}/A_{280}$  ratio and yield was measured by absorbance at 260 nm. (Reprinted, with permission, from Grunert and Worzella 2002, Promega Notes 81, p. 9, fig. 3694MA04\_2A [© 2003 Promega Corporation].)

amount of DNA from a blood or tissue sample. Methods have been developed for the purification of DNA in the ranges of either 100 ng or 1.0 µg from human whole blood. These applications have been adapted to several laboratory robotic workstations, including the Biomek FX and 2000 (Beckman Coulter) and Genesis (Tecan) systems.

#### METHOD 1: HIGH-THROUGHPUT ISOLATION OF GENOMIC DNA FROM 200 µL OF WHOLE BLOOD USING A LIQUID-HANDLING ROBOTIC WORKSTATION

This method is designed to process 200-µl blood samples in a 96-well format on a liquid-handling robot such as the Biomek FX and Biomek 2000 Laboratory Automation Workstations (Beckman Coulter). Single-plate processing time is less than an hour with a single-head instrument.

Blood is mixed with a detergent-chaotrope lysis/binding buffer to disrupt cell and nuclear membranes and denature proteins. Released DNA adsorbs to the surface of the

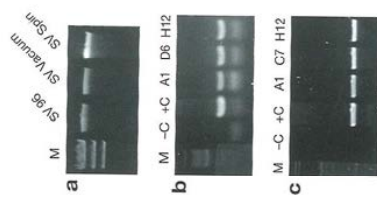


FIGURE 5. Analysis of genomic DNA from HeLa cells and mouse tail clippings isolated by using the Wizard SV and SV 96 Genomic DNA Purification Systems. (a) Agarose gel analysis of genomic DNA isolated from 5 × 10<sup>6</sup> HeLa cells. (b) PCR amplification of Factor V DNA from HeLa cells. (Lanes A1, C7, H12) Sample wells. 250-bp amplification products. (c) PCR amplification of IL-1β DNA from mouse tail samples. (Lanes A1, C7, H12) Sample wells. 1.2-kb amplification products. (Lane M) DNA markers. (-C) negative control. (Reprinted, with permission, from Grunert and Worzella 2002, Promega Notes 81, p. 9, fig. 3711TA02\_2A [© 2003 Promega Corporation].)

Magnesium particles contained in the lysis/binding buffer. The particle-adherent target complex is captured to the side of the well by an external magnetic field, and the lysate is removed as waste. Residual contaminants are removed with a series of washes, first with lysis/binding buffer, then with a salt wash solution, and finally with an alcohol/salt wash to remove the last traces of heme. After drying the particles, the DNA is eluted in TE buffer or water on a heat transfer plate.

#### MATERIALS

##### SPECIAL EQUIPMENT

- 3 single-position labware ALP (Beckman Coulter)
- 4 P250 tip rack assemblies (Beckman Coulter)
- 4 × 4 position labware ALP (Beckman Coulter)
- 96-well collection plate (Promega, A9161 or comparable)
- 96-well Tip Wash ALP (Beckman Coulter)
- Biomek FX Robotic Workstation fitted with a single pod and a 96-well pipetting head (Beckman Coulter)
- Deep-Well MagnaBot 96 Magnetic Separation Device (Promega)
- Deep-well multiwell plates (Marsh, AB-0932 [2.2 ml] and AB-0787 [1.2 ml] or comparable)
- Heat transfer block (Promega, Z3271 or comparable)
- Heating and Cooling ALP (Beckman Coulter)

MagnaBot Spacer, 1/8 inch (Promega)  
Orbital Shaker ALP (Beckman Coulter)  
Pyramid-bottom, 96-well reservoir (Innovative Microplates, S30014 or comparable)  
Tip Loader ALP (Beckman Coulter)

#### ADDITIONAL ITEMS

MagneSil Blood Genomic, Max Yield System (Promega; includes alcohol wash, anti-foam reagent, Lysis Buffer, Salt Wash, Elution Buffer, and MagneSil Paramagnetic Particles)

#### CELLS AND TISSUES

Whole blood, buccal swabs, or sample stains<=>

#### METHOD

1. Add 400  $\mu$ l of Lysis Buffer and 40  $\mu$ l of MagneSil particles to 200  $\mu$ l of blood. Use extensive robotic mixing to lyse the white blood cells. The released DNA binds the magnetic particles (PMPs).
2. Capture the particles with the MagnaBot magnetic array and discard the liquid.
3. Wash the PMPs extensively with 360  $\mu$ l of Lysis Buffer.
4. Capture the particles with the MagnaBot magnetic array and discard the liquid.
5. Wash the PMPs extensively with 360  $\mu$ l of Salt Wash.
6. Capture the particles with the MagnaBot magnetic array and discard the liquid.
7. Repeat steps 5 and 6 for a total of 2 washes with Salt Wash.
8. Wash the PMPs extensively with 360  $\mu$ l of Alcohol Wash Buffer.
9. Capture the particles with the MagnaBot magnetic array and discard the liquid.
10. Repeat steps 8 and 9 for a total of 3 washes with Alcohol Wash Buffer.
11. Dry the PMPs on a heat block to eliminate any residue of the Alcohol Wash Buffer.
12. Elute the DNA in 210  $\mu$ l of Elution Buffer, with extensive mixing, on a heat block set at 80°C. The temperature inside the wells will be -65°C.
13. Store the eluted DNA at -20°C.

▼ CAUTION  
See Appendix for appropriate handling of materials marked with <=>.

#### METHOD 2: DNA IQ ISOLATION OF GENOMIC DNA FROM STAINS AND BUCCAL SWABS

The DNA IQ System uses a novel approach for DNA isolation. The MagneSil chemistry is used to capture and release a consistent amount of DNA across a wide range of samples. The particles have a defined DNA capacity and, in the presence of excess DNA, will only bind a fixed amount of nucleic acid (Fig. 6). This property is used to isolate ~100 ng of DNA, independent of sample size. The DNA is eluted into 100  $\mu$ l of Elution Buffer to give a final concentration of 1 ng/ $\mu$ l. As a result, post-purification DNA quantitation is not necessary when using this system. An overview of the protocol is given in Figure 7.

#### MATERIALS

#### BUFFERS, SOLUTIONS, AND REAGENTS

Dithiothreitol (DTT)<=>, 1 M

▼ CAUTION  
See Appendix for appropriate handling of materials marked with <=>.

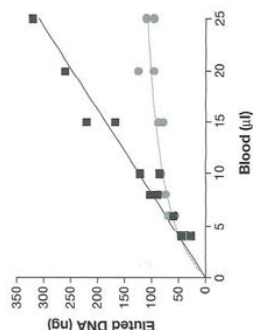


FIGURE 6. Effect of sample volume on the amount of DNA isolated using the DNA IQ System. A high-capacity resin shows a linear relationship between eluted DNA versus amount ( $\mu$ l) of blood extract (represented by squares). In contrast, the DNA IQ System Resin is saturated using the blood volumes tested and thus gives approximately the same amount of DNA regardless of sample size (represented by circles). (Reprinted, with permission, from Promega DNA IQ System-Database Protocol Technical Bulletin #TB297, fig. 3236MAC2\_1A [©2003 Promega Corporation].)

Ethanol, 95–100%<=>  
Isopropyl alcohol<=>  
Lysis Buffer<=>

Determine the total amount of Lysis Buffer to be used (Table 3). For each 100  $\mu$ l of Lysis Buffer (supplied with the DNA IQ System), add 1  $\mu$ l of 1 M DTT. Mix by inverting several times. Mark and date the label to indicate that DTT has been added. The Lysis Buffer can be stored at room temperature for up to one month.

#### SPECIAL EQUIPMENT

Aerosol-resistant micropipette tips  
DNA IQ Spin Baskets (Promega, V1221)  
Heat block  
MagneSphere Technology Magnetic Separation Stand (Promega)  
Microcentrifuge tubes, 1.5-ml conical

#### ADDITIONAL ITEMS

DNA IQ System (Promega; includes Resin, Lysis Buffer, 2x Wash Buffer, and Elution Buffer)  
Microtubes, 1.5 ml (Promega, V1231)

#### METHOD

1. Place sample (see Table 3) in a 1.5-ml microcentrifuge tube. Keep in mind that the recommended amount of resin can capture a maximum of ~100 ng of DNA.
2. Add the appropriate amount of prepared Lysis Buffer. Different samples require different volumes of Lysis Buffer; see Column 2 ("Lysis Buffer 1") of Table 3 for the appropriate volume to add at this point. Close the lid and place the tube in a heat block at 95°C for 30 minutes.

For small stains, an alternative approach is to place the stained material in a DNA IQ Spin Basket seated in 1.5-ml Microtubes, and add 100–150  $\mu$ l of Lysis Buffer to the basket.

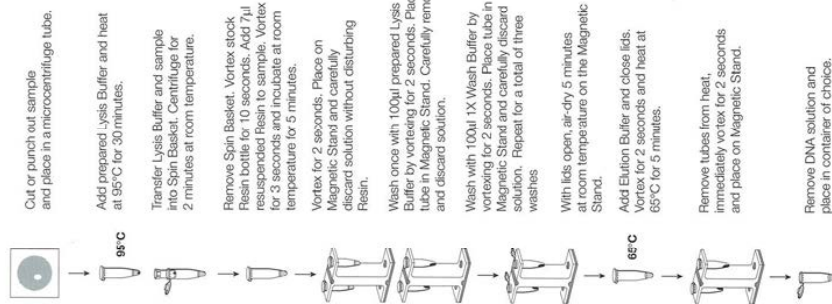


FIGURE 7. Schematic of DNA isolation from stains or swabs using the DNA IQ System. (Reprinted, with permission, from Promega DNA IQ System-Database Protocol Technical Bulletin #TB297, fig. 3237MA02\_1A[02003, Promega Corporation].)

Carefully close the lid and heat at 95°C for 30 minutes. Most of the Buffer should remain in the basket if the indicated Microtubes and Spin Baskets are used. Proceed to step 4. This does not work reliably with samples requiring more than 150 µl of Lysis Buffer.

3. Remove the tube from the heat block and transfer the Lysis Buffer and sample to a Spin Basket placed in a standard 1.5-ml conical microcentrifuge tube.

TABLE 3. Amount of DNA IQ Lysis Buffer required per sample

| Material                            | Lysis Buffer 1 | Lysis Buffer 2 | Total Buffer |
|-------------------------------------|----------------|----------------|--------------|
| Liquid blood                        | 100 µl         | 100 µl         | 200 µl       |
| Cotton swab                         | 250 µl         | 100 µl         | 350 µl       |
| 1/4th CEP swab                      | 250 µl         | 100 µl         | 350 µl       |
| 15–50 mm <sup>2</sup> S&S 903 paper | 150 µl         | 100 µl         | 150 µl       |
| 3–30 mm <sup>2</sup> FTA paper      | 150 µl         | 100 µl         | 150 µl       |

It is important to centrifuge Lysis Buffer with the stained matrix to obtain maximum recovery.

4. Centrifuge at room temperature for 2 minutes at maximum speed. Remove the Spin Basket.
5. Vortex the stock Resin bottle for 10 seconds at high speed or until thoroughly mixed. Add 7 µl of Resin to the DNA solution. Keep the stock Resin resuspended while dispensing to obtain uniform results.
6. Vortex the sample/Lysis Buffer/Resin mix for 3 seconds at high speed. Incubate at room temperature for 5 minutes.
7. Vortex the tube for 2 seconds at high speed and place it in the Magnetic Stand. Separation will occur instantly.
8. Carefully remove and discard all of the solution without disturbing the Resin on the side of the tube.
9. Add 100 µl of prepared Lysis Buffer. Remove the tube from the Magnetic Stand and vortex for 2 seconds at high speed.
10. Return the tube to the Magnetic Stand and discard all Lysis Buffer.
11. Add 100 µl of prepared 1X Wash Buffer. Remove tube from the Magnetic Stand and vortex for 2 seconds at high speed.
12. Return the tube to the Magnetic Stand and discard all Wash Buffer.
13. Repeat steps 11 and 12 two more times for a total of 3 washes. Make sure that all of the solution has been removed after the last wash.
14. Open the lid of the tube and air-dry the Resin in the Magnetic Stand for 5 minutes.
15. Add 100 µl of Elution Buffer.
16. Close the lid, vortex the tube for 2 seconds at high speed, and place the tube at 65°C for 5 minutes.
17. Remove the tube from the heat block and vortex for 2 seconds at high speed. Immediately place the tube on the Magnetic Stand.
18. Transfer the solution to a container of choice. The DNA concentration will be ~1 ng/µl, ready for use in an amplification reaction.

The DNA solution can be kept at 4°C for short-term storage, or at -20°C or -70°C for long-term storage.

#### POST-AMPLIFICATION CLEANUP OF PCR PRODUCTS

The purification of PCR products can be divided into two types: (1) direct purification from the PCR, sometimes referred to as solution purification, and (2) purification from agarose gels when separation from contaminating fragments is required.

A number of methods have been developed for solution purification. The most commonly used methods are based on ultrafiltration, precipitation, or binding and elution from silica. Ultrafiltration uses small plastic devices containing an ultrafiltration membrane that retains larger fragments and allows nucleotides and small primers to pass through. Several rounds of dilution and filtration may be required for efficient removal of contaminating primers and nucleotides. Precipitation may also be required to concentrate the sample. The need for centrifugation during this method makes processing of large numbers of samples cumbersome. For the same reason, this type of method can be difficult to automate.

Ethanol, isopropanol, and polyethylene glycol can be used to precipitate nucleic acids in the presence of various salts. These methods are low in cost and can meet various throughput needs ranging from single samples to 384 samples at a time. Multiple centrifugation steps are required, first to precipitate the nucleic acid and second to remove contaminating salts that can be inhibitory to enzymes used in some downstream applications. The multiple centrifugation steps make hands-off automation difficult. In contrast, the use of silica provides a balance between ease of use, cost per isolation, and the ability to automate the process.

When a target DNA must be isolated from other contaminating fragments, the mixture can be fractionated by agarose gel electrophoresis followed by extraction of the fragment of interest from the gel. Standard methods for extracting DNA from agarose gel slices include electrophoresis onto DEAE-cellulose membranes, electro-elution, anion-exchange chromatography, organic extraction, and enzymatic digestion with agarase. These purification methods work well in certain situations but frequently are limited by low recovery rates and poor quality of the recovered DNA. These methods can also be time-consuming and labor-intensive, and they often require further purification or extraction steps. Silica-based methods solve most of these problems.

## PROTOCOL 4 PURIFICATION OF PCR FRAGMENTS FROM AGAROSE GELS

The Wizard SV Gel and PCR Clean-Up System provides a silica-based method for the purification of PCR fragments from agarose gel slices or directly from PCR samples. In the gel-based method, the DNA band(s) of interest is excised from the gel and dissolved in a guanidine isothiocyanate solution. This mixture is passed through a binding membrane where DNA adsorbs to the silica surface. Nonaqueous material is removed with a wash solution, and the purified DNA is eluted in water. This chemistry is designed to purify fragments of 100 bp to 10 kb from standard or low-melting-point agarose in either Tris-acetate (TAE) or Tris-borate (TBE) buffer with 70–95% recovery. Processing time is 15–20 minutes, depending on the number of samples. The purified samples can be used for a number of downstream applications, including fluorescent DNA sequencing, cloning, labeling, amplification, restriction digestion, and *in vitro* transcription/translation without further processing.

### MATERIALS

#### BUFFERS, SOLUTIONS, AND REAGENTS

- Ethanol (95%)<1>
- Intercalating dye such as ethidium bromide<1>
- TAE electrophoresis buffer
- 40 mM Tris-acetate<1>, pH 8.2
- 1 mM EDTA<1>

▼ CAUTION  
See Appendix for appropriate handling of materials marked with <1>.

### OR

- TBE electrophoresis buffer
- 90 mM Tris-borate<1>, pH 8.3
- 2 mM EDTA

### NUCLEIC ACIDS AND OLIGONUCLEOTIDES

PCR fragments from agarose gel slices

### GELS

Agarose gel (standard or low-melt)

### SPECIAL EQUIPMENT

- Microcentrifuge tubes, 1.5-ml
- Scalpel or razor blade
- UV lamp, long-wavelength
- Vacuum manifold and vacuum adapters (Promega; for purification with vacuum)

### ADDITIONAL ITEMS

Wizard SV Gel and PCR Clean-Up System (Promega; includes SV Minicolumns, collection tubes, Membrane Binding Solution, Membrane Wash Solution, and nuclease-free H<sub>2</sub>O)

### CELLS AND TISSUES

PCR samples for purification

### METHOD

Standard safety apparel should be worn, especially when handling ethidium bromide-stained agarose gels. This includes gloves and a UV-blocking face shield to protect the eyes and face from UV light.

