Clinical Microbiology Newsletter

Stay Current... Stay Informed.

Vol. 34, No. 1 www.cmnewsletter.com January 1, 2012

Assessment of DNA Yield and Purity: an Overlooked Detail of PCR Troubleshooting

Kelly A. Boesenberg-Smith, PSM,[1] Mohammad M. Pessarakli, M.D.,[2] and Donna M. Wolk, Ph.D., D(ABMM),[3] [1]Quality Assurance Officer, Iowa State University College of Veterinary Medicine, [2]Department of Pathology, Keck School of Medicine, University of Southern California, Los Angeles, California, [3]University of Arizona College of Medicine, Department of Pathology, 1501 N. Campbell Ave., Tucson, Arizona

Abstract

Determination of nucleic acid concentration and purity is important for downstream applications, such as polymerase chain reaction (PCR) and multiplex PCR. There are several methods for quantifying and characterizing DNA, and results describing concentration and purity vary by the extraction method, as well as by the DNA quantification method used. Understanding the methods used for quantification and purity measurements of DNA may aid laboratory scientists when troubleshooting PCR and help them to determine the best extraction method for each application.

Introduction

Nucleic acid extraction methods

Efficient, effective nucleic acid extraction methods are of paramount importance in the molecular microbiology laboratory. There are very few procedures in biotechnology that do not require the use of nucleic acids in some fashion. This review will focus on DNA as the input for downstream nucleic acid amplification methods, such as polymerase chain reaction (PCR), real-time PCR, multiplex PCR, and DNA sequencing. These downstream applications play an important role, not only in research laboratories, but also in clinical laboratories, where they are used for microorganism detection, viral load testing, genetic testing, and epidemiological studies. It is not sufficient to simply extract these nucleic acids from their bacterial, viral, fungal, or human cell source; methods must be able to produce high nucleic acid yields from specimens while maximizing purity and freeing DNA from contaminants and inhibitors such as heme, heparin, and mucus (1,2).

Although laboratory needs for nucleic acids are numerous, the ability to extract pure DNA efficiently is extremely critical, yet is often overlooked. Assessment and documentation of DNA yield and purity can be a tedious process but is nevertheless an analytical component of PCR and is particularly important for multiplex PCR. Although countless hours are often spent attempting to enhance downstream PCR applications, microbiologists often disregard the fact that the source of their limitations and errors

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Corresponding Author: Kelly A. Boesenberg-Smith, PSM, Quality Assurance Officer, Iowa State University College of Veterinary Medicine, Veterinary Diagnostic and Production Animal Medicine, Veterinary Diagnostic Laboratory, 1600 S. 16th St., Ames, IA 50011. Tel.: 515-294-3884. Fax: 515-294-3564. E-mail: kellyisu@iastate.edu

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might actually be the difficulty of obtaining an inhibitor-free sample of sizeable nucleic acid strands from which to start.

When selecting an extraction method, there are many aspects to be considered. The purity of the nucleic acid sample obtained, the cost-effectiveness of the procedure, the duration of exposure to dangerous chemicals, and the amount of hands-on time and the number of transfer steps required must be taken into account. The scalability of the method, or its ability to adapt to a high-throughput environment, is also of great importance as laboratories adapt to increased workloads.

No matter the source or eventual use of the DNA, the goals of an extraction method are the same: release of the genetic material from its source (fluid, tissue, or microbe), stabilization of nucleic acids against degradation, removal of amplification inhibitors, concentration of the nucleic acid material into a useful volume of an aqueous solution compatible with downstream applications, and standardization of methods to support accurate, sensitive, and reproducible laboratory assays (3-6).

DNA extraction methods

There are three main categories of extraction methods. The first is the manual, more traditional, user-defined method involving the use of chemicals, such as phenol, chloroform, and ethanol, to isolate and precipitate nucleic acids (6). While these protocols represent the historical standard methods, they are generally ill-suited for clinical laboratory situations because of their often lengthy and manual nature. Numerous transfer steps can lead to contamination and potential exposure to phenol.

