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Full Length Research Paper

DNA purification and isolation of genomic DNA from bacterial species by plasmid purification system

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We have successfully adapted the Wizard® MagneSil™ plasmid DNA purification system to isolate genomic DNA from soil bacteria strains important in research and the agricultural industry. In this work, we describe the modified protocol for isolating genomic DNA from soil bacteria using manual and automated approaches on the Biomek® 2000. The isolated genomic DNA was then used to PCR amplify an 875 bp DNA fragment. We have developed a simplified protocol for the isolation and purification of PCR quality genomic DNA from soil bacteria using Wizard® MagneSil™ RED. Yields of genomic DNA ranged from 10 to 100^{+}ng. The experiments here outline both the manual and automated procedures and demonstrate robust PCR product amplification from minimal volume amounts of final eluate. We concluded that the plasmid purification system is a high quality, and suitable for molecular analyses such as PCR, restriction enzyme.

Key words: DNA, plasmid purification, PCR amplification.

INTRODUCTION

silica-coated paramagnetic particles to reversibly bind and purify DNA away from cell debris and proteins released upon directed cell lysis, alkaline denaturation s currently used to isolate and purify plasmid DNA (in bacteria) and to purify DNA from sequencing reactions as well as following PCR. Scientists working on agricultural studies, as well as ecologists, need to be able to routinely isolate genomic DNA from soil bacteria for gene structure studies as well as for taxonomical identification of bacterial species. Cullen and Hirsch (1998) developed methods for extracting DNA from soil suitable for PCR amplification to monitor GM rhizobia and extracting mRNA directly from soil to use in gene expression studies by Mendum et al. (1998).

DNA analysis by multiplex and real-time PCR, the importance of high-quality, purified DNA cannot be underestimated. Finding a suitable DNA isolation system to satisfy your downstream application needs is vital for the successful completion of experiments. This DNA purification chapter addresses general information on the basis of DNA isolation, plasmid growth and DNA quantitation as well as how purification by silica can help increase your productivity; so you spend less time purifying DNA and more time developing experiments and analyzing data. The basic steps of DNA isolation are disruption of the cellular structure to create a lysate, separation of the soluble DNA from cell debris and other insoluble material and purification of the DNA of interest from soluble proteins and other nucleic acids. Historically, this was done using organic extraction (e.g., phenol: chloroform) followed by ethanol precipitation. In the case of plasmid preparations, the multiple-day protocol typically involved cesium chloride banding followed by dialysis of the plasmid DNA. These methods were time consuming and used a variety of hazardous reagents. For ease-of-use, Promega offers an array of conveniently packaged DNA purification products that can isolate DNA in less than an hour using much safer methods. Disruption of most cells is done by chaotropic salts, detergents or alkaline denaturation, and the resulting lysate is cleared by centrifugation, filtration or magnetic clearing.

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DNA is purified from the soluble portion of the lysate. DNA fragment purification from an amplification reaction or restriction enzyme digestion involves a direct treatment of the solution to remove the enzyme and reaction buffer and for PCR products, reduce the amount of nucleotides and primers present. Historically, this was done with phenol: chloroform extraction followed by precipitation. However, safety issues and the expense associated make organic extraction a less convenient DNA purification method. Promega's option is adding chaotropic salt to the reaction volume and purifying the PCR products by silica chemistry. This method is quick and results in pure DNA ready for sequencing and cloning.

Basis for purification by silica

genomic, plasmid and PCR product purification are based on purification by silica. Regardless of the method used to create a cleared lysate, the DNA of interest can be isolated by virtue of its ability to bind silica in the presence of high concentrations of chaotropic salts (Chen and Thomas, 1980; Marko et al., 1982; Boom et al., 1990). These salts are then removed with an alcohol-based wash and the DNA eluted in a low-ionic-strength solution such as TE buffer or water. The binding of DNA to silica seems to be driven by dehydration and hydrogen bond formation, which competes against weak electrostatic repulsion (Melzak et al., 1996). Hence, a high concentration of salt will help drive DNA adsorption onto silica, and a low concentration will release the DNA.

More recent purification systems consist of two different formats: silica membrane column (e.g., the Pure Plasmid Midiprep System) and silica-coated MagneSil® Paramagnetic Particles (PMPs; e.g., Wizard® Magnetic 96 DNA Plant System). While both methods yield high-quality DNA, the silica membrane column is more convenient. For automated purification, either the 96-well silica membrane plates or the MagneSil® PMPs are easily adapted to a variety of robotic platforms. In order to process the DNA samples, the MagneSil® PMPs require a magnet for particle capture rather than centrifugation or vacuum filtration.