#### DISSOLVING THE GEL SLICE

1. Load the PCR samples on an agarose gel and run according to an established protocol.
2. Weigh a 1.5-ml microcentrifuge tube for each DNA fragment to be isolated; record the weight.
3. Visualize and photograph the DNA using a long-wavelength UV lamp and an intercalating dye such as ethidium bromide. To reduce nicking, irradiate the gel for the absolute minimum time possible (Grundemann and Schömig 1996; Zimmermann et al. 1998). Excise the DNA fragment of interest in a minimal volume of agarose using a clean scalpel or razor blade. Transfer the gel slice to the weighed microcentrifuge tube and record the weight. Subtract the weight of the empty tube from the total weight to obtain the weight of the gel slice.
4. Add Membrane Binding Solution at a ratio of 10 µl of solution per 10 mg of agarose gel slice.
5. Vortex the mixture and incubate at 50–65°C for 10 minutes or until the gel slice is completely dissolved. Vortex the tube every few minutes to increase the rate of agarose gel melting. Centrifuge the tube briefly at room temperature to ensure the contents are at the bottom of the tube. Once the agarose gel is melted, the gel will not resolidify at room temperature.
6. DNA may then be purified by centrifugation or vacuum filtration.

## DNA PURIFICATION BY CENTRIFUGATION

1. Place one SV Minicolumn in a Collection Tube for each dissolved gel slice.
2. Transfer the dissolved gel mixture to the SV Minicolumn assembly and incubate for 1 minute at room temperature.
3. Centrifuge the SV Minicolumn assembly at 10,000g for 1 minute. Remove the Minicolumn from the Spin Column assembly and discard the liquid in the Collection Tube. Return the Minicolumn to the Collection Tube.
4. Wash the column by adding 700  $\mu$ l of Membrane Wash Solution to the Minicolumn. Centrifuge the Minicolumn assembly for 1 minute at 10,000g. Empty the Collection Tube as before and place the Minicolumn back in the Collection Tube. Repeat the wash with 500  $\mu$ l of Membrane Wash Solution and centrifuge the Minicolumn assembly for 5 minutes at 10,000g.
5. Remove the Minicolumn assembly from the centrifuge, being careful not to wet the bottom of the column with the flowthrough. If the column becomes wet, empty the Collection Tube and re-centrifuge the column assembly for 1 minute.
6. Transfer the Minicolumn to a clean 1.5-ml microcentrifuge tube. Apply 50  $\mu$ l of nuclease-free H<sub>2</sub>O directly to the center of the column without touching the membrane with the pipette tip. Incubate at room temperature for 1 minute. Centrifuge for 1 minute at 10,000g.
7. Discard the Minicolumn and store the microcentrifuge tube containing the eluted DNA at 4°C or -20°C.

## DNA PURIFICATION BY VACUUM

1. Attach one Vacuum Adapter with a Luer-Lok fitting to one port of the manifold for each dissolved gel slice or PCR. Insert an SV Minicolumn into each Vacuum Adapter until it fits snugly in place.
2. Transfer the dissolved gel mixture or PCR to the Minicolumn and incubate for 1 minute at room temperature. Apply a vacuum to pull the liquid completely through the Minicolumn.
3. Wash the column by adding 700  $\mu$ l of Membrane Wash Solution to the Minicolumn. Make sure that any droplets remaining on the sides of the Minicolumn from the last step are washed away. Apply a vacuum to pull the liquid through the Minicolumn. Repeat this wash a second time with 500  $\mu$ l of Membrane Wash Solution.
4. Turn off the vacuum source and open an unused port to vent the manifold. Remove the minicolumn from the vacuum manifold and transfer the Minicolumn to a Collection Tube. Centrifuge the Minicolumn assembly for 5 minutes at 10,000g to remove any remaining Membrane Wash Solution from the column.
5. Transfer the Minicolumn to a clean 1.5-ml microcentrifuge tube. Apply 50  $\mu$ l of nuclease-free H<sub>2</sub>O directly to the center of the column, without contacting the membrane. Incubate at room temperature for 1 minute. Centrifuge for 1 minute at 10,000g.
6. Discard the Minicolumn and store the microcentrifuge tube containing the eluted DNA at 4°C or -20°C.

## PROTOCOL 5

## PURIFICATION OF PCR PRODUCTS FROM SOLUTION, SILICA MEMBRANE-BASED METHOD

The Wizard SV 96 PCR Clean-Up System provides a membrane-based system that can be used for high-throughput PCR cleanup in a 96-well multiwell plate format. In the Membrane Binding Solution, PCR products adsorb to the silica surface. Primers, low-molecular-weight double-stranded fragments such as primer-dimers, and other reaction components have no affinity for the solid phase and can be removed by vacuum filtration. Residual nonadherent material is removed by washing, and purified target DNA is eluted in water.

Fully automated methods have been developed for a number of automated liquid-handling robotic workstations, including the Biomek 2000 and FX. Recovery is independent of variable PCR conditions that may include different DNA polymerases, PCR additives, or the presence or absence of mineral oil. Fragments from 100 bp to 10 kb can be purified with up to 90% recovery. The purified PCR fragments are suitable for automated fluorescent DNA sequencing, cloning, labeling, restriction enzyme digestion, or DNA microarray analysis without further manipulation.

## MATERIALS

## BUFFERS, SOLUTIONS, AND REAGENTS

Ethanol <1%, 80% (75 ml/plate; freshly made before each use)

## NUCLEIC ACIDS AND OLIGONUCLEOTIDES

PCR samples in 96-well format (20–100  $\mu$ l per sample)

## SPECIAL EQUIPMENT

Vac-Man 96 Vacuum Manifold (Pomona) or suitable vacuum manifold  
Vacuum pump capable of 15–20 inches of Hg  
Vacuum trap for waste collection (1-liter size)  
Vacuum tubing

## ADDITIONAL ITEMS

Wizard SV 96 PCR Clean-Up System (Promega; includes Binding Plate, Collection Plate, DNA Elution Plate, Membrane Binding Solution, and nuclease-free H<sub>2</sub>O)

## METHOD

1. Prepare the vacuum manifold as shown in Figure 8. Place the Binding Plate in the vacuum manifold base. To ensure that the well numbers on the sample plate correspond to the numbers on the Binding Plate, orient the Binding Plate in the vacuum manifold with the numerical column headers toward the vacuum port. Attach the vacuum line to the vacuum port on the Manifold Base.
2. Add an equal volume of Membrane Binding Solution to each volume of PCR sample in a 96-well plate (e.g., add 100  $\mu$ l of Membrane Binding Solution to each 100  $\mu$ l of PCR sample).
3. Mix by pipetting, and transfer the entire sample volume to the wells of the Binding Plate sitting on the vacuum manifold. Incubate for 1 minute at room temperature.

**CAUTION**  
See Appendix for appropriate handling of materials marked with <1>.

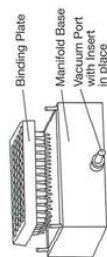
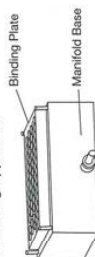
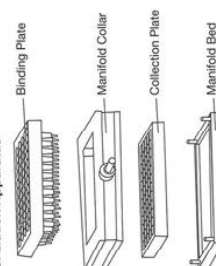
**a. PCR Product Binding Apparatus****b. Washing Apparatus****c. Elution Apparatus**

**FIGURE 8.** The Vac-Man 96 Vacuum Manifold with the Wizard SV 96 PCR Clean-Up System. *a*, *b*, and *c* show the manifold and plate combinations necessary to accomplish PCR product binding, washing, and elution, respectively, for manual PCR product cleanup. For automated cleanup on the Beckman Biomek 2000, the Beckman vacuum manifold (Beckman part no. 609670) and collar (Beckman part no. 609597) are required. (Reprinted, with permission, from Promega Wizard SV 96 PCR Clean-Up System Technical Bulletin #TB311, fig. 3744MA06.2A [© 2003 Promega Corporation].)

4. Apply a vacuum until the samples pass through the Binding Plate (~30 seconds). Release the vacuum.
5. Add 200  $\mu$ l of 80% ethanol to each well of the Binding Plate. Incubate for 1 minute at room temperature. Apply the vacuum until the ethanol passes through the plate (~30 seconds). Release vacuum.
6. Repeat step 5 for a total of 3  $\times$  200  $\mu$ l, 80% ethanol washes.
7. After the wells of the Binding Plate have emptied from the final wash, continue to apply the vacuum for an additional 4 minutes to allow the binding matrix to dry.
8. Turn off the vacuum. Release the vacuum line from the Manifold Base and snap it into the vacuum port in the Vacuum Manifold Collar. Remove the Binding Plate from the Manifold Base and blot by gently tapping onto a clean paper towel to remove residual ethanol.
9. Place a 96-well, U-bottom Collection Plate in the Manifold Bed and position the Vacuum Manifold Collar on top. Make sure to orient the U-bottom Collection Plate with the numerical column headers toward the vacuum port.

10. Position the Binding Plate on top of the Manifold Collar and the Collection Plate as shown in Figure 8. The Binding Plate tips must be centered on the Collection Plate wells, and both plates must be in the same orientation. Add 100  $\mu$ l of nuclease-free H<sub>2</sub>O to each well of the Binding Plate and incubate for 1 minute at room temperature. Apply the vacuum until the solution passes through the plate (~1 minute).

11. Release the vacuum and remove the Binding Plate. Carefully remove the Manifold Collar, making sure that the Collection Plate remains positioned in the Manifold Bed. If droplets are present on the walls of the Collection Plate, briefly centrifuge the plate to collect the droplets on the bottom of the wells. Eluate volumes may vary, but are roughly 75  $\mu$ l.

## PROTOCOL 6 PURIFICATION OF PCR PRODUCTS FROM SOLUTION, MAGNETIC PARTICLE-BASED METHOD

MagneSil is a silica-paramagnetic particle that acts as a mobile solid phase to capture double-stranded DNA fragments. The particle-adherent target complex is washed to remove residual contaminants, and purified DNA is eluted in water, ready for downstream fluorescent DNA sequencing and microarray analyses.

The MagneSil system can be scaled up for higher yield or scaled down for use in 384-well formats. PCR products greater than 150 bp in size are selectively isolated from primers and primer-dimers with high yields and purity. This technology has been adapted for use with a number of robotic workstations, including Beckman Coulter's Biomek 2000 and FX Laboratory Automation Workstations. The plate-processing times are less than 20 minutes with a single POD Biomek FX. Typical recovery is greater than 80% for a 1000-base product with negligible carryover of primers or nucleotides. Two methods have been developed to purify either a single plate or multiple plates of PCR samples: multiple-plate processing is achieved by the incorporation of a high-capacity stacker carousel to deliver consumables.

### MATERIALS

#### BUFFERS, SOLUTIONS, AND REAGENTS

Ethanol, 80% <1>

#### NUCLEIC ACIDS AND OLIGONUCLEOTIDES

PCR samples in 96-well format (up to 100  $\mu$ l/well)

#### SPECIAL EQUIPMENT

3 single-position labware ALP (Beckman Coulter)

4 P250 tip rack assemblies (Beckman Coulter)

4  $\times$  4 position labware ALP (Beckman Coulter)

96-well Tip Wash ALP (Beckman Coulter)

Heating and Cooling ALP (Beckman Coulter)

Liquid-handling robot such as the Biomek FX or Biomek 2000 Laboratory Automation workstation (Beckman Coulter)

MagnaBot 96 Magnetic Separation Device (Promega)

Nonpermeable plate sealer

Orbital Shaker ALP (Beckman Coulter)

Tip Loader ALP (Beckman Coulter)

Plate Clamp 96 (Promega)

## ADDITIONAL ITEMS

Wizard Magnesil PCR Clean-Up System (Promega; includes Magnesil YELLOW [paramagnetic particles], Wash Solution, nuclease-free  $H_2O$ , and Collection Plates)

## METHOD

The following protocol contains the steps and timing that are intended for use of the Magnesil PCR Clean-Up System in the development of an automated process using a robot. These same steps, including the specified reagent volumes, may also be used to perform the procedure manually. This system was designed to purify PCR products from 100- $\mu$ l sample volumes.

## DNA BINDING

1. Begin with completed PCR samples in a 96-well plate. Adjust the volume of each sample to 100  $\mu$ l with nuclease-free  $H_2O$ .
2. Resuspend Magnesil particles thoroughly by shaking. Add 100  $\mu$ l to each well.
3. Mix the contents of each well by pipetting up and down four times.
4. Incubate for 2 minutes at room temperature. Mix by pipetting up and down four times halfway through the incubation.
5. Transfer the mixed samples to a 96-well Collection Plate. Place the plate onto the MagnaBot 96 Magnetic Separation Device to magnetize the particles.
6. Remove and discard the supernatant.

## WASHING

1. Remove the Collection Plate containing the Paramagnetic Particles from the MagnaBot 96 Magnetic Separation Device. Add 200  $\mu$ l of Wash Solution to each well.
2. Mix the contents of each well by pipetting up and down four times.
3. Place the Collection Plate onto the MagnaBot 96 Magnetic Separation Device to magnetize the particles.
4. Remove and discard the supernatant.
5. Remove the Collection Plate from the MagnaBot 96 Magnetic Separation Device. Add 200  $\mu$ l of 80% ethanol to each well.
6. Mix the contents of each well by pipetting up and down four times.
7. Place the Collection Plate onto the MagnaBot 96 Magnetic Separation Device to magnetize the particles.
8. Remove and discard the supernatant.
9. Repeat steps 5–8 with 200  $\mu$ l of 80% ethanol, for a total of two ethanol washes.
10. Leave the plate to dry on the MagnaBot 96 Magnetic Separation Device for 5–10 minutes. Remove any residual ethanol from the wells by pipetting.

## ELUTION OF DNA

1. Remove the plate from the MagnaBot 96 Magnetic Separation Device. Add 100  $\mu$ l of nuclease-free  $H_2O$  to elute the PCR products from the Paramagnetic Particles. Mix each well by pipetting up and down four times. Incubate for 2 minutes at room temperature.
2. Place the Collection Plate onto the MagnaBot 96 Magnetic Separation Device to magnetize the particles.

3. Transfer the eluted PCR products to a clean Collection Plate.

4. If particles have been carried over with the eluate, place the Collection Plate onto the MagnaBot 96 Magnetic Separation Device and transfer the eluate to a clean Collection Plate. Alternatively, place the Collection Plate onto the MagnaBot 96 Magnetic Separation Device when withdrawing samples from the Collection Plate.

5. Seal the plate tightly with a nonpermeable plate sealer and store at 4°C for 1–2 days or at –20°C for long-term storage.

## PROTOCOL 7

## DYE TERMINATOR CLEANUP

Automated fluorescent sequencing using the ABI PRISM BigDye terminator chemistry is the method of choice for high-throughput DNA sequence determination. Sequence ladders are generated by the standard Sanger method (Sanger et al. 1977) using fluorescently labeled chain-terminator nucleotides (Prober et al. 1987). These BigDye terminators are dideoxynucleotide triphosphates linked to an energy transfer dye composed of a fluorescent donor and one of four d-Rhodamine-based acceptors (Rosenblum et al. 1997). Automated sequencers separate the labeled sequence extension products using a molecular sieve matrix and identify the terminal nucleotide based on its emission wavelength.

If not removed before analysis, certain reaction components will interfere with data collection. Buffer salts may also give rise to aberrant electrophoretic migration (Ruiz-Martinez et al. 1998) and unincorporated dye-labeled terminators, and related species interfere with the legitimate signal from sequencing extension products. Common purification methods based on gel filtration and precipitation by ethanol are problematic for high-throughput robotic applications because they can require multiple centrifugation and/or vacuum drying steps.

Magnesil particles can be applied to the high-throughput purification of BigDye terminator DNA sequencing reactions prior to automated sequence analysis. Procedures have been developed on several robotic workstations using the standard 96-well amplification plate format. Sequence quality is similar to or better than standard manual methods and offers the distinct advantage of a hands-off protocol. No user intervention is required from the time the amplification plate is put on the robot deck until the samples are loaded onto the DNA sequencer.

The Magnesil Sequencing Reaction Clean-Up System is designed to work with the ABI PRISM 310, 3100, 377, and 3700 DNA sequencers. Sequence quality has been assessed by accuracy (and/or PHRED score), read length, and start position. The system is qualified to provide sequence data that are >98% accurate over 600 bases with a start position of less than primer plus 25 bases when using the control template and primer included in the ABI PRISM BigDye terminator cycle sequencing reaction kit. An overview of the Magnesil Sequencing Reaction Clean-Up System protocol is given in Figure 9.

## MATERIALS

## BUFFERS, SOLUTIONS, AND REAGENTS

Elution/loading solution for sequencing gel<!-- (see Table 4) -->  
90% Ethanol wash solution<!-- -->

## SPECIAL EQUIPMENT

MagnaBot II Magnetic Separation Device (Promega)

**CAUTION**  
See Appendix for appropriate handling of materials marked with <!-- -->

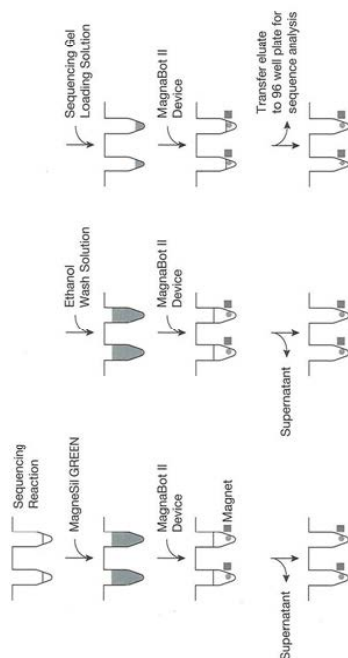


FIGURE 9. Schematic diagram of the Wizard MagnaSil Sequencing Reaction Clean-Up System protocol. (Reprinted, with permission, from Otto and Bjerke 2001, Promega Notes 78, p. 2, fig. 3269MA12.0A [©2003 Promega Corporation].)