The second extraction category, commercially available manual kits, has a long history of success in clinical laboratories and can employ a variety of nucleic acid isolation methods, including salt precipitation, cell lysis with protein precipitation, silica columns, and magnetic beads. Kit methods tend to vary greatly with respect to cost, processing time, type of input sample, amount of sample required, and the ability to recover nucleic acids. Although generally less labor-intensive and involving fewer transfer steps than user-defined phenol-chloroform methods, they are more costly because they include proprietary reagents and special plastics, and their manual nature does not lend itself to reproducibility or high-throughput requirements.

The final category, DNA extractions performed by automated laboratory instruments, became available near the turn of the millennium — a fairly recent addition to the laboratory. These methods seek to increase the uniformity of sample processing, sample throughput, efficacy of downstream procedures, and reproducibility between samples and batches and to decrease the total and hands-on time required for an extraction. Robotic platforms tend to work by two mechanisms: adhesion of the nucleic acids to a silica membrane and magnetic separation using silica-coated beads (i.e., magnetic glass beads), with extraction chemically based on a process which has come to be known as Boom technology, named for one of its inventors and licensed by bioMérieux to several manufacturers (1,2,7). Regardless of the microbial lysis method used, the nucleic acids of interest can be isolated by virtue of their ability to bind silica in the presence of high concentrations of chaotropic salts (2,8,9). These salts, guanidinium thiocyanate and guanidinium isothyacyanate, are then removed with an alcohol-based wash. Finally, the DNA is eluted in a low-ionic-strength solution, such as Tris-EDTA buffer or water. The binding of DNA to silica seems to be driven by hydrogen bond formation, which competes against weak electrostatic repulsion in the DNA strand, such that a high concentration of salt will help drive DNA adsorption onto silica and a low concentration will release the DNA.

For the sake of brevity, we will not discuss nucleic acid extraction methods that are specifically linked to a particular instrument, as they are many and beyond the scope of this review. Instead, we will use the most common stand-alone robotic extraction systems to illustrate the theory and general advantages and limitations of robotic extraction systems. The stand-alone robotic methods involve the use of specially-engineered instruments connected to a personal computer, special plastics, and kits with proprietary reagents to extract nucleic acids from a variety of samples. In general, comparisons of automated instruments have demonstrated that they are effective in isolating sample nucleic acids and produce results equivalent or superior to over those of manual methods (10). Although automated extraction method supplies are more expensive than their manual and kit counterparts, cost savings in personnel hours make automated methods economical for use in high-throughput environments. Automated methods are immensely beneficial to standardization due to fewer transfer steps and reduce exposure to dangerous chemicals. The three commercial suppliers of automated extraction systems commonly used in clinical laboratories are QIAGEN, bioMérieux, and Roche Applied Sciences.

QIAGEN (Germantown, MD) has two common types of nucleic acid extraction robots available. The QIACube uses silica gel membrane technology to bind DNA and RNA so that impurities and inhibitors can be washed away. The second family of QIAGEN robots, exemplified by BioRobots EZ1, EZ-XL, and M48 can process up to 6, 24, and 48 samples, respectively, and use silica and magnetic particle technology. Following pre-treatment steps, nucleic acid particles are bound to silica-coated magnetic beads in the presence of chaotropic salts (2). Bound nucleic acids are then separated by use of a magnet and washed to remove excess processing reagents. Performance of these robotic methods is well documented for use in clinical laboratories, with performance characteristics sufficient for most applications and a wide range of clinical commercialized kits for various specimen types.

The NucliSens miniMAG extraction instrument (miniMAG) and the automated NucliSens easyMAG are available from bioMérieux (Cary, NC). These systems also use Boom technology as the basis of their DNA extraction protocols and are known for recovery of high-purity nucleic acids. Although all commercial products generally produce equivalent results, there are rare reported differences for individual specimens and pathogen types for which the "MAG" systems are known to be listed among the best performers, particularly when

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the specimen matrix (i.e., the clinical specimen) presents a challenge or when multiplex target are amplified (11-15).

Roche Applied Science (Indianapolis, IN) sells an automated extraction platform that also uses magnet separation technology. The MagNA Pure Compact and MagNA Pure LC instruments can process up to 8 and 32 samples, respectively, and involve pre-treatment steps, binding of nucleic acids to magnetic beads in the presence of chaotropic salts, separation from solution by magnetism, and washing to remove excess reagents and impurities before eluting in aqueous buffer (2). The performance of these robotic methods is well documented for use in clinical laboratories, with performance characteristics sufficient for most applications.