Overview of plasmid DNA purification

The primary consideration for plasmid purification is separation of plasmid DNA from the chromosomal DNA and cellular RNA of the host bacteria. A number of methods have been developed to generate a cleared lysate that not only removes protein and lipids but also efficiently removes contaminating chromosomal DNA while leaving plasmid DNA free in solution. Methods for the preparation of cleared lysates that enrich for plasmid DNA include SDS-alkaline denaturation (Birnboim and Doly, 1979; Birnboim, 1983), salt-SDS precipitation (Hirt, 1967) and rapid boiling (Holmes and Quigley, 1981). The SDS-alkaline denaturation method, which is used in all Promega plasmid isolation systems, is a popular procedure for purifying plasmid DNA because of its overall versatility and consistency. This technique exploits the difference in denaturation and renaturation characteristics of covalently closed circular plasmid DNA and chromosomal DNA fragments. Under alkaline conditions (at pH 11), both plasmid and chromosomal DNA are efficiently denatured. Rapid neutralization with a high-salt buffer such as potassium acetate in the presence of SDS has two effects that contribute to the overall effectiveness of the method. First, rapid neutralization causes the chromosomal DNA to base-pair in an intrastrand manner, forming an insoluble aggregate that precipitates out of solution. There are several methods available to purify plasmid DNA from cleared lysate. These include:

1) Binding plasmid to silica in the presence of high concentrations of chaotropic salts (Chen and Thomas, 1980; Marko et al., 1982; Boom et al., 1990);
2) Differential precipitation of plasmid DNA from aqueous chaotropic salt/ethanol solutions (Hamaguchi and Geiduschek,1962; Wilcockson, 1973; Wilcockson, 1975);
3) Ion exchange chromatography over DEAE-modified cellulose membranes (van Huynh et al., 1993);
4) Precipitation with polyethylene glycol (Lis, 1980; Paithankar and Prasad, 1991); and
5) Organic extraction using phenol (Wang and Rossman, 1994).

Promega products like the Wizard® *Plus* SV Minipreps Systems combine the benefits of alkaline lysis with the rapid and easy purification by silica. This is done by using a silica-based membrane in a column format to bind the plasmid DNA contained in the cleared alkaline lysates. Purification is based on selective adsorption of DNA to the silica membrane in the presence of high concentrations of chaotropic salts, washes to efficiently remove contaminants, and elution of the DNA with low-salt solutions such as TE buffer or water.

Purified plasmid DNA is used in many applications from preparing vectors for cloning to generating templates for transcription or coupled transcription/translation reactions. Moreover, the silica-based purification systems from Promega minimize the amount of salts and other impurities carried over during isolation, which can negatively affect downstream applications, lower yield or prevent enzyme systems from synthesizing the product of interest.

Overview of genomic DNA isolation

Promega provides several systems designed to isolate genomic DNA from a variety of sources. One method, the

solution-based Wizard® Genomic DNA Purification Kit, is the most versatile system available from Promega. This purification system relies on a series of precipitation steps to purify high-molecular-weight DNA from a prepared lysate. It is an excellent choice when a pure population of dsDNA molecules is required for downstream applications such as Southern blotting, real-time PCR and restriction digestion. Alternatively, Promega offers genomic DNA isolation systems based on sample lysis by detergents and purification. These include both membrane-based systems (e.g., the single-column Wizard® SV Genomic DNA Purification Kit or the high-throughput, 96-well Wizard® SV 96 Genomic DNA Purification System) and the easily automated paramagnetic silica systems (e.g., MagneSil® Genomic, Large Volume System or the MagneSil® Blood Genomic, Max Yield System).

All of these systems purify genomic DNA that is amenable for use in many downstream applications. Although techniques like Southern blotting, which require microgram amounts of DNA, are still performed in molecular biology laboratories, most assessment of chromosomal DNA is done by PCR technology including monoplex or multiplex PCR, SNP analysis and real-time PCR. These latter techniques use nanogram amounts of DNA per reaction. Regardless of the system chosen, Promega genomic DNA purification kits not only yield DNA suitable for a wide range of DNA quantity specifications but provide the required amount of high-quality DNA with minimal contaminants.

Overview of DNA fragment purification from agarose gels and PCR amplifications

Applications such as cloning, labeling and sequencing DNA frequently require the purification of DNA fragments from agarose gels or amplification reactions. Promega provides multiple systems for DNA fragment purification, including two based on silica membrane technology (Wizard® SV Gel and PCR Clean-Up System and Wizard® SV 96 PCR Clean-Up System) and one based on MagneSil® PMPs (Wizard® MagneSil® Sequencing Reaction Clean-Up System). The Wizard® SV Gel and PCR Clean-Up System provides a reliable method to purify double-stranded, PCR-amplified DNA either directly from the reaction or from agarose. The quick protocol is simple to perform, and the PCR products are purified from contaminants, including primer dimers, PCR additives and amplification primers.

To purify PCR product from nonspecific amplification products, the reaction products can be separated in an agarose gel prior to purification. The agarose is dissolved by chaotropic buffer, freeing the DNA for binding to the silica SV membrane. After removal of contaminants by alcohol-based washes, the DNA bound to the SV column is eluted in water or TE buffer, free of salt or macromolecular contaminants. The Wizard® SV Gel and PCR Clean-Up System can also be used to purify DNA from enzymatic reactions such as restriction digestion and alkaline phosphatase treatment.

Overview of Personal Automation™ systems for purification

Automation is increasingly used to improve productivity for research, diagnostics and applied testing. Traditionally, automation refers to the use of large, specialized and costly equipment that requires extensive training to operate and maintain. Promega has developed ® 16 System which provides a flexible, reliable, compact and easy-to-use alternative to traditional automated systems.

