Improved Nucleic Acid Organic Extraction Through Use of a Unique Gel Barrier Material

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ABSTRACT

A novel density barrier material, Phase Lock Gel™, has been formulated and optimized for use in enhancing the separation between aqueous and organic phases during organic extraction/centrifugation of nucleic acids. Phase Lock Gel is inert, stable to heating, compatible with most enzymatic reactions of nucleic acids without sacrificing subsequent downstream applications, and can be easily and conveniently used with most plasmid DNA, genomic DNA, phage DNA and RNA organic extraction protocols. Use of Phase Lock Gel as an aqueous/organic phase barrier material can decrease processing time and improve nucleic acid recovery from organic extraction purifications by as much as 30%, all while minimizing user exposure to volatile organics.

INTRODUCTION

In molecular biology, purifying nucleic acid free from proteinaceous contaminants routinely involves extracting crude nucleic acid in aqueous solution with immiscible organic solutions comprised of phenol and/or chloroform. At the appropriate pH and ionic strength, the desired nucleic acid remains in the aqueous phase while denatured protein partitions into the organic phase. Unfortunately, after phase separation, some nucleic acids and proteins partition at the interface between the aqueous and organic phases. It is then difficult to recover nucleic acid in the aqueous phase free from denatured protein at the interface. Traditionally, a choice must be made: Leave behind a portion of the aqueous phase, thus sacrificing yield, in order to obtain purer nucleic acid uncontaminated by interface material, or recover as much aqueous phase as possible to improve yield and thereby risk a greater degree of contamination.

Phase Lock Gel™ (PLG™) is a third choice, one in which a nonreactive density barrier material migrates under centrifugal force to effectively isolate the aqueous phase from the organic and interface phases. The PLG barrier is durable enough that virtually 100% of the nucleic acid-containing aqueous phases can be recovered from above the PLG by pouring or pipetting. In this report we describe the use of PLG for the isolation of genomic DNA, plasmid DNA, DNA fragments from agarose gels and RNA, and present data regarding the quantity, quality and utility of the nucleic acid recovered.

MATERIALS AND METHODS

Genomic DNA Isolation

Genomic DNA (gDNA) was isolated by the proteinase K/sodium dodecyl sulfate (SDS)/phenol extraction method (5), described briefly below, with and without the use of PLG. Whole blood was obtained from normal rabbits, using EDTA as the anti-coagulant. NIH 3T3 (ATCC, Rockville, MD, USA) and F5B (an NIH 3T3 cell line stably transfected with the neo gene; gift from the W. French Anderson Laboratory, NIH) cells were grown to near confluency in 100-mm plates at 37°C and 5% CO₂ in complete growth medium and then harvested directly into the growth medium. The cells were pelleted by centrifugation at 250×g for 5 min at 4°C. The supernatants were aspirated and the cell pellets were resuspend-
ed into 1× Tris-buffered saline (TBS: 50 mM Tris-HCl, 200 mM NaCl, 3 mM KCl, pH 7.5). Both the blood samples and the suspended cells were mixed with an equal volume of 2× lysis buffer (0.65 M sucrose, 20 mM Tris-HCl, pH 7.8, 10 mM MgCl$_2$ and 2% Triton X-100) and incubated on ice. The nuclei were pelleted at 1100× g for 12 min at 4°C, resuspended into saline/EDTA (75 mM NaCl, 24 mM EDTA) and transferred to 15-mL disposable screw-cap centrifuge tubes with and without PLG Light (5 Prime → 3 Prime). Proteinase K and SDS were added to 0.5 mg/mL and 1%, respectively, and the mixtures were incubated at 37°C for 2 h. Samples were gently extracted with an equal volume of water-saturated phenol and centrifuged at 1500× g for 5 min. The recovered aqueous phases were extracted with water-saturated phenol:chloroform (1:1) as above. Virtually all of the final aqueous phases were recovered to fresh centrifuge tubes, KCl to 0.1 M and 1.25 vol 95% ethanol (EtOH) were added and the gDNA was spooled onto sterile Pasteur pipets. After washing in 70% EtOH, draining and dissolving in TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), the gDNA was quantitated by absorbance at 260 nm. Purity was ascertained by digesting 3 µg gDNA with 3 units restriction endonuclease as recommended by the supplier. Digested gDNAs were analyzed by 0.8% agarose gel electrophoresis and visualized by ethidium bromide (EtBr) staining. Additionally, EcoRI-digested NIH 3T3 and F5B gDNAs (3 µg each) were denatured, transferred to nitrocellulose and hybridized with a $^{32}$P-labeled neo gene probe [Southern (7) Hybridization Buffers protocol; 5 Prime → 3 Prime].