Determining DNA Purity and Yield

In addition to effective DNA extraction procedures, the accurate determination of nucleic acid concentration is extremely important to the modern molecular laboratory. Downstream applications often have a specific target or window of nucleic acid concentration that is required in order to perform optimally. While most automated extraction systems ensure that DNA purification is optimized for most protocols, there is variability systems, specimen types, and microbes. Sometimes, it is essential to know the exact concentration of a nucleic acid sample, as it helps to prevent unnecessary consumption, enhance reproducibility, enhance amplification of difficult targets, and standardize downstream protocols, such as sequencing. Inaccurate quantification can increase variability in downstream assays, which leads to reduced result confidence (16). Similar to requirements for extraction methods, high-throughput scalability and data analysis are important considerations for DNA quantification methods.

UV spectrometry

The standard nucleic acid quantitation method is ultraviolet (UV) spectrophotometry. In this method, the nucleic acid sample is placed into a quartz cuvette, which is then placed inside the UV spectrophotometer. UV light is passed through the sample at a specified path length, and the absorbance of the sample at specific wavelengths is measured, A_{260} (absorbance at 260 nm), to measure nucleic acid, and A_{280} to measure contaminating protein in the sample. Based upon the absorbance readings, the concentration of the sample is determined, and A_{260}/A_{280} ratios are calculated to indicate sample purity (17). The maximum absorbance of nucleic acids occurs at a wavelength of 260 nm. It is generally accepted that DNA or relative purity will yield an A_{260}/A_{280} ratio of ≥1.8 on a scale with a maximum of 2.0. Ratios can vary as a result of the nucleotide composition, as A_{260}/A_{280} ratios vary widely among different nucleotides: guanine, 1.15; adenine, 4.50; cytosine, 1.51; uracil, 4.00; and thymine, 1.47 (17).

Often overlooked, the A_{260}/A_{230} ratio is a key measure of relative purity. The absorption wavelength, A_{230}, represents the wavelength range that can identify the presence of several chemical contaminants. For instance, residual chaotropic salts, which can inhibit PCR, particularly multiplex PCR, are known to absorb in the 230-nm range and below. The A_{260}/A_{230} ratio should generally be greater than 1.4 on a scale of 2.0 in order to maximize multiplex PCR methods. It is a critical measurement to assess when high purity samples are required.

Traditional UV spectrophotometry is a common and simple DNA quantitation method because it does not require use of a large amount of purified sample. If clean disposable plastic cuvettes are used, nucleic acid extract can be used to measure the concentration and then reused in downstream applications. Further benefits are that the UV method does not require additional reagents or incubation time. Moreover, spectrophotometers are widely available. A drawback to spectrophotometry is that minimum sample volumes of at least 50 to 75 µl are a common requirement to obtain an accurate instrument reading. This can be problematic if the sample is of low initial concentration and diluting the sample is impractical or if a certain extraction method requires the elution of nucleic acids into low volumes. Additionally, this method is not as sensitive as others, as the lowest concentration of nucleic acid reliably detected is 1.5 µg/ml.

Common biological contaminants, such as proteins, RNA (if measuring DNA), and chaotropic salts from extraction procedures, can falsely elevate nucleic acid concentration estimations (16). Buffer salts, such as Tris, EDTA, and guanidine isothiocyanate, absorb strongly at 230 nm and bleed into the 260-nm absorbance range, which can falsely elevate A_{260}/A_{280} and A_{260}/A_{230} purity ratios for samples.

Additionally, free nucleotides present in a sample also have the ability to influence the UV quantitation method. Changes in sample pH also alter UV spectrophotometer readings. Small shifts in solution pH, resulting simply from the use of different water sources, can cause significant variability of RNA A_{260}/A_{280} ratios (18). Adjusting the pH of water used in UV analysis from 5.4 to 7.5 or 8.5 resulted in an increase in the A_{260}/A_{280} ratio from 1.5 to 2.0 (17).