The Maxwell® 16 System combines instrumentation, automated methods, prefilled reagent cartridges, service and support, providing everything needed for purification from a single source. The Maxwell® 16 System is designed for low- to moderate-throughput automated purification of 1 to 16 small samples. Currently, there are predispensed reagent cartridges in kits for genomic DNA purification, total RNA purification and recombinant protein purification. These multiple cartridges make the Maxwell® 16 Instrument flexible for laboratories that may use one or all of these different systems.

For genomic DNA purification, add blood, mouse tail, tissue, or bacteria samples directly to the prefilled

and hands-on labor of Proteinase K or other pre-processing aside from FFPE, and the purified genomic DNA sample is ready in about 30 min. The eluted DNA can be used in PCR and other applications. RNA purification follows a similar process, involving preparation of a DNA-free lysate followed by RNA purification. The eluted RNA can then be used in qRT-PCR and other applications. Recombinant polyhistidine- or HQ-tagged proteins can be purified from multiple sample types, including bacteria, mammalian cells, insect cells and culture medium. Purified protein is compatible with many common downstream applications including polyacrylamide gel electrophoresis and detection, functionality studies, Western blot analysis and mass spectrometry.

MATERIALS AND METHODS

Manual protocol

Genomic DNA was isolated from *Acetobacter* sp., *Citrobacter freundi*, *Rhyzobium leguminosarum*, *Azotobacter venelandi*, *Rhodospirillum rubrum*, *Enterobacter* sp., and *Escherichia coli*

Plasmid Purification System and other solutions. An 875 bp DNA fragment corresponding to a region of the 16S ribosomal DNA sequence was amplified from the genomic DNA by PCR using region-conserved primers.

Experiment 1: Lyse cells directly using solution.

Experiment 2: Treat cells first with Nuclei Lysis Solution (Cat.# A7943) containing 5 µl of RNAse A (Cat.# A7973). Then add

increase yield of DNA, as RNA competes with DNA for binding to Ma les:

1) 10 ml cultures of *Acetobacter* sp., *C. freundi*, *R. leguminosarum*, *A. venelandi*, *R. rubrum* and *Enterobacter* sp. *E. coli* JM109 (as control) were grown initially from slants using a 2 day incubation at 25°C followed by a 1:100 dilution (noted 1:100) and re-grown overnight;
2) Cultures were incubated with shaking in ATCC 111 Rhizobium X Medium or Nutrient broth with soil water medium (Carolina BA-15-3785);
3) Centrifuge 1 ml of each culture in a 1.5 ml microcentrifuge tube. Use a deep well 96 well plate for many manual samples, e.g. Costar® 431191 Deep Well);
4) Resuspend cell pellet in 120 µl Cell Resuspension Solution containing 3 mg/ml of lysozyme. Incubate 15 min at room temperature. For many manual samples, use 96 well U-bottom wash plate.

Protocol 1: Add 50
10 min at room temperature. Total volume is now 170 µl.

Protocol 2: Add 100 µl of Nuclei Lysis Solution and 5 µl RNaseA, vortex briefly to mix and incubate 5 min at room temperature. Add 50 µl MagneS for 10 min at room temperature. Total volume is 270 µl.

Protocols 1 and 2:
Technology Magnetic Separation Stand (Cat.# Z5332 or Z5342). Alternatively, use the MagnaBot® 96 Magnetic Separation Device (Cat.# V8151) for 96 well plate format. Remove the supernatant. Wash three times with 150 µl of 70% ethanol, removing supernatant each time. Dry at room temperature for 10 min, or 65°C for 5 min. Resuspend the captured DNA in 100 µl Nuclease-Free Water (Cat.# P1193), capture and collect the resuspended DNA into a 1.5 ml microcentrifuge tube (or 96 well plate). Measure $O.D._{260}$ and $O.D._{280}$ (optional).

PCR Amplification

Perform PCR using the 16S rDNA-specific primers (515F and 1390R). Primers were chosen to amplify highly conserved sequences among most bacterial species[1] . 16S Oligonucleotide Primers:

515F Forward: 5´-GTGCCAGCAGCCGCGGTAA-3´
1390 Reverse: 5´-AGGCCCGGAACGTATTCAC-3´

UV Treatment for PCR, to inhibit amplification of background DNA contamination in PCR solutions; treat the following materials and reagents with UV light for 20 min:

1) Master Mix without dNTPs,
2) Nuclease-Free Water (Cat.# P1193) with cap off,
3) GeneAmp® 0.5 ml amplification tubes,
4) Pipettes,
5) 1000P, 200P and 20P pipette tips with aerosol barrier,
6) Gloves,
7) Tube racks,
8) Ice in containers, and
9) Mineral oil (with cap off).