**Plasmid DNA Isolation**

Plasmid DNA was prepared by alkaline lysis (1) and spin column chromatography as described in the pZ523™ protocol (Reference 8; 5 Prime → 3 Prime). Briefly, pTZ19R plasmid/E. coli HB101 inoculated in 1 L Luria Broth containing 100 µg/mL ampicillin was incubated 14–16 h at 37°C with vigorous shaking. Bacteria were pelleted, supernatant was aspirated, and a cleared lysate was prepared (8). Crude pTZ19R DNA was precipitated from the cleared lysate with 0.6 vol 100% isopropanol and washed with 70% and 95% EtOH before resuspension in TE. The suspended DNA was transferred to a 15-mL disposable screw-cap centrifuge tube (with PLG Light), and the contaminating RNA was digested by incubation with 1:100 RNase PLUS™ (5 Prime → 3 Prime). The sample then was extracted twice with phenol:chloroform:isoamyl alcohol (50:49:1) and once with chloroform:isoamyl alcohol (49:1). Extractions were done sequentially in the

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**Figure 1.** Diagrammatic representation of aqueous and organic phase separation by PLG.
same PLG-containing tube by centrifuging at 1500 × g for 2 min to separate phases and reposition the barrier between extractions. The final aqueous phase was adjusted to 1.0 M NaCl and further processed by pZ523 spin column chromatography. Column eluate was precipitated and washed, and the pTZ19R DNA was resuspended in TE as above prior to restriction digestion and gel analysis.

**Isolation of DNA from LMP Agarose**

Lambda DNA, digested with HindIII, was electrophoretically resolved on a 1% low melting point (LMP) agarose gel in 1× TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.3). The gel was stained with 0.5 µg/mL EtBr, the bands were visualized by longwave UV light and the DNA fragment bands of interest were excised with a new razor blade. The slices were transferred to tared 1.5-mL microcentrifuge tubes with and without PLG Light in the tube, and the weight of each gel slice was determined. A volume of TE equivalent to 5× the gel slice weight was added, and the slices were melted at 65°C. The samples then were extracted with an equal volume 0.1 M Tris-HCl (pH 8.0)-saturated phenol and centrifuged at 12 000 × g for 2 min to separate the phases. The aqueous phases were recovered to fresh 1.5-mL tubes (with and without PLG), extracted with an equal volume of room-temperature phenol:chloroform:isoamyl alcohol and centrifuged. After transfer to fresh 1.5-mL tubes (with and without PLG), the aqueous phases were further extracted with an equal volume of room-temperature chloroform:isoamyl alcohol. Post-centrifugation aqueous phases were recovered to fresh 1.5-mL tubes (no PLG) and admixed with 0.25 vol 10 M ammonium acetate and 2.5 vol 95% EtOH. The samples were incubated, centrifuged and washed, and the dried RNA was dissolved in RNase-free water. Two-and-one-half volumes of RNA gel loading buffer containing EtdBr (5 Prime → 3 Prime) were mixed with each sample and incubated at 65°C for 10 min. The RNA was then analyzed by formaldehyde/1% agarose gel electrophoresis.

**RESULTS AND DISCUSSION**

**Mechanism of PLG Action**

As shown in Figure 1, PLG present during aqueous/organic extractions utilizing phenol and/or chloroform as the organic solution migrates under centrifugal force to form a barrier between the immiscible phases. The organic phase and interface material are effectively trapped below the nonreactive, hydrophobic PLG, and the aqueous nucleic acid-containing phase is easily recovered quantitatively. As PLG function is density dependent, proper phase separation is contingent

![Figure 2. Isolation and analysis of gDNA.](image1)

(A) EcoRI digests of 3 µg (each) NIH 3T3 cell gDNA (lane 2) and neo gene-containing F5B cell gDNA (lane 3) resolved by 1% agarose–1× TBE gel electrophoresis. Lanes 1 and 4: Lambda DNA–HindIII digest. (B) Autoradiograph of a Southern blot of the Panel A gel hybridized with a 32P-labeled neo gene probe.

![Figure 3. Isolation and analysis of plasmid DNA.](image2)

Plasmid pTZ19R was extracted in the presence of PLG Light and purified by pZ523 chromatography. Overnight digestion with PstI (lane 3), RsaI (lane 4), PvuII (lane 5), EcoRI (lane 6) and BamHI (lane 7) along with uncut (lane 2) and lambda DNA–HindIII digest samples (lanes 1 and 8) were resolved by 1% agarose–1× TBE gel electrophoresis.
upon the composition of both the aqueous and the organic solutions. To provide optimum performance, two different density formulations of PLG are available: Light and Heavy, for applications involving phenol extraction and high density aqueous phases, respectively.