Plate Clamp 96 (Promega)  
Plate Stand (Promega)

### ADDITIONAL ITEMS

96-well plate  
GREEN [paramagnetic particles])  
Wizard MagnaSil Sequencing Reaction Clean-Up System (Promega; includes MagnaSil)

## METHOD

1. Assemble the 96-well plate into the Plate Clamp 96 (Fig. 10a,b).
2. Use the Plate Stand (Fig. 10c) to position the plate on the robotic deck.
3. Resuspend the MagnesiII particles by vigorously shaking the bottle. Add 180  $\mu$ l of the MagnesiII particles to each 20- $\mu$ l sequencing reaction.
4. Incubate at room temperature for 5 minutes. Mix by pipetting at 0, 2.5, and 5 minutes.

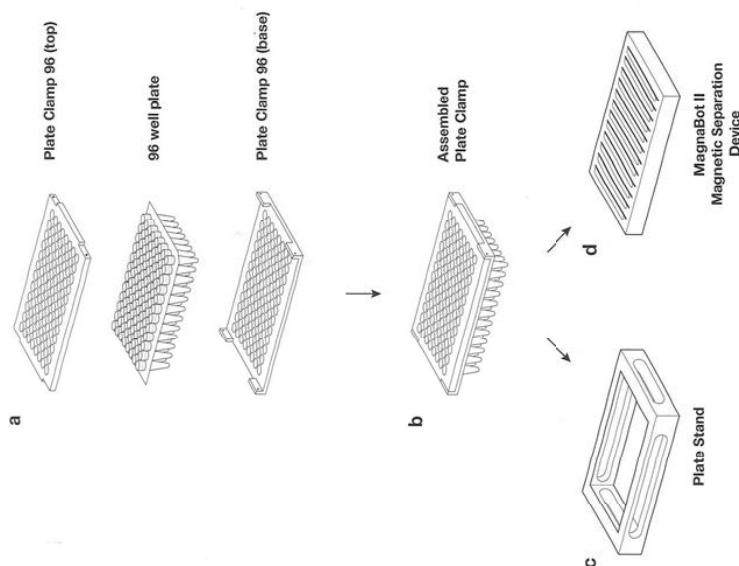


**FIGURE 10.** Accessories for the Wizard Magnesil Sequencing Reaction Clean-Up System. To eliminate plate warping, the 96-well plate is placed into the Plate Clamp 96 (a and b). During the Clean-Up procedure, the Plate Clamp assembly can be held by the Plate Stand (c) or placed onto the MagnaBot II. Device for magnetic separation of the Magnesil Paramagnetic Particles (d). (Reprinted, with permission, from Promega Wizard Magnesil Sequencing Reaction Clean-Up System Technical Bulletin #TB287, July, 3222MA01\_2A [©2003 Promega Corporation].)

5. Place the plate on to the MagnaBot II Magnetic Separation Device (Fig. 10c) to capture the particles.
6. Remove and discard the liquid. Avoid removing any particles.
7. Remove the plate from the MagnaBot II Device and place on Plate Stand.
8. Add 100  $\mu$ l of 90% ethanol to each sample.

TABLE 4. Recommended elution/loading solution volumes for the MagnaSil Sequencing Reaction Clean-Up System protocol

| Instrument | Elution/Loading solution                                     | Volume        |
|------------|--------------------------------------------------------------|---------------|
| ABI 377    | formamide/blue dextran/ED7A                                  | 6 $\mu$ l     |
| ABI 310    | template suppression reagent supplied with ABI POP-6 polymer | 10–20 $\mu$ l |
| ABI 3100   | Hi-Di formamide                                              | 10–20 $\mu$ l |
| ABI 3700   | formamide or water                                           | 10 $\mu$ l    |

9. Incubate at room temperature for 5 minutes. Mix at 0, 2.5, and 5 minutes.
10. Place the plate on the MagnaBot II Device to capture particles.
11. Remove and discard the liquid. Avoid removing any particles.
12. Repeat steps 7–11 for a total of two washes.
13. Allow the particles to air-dry for ~10 minutes at room temperature.
14. Add appropriate elution/loading solution (Table 4).
15. Incubate at room temperature for 1–2 minutes.
16. Place the plate on the MagnaBot II Device to capture particles.
17. Transfer purified sequencing reactions to a clean 96-well plate. Be careful to avoid removing any particles.

## SUMMARY

The purification of nucleic acids is an integral part of the PCR process, providing the source nucleic acid to be amplified and the methods to clean up amplified product after PCR. The choice of purification method depends on the efficiency of the amplification reaction, PCR fragment size, the researcher's needs for template purity, purification scale, and number of samples to be handled, and the need for automation. Today's methods have eliminated the need for hazardous organic extractions, have reduced time requirements, and have provided increased convenience and reproducibility. Methods based on binding and elution from silica, as well as solution extraction methods, have been developed to handle the majority of researchers' current needs. Advances in technology such as capillary sequencing, continuous assay miniaturization, and the push for more automation will place higher demands on the nucleic acid purification systems of the future.

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Michael A. Connolly, Peter A. Clausen, and James G. Lazar

*Marligen Bioscience, Inc., Jhansville, Maryland 21754*

Francis Crick and Leslie Orgel in 1963 proposed that RNA was the carrier of genetic information (Crick 1968; Orgel 1968). Since that time, more than 350,000 peer-reviewed studies have been published that rely, at least in part, on the analysis of RNA. The total RNA population in a cell or tissue comprises several different RNA species including ribosomal (rRNA), transfer RNA (tRNA), small nuclear RNA (snRNA), and messenger RNA (mRNA). Table 1 lists these types of RNA and their relative abundance in a typical cell.

Specific protocols have been developed to isolate the various species of RNA, and these generally begin with the isolation of total RNA. Relative to rRNA, snRNA, and tRNA, mRNA continues to hold the vast majority of interest on the basis of its role as the template for protein synthesis. Although total RNA is often the desired starting material for downstream applications designed to analyze mRNA populations, protocols have been developed to isolate mRNA directly from cells and tissues. This chapter provides protocols that are used for preparing total RNA from cell and tissue sources as well as protocols for the purification of mRNA.

#### YIELD AND QUALITY OF RNA

Independent of the protocol, the primary goal of RNA isolation is to obtain a yield and quality of RNA that will enable successful downstream applications. Yield is typically assessed by measuring the absorbance of the RNA solution at a wavelength of 260 nm. RNA concentration (and therefore yield) is calculated from the  $A_{260}$  value by assuming that one  $A_{260}$  unit represents an RNA concentration of 40 µg/ml. RNA quality is assessed by evaluating the  $A_{260}/A_{280}$  ratio and by visualization of RNA samples on agarose gels. The  $A_{260}/A_{280}$  ratio is an indicator of RNA purity and should fall between 1.9 and 2.2. Lower ratios generally indicate contamination with protein, phenol, or guanidinium salts. Formaldehyde gels can provide an additional indication of yield and sample quality. Dyes such as ethidium bromide are used to detect RNA in gels, and yield can be estimated by comparison with a standard by direct visualization or with computer imaging software. The rate of migration of RNA molecules in formaldehyde gels is directly related to their size. Gel analysis can be used to estimate the average size distribution of RNA in a sample and can also be used to assess the level of degraded RNA present within a sample. Visualization of RNA on formaldehyde gels is sensitive to ~5 nanograms and makes gel analysis especially useful for quality control when there are not sufficient quantities of RNA for spectrophotometric analysis.

TABLE 1. RNA species and their relative abundance

| RNA type | Relative abundance<br>% | Picogram/<br>cell |
|----------|-------------------------|-------------------|
| rRNA     | 80                      | 10–30             |
| sRNA     | 15                      | 2–5               |
| mRNA     | 5                       | 0.5–2             |

Modified from Stryer (1988).

The two variables that most often affect RNA yield and quality are the condition of the cells or tissue samples being analyzed and the presence of RNase activity. For example, quiescent cells tend to contain less mRNA relative to cells in log-phase growth, and cells that are overgrown or cultured to confluence tend to yield more degraded RNA. Some sources of cells and tissues, such as pancreas, contain high levels of endogenous RNase activity that can be highly problematic for the isolation of intact RNA. When using frozen samples, even small amounts of sample thawing prior to addition or contact with chaotropic agents may cause leakage of compartmentalized RNase activity and the subsequent degradation of the RNA. Extra care must be taken to avoid this possibility when dealing with larger cell pellets and tissue samples that must dissociate before complete lysis is possible.

### EXOGENOUS RNASE CONTAMINATION

Exogenous RNase contamination is another hazard to handling RNA in the laboratory. Exogenous RNase activity can be present on hands, bench tops, and laboratory equipment, but the risk of contamination can be easily managed by following basic RNA handling procedures.

The risk of introducing exogenous RNase activity is minimized by appropriate cleaning of bench tops, plasticware, and laboratory equipment, and also by the appropriate handling of samples. Skin is an excellent source of RNase activity, and gloves should be worn at all times. Mild detergent or chemicals such as RNaseAway (Molecular BioProducts) can be used to decontaminate laboratory surfaces and equipment prior to handling RNA samples. Although most unopened plasticware is rendered RNase-free by the molding process, the use of materials that are certified as RNase-free is advisable. Glassware to be used for RNA work should be cleaned and then baked at 180°C for at least 8 hours before use (Sambrook et al. 1989). Solutions should be prepared using chemicals that are free of RNase activity, and water should be treated with diethylpyrocarbonate (DEPC) to inactivate RNase (Sambrook et al. 1989). However, DEPC readily reacts with amines and is not compatible with certain reagents such as Tris-based buffers. A wide variety of chemicals, reagents, and buffers certified as “RNase-free” are now commercially available and help make RNA handling much simpler than in the past.

### SAMPLE PROCESSING

With the exception of paraffin-embedded or fixed tissues, the initial sample-processing steps for RNA purification are similar for most protocols. It is preferable to use fresh cells (60–80% confluency) or tissues as the starting material for RNA isolation. If samples must be stored for later processing, manageable-sized cell pellets or tissues should be snap-frozen using a dry ice bath or liquid nitrogen. When processing frozen material, extreme care must be taken to avoid even minimal amounts of thawing, which could detrimentally affect the integrity of an RNA preparation. All protocols for isolating RNA utilize lysis buffers contain-

ing denaturants such as phenol, sodium dodecyl sulfate (SDS), and guanidinium salts that effectively inhibit RNase. These lysis buffers can be used to lyse cells directly. However, fresh or frozen tissues must be ground to a fine powder prior to lysis. Grinding is performed under liquid nitrogen using a prechilled (liquid nitrogen) mortar and pestle. In most cases, a finer grind provides a better RNA yield from both plant and animal tissues due to the higher surface area and faster kinetics for inactivation of RNase. As an alternative to the conventional snap-freeze, Ambion Inc offers RNAlater, a storage solution for cells and tissues that will reportedly preserve RNA in tissues and cells prior to processing. Although the authors have not used this product, RNAlater has been reported to stabilize RNA in cells prior to freezing. Grotzer et al. (2000) were able to isolate total RNA from tissue samples stored for up to 7 days at room temperature. No significant differences were seen between the samples stored in RNAlater at room temperature and samples snap-frozen and stored at -70°C. RNAlater may provide the capability for long-term storage of samples at -20°C and short-term (up to 1 month) storage at 4°C and may also obviate the need for processing in liquid nitrogen prior to lysis.

For protocols other than those relying on phenol-based phase separation, homogenization of the sample using a rotor-stator tissue homogenizer or similar device is recommended. Homogenization facilitates contact of all cell surfaces with lysis buffer and facilitates rapid inactivation of RNases. Homogenization also shears genomic DNA, which results in reduced sample viscosity and is of particular importance in protocols that employ solid-phase RNA-binding matrices that are readily clogged by viscous samples. When processing larger samples (>10<sup>6</sup> cells or 10 mg of tissue) on solid-phase binding matrices, homogenization is critical to obtain a good yield and quality of RNA.

### RNA ISOLATION STRATEGIES

Following the initial processing of samples, RNA is generally isolated using one or more of three strategies: phenol-based phase separation, silica binding, and oligo(dT) capture. Phenol-based strategies have evolved from the method developed by Chomczynski and Sacchi (1987). Using phenol-based phase separation, cellular proteins and lipids are extracted into an organic phase, and RNA is precipitated from the aqueous phase. These protocols involve gentle homogenization in buffered phenol solution, phase separation, precipitation and washing of RNA, and resuspension of the RNA pellet. Current silica-binding methods are derived from a combination of the Chirgwin et al. (1979) RNA isolation protocol and a modification to the Vogelstein and Gillespie (1979) technique for purifying DNA. These methods rely on homogenization in a chaotropic lysis buffer, selective binding of RNA to silica in the presence of high salt, and elution of RNA using water or low-salt buffer. These protocols eliminate the need for hazardous chemicals like phenol and chloroform that require special handling and enable rapid (<30 minutes) isolation of highly purified RNA.

A number of protocols for obtaining messenger RNA (mRNA) directly from cells and tissues are also available. These protocols are adaptations of the strategy described by Berger and Kimmel (1987) and Sambrook et al. (1989) that uses oligo(dT) to capture mRNA through binding of the polyadenylated tails that are present on most mRNA molecules. The direct isolation of mRNA typically requires homogenization in lysis buffer, shearing of genomic DNA, binding of polyadenylated RNA to oligo(dT), and washing and elution of the RNA sample. Using oligo(dT) capture, a single round of selection on oligo(dT) will provide a sample enriched in the poly(A) fraction of RNA, and additional rounds of oligo(dT) purification are recommended to obtain samples of pure mRNA.

Phenol-based, silica-based, and oligo(dT)-based protocols have been used successfully to isolate RNA from animal cells and tissues, but have met with varying degrees of success in obtaining RNA from plant cells and tissues. Frequently these reagents do not produce a high yield or quality of RNA because plants often contain high levels of starches, polysaccharides, resins, or polyphenolic compounds that interfere with the isolation of pure RNA or that may copurify with the RNA. One adaptation used to isolate RNA from difficult plant tissues is to add polyvinylpyrrolidone (PVP) to the chaotropic lysis solution. PVP is used to bind and precipitate contaminating polysaccharides and polyphenolics, but this approach also has met with limited success. More recently, specific reagents and methods have emerged that are effective for isolating RNA from difficult plant tissues such as spruce, potato tuber, grape, and seeds (Hughes and Galau 1988; Wan and Wilkins 1994; Geuna et al. 1998; Scott et al. 1998; Wang et al. 1999; Lönnenborg and Jensen 2000; Gao et al. 2001; Hossein 2001). Methods representative of the three basic strategies for the isolation of RNA and specific protocols for obtaining RNA from plants and tissues are detailed below.

In all RNA purification strategies, the final step in purification is to redissolve or elute RNA samples in a low-salt buffer or water. In the absence of chaotropic agents, the RNA samples are susceptible to the activity of RNase. For this reason, RNase inhibitors such as RNasin (Promega) or vanadyl-ribonucleoside complexes can be used to ensure long-term integrity of purified RNA (Beiger and Kimmel 1987). If the use of RNase inhibitors is desirable, it is important to consider the compatibility of inhibitors with downstream applications. For example, vanadyl-ribonucleoside complexes inhibit *in vitro* translation of RNA and are not recommended for preserving samples intended for this purpose.

Purified RNA preparations invariably contain at least trace amounts of DNA, which may affect downstream applications such as reverse transcriptase (RT)-PCR or microarray analysis. DNase I is typically used to digest contaminating genomic DNA, while not affecting the RNA. Traditionally, digestion of DNA is done as a final step after the elution or resuspension of purified RNA. More recently, silica-based protocols have been developed that streamline the process by incorporating the DNase digestion on a column containing the bound RNA. However, the authors' experience, using purified RNA for real-time PCR, is that on-column digestion is not as effective as post-elution DNase treatment for removing 100% of contaminating DNA.

Several published protocols are available for the isolation of total RNA from cells and tissues (Chirgwin et al. 1979; Auffray and Rougeon 1980; Favaloro et al. 1980; Birnboim 1988), and many RNA purification kits and reagents are commercially available. Although there are a limited number of manufacturers of phenol-based RNA purification reagents, there are more than 25 manufacturers of silica- and oligo(dT)-based kits. Protocols that are representative of phenol-based, silica-based, and oligo(dT)-based strategies for RNA isolation are presented below, beginning with the guanidinium isothiocyanate/cesium chloride ultracentrifugation method. This protocol is a modification of the original method developed by Chirgwin et al. (1979). All other protocols presented in this chapter were selected on the basis of the authors' experience both in terms of "ease of use" and their relative superiority for consistently providing the highest yield and quality of RNA.

## PROTOCOL 1 PREPARATION OF RNA USING GUANIDINIUM ISOTHIOCYANATE/CESIUM CHLORIDE ULTRACENTRIFUGATION

This is a modification of the protocol outlined by Chirgwin et al. (1979). Adaptations of the reagents defined by Chirgwin et al. are found in many kits, and reagents are currently available for the isolation and purification of RNA.