NanoDrop spectrometry

An extension and improvement of the UV spectrophotometer is the NanoDrop ND-1000 instrument and its associated models. The NanoDrop technology is similar in principle to a conventional spectrophotometer but has many additional capabilities. The use of sample surface tension is the enabling technology of the instrument, which requires only 1 to 2 µl of sample. The sample is retained on an optical fiber, which assesses the UV absorbance of the sample across an absorbance range of 220 nm to 750 nm. The instrument is accompanied by special software to enable analysis of signal from small quantities of sample.

Although the NanoDrop quantitation method is still susceptible to many of the pitfalls of the traditional UV spectrophotometer method, the NanoDrop instrument is powerful because, in addition to calculating the concentration of the sample and its A_{260}/A_{280} ratio, it displays the entire absorbance spectrum of the sample in graphical form (Fig. 1). This allows contaminants to be more readily detected and potentially identified based on their absorbance wavelengths; these contaminants could have been unnoticed if readings were performed with a traditional UV spectrophotometer. Figure 1 depicts two NanoDrop curves, one generated prior to sample cleanup and salt removal, and the second generated after removal of excess guanidine isothiocyanate

salts. The arrow represents removal of contaminants, which can limit the amplification of DNA in a PCR reaction, particularly in multiplex PCR.

An additional benefit of NanoDrop technology is its capability to determine a wide range of sample concentrations without requiring serial dilutions (19). The minimum volume required than for an accurate instrument reading is also far less than for a conventional spectrophotometer. When using this method, it is of utmost importance to adequately mix the nucleic acid sample before removing an aliquot for measurement to ensure the aliquot is representative of the entire sample.

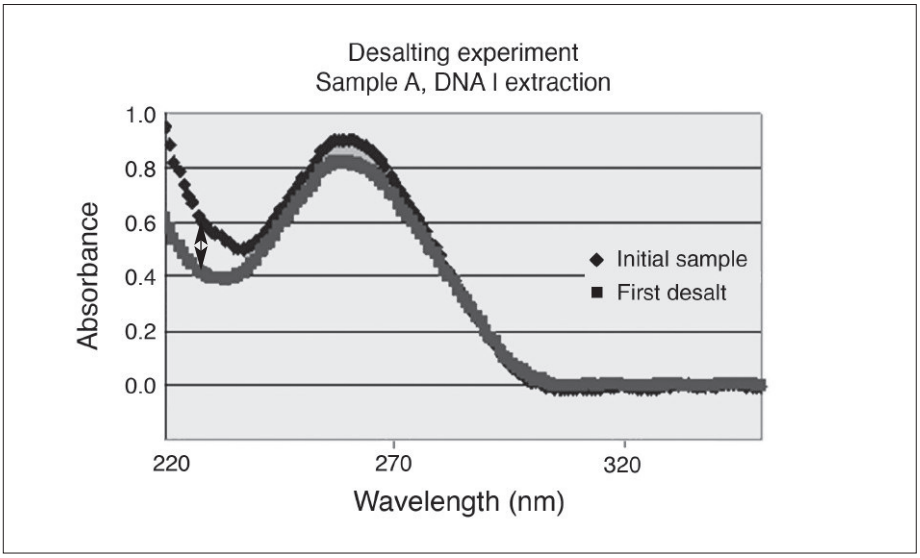

Figure 1. Typical NanoDrop curves generated from DNA extract. The top line represents the original curve prior to removal of guanidinium salts; the second curve represents the same sample after salt removal. The bottom curve is more representative of the true DNA concentration without false elevation due to absorbance by salts. The arrows represent salt and contaminant removal, which can enhance DNA amplification by PCR.

Fluorometric methods

Fluorometric methods are a third means of nucleic acid quantitation and are widely regarded as one of the most sensitive methods available (20). These methods use dyes, which intercalate and bind nucleic acid grooves, bind non-specifically, or selectively bind certain types of nucleic material. Differences in spectral characteristics of nucleic acid-bound fluorophores allow sample concentrations to be determined, because dyes are excited at one wavelength of light and emit another wavelength of light, with intensity then measured.