PCR Procedure

PCR is used to amplify a specific region of a DNA strand (the DNA target). Most PCR methods typically amplify DNA fragments of up to ~10 kilobase pairs (kb), although some techniques allow for amplification of fragments up to 40 kb in size:

1) 5 µl genomic DNA sample, or nuclease-free water for no-template control reaction.
2) 3.5 µl 515F primer (13.8 pmol/µl stock, final concentration 50 pmol or water for no-primer control).
3) 3.5 µl 1390R primer (13.8 pmol/µl stock, final concentration 50 pmol or water for no-primer control).
4) 7 µl of nuclease-free water for no-primer control reaction.
5) 38 µl of PCR Master Mix.
6) 50 µl final volume.
7) Samples were overlaid with a drop of mineral oil.

PCR Cycle: Program for isolation of genomic DNA from bacteria is as follows:

1) 2 min 94°C;
2) 30 cycles: 30 s 94°C, 1 min 60°C, 1 min 72°C;
3) 7 min 72°C; and
4) Overnight 4°C.

Automated protocol and biomek ® 2000 program for isolation of genomic DNA from bacteria

1) Resuspend bacteria cell pellet in 120 µl of Cell Resuspension Solution containing 3 mg/ml lysozyme and incubate at room temperature for 10 min;
2) 100 µl of Nuclei Lysis Solution is added to the resuspended bacterial cell pellet and incubated at room temperature for 2 min to allow for cell lysis;
3) 50
and mixed to capture bacterial genomic DNA. The cell lysate -well U-bottom wash plate. Once the lysate has been transferred to the wash plate, the plate is moved to the MagnaBot® and the ed from cell lysate.
The cell lysate is removed to the waste reservoir;
4)
are then washed three times with 70% ethanol, using the Wash 8 tool that is connected to a bottle containing 25 ml of 70% ethanol. -dried to remove residual ethanol;
5) Finally, the genomic DNA is eluted in 100 µl of nuclease-free water and placed into the 96-well elution plate (Figure 1).

The tools required include the Wash 8, MP200 and Gripper all placed at position A1. In addition, a 6-port valve is required. Positions A5, A6 and B6 remain empty. A MagnaBot® 96 Magnetic Separation Device (Cat.# V8151) is placed at position A2. Boxes of P250 tips are placed at positions A3 and A4. Position B1 contains a reservoir for waste disposal. A 96-deep well plate containing pelleted bacterial culture with media removed is placed at position B2. At positions B3 and B5 are a 96 well U-bottom plate, one for the wash steps of the protocol and the other for purified sample elution. At position B4 are reservoirs containing the indicated volumes of reagents. Add 3 mg/ml lysozyme to the Cell Resuspension Solution (Cat.# A7114). To the 4 ml of Nuclei Lysis Solution (Cat.# A7941) add 133 µl of RNase A (Cat.# A7973). Finally, a 25 ml 70% ethanol bottle is attached to the 6-port valve.

RESULTS AND DISCUSSION

We successfully isolated genomic DNA from soil bacteria

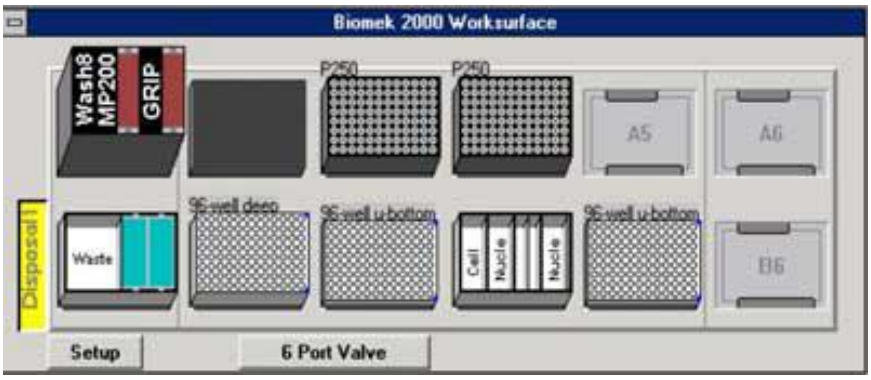

Figure 1. Initial deck configuration of the Biomek® 2000.