### MATERIALS

#### BUFFERS, SOLUTIONS, AND REAGENTS

Chloroform<sup>1</sup>

CsCl solution, 5.7 M (5.7 M CsCl, 25 mM sodium acetate<sup>1</sup>, pH 6.0, 1 mM EDTA, pH 8.0)

Diethyl procarbonate (DEPC)<sup>1</sup>-treated H<sub>2</sub>O

Dithiothreitol (DTT) <sup>1</sup>, 0.2 mM

EDTA, 1 mM, pH 8.0

Ethanol 70%<sup>1</sup> (v/v)

Guanidine isothiocyanate lysis buffer (4 M guanidine isothiocyanate <sup>1</sup>, 25 mM sodium acetate, pH 6.0, 1 mM EDTA, pH 8.0)

Isopropanol<sup>1</sup>

N-Laurylsarcosine 20%<sup>1</sup> (w/v)

Lithium chloride (LiCl)<sup>1</sup>, 8 M

Liquid nitrogen<sup>1</sup>

β-Mercaptoethanol<sup>1</sup>, 10 M

Phenol<sup>1</sup>, H<sub>2</sub>O-saturated, pH 4.3

#### CENTRIFUGES AND ROTORS

Beckman Ti50 rotor or equivalent

#### SPECIAL EQUIPMENT

Mortar and pestle, washed in DEPC-treated H<sub>2</sub>O, prechilled

Polyallomer ultracentrifuge tubes or equivalent

Polytron or equivalent homogenizer

#### CELLS AND TISSUES

Source cells/tissues, frozen in liquid nitrogen<sup>1</sup>

#### METHOD

1. Grind frozen tissue samples under liquid nitrogen using a mortar and pestle to produce a fine powder. Homogenize the tissues or cells (up to 1 × 10<sup>6</sup>) in 7 ml of guanidine lysis buffer using a Polytron or other suitable apparatus.
2. Place 4 ml of 5.7 M CsCl solution in a polyallomer ultracentrifuge tube.
3. Add 1 ml of the 5.7 M CsCl solution, 120 μl of 10 M 2-mercaptoethanol, and 240 μl of N-laurylsarcosine to the lysate.
4. Mix the cesium chloride/lysate mixture and carefully layer it over the cesium chloride cushion in the centrifuge tube.
5. Centrifuge at 180,000g for 21 hours at 20°C.
6. Remove the cesium chloride supernatant, being careful not to disrupt the RNA pellet in the bottom of the tube.
7. Gently redissolve the RNA pellet in 750 μl of guanidine isothiocyanate lysis buffer.
8. Add an equal volume of phenol and mix.
9. Centrifuge for 15 minutes at 10,000g.
10. Transfer the aqueous phase to a fresh tube. Repeat the phenol extraction of the aqueous phase until the interface is clear.

▼ CAUTION  
See Appendix for appropriate handling of materials marked with <sup>1</sup>.

11. Add 1 volume of chloroform to the clear aqueous phase.
12. Mix and centrifuge at 10,000g for 10 minutes.
13. Transfer the aqueous phase to a clean tube. Add an equal volume of isopropanol and 1/10 volume of 8 M lithium chloride. Centrifuge at 10,000g for 10 minutes.
14. Remove the supernatant. Wash the RNA pellet with 750  $\mu$ l of 70% ethanol and centrifuge at 10,000g.
15. Remove the ethanol, air-dry the pellet, and redissolve in 50–500  $\mu$ l of 0.2 mM DTT.
16. Store the RNA at  $-70^{\circ}\text{C}$ .

## PROTOCOL 2 PREPARATION OF RNA FROM PARAFFIN-EMBEDDED FIXED TISSUE

This protocol is a modification of the Fisher protocol published by Godfrey et al. (Fisher 1988; Godfrey et al. 2000). Additional protocols for the purification of RNA from fixed tissues have been published previously (Stania and Schneider 1991; Cairns et al. 1997; Kraft et al. 1997; Coombs et al. 1999; Masuda et al. 1999). Commercial kits for the purification of RNA from paraffin-embedded tissues are available from Ambion and Roche.

### MATERIALS

#### BUFFERS, SOLUTIONS, AND REAGENTS

- Deionized formamide<1>
- Diethyl pyrocarbonate (DEPC)<1>-treated  $\text{H}_2\text{O}$
- Digestion buffer (1 M guanidine isothiocyanate)<1>, 25 mM 2-mercaptoethanol<1>, 0.5% N-lauroylsarcosine<1>, 20 mM Tris-HCl, pH 7.5
- Ethanol<1>, 100% and 70%
- Glycogen
- Isopropanol<1>
- Phenol<1> (pH 4.3); chloroform<1> (70:30%)
- Proteinase K<1> (60 mg/ml at 20 units/mg)
- Trizol (Invitrogen)<1>
- Xylene<1>

#### SPECIAL EQUIPMENT

- Microfuge tubes, 2 ml, RNase-free
- Incubator, preset to  $37^{\circ}\text{C}$
- Water bath or incubator, preset to  $55^{\circ}\text{C}$

#### CELLS AND TISSUES

- Paraffin-embedded tissue samples, cut to 5–50  $\times$  5- $\mu\text{m}$  sections

### METHOD

1. Place tissue sections in 2.0-ml microfuge tubes and add 1.8 ml of xylene to the sample. Incubate at  $37^{\circ}\text{C}$  for 20 minutes.
2. Centrifuge the sample briefly and remove the supernatant. Add fresh xylene and repeat the incubation and centrifugation.
3. Wash the tissue with 0.5 ml of ethanol. Remove the ethanol and air-dry.
4. Add 80  $\mu$ l of proteinase K (60 mg/ml at 20 units/mg) and 720  $\mu$ l of digestion buffer. Vortex and incubate overnight at  $55^{\circ}\text{C}$ .

## PROTOCOL 3

### PURIFICATION OF RNA USING TRIZOL

There are a limited number of manufacturers of phenol-based reagents for RNA isolation. These reagents are based on the Chomczynski protocol (Chomczynski and Sacchi 1987) and make use of guanidine buffers as lysis reagents and phenol chloroform for extraction of proteins and lipids. Today, these protocols are most useful for obtaining RNA from tissues rich in RNase, such as pancreas. Presented here is a summary of the protocol that accompanies the Trizol reagent sold by Invitrogen. This protocol was adapted, with permission, from Invitrogen.

### MATERIALS

#### BUFFERS, SOLUTIONS, AND REAGENTS

- Trizol<1> (Invitrogen)
- Chloroform<1>
- Isopropanol<1>
- Ethanol<1>, 75%, in diethyl pyrocarbonate (DEPC)<1>-treated  $\text{H}_2\text{O}$
- DEPC-treated  $\text{H}_2\text{O}$

#### CELLS AND TISSUES

- 50- to 100-mg source tissue, finely ground

### METHOD

1. Add 1 ml of Trizol to 50–100 mg of finely ground tissue in a microfuge tube. Incubate for 5 minutes at room temperature.  
It is important that the tissue be of a fine grind to aid in the disruption of the tissue.
2. Add 200  $\mu$ l of chloroform for each 1 ml of Trizol. Mix by inverting the tube several times.

▼ CAUTION  
See Appendix for appropriate handling of materials marked with <1>.

Vortexing is not recommended because it may result in shearing of genomic DNA and excessive DNA contamination of the RNA preparation.

3. Incubate for 3 minutes and centrifuge at 1500g for 15 minutes.
4. Transfer the aqueous phase to a fresh tube. Add 500  $\mu$ l of isopropanol for each 1 ml of Trizol. Incubate for 10 minutes.
5. Centrifuge at 1500g for 10 minutes. RNA should be visible at the bottom of the tube.
6. Remove the supernatant, being careful not to disrupt the pellet.
7. Add 1 ml of 75% ethanol for each 1 ml of Trizol used. Resuspend the pellet. Centrifuge at 1500g at 4°C for 5 minutes.
8. Remove the alcohol and air-dry the pellet.
9. Redissolve in DEPC-treated  $H_2O$ .

When using phenol-based reagents, it may be necessary to subject purified RNA to a second round of phenol-chloroform extraction to obtain a sufficient level of purity.

#### PROTOCOL 4 PREPARATION OF RNA FROM PLANT TISSUE USING TRIZOL

The following protocol is a modification of the Trizol protocol to be used for the isolation of RNA from plant tissues. A high-salt isopropanol precipitation step has been added to precipitate RNA selectively while maintaining polysaccharides and proteoglycans in solution (Sewall and McRae 1998).

##### MATERIALS

###### BUFFERS, SOLUTIONS, AND REAGENTS

Trizol<sup>®</sup> (Invitrogen)  
Chloroform<sup>®</sup>  
Isopropanol<sup>®</sup>  
Ethanol <sup>®</sup>, 70% in diethyl pyrocarbonate (DEPC) <sup>®</sup>-treated  $H_2O$   
DEPC-treated  $H_2O$   
Liquid  $N_2$  <sup>®</sup>  
NaCl, 1.2 M  
Sodium citrate<sup>®</sup>, 0.8 M

###### SPECIAL EQUIPMENT

Rotor-stator homogenizer (or equivalent)  
Mortar and pestle, washed in DEPC-treated  $H_2O$ , prechilled in liquid  $N_2$   
50-ml conical tube, e.g., Falcon

###### CELLS AND TISSUES

Appropriate plant tissue

###### METHOD

1. Grind ~1 g of plant tissue in a  $N_2$ -chilled mortar using enough liquid  $N_2$  to cover the plant tissue.  
It is important to grind the tissue to obtain a very fine powder.
2. Transfer the powder to a 50-ml conical tube containing 10 ml of Trizol per gram of tissue.
3. Homogenize the tissue using a rotor-stator homogenizer for 30 seconds.
4. Centrifuge the lysate at 1500g for 15 minutes at 4°C to remove debris.

5. Transfer the supernatant to a fresh tube. Add 200  $\mu$ l of chloroform per ml of Trizol used.
6. Mix by inverting the tube several times. Incubate for 5 minutes at room temperature.
7. Centrifuge at 1500g for 20 minutes at 4°C. Transfer the aqueous phase to a fresh 50-ml tube.

8. Add 0.5 volume of isopropanol and 0.5 volume of a 0.8 M sodium citrate, 1.2 M sodium chloride solution. For example, to a 500- $\mu$ l volume of aqueous phase, add 250  $\mu$ l of isopropanol and 250  $\mu$ l of sodium citrate solution.

9. Mix and incubate at room temperature for 10 minutes. Overnight incubation at -20°C may improve the RNA yield.

10. Centrifuge at 4°C for 20 minutes at 1500g.

11. Remove the supernatant and add 1 ml of 70% ethanol for each 1 ml of Trizol used for the initial extraction.

12. Centrifuge at 1500g for 15 minutes. Carefully remove the supernatant being careful not to disrupt the pellet. Air-dry the pellet.

13. Redissolve the RNA in DEPC-treated  $H_2O$ .

A typical yield from 1 g of mature leaf tissue is ~500  $\mu$ g of RNA.

This protocol may not work well with plant tissues that contain polyphenolic compounds or resins. The Concert Plant Reagent (Invitrogen) is recommended for difficult plant tissues that are rich in polyphenolics, resins, or starch.

#### PROTOCOL 5 PURIFICATION OF RNA FROM PLANT TISSUE USING THE CONCERT PLANT

Summarized below is the protocol included with the Concert Plant Reagent (Invitrogen). This protocol is not phenol based, but does require the addition of chloroform. This reagent is intended for the isolation of RNA from a wide variety of plant tissues including blue spruce needles, potato tuber, corn seeds, and cotton leaves. This protocol was adapted, with permission, from Invitrogen.

##### MATERIALS

###### BUFFERS, SOLUTIONS, AND REAGENTS

Concert Plant RNA Reagent<sup>®</sup> (Invitrogen), prechilled to 4°C  
NaCl, 5 M (RNase-free)  
Chloroform<sup>®</sup>  
Isopropanol<sup>®</sup>  
Ethanol<sup>®</sup>, 75% in DEPC-treated  $H_2O$   
DEPC-treated  $H_2O$

###### CELLS AND TISSUES

Appropriate plant tissue (see step 1), frozen and finely ground

###### METHOD

1. Add 0.5 ml of cold (+4°C) Concert Plant RNA Reagent for up to 0.1 g of frozen, ground plant tissue. Mix by vortexing briefly until the sample is suspended in the reagent.

TABLE 2. Comparison of selected commercially available total RNA purification kits

| Vendor Product name | Qiagen RNeasy Mini                           | Ambion RNAsqueous                            | Promega SV Total RNA Isolation system        | Marligen Rapid RNA Purification system        | Stratagene StrataPrep Total RNA Miniprep system | Invitrogen Micro-to-Mid RNA purification system |
|---------------------|----------------------------------------------|----------------------------------------------|----------------------------------------------|-----------------------------------------------|-------------------------------------------------|-------------------------------------------------|
| Lysis method        | guanidine isothiocyanate silica-gel membrane | guanidine isothiocyanate glass fiber filter  | guanidine isothiocyanate silica membrane     | guanidine isothiocyanate silica membrane      | guanidine isothiocyanate silica-based fiber     | guanidine isothiocyanate silica fiber           |
| Binding matrix      | cells or tissue                              | cells or tissue                              | cells or tissue                              | cells or tissue                               | cells or tissue                                 | cells or tissue                                 |
| Starting material   | 1 x 10 <sup>6</sup> cells or 30 mg of tissue | 1 x 10 <sup>6</sup> cells or 75 mg of tissue | 5 x 10 <sup>6</sup> cells or 60 mg of tissue | 1 x 10 <sup>6</sup> cells or 200 mg of tissue | 1 x 10 <sup>6</sup> cells or 40 mg of tissue    | 1 x 10 <sup>6</sup> cells or 200 mg of tissue   |
| Capacity            | 100 µg                                       | 100 µg                                       | 100 µg                                       | 600 µg                                        | 120 µg                                          | 600 µg                                          |
| Yield               |                                              |                                              |                                              |                                               |                                                 |                                                 |

## PROTOCOL 6 PURIFICATION OF TOTAL RNA USING THE MARLIGEN RAPID TOTAL RNA SYSTEM

The Rapid Total RNA System offered by Marligen Biosciences provides reproducible yields and quality over a broad range of sample sizes.

### MATERIALS

#### BUFFERS, SOLUTIONS, AND REAGENTS

Ethanol<1>, 70% and 100%

2-Mercaptoethanol<1>

#### SPECIAL EQUIPMENT

Rotor-stator, or equivalent homogenizer

#### ADDITIONAL ITEMS

Marligen Rapid Total RNA System<1> (Marligen Biosciences)

### METHOD

1. Prepare Lysis Solution by addition of 2-mercaptoethanol.
2. Add Lysis Solution to cell or tissue samples according to manufacturer's protocol. (Lysis volume is dependent on the size of the sample being processed.) Homogenize with a rotor-stator or similar device.
3. Add one volume of 70% ethanol to the lysate. Homogenize lysate to disperse precipitate.
4. Apply lysate to spin column provided with the kit and centrifuge at 12,000–16,000g in a microfuge.
5. Wash once with 700 µl of Wash Buffer 1 and centrifuge at 12,000–16,000g for 1 minute.
6. Wash twice with 500 µl of Wash Buffer 2 and centrifuge at 12,000–16,000g for 1 minute.

▼ CAUTION  
See Appendix for appropriate handling of materials marked with <1>.

2. Incubate the tube on its side for 5 minutes at room temperature.
3. Centrifuge for 2 minutes at 12,000g to remove debris.
4. Transfer the supernatant to a new RNase-free tube and add 0.1 ml of 5 M NaCl. Mix the tube by tapping it on the bench.
5. Add 0.3 ml of chloroform and mix by inverting the tube several times.
6. Centrifuge the sample at 4°C for 10 minutes at 12,000g.
7. Transfer the aqueous phase to a fresh RNase-free tube. Add an equal volume of isopropanol to the aqueous phase. Mix by inverting the tube several times. Incubate at room temperature for 10 minutes.
8. Centrifuge the sample for 10 minutes at 12,000g at 4°C.
9. Carefully remove the supernatant, being careful not to disrupt the pellet.
10. Add 1 ml of 75% ethanol to the pellet. Centrifuge at room temperature for 1 minute at 12,000g.
11. Remove the supernatant, being careful not to disrupt the pellet. Centrifuge briefly and remove any remaining liquid with a pipette.
12. Redissolve the pellet in 10–30 µl of DEPC-treated H<sub>2</sub>O. If any cloudiness is observed, centrifuge the solution at 12,000g for 1 minute at room temperature. Transfer the supernatant to a fresh RNase-free tube and store at –70°C.

**COMMERCIALLY AVAILABLE KITS FOR ISOLATION OF TOTAL RNA**  
A number of silica-based kits are commercially available for the isolation of total RNA from animal cells and tissue. Table 2 provides a listing of some of these kits. Protocol 6 summarizes the protocol that is supplied with Rapid Total RNA System from Marligen.