Isolation of DNA

Genomic, plasmid and LMP agarose gel-resolved DNAs were isolated and purified by aqueous-extraction and hybridization in the presence of PLG. Yields of approximately 130 µg and 240 µg gDNA from 5 mL normal rabbit whole blood or one 100-mm plate of confluent NIH 3T3 or F5B cells (ca. 10^9 cells), respectively, are routinely obtained: 20%–30% higher yields than those obtained in the absence of PLG (data not shown). Resultant gDNA is of high purity, with A\textsubscript{260}/A\textsubscript{280} ratios of 1.75–1.80, and, as demonstrated in Figure 2, is suitable for restriction digestion and Southern blot analysis. EcoRI digests of NIH 3T3 and F5B gDNAs shown in Figure 2A, lanes 2 and 3, were complete within 30 min, and further incubation for 12 h did not change the gel profile. These gDNAs were equally good substrates for the other restriction endonucleases tested (data not shown). Figure 2B shows that a single, discrete 1.5-kbp band in the digested F5B (neo gene-containing cells) gDNA (lane 3) hybridized with a \textsuperscript{32}P-labeled neo gene probe, while no hybridization is evident with the digested NIH 3T3 gDNA negative control (lane 2).

Inclusion of PLG Light during aqueous-organic extraction of precipitated, resuspended and RNase-treated cleared lysate plasmid DNA decreased the organic extraction processing time and, as shown in Figure 3, yielded pure pTZ19R DNA that digested cleanly and completely with the restriction endonucleases tested. Use of PLG Heavy in a rapid aqueous-organic extraction microprep plasmid DNA purification protocol resulted in high-quality plasmid DNA sufficient for direct screening, hybridization probe synthesis, PCR amplification or sequencing (4). Additionally, with inclusion of PLG in the extraction tube(s) as the only procedural modification, phase separation was improved in standard protocols (6) for the preparation of DNA from M13-type phage and lambda phage (data not shown). PLG also facilitated phase separation in a high-throughput M13 DNA preparation method (9).

Aqueous-organic extraction is a standard technique used to purify DNA fragments from LMP agarose gels (6). Figure 4 shows a comparison of the DNA recoveries between this method performed without (lanes 2–7) and with (lanes 8–13) PLG present during extraction. The recovery of lambda DNA fragments ranging from 0.564 through 23.1 kbp in size is significantly improved through the use of the PLG barrier material. As the standard procedure was unmodified except for the PLG use, the improved yield probably results from compaction and segregation of interface material in conjunction with increased aqueous volume recovery.

Isolation of RNA

Due to its rapid chaotropic inactivation of RNase, guanidinium isothiocyanate lysis of cells and tissues followed by organic extraction is a very popular total RNA isolation method (2,3). However, some lysates produced by this method are difficult to process through the protein extraction steps with high RNA yield. By acting as a density barrier, PLG minimizes or eliminates problems with the denatured protein interface material. Figure 5 shows that total RNA isolated from a cell monolayer (lanes 1–3), washed and pelleted cells (lanes 4–6) and rabbit liver (lanes 7 and 8) has prominent 18S and 28S ribosomal RNA bands and, therefore, likely was intact and of high quality. Total RNA yields on the order of 15–45 µg per 10^9 cells and 600–700 µg per 100 mg rabbit liver were routinely achieved using the PLG modification.

Compatibility

Many enzyme reactions with nucleic acid substrates are extracted with phenol, phenol:chloroform:isoamyl alcohol and/or chloroform:isoamyl alcohol to purify the nucleic acid prior to the next procedure. Inclusion of PLG in the reaction tubes did not interfere with the following restriction and modification enzymes: AccI, AscI, BamHI, BglII, Clal, EcoRI, EcoRV, HincII, HindIII, HpaI, KpnI, NcoI, NdeI, PstI, PvuII, Rsal, SalI, Sau3AI, Smal, SphI, SstI, XmrI, calf intestinal alkaline phosphatase, reverse transcriptase, terminal deoxynucleotidyl transferase, T4 DNA polymerase, T4 polynucleotide kinase, T4 DNA ligase, E. coli DNA polymerase, E. coli RNase H and E. coli DNA ligase. Thus, PLG is inert and compatible with enzyme activity.

SUMMARY

The technique of purifying nucleic acids in aqueous solution by organic extraction has been improved in yield, purity,
speed and safety through the use of a density barrier material, Phase Lock Gel. This inert material migrates under centrifugal force to form a durable seal at the aqueous/organic interface, effectively isolating the nucleic acid-containing aqueous phase away from the organic and the interface/denatured protein phases. PLG was shown to be compatible with standard organic extraction procedures for gDNA, plasmid DNA, DNA from agarose gels, phage DNA and total RNA with minimal modification to the original methods. Further utility of PLG was demonstrated by performing many common restriction/modification reactions in the presence of the non-interfering PLG and then extracting.

ACKNOWLEDGMENTS

We thank Michael Connors, Ph.D., Joseph Lowndes, Ph.D., Katherine Wood and James Cypser for their technical assistance. We thank J. Michael Hurley, Ph.D., Nara Schramm, Ph.D., and Robert W. Morris of 5 Prime → 3 Prime, Inc. for their critical review of this manuscript and constructive comments.

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