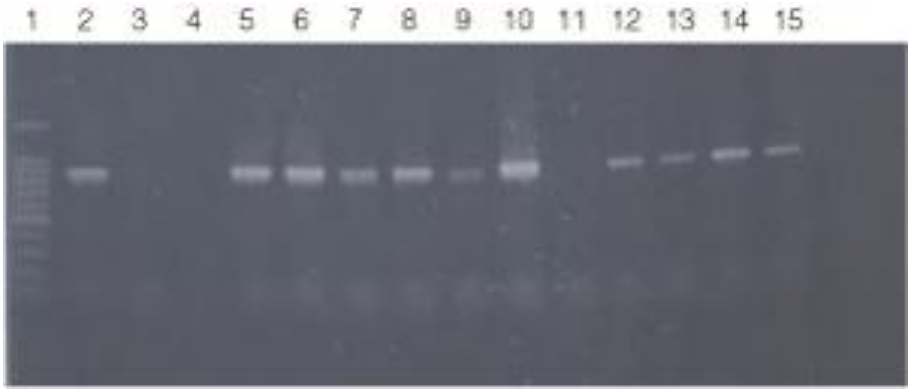

Figure 2. PCR amplification of bacterial genomic DNA from various soil bacteria, using manual protocol 1. The PCR DNA fragment is 875 bp. Lane 1, 100 bp DNA Ladder (Cat.# G2101); lane 2, *E. coli* JM109 positive control lane 3, blank; lane 4, no-template negative control; lane 5, *Acetobacter* sp. (tube #1); lane 6, *Citrobacter freundi* (tube #2); lane 7, *Citrobacter freundi* 1:100 (tube #3); lane 8, *Rhyzobium leguminosarum* (tube #4); lane 9, *Rhyzobium leguminosarum* 1:100 (tube #5) lane 10, *Azotobacter venelandi* (tube #6); lane 11, *Azotobacter venelandi* 1:100 (tube #7); lane 12, *Rhodospirillum rubrum* (tube #8); lane 13, *Rhizobium leguminosarum* (tube #9); lane 14, *Enterobacter* sp. (tube #10); lane 15, *Citrobacter freundi* (tube #11). Bacteria identified in lanes 12-15 were grown in nutrient broth with soil water medium (Carolina BA-15-3785). Bacteria identified in all other lanes were grown in ATCC 111 Rhizobium X Medium.

using modified manual and automated protocols with the Wizard®
amplify by PCR an 875 bp fragment from the 16S bacterial ribosome DNA sequence. In both protocols, PCR amplification was robust across all samples after only 30 amplification cycles and using minimal amounts of genomic DNA product. UV light treatment of tools and reagents used for setting up and performing PCR effectively, prevented the background amplification of the 16S ribosomal DNA sequence from either the Taq DNA polymerase or from other non-specific contaminating bacteria. This is shown in Figure 2 (lane 4), in Figure 3 (lane 3), in Figure 4 (lane 3) and in Figure 5 (lane 3). PCR amplification was also performed with varying amounts of genomic DNA as demonstrated in Figure 5. Robust amplification was seen with either 1.0 $O.D._{600}$ units or 0.5 $O.D._{600}$ units of starting bacterial culture [For those samples with data for 0.5 $O.D._{600}$ units only, cultures did not grow to an $O.D._{600}$ of 1.0, thus not permitting those experiments]. Use of Nuclei Lysis Solution and RNAse A appeared to increase the PCR signal intensity (compare Figures 2 and 3). The automated protocol produced clean sharp bands in PCR.

Evaluation of methods for determining DNA yield and purity (DNA purification – protocol)

DNA yield can be assessed using three different methods: absorbance (optical density), agarose gel electrophoresis and fluorescent DNA-binding dyes. Each technique is described and includes information on

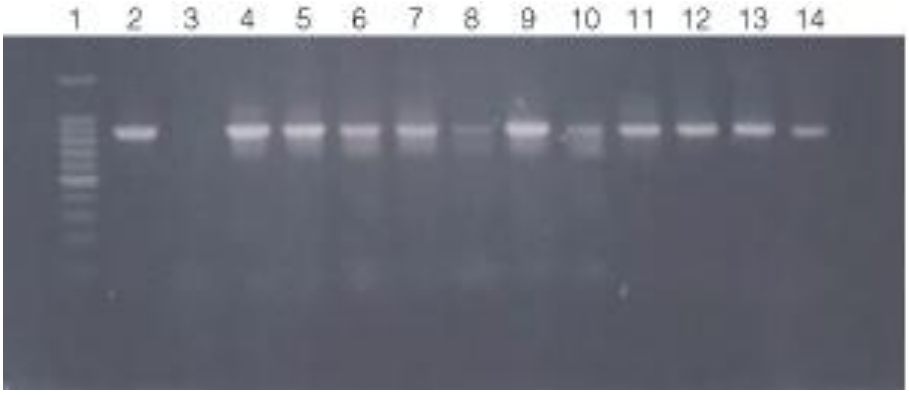

Figure 3. PCR amplification of bacterial genomic DNA from various soil bacteria, using manual protocol 2. The PCR DNA fragment is 875 bp. Lane 1, 100 bp DNA ladder (Cat.# G2101); lane 2, *E. coli* JM109 positive control; lane 3, No template negative control; lane 4, *Acetobacter* sp. (tube #1); lane 5, *Citrobacter freundi* (tube #2); lane 6, *Citrobacter freundi* 1:100 (tube #3); lane 7, *Rhyzobium leguminosarum* (tube #4); lane 8, *Rhyzobium leguminosarum* 1:100 (tube #5); lane 9, *Azotobacter venelandi* (tube #6); lane 10, *Azotobacter venelandi* 1:100 (tube #7); lane 11, *Rhodospirillum rubrum* (tube # 8); lane 12, *Rhizobium leguminosarum* (tube #9); lane 13, *Enterobacter* sp. (tube # 10); lane 14, *Citrobacter freundi* (tube #11). Bacteria identified in lanes 1 to 15 were grown in nutrient broth with soil water medium (Carolina BA-15-).