Several companies (Ambion, Marligen, Promega, Qiagen, and Stratagene, to name a few), in addition to single-tube formats, offer 96-well plate formats for the high-throughput purification of total RNA. These high-throughput systems generally do not accommodate the large sample sizes that can be processed using single spun columns. The static capacity for most 96-well plate systems is 1 x 10<sup>6</sup> cells, with an expected yield of between 10 and 30 µg. In general, 96-well plates can be processed using vacuum and centrifugation. Vacuum protocols are well suited for use with robotic platforms but require a vacuum manifold that can accommodate the plate format. Vacuum manifolds that can be used with 96-well plate formats are available from BioRad, Wharman, and Qagen. Processing plates using centrifugation make the systems difficult for use on robotic platforms, but they work well for manual processing. The centrifugation method also requires the centrifuge to accommodate the plate "stack," consisting of the RNA purification plate and the sample collection plate. In most cases, special plate carriers are required to accommodate the plate stack. In the authors' experience, processing RNA purification plates using a centrifugation protocol will slightly improve the quality and quantity of the RNA obtained compared to the vacuum method.

7. Centrifuge the spun column at 12,000–16,000g for 2 minutes to remove residual wash buffer.
8. Elute RNA from the column with  $H_2O$  according to manufacturer's recommendations. Store the RNA at  $-70^{\circ}C$ .  
Smaller samples can be eluted in 45  $\mu l$  of  $H_2O$ , whereas larger samples should be eluted in 200  $\mu l$ .

#### COMMERCIALLY AVAILABLE KITS FOR THE ISOLATION OF mRNA

There are numerous kits available for the isolation of mRNA directly from cells and tissues, some of which are compared in Table 3. The majority of these kits rely on oligo(dT), coupled to a solid phase, to bind the polyadenylic acid (poly(A)) residues that reside at the 3' terminus of the mRNA molecule (Berger and Kimmel 1987; Sambrook et al. 1989). Ambion's MicroPoly(A)Pure can be used to obtain reproducible yields of high-quality Poly(A) RNA, and this kit protocol is summarized in Protocol 7.

### PROTOCOL 7 ISOLATION OF mRNA USING THE MICRO POLY(A) PURE KIT FROM AMBION

#### MATERIALS

##### BUFFERS, SOLUTIONS, AND REAGENTS

Ethanol <1%, 100%

##### SPECIAL EQUIPMENT

Tissue homogenizer; *optional*, see step 4

Water bath, preset to  $70^{\circ}C$

##### ADDITIONAL ITEMS

Micro Poly(A) Pure Kit (Ambion)

##### CELLS AND TISSUES

Source cells

#### METHOD

1. Prewarm the Elution Buffer to  $70^{\circ}C$ .
2. Pellet cells at 250g and then remove the supernatant.
3. Wash the cell pellet once in PBS by gentle resuspension and centrifugation. Discard the supernatant.
4. Add 250  $\mu l$  of Lysis Solution. Homogenize with a tissue homogenizer or by vigorous vortexing or pipetting. Measure the volume of the lysate. This is the "Starting Volume."
5. Add 2 Starting Volumes of dilution buffer to the lysate. Mix by inversion or shaking for 10 seconds.
6. Centrifuge at 12,000g at  $4^{\circ}C$  for 15 minutes. Transfer the supernatant to a fresh tube.
7. Add one vial of oligo(dT) cellulose to the sample. Mix by inversion. Incubate for 30–60 minutes with gentle agitation at room temperature.

TABLE 3. Comparison of commercially available mRNA purification kits

| Vendor       | Product name                       | Isolation method                                                                      | Starting material                                           | Yield                                                                            |
|--------------|------------------------------------|---------------------------------------------------------------------------------------|-------------------------------------------------------------|----------------------------------------------------------------------------------|
| Active Motif | mTRAP Midi                         | lysis in guanidine isothiocyanate, poly(T) peptide binding                            | $2 \times 10^6$ cells or 200 mg of tissue (Midi)            | 100–200 $\mu g$                                                                  |
| Ambion       | Poly(A)Pure                        | lysis in guanidine isothiocyanate, d(T) binding, spin column purification             | $2 \times 10^6$ cells or 1 g of tissue                      | not reported                                                                     |
| Invitrogen   | FastTrack 2.0                      | Micro- FastTrack 2.0, detergent lysis, batch d(T) binding                             | $3 \times 10^6$ cells or 1 g of tissue                      | 1.8 $\mu g$ for $5 \times 10^6$ HeLa cells                                       |
| Promega      | PolyAT tract mRNA Isolation System | magnetic separation, lysis in guanidine isothiocyanate, spin column                   | 5 mg of total RNA                                           | 40 $\mu g$ for $1 \times 10^6$ S19 cells                                         |
| Qiagen       | Oligotex mRNA Purification System  | magnetic separation, lysis in guanidine isothiocyanate, oligotex binding, spin column | $1 \times 10^5$ cells or 1 g of tissue                      | 1–2% of total RNA                                                                |
| Roche        | mRNA Isolation kit                 | detergent lysis, binding to oligo d(T), push column                                   | 2.5 mg of total RNA, $1 \times 10^6$ cells or 1 g of tissue | 30–200 $\mu g$                                                                   |
| Stratagene   | Poly(A) Quick mRNA Isolation Kit   | batch binding to d(T), push column                                                    | 500 $\mu g$ of total RNA                                    | 25 $\mu g$ of mRNA from 2.5 mg of total RNA, 14 $\mu g$ of $1 \times 10^6$ cells |

▼ CAUTION  
See Appendix for appropriate handling of materials marked with <S>.

8. Centrifuge at 4000g for 3 minutes at room temperature.
9. Remove the supernatant. Save the supernatant until mRNA isolation is verified.
10. Add 1 ml of Binding Buffer to the oligo(dT) cellulose pellet. Mix by inversion. Pellet by centrifugation at 4000g for 3 minutes. Remove the Binding Buffer and repeat this step two times.
11. Add 1 ml of Wash Buffer to the pellet. Mix by inversion. Pellet by centrifugation at 4000g for 3 minutes. Remove the Wash Buffer and repeat this step two times.
12. Place a spun column in a wash tube. Resuspend the oligo(dT) cellulose in 400  $\mu$ l of Wash Buffer. Transfer the solution to a spun column and centrifuge briefly at 4000g. Discard the flowthrough.
13. Add 500  $\mu$ l of Wash Buffer to the spun column. Mix the resin with a pipette tip, being careful not to damage the membrane in the column. Centrifuge briefly at room temperature. Discard the flowthrough and repeat this step. Save the flowthrough from the last wash.
14. Determine the  $A_{260}$  of the flowthrough. If the  $A_{260}$  is less than 0.05, continue with the protocol. If the  $A_{260}$  is greater than 0.05, repeat washes with 500  $\mu$ l of Wash Buffer until the  $A_{260}$  is less than 0.05.
15. Place the spun column into a fresh microfuge tube. Add 100  $\mu$ l of 70°C Elution Buffer to the spun column. Centrifuge immediately at 5000g. Add an additional 100  $\mu$ l of warm Elution Buffer to the spun column and centrifuge briefly at 5000g. Discard the spun column.
16. Add 20  $\mu$ l of 5 M ammonium acetate, 1  $\mu$ l of glycogen, and 550  $\mu$ l of 100% ethanol to the eluted mRNA. Incubate overnight at -20°C.
17. Recover the mRNA by centrifugation at 12,000g or more for 20 minutes at 4°C.
18. Remove the supernatant carefully. Centrifuge again briefly and remove any remaining fluid using a fine-tipped pipette.
19. Dissolve the pellet in 1–20  $\mu$ l of DEPC-treated H<sub>2</sub>O/EDTA provided with the kit. Vortex vigorously to completely redissolve the pellet. Store the RNA at -70°C.

## SUMMARY AND TROUBLESHOOTING

Commercial reagents and kits are available for the isolation of high-quality RNA. Due to the ease and reproducibility associated with the silica-based strategies, many laboratories have adopted these systems as the method of choice to isolate RNA from animal cells and tissues. Although these systems frequently are not suitable for the isolation of RNA from plant tissues, novel reagents such as the Concert Plant Reagent fill this void. The reagents and protocols presented in this chapter can be used to provide quality RNA contingent on suitable condition of the starting materials and adherence to the recommended protocols. As a primary concern for any RNA isolation procedure, careful sample handling and adequate preparation of work surfaces minimize the potential for the introduction of RNases into samples. RNA obtained using these protocols is of a quality suitable for downstream applications such as northern blotting, RNase protection assays, *in vitro* translation, cDNA synthesis, microarray analysis, and quantitative RT-PCR.

Depending on the particular downstream application, it is important to consider that contamination of purified RNA with small to modest amounts of genomic DNA is inherent to all strategies used for the purification of RNA. In cases where downstream applications

will be affected by contaminating DNA, DNase I treatment of samples is recommended. Typically, DNase I treatment is performed on the final RNA sample that has been redissolved or eluted in H<sub>2</sub>O. In the case of the silica-based strategies, protocols including on-column solid-phase DNase I treatment have been developed in an effort to streamline the DNA removal. Nonetheless, in the authors' experience, on-column DNase I treatment is not effective for the complete removal of contaminating DNA. Therefore, samples to be used for applications that demand RNA completely devoid of contaminating DNA should be treated with DNase I after elution of the RNA. Additional protocols for "on-column" and post-elution treatment of RNA with DNase I are presented here.

## ADDITIONAL PROTOCOL 1 POST-ELUTION REMOVAL OF CONTAMINATING DNA

The following protocol is a modified version of the Dilworth and the Huang protocols (Dilworth and McCarey 1992; Huang et al. 2000). It can be used to remove contaminating DNA from RNA prepared using most commercial kits and reagents.

### MATERIALS

#### BUFFERS, SOLUTIONS, AND REAGENTS

Diethyl pyrocarbonate (DEPC) <1>-treated H<sub>2</sub>O

EDTA, 25 mM

#### ENZYMES AND ENZYME BUFFERS

DNase I, amplification grade (RNase-free)

DNase I buffer, 10x

#### NUCLEIC ACIDS AND OLIGONUCLEOTIDES

RNA sample

#### SPECIAL EQUIPMENT

Water bath, preheated to 65°C

### METHOD

1. To an RNase-free tube add the following:

RNA

up to 80  $\mu$ g

DNase I buffer, 10x

1  $\mu$ l

DNase I (1 unit/ $\mu$ l)

1  $\mu$ l

Bring total volume to 10  $\mu$ l with DEPC-treated H<sub>2</sub>O.

2. Incubate at room temperature for 15 minutes.
3. Inactivate DNase I by adding 1  $\mu$ l of 25 mM EDTA solution and heating for 10 minutes at 65°C.

Samples treated in this manner are suitable for direct use in RT-PCR.

## ADDITIONAL PROTOCOL 2 ON-COLUMN REMOVAL OF CONTAMINATING DNA

This protocol can be used with RNA isolation strategies that utilize silica-based RNA-binding columns, e.g., Protocol 6. However, 100% DNA removal may not be achieved.

## MATERIALS

## ENZYMES AND ENZYME BUFFERS

DNase I, amplification grade (RNase-free)

DNase I buffer, 10x

## CELLS AND TISSUES

Cell lysate obtained using a Total RNA Purification Kit (such as the Marligen Rapid Total RNA Purification System)

## METHOD

1. Load the lysate onto a spin column included in a total RNA Purification kit (such as the Marligen Rapid Total RNA Purification System).
2. Following the loading of the RNA purification membranes with lysates, add 50  $\mu$ l of DNase I solution containing 5 units of DNase I in 1x DNase I buffer to each spin column.
3. Incubate at room temperature for 15 minutes.
4. Continue with the Marligen purification protocol at step 6. The subsequent washing steps in the protocol will remove the DNase I.

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## In Situ Localization of Gene Expression Using Laser Capture Microdissection

Motoko Morimoto,<sup>1\*</sup> Masahiro Morimoto,<sup>2,3\*</sup> Jeannette Whitmire,<sup>1</sup>  
Robert A. Star,<sup>4</sup> Joseph F. Urban, Jr.,<sup>3</sup> and William C. Gause<sup>1</sup>

<sup>1</sup>Department of Microbiology and Immunology, Uniformed Services University of the Health Sciences, Bethesda, Maryland 20814-4796; <sup>2</sup>Department of Veterinary Pathology, Yamaguchi University, Yamaguchi 753-8515, Japan; <sup>3</sup>Nutrient Requirements and Functions Laboratory, Beltsville Human Nutrition Research Center, U.S. Department of Agriculture, Beltsville, Maryland 20705-2350; <sup>4</sup>Renal Diagnostics Unit, and Laboratory of Pathology, National Institutes of Health, Bethesda, Maryland 20892-1268

The lymph nodes of the immune system are complex organs with specific microenvironments in which lymphoid lineages differentiate and become committed to specific effector functions. Elucidation of the proteins and genes expressed by lymphocytes in situ can potentially provide important information regarding their function in their natural milieu. The technique of laser capture microdissection (LCM), which was originally developed to study gene expression in tumor cells (Emmert-Buck et al. 1996; Bonner et al. 1997; Simone et al. 1998), is well-suited for examination of cell populations in lymphoid organs or other heterogeneous tissue microenvironments. Other techniques that examine cell function in situ include immunohistology and in situ hybridization. These techniques are frequently not sufficiently sensitive to detect physiological up-regulation of proteins or specific mRNA expression (Fend and Raffeld 2000), and they usually cannot be used to detect more than one or two expressed molecules at a time.

Various microdissection techniques have been used to remove single cells from tissue sections for further analysis (Isenberg et al. 1976; Meier-Ruge et al. 1976; Whetsell et al. 1992). For example, micropipettes have been successfully used to remove single cells from germinal centers of B-cell follicles in the lymph node to study B-cell differentiation (Kuppers et al. 1993). However, these techniques are laborious and difficult to perform and thus are not practical in many laboratories. The development of LCM provides a rapid and readily learned technique for removal of specific cells or tissue regions from a tissue section on a microscope slide. RNA or protein can be purified and used in a variety of assays in the laboratory, including real-time reverse transcriptase (RT)-PCR, allowing the analysis of the expression of a number of different genes from the same captured sample.

\*These authors contributed equally to this work.

## BASIC TECHNIQUE

The basic technique involves the use of a microscope and a laser to select single cells or regions of tissue from a thin section on a microscope slide (Fig. 1). A thermoplastic membrane, attached to a cap, is placed over the target area and a laser pulse is delivered to the thermoplastic membrane, which then melts onto the selected area. When the cap is removed, the targeted cell or tissue remains attached to the membrane, allowing it to be removed selectively from the rest of the tissue section on the slide. The cap can then be transferred to a solution for isolation of DNA, RNA, or protein. Our laboratory and many others currently perform microdissection on a PixCell II (Arcturus Engineering, Mountain View, CA). Here we present a detailed protocol that yields high-quality mRNA isolated from captured cells or regions in tissue sections.

## METHODS STRATEGY

There are five basic steps involved in the analysis of gene expression of cells and tissues using the LCM technique, as shown in Figure 2:

1. Preparation of frozen tissue blocks and sectioning
2. Staining of the slides (if necessary, immunohistology)
3. Performing LCM
4. RNA extraction from small numbers of cells and cDNA synthesis
5. Real-time PCR



FIGURE 1. Laser-capture microdissection (LCM) system. The Arcturus LCM system allows rapid isolation of targeted cell populations from tissue sections. (A) The capture process is visualized with a specially adapted microscope. (B) Using this microscope, a plastic cap coated with a thermoplastic film is brought in close proximity to the tissue region of interest. A laser passes through the cap and melts the film, which adheres to the tissue. When the cap is lifted, the selected cells remain attached and captured for analysis. (Reprinted, with permission, from Arcturus, [www.arctur.com](http://www.arctur.com).)

## PROTOCOL 1 PREPARATION OF FROZEN TISSUE BLOCKS AND SECTIONING

For optimal results, freshly obtained tissue should be used, because storage of tissue blocks at  $-80^{\circ}\text{C}$  for more than 24 hours reduces the RNA integrity and yield. Although several groups have reported good recovery of undegraded RNA from paraffin-embedded tissue by LCM (Schutze and Lahr 1998; Shibutani et al. 2000; Specht et al. 2001), we recommend frozen blocks.

## MATERIALS

## BUFFERS, SOLUTIONS, AND REAGENTS

- Acetone  $<1>$
- Distilled  $\text{H}_2\text{O}$
- Dry ice  $<1>$
- Phosphate-buffered saline (PBS)
- Tissue-Tek O.C.T. Compound (Sakura Finetek U.S.A., Torrance, CA)

## SPECIAL EQUIPMENT

- Air-tight container
- Beaker
- Cryostat
- Dissecting tools
- Flat plate
- Peel-A-Way Disposable Histology Molds (Pelco International, Redding, CA)
- Plain uncoated slides
- Sakura Low Profile blades (IMEB, San Marcos, CA)

## CELLS AND TISSUES

Tissue, freshly obtained (see step 2)

## METHOD

## Preparation of Frozen Tissue Blocks

1. Cover the cryomold with embedding medium (Tissue-Tek O.C.T. Compound is recommended). Prepare the dry ice-acetone bath (a simple mixture of acetone and dry ice in beaker).