Ethidium bromide, a standard fluorometric dye, binds double-stranded DNA (dsDNA) by intercalation (20) but has a high degree of intrinsic fluorescence, which limits the sensitivity of the assay (21). Another dye, Hoechst 33258, binds dsDNA by intercalation of the minor DNA groove (20) but has limited use, because it requires high salt concentrations to detect dsDNA when RNA is present and low salt concentration to detect dsDNA when single-stranded DNA is present (21). Many fluorometric dyes used to quantitate DNA also bind RNA but unfortunately lack the fluorescence intensity and assay linearity needed for a sensitive RNA detection method. (22).

PicoGreen, a fluorometric dye from Life Technologies (Carlsbad, CA), selectively binds dsDNA by a yet unknown mechanism. It exhibits low intrinsic fluorescence when unbound, has been shown to be more sensitive than other dye methods, and is able to detect nucleic acid concentrations over a four-fold range of 25 pg/ml to 1 μg/ml. Quantitating dsDNA with PicoGreen is powerful, as it has demonstrated a 1,980-fold fluorescence enhancement compared to only a 95-fold enhancement when Hoechst 33258 is used. A limitation is that the PicoGreen dye is also sensitive to chaotropic salts, which tend to decrease signal intensity, and to organic solvents, which tend to increase signal intensity (21). Other drawbacks of fluorometric dye methods like PicoGreen include the need for access to relatively costly equipment capable of fluorometric readings, expensive proprietary reagent kits required to perform quantitation assays, lengthy assay set-up and dye incubation time, and sample volume consumption. These shortcomings, however, may be outweighed by the benefits of fluorometric methods over UV spectroscopy. The low sample volume requirement is a marked benefit if DNA samples are limited. The high degree of assay sensitivity is also important, because very small sample concentrations can be adequately detected and accurately quantitated so as not to unnecessarily waste sample, saving the maximum amount for downstream applications.

Real-time PCR

Another powerful nucleic acid quantitation method is real-time PCR, and a variety of methods yield relative (not absolute) quantities with ease (23). Real-time PCR is similar to traditional PCR in that primers are used to anneal to denatured target DNA in order to successfully extend and amplify it exponentially during thermal cycling. Where real-time PCR differs is the use of specialized fluorometric probes that, similar to primers, bind target DNA during the annealing phase of PCR. The fluorometric probes are then displaced and cleaved, which allows the emission of fluorescent dye upon target extension. The dye can ultimately be detected in order to determine the success of the amplification reaction, and the initial quantity of nucleic acid present in the sample is depicted in graphical form.

The main advantage of real-time PCR is the ability to assess how much of the available nucleic acid sample is amplifiable versus that which is present. The concentration of the unknown nucleic acid sample is determined by comparing a standard curve of known concentrations to the amplification plot of the sample. Other benefits include the detection of PCR inhibitors within the reaction mixture and the specificity inherent in the assay by use of fluorometric probes. Traditional PCR allows analysis at only two points in time: before the reaction, when the amount of amplifiable nucleic acid is unknown, and after the reaction, following the amplification plateau. Drawbacks to this method include the expensive pro-

prietary reagents, primers, and probes needed to perform assays; the specialized instrumentation necessary to amplify nucleic acid and analyze fluorometric data; the lengthy assay time required; and the fact that sample volume is expended in order to determine its concentration.

A unique example of a real-time PCR assay that quantitates human DNA is the Quantifiler Human DNA Quantification Kit by Applied Biosystems, which quantitates the total amount of amplifiable human DNA present in a nucleic acid sample and is designed to be used with the company's real-time PCR detection systems, including the ABI 7900HT. The proprietary kit contains primers used to target the human telomerase reverse transcriptase gene, which amplifies an intron sequence, and a probe labeled with FAM dye in order to detect the amplified sequence (24,25).

Summary

In a clinical microbiology setting where molecular procedures continue to play a prominent role in patient diagnoses, extraction methods are of great importance. Proper extraction maximizes the use of precious sample quantities and provides the most desirable nucleic acid material for use in the downstream molecular applications. Clinical microbiologists should be familiar with methods used to quantify DNA yield and purity and should consider these sample characteristics when optimizing PCRs, particularly for multiplex molecular methods.

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