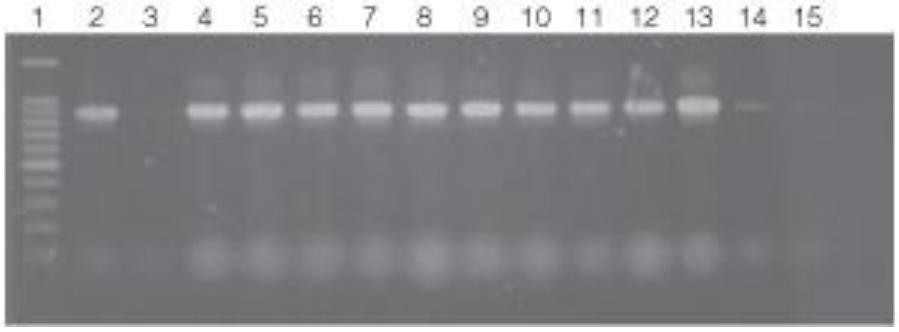

Figure 4. PCR amplifications of bacterial genomic DNA from various soil bacteria using the automated Biomek® 2000 protocol. The PCR DNA fragment is 875 bp. Lane 1, 100 bp DNA Ladder (Cat.# G2101); lane 2, *E. coli* JM109 positive control DNA isolated using manual protocol; lane 3, negative control no template; lanes 4 and 5, *E. coli* (Biomek® protocol); lanes 6 and 7, *Enterobacter* sp.; lanes 8 and 9, *Azotobacter venelandi*; lanes 10 and 11, *Rhyzobium leguminosarum*; lanes 12 and 13 *Acetobacter*; lanes 14 and 15 *Citrobacter freundi*. All bacteria were grown in ATCC 111 Rhizobium X Medium.

necessary accessories (e.g., equipment). While all methods are useful, each has caveats to consider when choosing a quantitation system. The most common technique to determine DNA yield and purity is also the easiest method absorbance. All that is needed for measurement is a spectrophotometer equipped with a UV lamp, UV-transparent cuvettes (depending on the instrument) and a solution of purified DNA. Absorbance readings are performed at 260 nm (A_{260}) where DNA absorbs light most strongly, and the number generated allows one to estimate the concentration of the solution (see Estimation of DNA Concentration, Yield and Purity by Absorbance for more details). To ensure the numbers are useful, the A_{260} reading should be between 0.1 to 1.0.

However, DNA is not the only molecule that can absorb UV light at 260 nm. Since RNA also has a great absorbance at 260 nm, and the aromatic amino acids present in protein absorb at 280 nm, both contaminants, if present in the DNA solution, will contribute to the total measurement at 260 nm. Additionally, the presence of guanidine will lead to higher 260 nm absorbance. This means that if the A_{260} number is used for calculation of yield, the DNA quantity may be overestimated (Adams, 2003). To evaluate DNA purity by spectrophotometry, measure absorbance from 230 to 320 nm in order to detect other possible contaminants present in the DNA solution [detailed in the *MagneSil® Genomic, Large Volume System Technical Bulletin*]. The most common

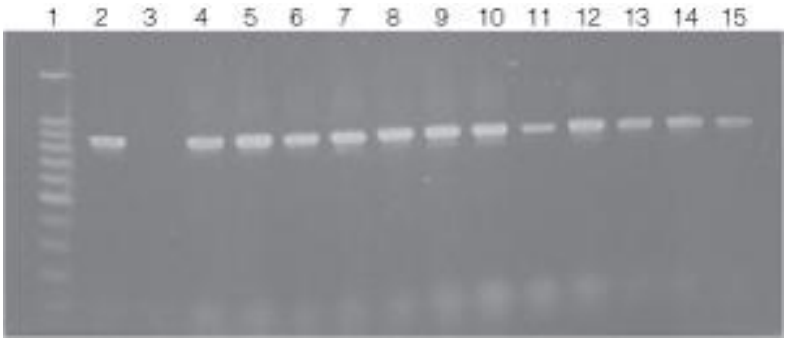

Figure 5. PCR amplification of bacterial genomic DNA from various soil bacteria using the automated Biomek® 2000 protocol. The PCR DNA fragment is 875 bp. Genomic DNA was isolated from cultures grown to both 0.5 and 1.0 $O.D._{600}$ units. Lane 1, 100 bp DNA Ladder (Cat.# G2101); lane 2, *E. coli* JM109 positive control DNA isolated using the manual protocol; lane 3, negative control no template; lane 4, *E. coli* (Biomek® protocol) $O.D._{600}$ (0.5); lane 5, *E. coli* (Biomek® protocol) $O.D._{600}$ (1.0); lane 6, *Enterobacter* $O.D._{600}$ (0.5); lane 7, *Enterobacter* $O.D._{600}$ (1.0); lanes 8 and 9, *Azotobacter venelandi* $O.D._{600}$ (0.5); lane 10, *Rhyzobium leguminosarum* $O.D._{600}$ (0.5); lane 11, *Rhyzobium leguminosarum* $O.D._{600}$ (1.0); lanes 12 and 13, *Acetobacter* $O.D._{600}$ (0.5); lanes 14 and 15, *Citrobacter freundi* $O.D._{600}$ (0.5). All bacteria were grown in ATCC 111 Rhizobium X Medium.

purity calculation is determining the ratio of the absorbance at 260 nm divided by the reading at 280 nm. Good-quality DNA will have an A_{260}/A_{280} ratio of 1.7 to 2.0. A reading of 1.6 does not render the DNA unsuitable for any application, but lower ratios indicate more contaminants are present. However, the best test of DNA quality is functionality in the application of interest (e.g., real-time PCR).