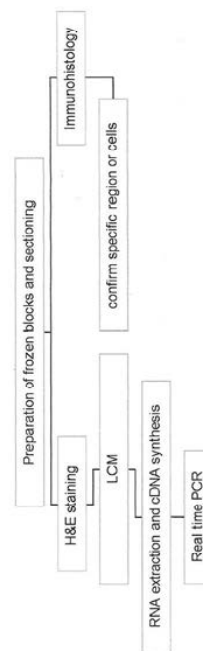


FIGURE 2. Flow chart of the combination of LCM and quantitative real-time PCR for analysis of gene expression in situ. This approach uses navigational LCM, as described in the text, to locate the cells or tissue region of interest.

2. Sacrifice the animals and remove the desired tissues. Mix the tissues with embedding medium on a flat plate.
3. Place the tissue against the bottom of the cryomold. To facilitate cutting with the cryostat, place the desired cutting face flush against the bottom of the cryomold.
4. Touch the bottom of the cryomold to the surface of the dry ice-acetone bath, which will gradually freeze the sample in 3–4 minutes. The sample will turn white when frozen. Do not drop cryomold into acetone during the freezing procedure.
5. Remove the block from the cryomold and place it at  $-80^{\circ}\text{C}$  until ready for sectioning. If the blocks are stored for a prolonged period, place them in an airtight container with frozen distilled  $\text{H}_2\text{O}$  to maintain humidity.

#### Sectioning and Slide Storage

1. Attach a frozen block of tissue to the chuck in the cryostat with O.C.T. Compound, using standard frozen tissue-sectioning methods.
2. Allow the block to equilibrate to the cryostat temperature ( $-20$  to  $-25^{\circ}\text{C}$ ) for about 15–20 minutes.  
If the block is too cold during cutting, this time may need to be extended.
3. Cut thin sections (1–5  $\mu\text{m}$ ) for LCM sampling and place them on a plain uncoated glass slide.  
Tissue adheres too strongly to coated slides, making LCM sampling difficult.
4. After placing the section on the slide, immediately place the slides on dry ice. If LCM is to be performed that day, the slides should be kept on dry ice. Alternatively, they may be stored in paper slide boxes at  $-80^{\circ}\text{C}$  until needed.

### PROTOCOL 2 H&E STAINING OF THE SLIDES FOR LCM

Before performing LCM, the sectioned tissue sample is fixed, stained if necessary, and dehydrated. We typically stain with H&E for gut and lymph node tissue. In addition, we prepare serial slides for immunohistochemistry to distinguish specific regions or cells. There is also a commercially available staining kit, HistoGene LCM Frozen Section Staining Kit (KIT0401, Arcturus, Mountain View, CA) that can process 32 slides. Once the slide is ready, LCM should be performed within 45 minutes. All reagents, tubes, and other materials for the RNA extraction step should be prepared before staining.

#### MATERIALS

##### BUFFERS, SOLUTIONS, AND REAGENTS

- 100% ethanol<1>
- 70% ethanol<2>
- Eosin Y (Sigma)
- Mayer's hematoxylin (Sigma)
- DEPC-treated Ultraspec Water<3> (Biotecx Laboratories, store at  $4^{\circ}\text{C}$ )
- Xylene <4>, mixed (Sigma)

##### SPECIAL EQUIPMENT

- Conical tubes, 50-ml
- Forceps
- Glass jars (for xylene)

▼ CAUTION  
See Appendix for appropriate handling of materials marked with <1>.

#### CELLS AND TISSUES Slides from Protocol 1

##### METHOD

Staining should be performed as closely as possible to the scheduled LCM transfer time using solution baths that are replaced regularly. In particular,  $\text{H}_2\text{O}$  and the last ethanol and xylene baths should be fresh every time. Keep in mind that the extent of RNA degradation is in proportion to the time the tissue is in aqueous phase after fixation (5 minutes is the limit for obtaining good RNA quality). Use DEPC-treated  $\text{H}_2\text{O}$  for all steps of this protocol. The last ethanol and xylene steps can be modified depending on the humidity. If the humidity is high, we recommend dipping the slide in 100% ethanol three times and keeping it in the second xylene wash for 2 minutes. If the humidity is low, skip the third ethanol wash and shorten the xylene washes to 30 seconds.

Proceed rapidly through each of the following steps, wiping off the edges and back side of the slide between solutions. Sometimes the slides repel the solution, making it necessary to dip the slide repeatedly. Use forceps to hold the slides.

1. Prepare the staining solution set (Fig. 3).
2. Fix in 70% ethanol (30 seconds).
3. Purified  $\text{H}_2\text{O}$  wash (don't substitute PBS for  $\text{H}_2\text{O}$ ; if PBS is used, tissue will not adhere well to LCM membrane). Dip up and down (5–30 seconds).
4. Mayer's hematoxylin (30 seconds).
5. Purified water wash (don't substitute PBS for water; if PBS is used, tissue will not adhere well to LCM membrane). Dip up and down (5–30 seconds).
6. 70% ethanol wash (30 seconds).
7. 100% ethanol wash (30 seconds).
8. Eosin Y (30 seconds).

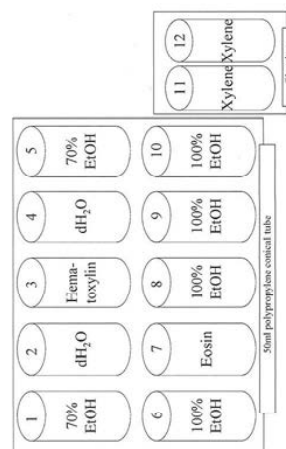


FIGURE 3. Rapid Hematoxylin & Eosin staining. In this technique, the slides are incubated for a minimal period to reduce exposure to aqueous solutions, which is associated with RNA degradation. After the slides are removed from the freezer, they are maintained at room temperature until ice crystals at the slide edge start melting, and then immersed in 70% ethanol (the first 50-ml conical tube), (see Protocol 2 for further details.)

9. 100% ethanol wash (30 seconds), two or three times.
  10. Xylene wash for 0.5–2 minutes twice.
- The sample is now ready for LCM.

### PROTOCOL 3 PERFORMING LCM

The workspace for LCM methods must be clean, dry, and RNase-free. Dispenser, forceps, and other equipment should be wiped with RNaseAway. If humidity is high, a dehumidifier may be required to maintain dry conditions. The laser beam of the PixCell II must be properly focused to obtain a good specimen. Because differences in tissue thickness affect laser focus, tissue sections must be of uniform thickness.

Coupling immunohistological staining with LCM is an obvious advantage, permitting capture of specific cell populations recognized by antibodies *in situ*. Its use for analysis of cytokine gene expression is limited by the loss of undegraded mRNA during the staining procedures (Jin et al. 1999; Kohda et al. 2000). Immunofluorescence staining is preferred because it avoids the use of enzymes and multiple incubations in aqueous solutions required for many immunohistochemical procedures. Recently, a rapid immunofluorescence LCM that minimizes mRNA loss was developed (Murakami et al. 2000). Another approach is to use serial sections on different slides, with one slide dedicated to LCM and the other to immunohistological staining (Wong et al. 2000).

The LCM slide is usually stained with H & E so that the cell populations which have been specifically identified through immunohistological staining can be generally localized. This approach, called intervening or navigated LCM, becomes cumbersome and inaccurate because smaller numbers of cells are selected or the cells are in heterogeneous tissue microenvironments. However, this LCM method can be very effectively used when only one cell type in the region selected expresses a particular gene or when the region selected is predominantly of one cell type.

#### MATERIALS

##### BUFFERS, SOLUTIONS, AND REAGENTS

RNaseAway (7000, Molecular BioProducts, San Diego, CA)

##### SPECIAL EQUIPMENT

Adhesive tape  
CapSure LCM Caps (Arcturus Engineering;  
Dehumidifier  
Dispenser, P200, P2  
Forceps  
LCM system  
PixCell II (Arcturus Engineering)  
RNase-free tube, 0.5 ml

##### METHOD

Perform LCM following the manufacturer's instruction. A brief description follows.

1. Place sample slide on the translation stage of the PixCell II apparatus.
2. Manually position the slide to locate the transfer area.
3. Turn on the vacuum chuck to hold the slide in place.

4. Place the LCM cap on the slide by rotating the placement arm.
5. Rotate the laser interlock keyswitch and wait 10 seconds.
6. Push the laser enable button.
7. Proceed to a higher magnification and adjust the focus.

The laser beam should be located in the center of the field of view. Note that the laser beam is invisible through the microscope. When the laser is enabled, you should carry out microdissection watching the monitor. Select the smallest spot size (7.5  $\mu$ m) and fire a test pulse outside tissue area to adjust focus of the laser beam.

8. Use the joystick to move to other areas on the tissue and capture other cells of interest.

After LCM, the LCM cap should be removed immediately and inserted onto an RNase-free 0.5-ml tube containing RNA isolation solution.

#### ▣ TROUBLESHOOTING

- If it is difficult to focus the laser beam, dust may be present on the LCM cap. Dust can be removed from LCM caps with adhesive tape. Otherwise, you can try to shoot another area. If the thickness of the section varies, refocusing may be necessary.

### PROTOCOL 4 RNA EXTRACTION FROM LIMITED CELLS

RNA samples can be kept at  $-80^{\circ}\text{C}$  for 6 months. Total cellular RNA is extracted using the Stratagene RNA Isolation kit following the manufacturer's instructions. Alternatively, the PicoPure RNA Isolation Kit is available and is particularly useful for RNA extraction from a few cells. Quantitation of isolated RNA through UV spectrometry is often not practical, because reagents in the RNA isolation kits affect absorbance values and because the total amount of RNA from LCM samples is frequently too little to detect with conventional spectrometers. Instead, one can use ribosomal RNA values as a standard to normalize for differences in undegraded RNA between samples. Collecting the same number of laser spots per sample can also help to maintain similar RNA quantities between samples.

Reverse transcription is performed immediately following the RNA extraction, and first-strand cDNA is synthesized with a random hexamer and SuperScript II RNase H<sup>-</sup> reverse transcriptase (see Protocol 5). Once the RNA is collected and purified, real-time PCR commercial kits (PE Applied Biosystems, Foster City, CA) specific for different cytokines or ribosomal RNA are used, and all data are normalized to constitutive ribosomal RNA values (see Protocol 6). The Applied Biosystems 7700 sequence detector (PE Applied Biosystems) is used for amplification of target mRNA, and quantitation of differences between treatment groups is calculated according to manufacturer's instructions.

#### MATERIALS

##### BUFFERS, REAGENTS, AND SOLUTIONS

75% Ethanol  $\leq$  with Ultraspec Water  
Glycogen, 5 mg/ml (Ambion, Austin, TX)

Stratagene RNA Isolation kit (200345, Stratagene Cloning Systems, La Jolla, CA)

Includes Denaturing solution; phenol  $\leq$  saturated with  $\text{H}_2\text{O}$  (store at  $4^{\circ}\text{C}$ ),  $\beta$ -mercaptoethanol  $\leq$  (store at  $4^{\circ}\text{C}$ ), chloroform  $\leq$  isoamyl alcohol  $\leq$ , 2 M sodium acetate, isopropanol  $\leq$  OR use PicoPure RNA Isolation Kit (KIT0202, Arcturus, Mountain View, CA)

**▼ CAUTION**  
See Appendix for appropriate handling of materials marked with  $\leq$ .

DEPC-treated Ultraspec Water <1> (Biotecx Laboratories, Houston, TX) (store at 4°C)

#### SPECIAL EQUIPMENT

Pipette  
RNase-free tube, 1.5 ml  
CELLS AND TISSUES  
LCM caps from Protocol 3

#### METHOD

1. Add 200  $\mu$ l of Denaturing solution and 1.6  $\mu$ l of  $\beta$ -mercaptoethanol to a 0.5-ml tube.
2. Insert the LCM cap into the tube and vortex for 5 seconds.
3. Invert the tube and incubate for 5 minutes.
4. Remove the LCM cap.

Samples processed to this point can be stored at -80°C up to 6 months.

5. Transfer the solution to a 1.5-ml tube.
6. Add 20  $\mu$ l of 2 M sodium acetate. Mix gently.
7. Add 220  $\mu$ l of H<sub>2</sub>O-saturated phenol (bottom layer). Mix gently.
8. Add 60  $\mu$ l of chloroform:isoamyl alcohol. Mix vigorously.
9. Put on wet ice for 15 minutes.
10. Centrifuge at 14,000 rpm for 30 minutes at 4°C.
11. Transfer upper layer to a new tube.
12. Add 2  $\mu$ l of glycogen (final 10–150  $\mu$ g/ml).
13. Add 200  $\mu$ l of cold isopropanol.
14. Place the tube in a -80°C freezer overnight.
15. If the sample is frozen solid, allow it to melt a little until it is a slurry. This will facilitate formation of a more compact pellet. Centrifuge at 14,000 rpm for 30 minutes at 4°C.
16. Remove the supernatant and wash the pellet with 400  $\mu$ l of 75% ethanol.
17. Centrifuge at 14,000 rpm for 5 minutes at 4°C.
18. Remove the supernatant. Use smaller pipette to remove the supernatant completely.
19. Air-dry for 5–10 minutes.
20. Resuspend the RNA with 25  $\mu$ l of Ultraspec Water.

### PROTOCOL 5 cDNA SYNTHESIS

#### METHOD

BUFFERS, SOLUTIONS, AND REAGENTS

0.1 M Dithiothreitol <1>  
dNTPs (2.5 mM each dNTP)  
Random primer (20–40 units/ml) (1034731, Roche Applied Science, Indianapolis, IN)  
RNA sample from Protocol 4  
RNasin (Promega)

▼ CAUTION  
See Appendix for  
appropriate han-  
dling of materials  
marked with <1>.

5x RT buffer  
RT master mix (x sample number)

5x RT buffer 10  $\mu$ l  
dNTP 5  $\mu$ l  
0.1 M DTT 4  $\mu$ l  
random primer 4  $\mu$ l  
RNasin 1  $\mu$ l  
SuperScript II (18064-014, Invitrogen, Carlsbad, CA)

Store reagents at -20°C.

#### SPECIAL EQUIPMENT

PCR tube, 0.2 ml

Thermal cycler e.g., Applied Biosystems 7700 sequence detector; PE Applied Biosystems, Foster City, CA)

#### METHOD

1. Dispense 24  $\mu$ l of RT reaction mixture to each tube.
2. Add 25  $\mu$ l of RNA sample from Protocol 4.
3. Denature at 70°C for 5 minutes and then quench on ice for 5 minutes.
4. Add 1  $\mu$ l of SuperScript II (final reaction volume, 50  $\mu$ l).
5. Incubate the mixture at 37°C for 60 minutes, denature at 90°C for 5 minutes, and quench on ice for 5 minutes.
6. The cDNA samples should be stored at -20°C until use.

### PROTOCOL 6

#### REAL-TIME PCR

Once the RNA is collected and purified, real-time PCR commercial kits specific for different cytokines or ribosomal RNA are used, and all data are normalized to constitutive ribosomal RNA values. The Applied Biosystems 7700 sequence detector is used for amplification of target mRNA, and quantitation of differences between treatment groups is calculated according to manufacturer's instructions.

#### MATERIALS

BUFFERS, SOLUTIONS, AND REAGENTS

cDNA sample from Protocol 5  
20x 18S rRNA primers and probe (Applied Biosystems)  
20x Target primers and probe (Applied Biosystems)  
2x TaqManUniversal PCR Master Mix (4304437, Applied Biosystems, Foster City, CA)  
DEPC-treated Ultraspec Water <1> (Biotecx Laboratories) (Store at 4°C.)

Store reagents at -20°C.

#### SPECIAL EQUIPMENT

Applied Biosystems 7700 sequence detector (PE Applied Biosystems, Foster City, CA)

# METHOD

1. Dilute the cDNA sample from Protocol 5 1/100.
2. Use 1 µl of diluted solution for the control reaction and 10 µl for the target reaction.

| Solutions            | Control | Target  |
|----------------------|---------|---------|
| Water                | 21.5 µl | 12.5 µl |
| 20x Master Mix       | 25 µl   | 25 µl   |
| 2x primers and probe | 2.5 µl  | 2.5 µl  |
| cDNA sample          | 1 µl    | 10 µl   |
| Total                | 50 µl   | 50 µl   |

3. PCR cycle conditions:
  1. 50°C for 2 minutes
  2. 95°C for 10 minutes
  3. PCR cycle conditions – n cycles
    - a. 95°C for 15 seconds
    - b. 60°C for 1 minute

# ANALYSIS OF RESULTS

The results from a real-time PCR analysis of ribosomal RNA isolated from LCM-captured mouse lymph node samples are shown in Figure 4. This procedure used a 30-µm cap diameter. A linear correlation between cycle number and number of spots was obtained, indi-

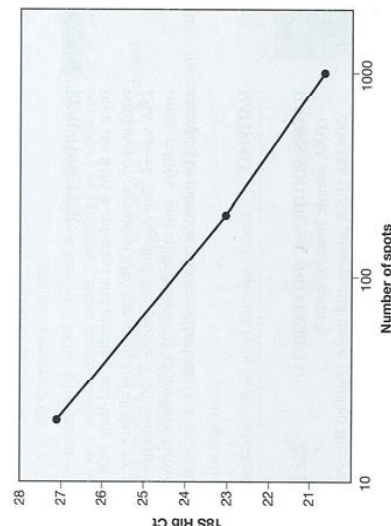


FIGURE 4. The relationship between number of spots (captured samples) and real-time PCR amplification of ribosomal RNA. Cells were captured from the lymph node using 30-µm-diameter laser spots. Total RNA was extracted and reverse-transcribed with random primer and SuperScript II reverse transcriptase. The numbers of spots pooled into each amplification reaction are listed on the x axis, and the threshold level of detection of the ribosomal RNA signal is graphed on the y axis.

cating that one can equilibrate RNA by taking an equal number of spots per sampling area. To show the practicality of the method, results of the cytokine gene expression in localized microenvironments in the gut after infection of mice with the intestinal nematode parasite *Heligmosomoides polygyrus* are shown in Figure 5. Figure 5A is a micrograph of a cyst containing developing larvae at day 4 after a primary inoculation. Figure 5B shows the region after tissue samples (the area immediately around the cyst) have been collected by LCM. To examine the gene expression of individual microenvironments in the small intestine of nematode-infected mice, cells were captured from (1) the lamina propria; (2) the Peyer's patch; (3) the area immediately surrounding the cyst; and (4) the area inside the cyst. RNA was purified from pooled LCM samples, reverse-transcribed, and interleukin (IL)-4, IL-13, and ribosomal RNA were assessed by real-time RT-PCR. All data were normalized to constitutive ribosomal RNA values. Nematode-infected mice that received anti-CD4 antibodies at day 7 after inoculation showed markedly reduced elevations in both IL-4 and IL-13 in the region surrounding the cyst (Fig. 6).