Strong absorbance around 230 nm can indicate that organic compounds or chaotropic salts are present in the purified DNA. A ratio of 260 to 230 nm can help evaluate the level of salt carryover in the purified DNA. The lower the ratio, the greater the amount of thiocyanate salt present, for example. As a guideline, the A_{260}/A_{230} is best if greater than 1.5. A reading at 320 nm will indicate if there is turbidity in the solution, another indication of possible contamination. Therefore, taking a spectrum of readings from 230 to 320 nm is most informative. Agarose gel electrophoresis of the purified DNA eliminates the issues associated with absorbance readings. To use this method, a horizontal gel electrophoresis tank with an external power supply, analytical-grade agarose, an appropriate running buffer and an intercalating DNA dye along with appropriately sized DNA standards are needed for quantitation. A sample of the isolated DNA is loaded into a well of the agarose gel and then exposed to an electric field. The negatively charged DNA backbone migrates toward the anode. Since small DNA fragments migrate faster, the DNA is separated by size. The percentage of agarose in the gel will determine what size range of DNA will be resolved with the greatest clarity (Sambrook et al., 1989). Any RNA, nucleotides and protein in the sample migrate at different rates compared to the DNA, so the band(s) containing the DNA will be distinct.

Concentration and yield can be determined after gel eletrophoresis is completed by comparing the sample DNA intensity to that of a DNA quantitation standard.

For example, if a 2 µl sample of undiluted DNA loaded on the gel has the same approximate intensity as the 100 ng standard, then the solution concentration is 50 ng/µl (100 ng divided by 2 µl). Standards used for quantitation should be labeled as such and be the same size as the sample DNA being analyzed. In order to visualize the DNA in the agarose gel, staining with an intercalating dye such as ethidium bromide or SYBR® Green is required. Because ethidium bromide is a known mutagen, precautions need to be taken for its proper use and disposal (Adams, 2003). DNA-binding dyes compare the unknown sample to a standard curve of DNA, but genomic, fragment and plasmid DNA will each require their own standard curves and cannot be used interchangeably. If the DNA sample has been diluted, you will need to account for the dilution factor when calculating final concentration. Hoechst bisbenzimidazole dyes or PicoGreen® selectively bind double-stranded DNA (dsDNA). To use this method, a fluorometer to detect the dyes, dilution of the DNA solution and appropriate DNA standards are required. However, there are size qualifications: the DNA needs to be at least 1 kilobase in length for Hoechst and at least 200 bp for PicoGreen® for successful quantitation. The range of measurement is 10 to 250 ng/ml for Hoechst, 25 pg/ml to 1µg/ml for PicoGreen®, and the dyes are sensitive to GC content. In addition, the usual caveats for handling fluorescent compounds apply photobleaching and

quenching will affect the signal. While the dyes bind preferentially to dsDNA, RNA and nucleotides may contribute to the signal.

Estimation of DNA concentration, yield and purity by absorbance

DNA concentration can be estimated by adjusting the A_{260} measurement for turbidity (measured by absorbance at A_{320}), multiplying by the dilution factor, and using the relationship that an A_{260} of 1.0 = 50 µg/ml pure DNA:

Concentration (µg/ml) = (A_{260} reading A_{320} reading) × dilution factor × 50 µg/ml

Total yield is obtained by multiplying the DNA concentration by the final total purified sample volume:

DNA Yield (µg) = DNA Concentration × Total Sample Volume (ml)

A_{260}/A_{280} ratio can be used as an estimate of DNA purity [with a number of important limitations (Wilfinger et al., 1997; Glasel, 1997; Manchester, 1995)]. An A_{260}/A_{280} ratio between 1.7 and 2.0 is generally accepted as representative of a high-quality DNA sample. The ratio can be calculated after subtracting the non-nucleic acid absorbance at A_{320}:

DNA Purity (A_{260}/A_{280}) = (A_{260} reading A_{320} reading) ÷ (A_{280} reading A_{320} reading)

Note that the spectrophotometer is most accurate when measurements are between 0.1 to 1.0.