# DISCUSSION

Various assays have been used to measure protein and mRNA expression in situ, and each has distinct advantages and disadvantages. Immunohistological techniques (immunohistochemistry and immunofluorescence) can measure protein directly, but, because many proteins are rapidly made, secreted, and utilized, sufficient quantities may not be present at a given time point to be detected. Furthermore, the technique is dependent on the availability of specific antibodies effective in staining tissue sections. In situ hybridization can localize mRNA expression and, coupled with IH or IF, can detect specific cell populations expressing the target mRNA. However, in situ hybridization is often not sufficiently sensitive to detect tightly regulated mRNA species. In addition, expression of only one or two genes can be analyzed in a single assay, making analysis of multiple mRNA species cumbersome.

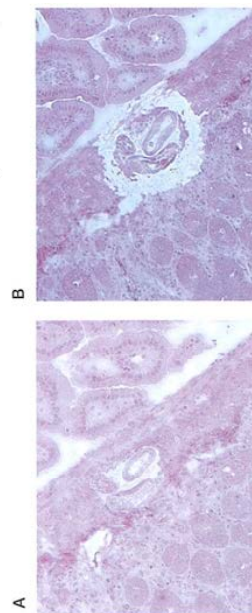


FIGURE 5. Laser capture microdissection a: the host-pathogen interface. Mice were inoculated orally with the intestinal nematode parasite, *H. polygyrus*. At day 8 after inoculation, tissues were embedded in O.C.T., frozen, and sectioned with a cryostat. LCM was performed to examine the localized immune response in the microenvironment surrounding the invading parasite. Intestinal cyst containing the nematode is shown in serial sections: (A) before LCM, (B) after LCM. The cells were taken from the area immediately around the cyst in this picture. The cells were also captured from the lamina propria, Peyer's patch, and inside the cyst.

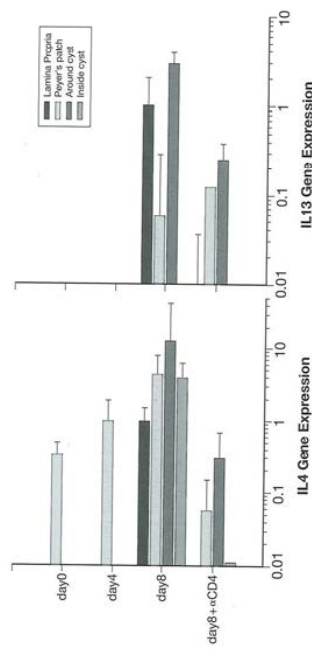


FIGURE 6. RT-PCR analysis of LCM-captured samples at the host-pathogen interface during primary immune response to an intestinal nematode parasite. IL-4 and IL-13 mRNA were detected in the lamina propria, and in the localized region around and inside the cyst by day 8 after inoculation. Anti-CD4 antibody treatment at day 7 after inoculation inhibited elevations in both IL-4 and IL-13. Mice were inoculated orally with the infectious parasite, as described in Fig. 5. All data were normalized to ribosomal RNA and expressed relative to day-8 lamina propria. The results are expressed as the mean and standard error for each treatment group. Similar results were obtained in two experiments.

The recently developed LCM technique adds an important methodology that greatly extends *in situ* analyses of tissue sections. The ability to remove targeted cell populations selectively from their tissue microenvironment for nucleotide and protein analysis allows the use of sensitive laboratory techniques available for identifying and quantitating cellular molecules in solution (Nagle 2001; Wittliff and Erlander 2002). Real-time PCR is well-suited for gene expression of samples captured by LCM (Specht et al. 2000; Trogan et al. 2001; Vincent et al. 2002). After cDNA synthesis, multiple targets can be analyzed from a single sample, and the sensitivity of the assay allows successful amplification, even with small amounts of target material. When used as described in the accompanying protocols, the LCM technique can collect significant quantities of undegraded mRNA. However, slight changes in these protocols, particularly involving the sectioning and storage of tissue sections, can result in a considerable loss in RNA integrity. Combining LCM with immunohistochemistry staining can be done under certain conditions, particularly with IF, but it is still not widely used due to significant loss of mRNA. The ability to capture IF-stained cells directly, rather than being limited to using navigational LCM, will greatly extend the capability of this technique.

Another area of refinement will be the diameter of the focused laser beam. The current minimum diameter is 7.5  $\mu$ m, but most studies have suggested that the actual area taken is about twice that diameter (L. Barisoni and R.A. Star, in prep.), which in many cases can result in several cells, perhaps heterogeneous, being captured at a single site. There are adjustments that can be made to the current LCM microscope (PixCell II) which may further tighten the beam, including additional focusing lenses and specially designed caps, all of which are less than optimal. A new LCM microscope has recently been developed by Arturus (PixCell Ite) that has a narrower laser beam and improved image quality. More studies are required to determine whether this apparatus can be used routinely to collect single cells, but if it does have this capability, it would be a marked improvement.

The LCM technique has developed in recent years into a versatile and robust tool that can be routinely used in the laboratory after the initial fairly expensive purchase of the equipment. It greatly extends our capability for analyzing both protein and gene expression in the tissue microenvironment (Liotta and Kohn 2001).

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## WWW RESOURCE

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## Strategies for Overcoming PCR Inhibition

Peter Rådström,<sup>1</sup> Charlotta Löfström,<sup>1,2</sup> Maria Lövenklev,<sup>1</sup> Rickard Knutsson,<sup>1</sup> and Petra Wolffs<sup>1</sup>

<sup>1</sup>*Applied Microbiology, Center for Chemistry and Chemical Engineering, Lund Institute of Technology, Lund University, SE-221 00 Lund, Sweden;* <sup>2</sup>*Analyticon Nordic AB, SE-531 19 Lidköping, Sweden*

The use of conventional and real-time PCR is to some extent restricted by the presence of PCR inhibitors. This is particularly so when the techniques are applied directly to complex biological samples such as clinical, environmental, or food samples for the detection of microorganisms. PCR inhibitors may originate from the sample itself, or as a result of the method used to collect or otherwise prepare the sample (Rossen et al. 1992). Either way, inhibitors can dramatically reduce the sensitivity and amplification efficiency of PCR. Altered amplification efficiency will change the kinetics of an amplification reaction, generating ambiguous data in quantitative PCR. Some inhibitors, originating from clinical, environmental, and food samples, are shown in Table 1 (for a comprehensive list, see Wilson 1997). Most of these inhibitors are known to interact with the structure and function of proteins, and the thermostable DNA polymerase is doubtless the most important target site of the inhibitors. The polymerase can be degraded by proteinases, denatured by phenol or detergents, and inhibited by the blocking of the active site by the inhibitor, as is the case with heme (Akane et al. 1994). Furthermore, it is well known that the polymerization activity of DNA polymerase varies with, for example, ionic strength, pH, sulphydryl, and the presence of other chemical agents. However, there is a need to specifically and systematically identify and study the mechanisms of PCR inhibitory compounds.

PCR inhibition can be categorized into (1) inhibition caused by inhibitory molecules and particles in the sample and (2) sample-independent inhibition, including random amplification failure, probably caused by human factors (Cone et al. 1992). The latter category is rather uncommon, and it is difficult to discriminate between a failure in sample and reagent handling and an actual failure of amplification. The inhibitory mechanisms at work in conventional PCR may include (1) inactivation of the thermostable DNA polymerase, (2) degradation or capture of the nucleic acids, and/or (3) interference with the release of target nucleic acids from cells (Wilson 1997). However, the list of inhibitors and the mechanisms by which they may act has grown since the introduction of real-time PCR. New inhibitors may interfere with fluorescent double-stranded DNA-intercalating dyes, such as SYBR Green I, or with different types of fluorogenic sequence-specific probes used in these complex reactions. Inhibitory compounds may also generate background fluorescence or scatter the emission from the fluorogenic molecules.

TABLE 1. PCR inhibitors

| Inhibitor          | Proposed mechanism                                     | Reference                     |
|--------------------|--------------------------------------------------------|-------------------------------|
| Proteinases        | degradation of DNA polymerase                          | Powell et al. 1994            |
| IgG                | binding to nucleic acids                               | Abu Al-Soud et al. 2000       |
| Exopolysaccharides | binding to DNA polymerase                              | Monteiro et al. 1997          |
| Lactoferrin        | release of iron ions                                   | Abu Al-Soud and Rådström 2001 |
| Calcium ions       | competing with Mg <sup>2+</sup> as polymerase cofactor | Bickley et al. 1996           |
| Phenol             | denaturation of DNA polymerase                         | Katicher and Schwartz 1994    |
| EDTA               | chelation of Mg <sup>2+</sup> ions                     | Rossen et al. 1992            |
| Heparin            | binding to nucleic acids                               | Sainsangi et al. 1994         |

## SAMPLE PREPARATION

A great deal of effort is being focused on the pretreatment of samples to reduce PCR inhibition, with little understanding of the mechanisms by which the inhibitors act (for review, see Lantz et al. 1994a). The treatments aim either to reduce the effect of the inhibitors, to remove them, or to provide a combination of both. For example, aqueous two-phase systems (Lantz et al. 1994b), boiling (Magistrado et al. 2001), density gradient centrifugation (Lindqvist et al. 1997), dilution (Abu Al-Soud et al. 1998), DNA extraction (Dahlenborg et al. 2001), filtration (Lantz et al. 1999), immunological techniques (Nogya et al. 2000), and PCR-compatible enrichment media (Payne and Kroll 1991; Knutson et al. 2002b) are some of the techniques that are presently used. Particular attention should be paid to the final concentration of target molecules and inhibitory substances. The optimal method of sample preparation will (1) concentrate the target molecules to suit the practical operating range of the PCR, (2) remove or reduce the effects of PCR-inhibitory substances, (3) minimize sample variation, and (4) be simple to perform.

Many of the currently used sample preparation methods are technically challenging and time-consuming. Rapid and robust sample preparation methods are needed before PCR can be used for routine analysis to facilitate detection of targets in recalcitrant biological samples.

## CHOICE OF DNA POLYMERASE AND AMPLIFICATION FACILITATORS

As an alternative to optimizing sample preparation, PCR inhibition can sometimes be overcome by using inhibitor-tolerant thermostable polymerases or including amplification facilitators in the reaction (Abu Al-Soud and Rådström 1998, 2000). Thermostable DNA polymerases vary in their tolerance of inhibitors. For example, the DNA polymerases from *Thermus aquaticus*, AmpliTaq Gold and Taq, are completely inactive in the presence of 0.004% (v/v) human blood, whereas the HotTub, Pwo, rTth, and Tfi DNA polymerases are unaffected by the presence of at least 20% (v/v) blood (Abu Al-Soud and Rådström 1998). A number of thermostable DNA polymerases with various properties are now commercially available (Table 2). Table 3 presents a survey of PCR applications that have benefited from a careful choice of DNA polymerase and/or inclusion of facilitators in the reaction mix.

Amplification facilitators have been found to improve the specificity of PCR and to allow the amplification of GC-rich regions (Sarkar et al. 1990; Varadaraj and Skinner 1994). Facilitators can increase the fidelity of DNA synthesis (Wu and Yeh 1973), as well as enhance amplification efficiency in the presence of PCR-inhibitory substances (Abu Al-

TABLE 2. The thermostable DNA polymerases

| Polymerase                            | Source                        | Molecular weight (kD) | Thermal stability <sup>a</sup> | Exonuclease activity | References                                            |
|---------------------------------------|-------------------------------|-----------------------|--------------------------------|----------------------|-------------------------------------------------------|
| AmpliTag <sup>c</sup>                 | <i>Thermus aquaticus</i>      | 94                    | 95°C/40 min                    | 5'-3'                | Barnes 1994                                           |
| Deep Vent <sup>®</sup>                | <i>Pyrococcus</i> sp. GB-D    | 91                    | 100°C/3.4 h                    | 3'-5'                | Barnes 1994; Takagi et al. 1997                       |
| DYNazyme <sup>d</sup>                 | <i>Thermus brockianus</i>     | 90                    | 100°C/2 h                      | 3'-5'                | Klimeczak et al. 1986                                 |
| Pwo <sup>e</sup>                      | <i>Pyrococcus furiosus</i>    | 90                    | 100°C/2 h                      | 3'-5'                | Perlier et al. 1996                                   |
| Stoffel fragment <sup>f</sup>         | <i>Thermus aquaticus</i>      | 61                    | 95°C/80 min                    | -                    | Lawyer et al. 1989; Vainshtein et al. 1996            |
| Tfi/HotTub <sup>g</sup>               | <i>Thermus flavus</i>         | 94                    | 70°C/2 h                       | 5'-3'                | Kalichin et al. 1981                                  |
| rTth <sup>h</sup>                     | <i>Thermus thermophilus</i>   | 94                    | 95°C/20 min                    | 5'-3'                | Rüttimann et al. 1985; Wendt et al. 1990              |
| Ultima <sup>i</sup>                   | <i>Thermotoga maritima</i>    | 70                    | 97°C/50 min                    | 3'-5'                | Perlier et al. 1996                                   |
| Vent <sup>®</sup> (Exo-) <sup>j</sup> | <i>Thermococcus litoralis</i> | 90                    | 100°C/2 h                      | -                    | Marilla et al. 1991; Barnes 1994; Perlier et al. 1996 |
| Tth <sup>k</sup>                      | <i>Thermus thermophilus</i>   | 95                    | 95°C/40 min                    | 5'-3'                | Rüttimann et al. 1985                                 |
| Hot start                             |                               |                       |                                |                      |                                                       |
| AmpliTag Gold <sup>c</sup>            | <i>Thermus aquaticus</i>      | 94                    | 95°C/40 min                    | 5'-3'                | Ketelmann-Beizing et al. 1998                         |
| PlatinumTaq <sup>b</sup>              | <i>Thermus aquaticus</i>      | 94                    | 95°C/40 min                    | 5'-3'                | Westall et al. 1995, 1998                             |
| FastStart                             | <i>Thermus aquaticus</i>      |                       |                                |                      |                                                       |
| Enzyme mixture                        |                               |                       |                                |                      |                                                       |
| Expand PCR system <sup>l</sup>        | <i>Thermus aquaticus</i>      | 95                    | 100°C/3 min                    | 5'-3'                | Barnes 1994                                           |
| ELONGase <sup>m</sup>                 | <i>Pyrococcus</i> sp. GB-D    | 91                    | 100°C/3.4 h                    | 3'-5'                | Westall et al. 1995                                   |
| TaqPlus PCR System <sup>n</sup>       | <i>Thermus aquaticus</i>      | 94                    | 95°C/40 min                    | 5'-3'                |                                                       |
|                                       | <i>Pyrococcus furiosus</i>    | 90                    | 100°C/2.9 h                    | 3'-5'                |                                                       |

<sup>a</sup>These data were obtained from published data and from commercial sources.

<sup>b</sup>Deep Vent<sup>®</sup> is the registered trademark of New England Biolabs, Beverly, Maryland.  
<sup>c</sup>AmpliTag, AmpliTaq Gold, Stoffel fragment, and rTth are registered trademarks of Applied Biosystems, Foster City, California. rTth can be used as reverse transcriptase in the presence of "in" and as DNA polymerase after chelation of Mn<sup>2+</sup> by EGTA and addition of Mg<sup>2+</sup>.  
<sup>d</sup>DYNazyme is a registered trademark of Finnzymes Oy, Riihimäki, Finland.  
<sup>e</sup>Pwo, Tth, and Expand PCR system are registered trademarks of Stratagene, La Jolla, California.  
<sup>f</sup>Stoffel fragment is a registered trademark of Promega, Madison, Wisconsin.  
<sup>g</sup>HotTub is a registered trademark of Amersham Pharmacia Biotech, Cleveland, Ohio.  
<sup>h</sup>ELONGase and PLATINUMTaq are registered trademarks of Life Technologies, Inc., Rockville, Maryland.