Plasmid DNA purification (bacterial growth and culture conditions)

Successful isolation of quality plasmid DNA begins with culture preparation. A number of factors can influence the growth of bacterial cells. Bacterial growth in liquid culture occurs in three phases: 1) a short lag phase in which the bacteria become acclimated to the media and begin to divide; 2) a log phase, characterized by exponential growth in which most strains of *E. coli* will divide every 20 to 30 min; and 3) a stationary phase in which growth slows and eventually stops in response to the lack of nutrients in the medium. No net increase in biomass will occur in the stationary phase, but plasmid replication will continue for several hours after reaching stationary phase. Most strains of *E. coli* will reach a concentration of 1.0 to 4.0 × 10^9 cells/ml of culture at this stage, depending on culture media and aeration conditions. Depending on inoculation size and the size of the culture, stationary phase will be reached in 6 to 8 h. Aeration and temperature are of critical importance. The culture volume should be less than or equal to 1/4 the volume of the container (e.g., 250 ml medium in a 1 L flask); using 1/10 the container volume (e.g., 100 ml medium in a 1,000 ml flask) produces optimal results. The culture tube or flask should be placed in an orbital shaker (approximately 250 rpm) to ensure adequate aeration (Ausubel et al., 1989). Since most strains of *E. coli* grow best at 37°C, this incubation temperature is recommended unless the strain of interest requires different conditions for optimal growth.

Different culture media will also have a profound effect on the growth of different bacterial strains. Promega plasmid DNA purification systems are appropriate for bacterial cultures grown in 1X Luria-Bertani (LB) medium. However, use of LB-Miller medium containing more NaCl will produce significantly greater yields and is highly recommended. Richer media such as 2X YT, CIRCLEGROW® or Terrific Broth may be used to increase plasmid yields by increasing the biomass for a given volume of culture. Keep the biomass in a range acceptable for the plasmid isolation system used, as overloading may result in poor purity and yield of the plasmid DNA. Culture incubation time affects both the yield and quality of plasmid DNA isolated. Bacterial cultures grown to insufficient density will yield relatively low amounts of DNA. Overgrown cultures may result in suboptimal yields and excessive chromosomal DNA contamination due to autolysis of bacterial cells after they have reached stationary phase. We do not recommend the use of cultures grown longer than 18 to 20 h.

Plasmid copy number

One of the most critical factors affecting the yield of plasmid from a given system is the copy number of the plasmid. Copy number is determined primarily by the region of DNA surrounding and including the origin of replication in the plasmid. This area, known as the replicon, controls replication of plasmid DNA by bacterial enzyme complexes. Plasmids derived from pBR322 (Cat.# D1511) contain the ColE1 origin of replication from pMB1. This origin of replication is tightly controlled, resulting in approximately 25 copies of the plasmid per bacterial cell (low copy number). Plasmids derived from pUC contain a mutated version of the ColE1 origin of replication, which results in reduced replication control and approximately 200 to 700 plasmid copies per cell (high copy number).

Some plasmids contain the p15A origin of replication, which is considered a low-copy-number origin. The presence of the p15A origin of replication allows for replication of that particular plasmid in conjunction with a plasmid containing the ColE1 origin of replication. A compatibility group is defined as a set of plasmids whose members are unable to coexist in the same bacterial cell.

They are incompatible because they cannot be distinguished from one another by the bacterial cell at a

stage that is essential for plasmid maintenance. The introduction of a new origin, in the form of a second plasmid of the same compatibility group, mimics the result of replication of the resident plasmid. Thus, any further replication is prevented until after the two plasmids have been segregated to different cells to create the correct prereplication copy number (Lewin, 2004). Most plasmids provided by Promega, including the pGEM® Vectors, are considered to be high-copy-number. The only exception is the pALTER®-MAX Vectors.

Some DNA sequences, when inserted into a particular vector, can lower the copy number of the plasmid. Furthermore, large DNA inserts can also reduce plasmid copy number. In many cases, the exact copy number of a particular construct will not be known.

However, many of these plasmids are derived from a small number of commonly used parent constructs. Many plasmid isolation systems indicate they are transfection-quality (

as some cultured cells are sensitive to the amount of endotoxin and other contaminants present in the plasmid preparation. Endotoxin is a lipopolysaccharide cell wall component of the outer membrane of Gram-negative bacteria (that is all *E. coli* strains) that can copurify with the plasmid DNA regardless of the purification system used. The amount of this molecule varies by bacterial strain, growth conditions and isolation method. In the

Removal Wash solution that reduces the amount of endotoxin, proteins and other contaminants eluted with the plasmid DNA. For many common cell lines like 293 and HeLa, the amount of endotoxin present for routine transfections has a minimal effect on the efficiency of transfection (Butash et al., 2000).

Many factors influence transfection efficiency and/or cellular death including the type and amount of transfection reagent, cell confluency, DNA amount and incubation time with the reagent: DNA complex. Each of these factors will need to be optimized for each cell line-plasmid combination transfected in order to minimize cell death and maximize transfection efficiency. In our experience, transfection experiments with HeLa and NIH/3T3 cells demonstrated that there was little DNA preparation difference with four different plasmid isolation systems used (based on silica membrane, anion exchange and silica resin) when comparing efficiencies using the same transfection reagent. However, the transfection reagent used for DNA uptake had a significant effect on transfection efficiency and cell death. For general considerations for optimization, consult the Protocols and Applications Guide chapter on Transfection.

Conclusions

Isolation of genomic DNA from soil bacteria is an important first step for agricultural scientists and ecologists involved in molecular biology research. We

System to permit isolation and purification of PCR-quality genomic DNA from soil bacteria. The adapted protocols provided here are quick, simple and flexible. Furthermore, these protocols avoid organic solvents, which are messy as well as hazardous.

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