Soud and Rådström 2000). Bovine serum albumin (BSA), for example, has proved to be a particularly potent facilitator by relieving the inhibitory effects of hemoglobin and lactoferrin (Abu Al-Soud and Rådström 2001). The ability of BSA to relieve inhibition may be related, at least in part, to its ability to bind inhibitors (Akane et al. 1994). Thus, the PCR-inhibitory effects of biological samples can be reduced, or even eliminated, by the use of an appropriate combination of DNA polymerase and amplification facilitator(s). The following sections discuss optimization strategies for overcoming PCR inhibition in conventional and real-time PCR.

## CONVENTIONAL PCR PCR-inhibition Studies

PCR inhibitors originating from the sample itself or from sample preparation, e.g., residual phenol from the DNA purification step, have been shown to affect both the yield and specificity of PCR (Katcher and Schwartz 1994). The effect of PCR inhibitors can be studied by increasing the concentration of the purified template DNA, and/or by adding different concentrations of the PCR-inhibitory substance. Increasing the concentration of target DNA may overcome the effect of inhibitors that interfere with DNA and/or bind reversibly to the DNA-binding domain of the DNA polymerase. Adding different concentrations of inhibitory substances such as blood, feces, or heme to reactions allows their inhibitory effect to be evaluated. Characterization of PCR-inhibitory effects is necessary for the development of more efficient sample preparation methods, which will eliminate the need for extensive processing of biological samples prior to diagnostic PCR.

## Detection Limit and Detection Probability

In diagnostic PCR applications, "sensitivity" and "detection limit" are loosely defined and often used interchangeably. The terms are related and refer to the lower concentration range of amplification of target DNA/cells. Both sensitivity and detection limits are strongly affected by reaction conditions, the quality of the target DNA, and the presence of extraneous matter (Schmidt 1997). When studying the sensitivity and detection limit of a reaction, it should be borne in mind that PCR employs very small sample volumes. When analyzing a sample for the presence of a microorganism, 5 µl might be a typical sample volume. If the detection limit of the assay is stated to be 1 CFU/ml, the probability of the target being present in a 5-µl PCR sample is very low; i.e., one positive reaction out of 200 (assuming no concentration step is included in the pre-PCR processing of the sample). To improve the reliability of the assay, the detection limit should be viewed in association with the probability of detecting the target DNA/cell at a certain concentration. For instance, in Figure 1, the vertical line illustrates the detection level at 95% probability.

Mathematical and statistical models can be used to define the method detection limit (MDL) of an assay, which provides an idea of how well an assay works and how well it can be reproduced. It is determined from replicate analyses and is based on statistical analyses, which incorporate an acceptable level of risk of false positive or false negative results. The advantage of using models is that the results can be interpreted objectively. This is especially important for the identification of false negatives in microbiological assays. A model was used to determine the MDL of a PCR detection assay for *Cryptosporidium parvum* (Walker et al. 1998). However, other models have also been used to describe the detection probability of a PCR assay. Since conventional PCR, based on agarose gel electrophoresis, is a qual-

TABLE 3. Examples of DNA polymerases and/or PCR facilitators used in different PCR-based applications

| Application               | Objective                                             | DNA polymerase                    | PCR facilitator                  | Reference                           |
|---------------------------|-------------------------------------------------------|-----------------------------------|----------------------------------|-------------------------------------|
| Diagnostic PCR            | overcome inhibition from meat, blood, and feces       | <i>rTh</i> (Applied Biosystems)   | BSA, gp32, Tween 20              | Abu Al-Soud and Rådström 1998; 2000 |
| Reverse transcription-PCR | overcome inhibition from phenol                       | <i>Tth</i> (Amersham)             | —                                | Katcher and Schwartz 1994           |
|                           | increase sensitivity                                  | <i>rTh</i> (Applied Biosystems)   | —                                | Schwab et al. 2001                  |
|                           | reduce intra-PCR variability and increase sensitivity | <i>Pwo</i> (Roche Diagnostics)    | —                                | Mullan et al. 2001                  |
| Cloning and sequencing    | perform PCR of GC-rich sequences                      | 3 different DNA polymerases       | Betaine                          | Henke et al. 1997                   |
|                           | reduce amplification errors                           | <i>Pfu</i> (Stratagene)           | —                                | Cline et al. 1996; Shaikhani 2002   |
|                           | improve amplification capacity                        | DynaZyme (Finnzymes)              | —                                | Zsolnai and Fesus 1997              |
| Genotyping                | increase yield and specificity                        | AmpliTaqGold (Applied Biosystems) | —                                | Moretti et al. 1998                 |
|                           | reveal polymorphism                                   | DynaZyme EXT (Finnzymes)          | Betaine                          | Diakou and Doyas 2001               |
|                           | overcome inhibition from sludge                       | AmpliTaqGold (Applied Biosystems) | Polyvinylpyrrolidone (PVP, gp32) | Monpocho et al. 2000                |
| Real-time PCR             | overcome inhibition from buffered peptone water (BRV) | <i>rTh</i> (Applied Biosystems)   | Glycerol                         | Knutsson et al. 2002c               |
|                           | enable detection                                      | 15 different DNA polymerases      | —                                | Kreuzer et al. 2000                 |
|                           | increase sensitivity                                  | Platinum Tag (Gibco BRL)          | —                                | Hein et al. 2001                    |

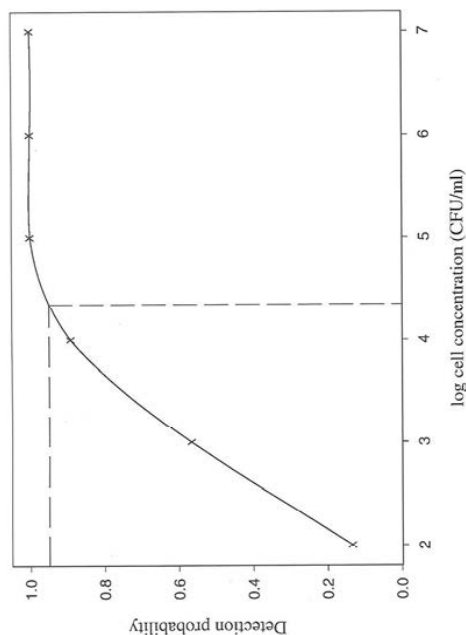


FIGURE 1. Detection probability of target cells (log CFU/ml) using a PCR assay. The PCR detection is described by a logistic regression model, estimated from a number of replicate experiments, and represented by the solid line. By plotting the concentration of target against the observed relative frequencies (X) of positive PCR detection (calculated as the number of positive samples divided by the total number of replicates), it can be verified that the model applied fits the observed data. The model can then be used to describe the detection probability of the PCR studied and to calculate the detection probability at various target concentrations or to determine the probability of detecting a certain amount of target, i.e., the dashed line indicates the concentration of cells (log CFU/ml) at a detection probability of 0.95.

itative method, the response is binary, being 1 for detection and 0 for no detection. By assuming that the response is a binary random variable and that it follows a binomial distribution, a logistic regression model has been applied to describe the detection probability of a *Yersinia enterocolitica* PCR assay (Knutsson et al. 2002a).

## PCR Optimization

In addition to characterizing the general effects of inhibitors and the detection probability of PCR assays, the individual demands of each PCR-based application must be met. For instance, in applications that require repeated amplification of the same target sequence, the prevention of carryover contamination is of great importance. Furthermore, studying the nucleic sequence of a genome in, for example, sequencing applications requires highly accurate PCR. Other examples are the need for high sensitivity or specificity. To ensure a reliable method, all the steps in the PCR procedure should be optimized for the intended use.

The choice of a suitable DNA polymerase and buffer has been shown to affect many PCR-based applications, such as diagnostic PCR, genotyping using restriction fragment length polymorphism (RFLP) (Zsolnai and Fesus 1997), short tandem repeats (STR)

(Moretti et al. 1998), and randomly amplified polymorphic DNA (RAPD) (Diakou and Dovas 2001), as well as cloning, sequencing, RT-PCR (Jaag and Raman 1994), quantitative PCR, and real-time PCR (Table 2) (Kreuzer et al. 2000). The various DNA polymerases differ in many features that are important for PCR, such as susceptibility to PCR inhibitors (Abu Al-Soud and Rådström 1998), fidelity (Cline et al. 1996), the ability to incorporate modified bases (Sakaguchi et al. 1995), and termination of primer extension (Modin et al. 2000). As discussed earlier, thermostable DNA polymerases differ in their tolerance of inhibitors (Abu Al-Soud and Rådström 1998), and the polymerase of choice will depend on the application being used. It is also important to optimize the buffer conditions of a reaction. Using the most suitable polymerase under the correct buffer conditions is critical to the success of RT-PCR (Brooks et al. 1995). For cloning and sequencing applications, where high fidelity is required, *Pfu* DNA polymerase has been found to reduce amplification errors (Shafikhan 2002).

## REAL-TIME PCR

### Amplification Efficiency and Linear Range

The development of real-time PCR has provided new opportunities to study the PCR process, because it is now possible to follow the amplification of the product by monitoring accumulation of a fluorescent marker which is incorporated into the amplicon during the reaction. In contrast to end-point measurements in conventional PCR, real-time PCR measures the first significant increase in fluorescence, or the point at which the fluorescence crosses a certain threshold level. This level is known as the  $C_p$  (Wittwer et al. 1997; Kyger et al. 1998) or  $C_t$  value (Heid et al. 1996). By measuring the threshold values for samples of known concentration, standard curves can be produced (Fig. 2). From these standard curves, new information can be derived. These data illustrate the linear range of amplification; i.e., the range over which the logarithm of the target concentration versus the threshold value forms a linear relationship. Furthermore, the slope of the standard curve over the linear range can be used to determine the amplification efficiency (AE), using the following equation:

$$AE = 10^{-1/\text{slope}} - 1$$

## Quantitative PCR

For absolute quantitative real-time PCR, comparison with a standard curve is required, whereas for relative quantification, comparison with a reference target/gene is sufficient (Orlando et al. 1998). Small differences in amplification efficiency can have a significant impact on the productivity of a PCR. For example, a 5% difference in amplification efficiency between two targets with identical starting concentrations can lead to a twofold difference in amplicon concentration after just 26 cycles (Freeman et al. 1999). Differences in amplification efficiency between the target and reference will lead to mistakes in relative quantification unless corrections are made according to newly developed methods (Pfaffl 2001). The length of the standard curve, i.e., the linear range of amplification, influences mainly absolute quantification. As with any method that employs a standard curve, it is important that the linear range be as wide as possible, because for correct quantification, the unknown samples must fall within this window.

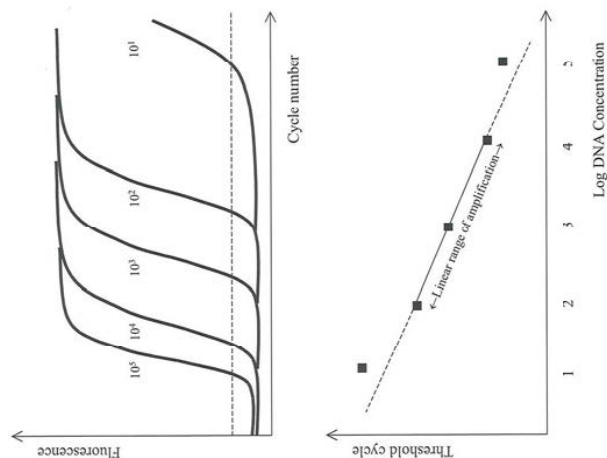


FIGURE 2. Schematic overview of the generation of data and standard curves by real-time quantitative PCR.

## PCR Optimization

Real-time PCR offers several novel ways to study PCR inhibition and the effects of sample preparation. By studying the amplification efficiency and the linear range of amplification, possible PCR inhibition can be easily spotted. For example, by comparing these two parameters for a DNA dilution series in water and another in carcass rinse from poultry, it is clear that the reaction is less efficient and also the linear range of amplification is narrower in carcass rinse (Fig. 3A,B). In addition to studying the pre-PCR processing effect of removing inhibitors or reducing the inhibition (Fig. 3C), the effects of target concentration and homogenization of the sample should also be studied.

The effects of PCR inhibitors on DNA synthesis may be investigated using a reaction system based on a single-stranded poly(dA) template with an oligo(dT) primer annealed to the 3' end (Abu Al-Soud and R  dstr  m 2001). For objective characterization of PCR inhibitors, the use of mathematical models is recommended. Models enable (1) comparison of the effects of different inhibitors, (2) kinetic analysis of the DNA polymerase in the presence and absence of inhibitors, and (3) evaluation of the effect of amplification facilitators. The model developed by Kn  tsson et al. (2002c) was used to study the effect of buffered

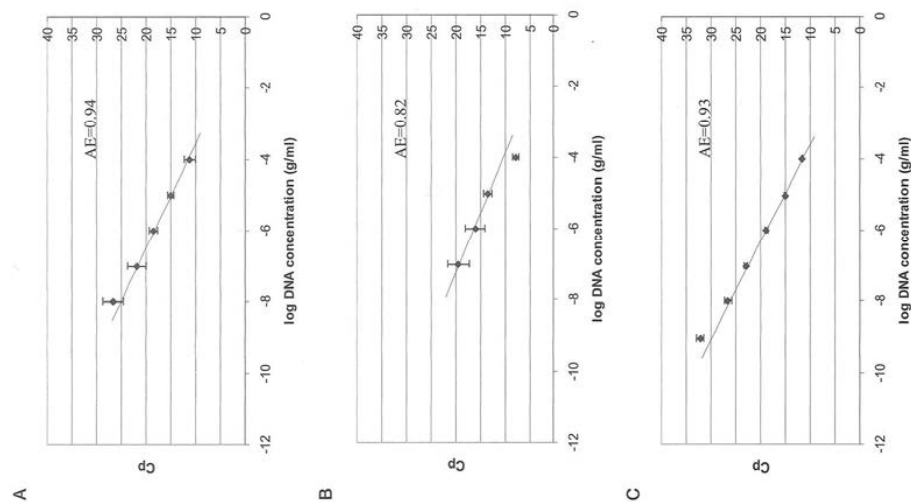


FIGURE 3. Standard curves made testing 12 samples with DNA concentrations between 1 mg/ml and 1   g/ml. All the results that gave positive product peaks between 88  C and 92  C in the melting curve analysis are shown. Results show the linear range of amplification and the amplification efficiency: (A) Taq DNA polymerase with DNA samples in water, (B) Taq DNA polymerase with DNA samples in tenfold diluted carcass-rinse of chicken, (C) Taq DNA polymerase with DNA samples in tenfold diluted carcass-rinse of chicken.

peptone water (BPW) on real-time PCR performance. Results showed that BPW partially inhibited AmpliTaq Gold mixture, whereas *rhtA* maintained the same PCR performance as the positive control.

Due to the complexity of the real-time PCR mix, and the associated high sensitivity to PCR inhibition, nearly all sample preparation methods for this technique involve DNA purification, perhaps the most rigorous of sample treatments. However, even PCR using purified DNA templates can be enhanced by using an alternative DNA polymerase and/or changing the buffer composition (P. Wolffs and P. Rådström, unpubl.). The performance of real-time PCR using TaqMan probes relies completely on the exonuclease activity of the DNA polymerase. This means that only certain DNA polymerases are suitable for use in this technique (Kreutzer et al. 2000).

## SUMMARY

To design a reliable and sensitive conventional or real-time PCR method, it is crucial to select the optimal DNA polymerase(s) and/or amplification facilitator(s) for the chemicals present in the samples during amplification. PCR optimization currently tends to focus on primer design, buffer, and thermocycling conditions to obtain specific PCR products. The amplification mixture can be modified by the addition of PCR facilitators, or by the choice of the appropriate polymerase, to improve proofreading activity, PCR yield, length of amplicon, etc., or to suit specific applications, such as cloning of PCR products, *in vitro* mutagenesis, *in situ* PCR, multiplex PCR, PCR ELISA, reverse-transcription PCR. However, less effort is being devoted to overcoming the effects of PCR-inhibitory compounds. Ideally, the number of steps required to generate PCR samples should be minimized. The use of appropriate DNA polymerases and amplification facilitators, in combination with optimized sampling techniques, reduces the amount of sample handling involved in the analysis. In the future development of PCR technology, research in pre-PCR processing is likely to expand in response to the growing demand for rapid, robust, and simple PCR protocols. A future challenge in pre-PCR processing strategies is to design PCR protocols that integrate sampling and DNA amplification in an automated manner. Furthermore, to monitor PCR performance in the presence of biological compounds, mathematical models are required for the objective interpretation of PCR results. Once the validation parameters of the PCR assay of interest have been studied, the specific sample matrix can be characterized with respect to PCR interference. After obtaining this information, pre-PCR processing strategies can be designed to optimize the protocol for a specific sample.

## ACKNOWLEDGMENTS

This work was supported by grants from the Swedish Agency for Innovation Systems (VINNOVA), the Foundation of the Swedish Farmers Supply and Crop Marketing Cooperation (SL-stiftelsen), the Swedish Foundation for Strategic Research, and the Commission of the European Community within the program, "Quality of Life and Management of Living Resources," QLRT-1999.00226.

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