

Reports

A single protocol for extraction of gDNA from bacteria and yeast

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BioTechniques 58:120-125 (March 2015) doi 10.2144/000114263

Keywords: DNA extraction; DNA amplification; bacteria, yeast; PCR

Supplementary material for this article is available at www.BioTechniques.com/article/114263.

Guanidine thiocyanate breakage of microorganisms has been the standard initial step in genomic DNA (gDNA) extraction of microbial DNA for two decades, despite the requirement for pretreatments to extract DNA from microorganisms other than Gram-negative bacteria. We report a quick and low-cost gDNA extraction protocol called EtNa that is efficient for bacteria and yeast over a broad range of concentrations. EtNa is based on a hot alkaline ethanol lysis. The solution can be immediately centrifuged to yield a crude gDNA extract suitable for PCR, or it can be directly applied to a silica column for purification.

Molecular biology techniques such as PCR, mass spectrometry, and sequencing have been optimized in order to speed up the detection and analysis of microorganisms (1). DNA extraction methods, although central to these procedures, have seen little progress since the introduction of guanidine thiocyanate treatment and silica column purification (2). Although this approach is satisfactory for extraction of DNA from Gram-negative bacteria, enzymatic or mechanical pretreatments are necessary to extract Gram-positive or acid-fast bacteria and yeasts (3,4). This is particularly problematic when different types of microorganisms may be present, such as in blood or urine, and especially when it is important to extract the microorganisms with equal efficiency so as not to bias relative populations in studies of microbiota. Previous studies have shown that differences in the structure of the cell wall are at the basis of these problems. The thicker layer of peptidoglycan in the cell walls of Gram-positive bacteria makes them more difficult to break than Gram-negative bacteria (5). Yeast, mycobacteria, and spores also have more complicated cell walls than Gram-negative

and Gram-positive bacteria and are even more difficult to break. Clinical samples may contain all of these types of microorganisms. For many of these microbes, the DNA yield and quality can vary considerably depending on the DNA extraction method used (6), and thus different methods or conditions must be employed simultaneously for each sample.

Various strategies have been attempted to enhance guanidine thiocyanate lysis of microorganisms, including mechanical, enzymatic, and chemical treatments alone or in combination. Probably the most universal protocols use mechanical lysis with beads, followed by silica column purification (3,4,7). Such treatments, however, can shear genomic DNA (gDNA) into small fragments that limit the size of potential PCR amplicons and may also create a bias when different sized amplicons are envisioned (8). This problem becomes more complex when samples contain unknown amounts of different types of microorganisms because different types of beads and times of beating may be required for different microbes and even different numbers of microbes (9).

Chemical and enzymatic lysis protocols are usually specific for different types of microorganisms and may also be more time-consuming because they require several steps including enzymatic digestion (10). Many procedures employ toxic substances (e.g., guanidine thiocyanate) that must be safely disposed of or ionic detergents (e.g., SDS) that can react with the sample and must be removed or neutralized to avoid inhibition of subsequent PCR.

We describe a procedure that is based on an unpublished technique developed by Eric Frost and Sylvie Deslandes to extract *Staphylococcus aureus* DNA to be screened for the presence of the *mecA* gene. Their procedure has been used in the clinical microbiology laboratory of the Centre Hospitalier Universitaire de Sherbrooke (CHUS) since 2004. Frost and Guay also used this procedure to successfully extract DNA from *Mycobacterium tuberculosis* for PCR analysis. It involved suspending pelleted bacteria in 61% ethanol, 0.1 M NaOH and heating the suspension for 10 min at 70°C. The single-stranded DNA (ssDNA) released was then pelleted in a microcentrifuge and suspended for PCR.

METHOD SUMMARY

The EtNa method releases single-stranded DNA from bacteria and yeast with similar efficiency by heating in an ethanol alkaline solution. Centrifugation yields a crude DNA pellet, while direct addition to silica columns allows purification. This procedure could be used when the identity of the microbes is unknown, and it is important not to overlook or bias particular microorganisms.

The present report describes improvements to this procedure to break Gram-negative and Gram-positive bacteria as well as yeasts and to use the method as a first step in DNA purification on silica columns. We compare this procedure with commercial kits and research methods including our adaptation of Chomczynski's (11) alkaline polyethylene glycol strategy. This procedure, which we call *EtNa* (Et for ethanol and Na for NaOH), does not require any hazardous chemicals, ionic detergents, enzymes, or mechanical steps but is nonetheless effective for bacteria and yeast.

Materials and methods

Bacterial strains and culture conditions The following bacteria and yeast were obtained from the ATCC (Manassas, VA): *Enterococcus faecalis* (ATCC 51299), *Proteus mirabilis* (ATCC 12453), *Streptococcus agalactiae* (ATCC 12326), *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermidis* (ATCC 12228), *Pseudomonas aeruginosa* (ATCC 27853), *Candida albicans* (ATCC 60193), and *Saccharomyces cerevisiae* (ATCC 204508). The microbes were grown overnight on sheep blood agar (Oxoid, Nepean, Canada) at 37°C. One colony was then suspended in Nutrient Broth (Sigma-Aldrich, Oakville, Canada) and incubated at 37°C to a density corresponding to McFarland standard 2.0 (Thermo Scientific, Ottawa, Canada). The number of bacteria (CFU/mL) was confirmed by the plate count method.

Extraction procedures

***EtNa* gDNA extraction.** Bacteria and/or yeast in 100 µL broth or saline were added to a 1.5-mL microcentrifuge tube. Four hundred fifty-five microliters of a 240 mM NaOH, 2.7 mM EDTA, 74% ethanol solution were added to the tube and mixed gently to give final concentrations of 200 mM NaOH, 2.25 mM EDTA, 61% ethanol. The tube was then heated to 80°C for 10 min and centrifuged at 16,060 × g for 10 min. The supernatant was removed, and 100 µL of an optimized suspension solution (see Supplementary Material) containing 0.1 mM EDTA, 50 mM Tris-HCl, pH 8.0, 1% Triton-X-100, and 0.5% Tween-20 was added to solubilize the denatured DNA; we called the resulting suspension *EtNa crude extract*. Alternatively, after heating, the mixture was added directly to a QiaAmp DNA Mini Kit column (Qiagen, Toronto, Canada) for purifi-

cation of gDNA. EtNa extraction replaced AL- or ATL-mediated bacterial lysis and allowed binding to the column. Subsequent washing steps, starting with buffer AW-1, were followed according to the manufacturer's instructions. The purified gDNA was eluted in 100 µL TE and is referred to as *EtNa pure extract*. A detailed protocol can be found in the Supplementary Material.

Alkaline polyethylene glycol (PEG)-based methods. As in the original method (11), 100 µL of a reagent composed of 60 g PEG 200 in 0.93 mL 2 M KOH and 39 mL H₂O was added to 1–10 µL of liquid or 1–10 mg of solid sample. The mixture was incubated for 15 min at room temperature for Gram-negative bacteria and 10 min at 70°–90°C for Gram-positive bacteria.

Modified PEG-NaOH gDNA extraction. Extraction was performed in a 1.5-mL tube containing 100 µL of bacteria in a broth culture or from a colony suspended in saline. It was centrifuged at 6082 × g for 4 min. Ninety microliters of the supernatant were withdrawn, and the bacteria were suspended in the remaining 10 µL. Ninety microliters of a PEG-NaOH reagent solution composed of 60 mL PEG 200, 5 mL 2 M NaOH, and 25 mL H₂O were added. The mixture was heated at 80°C for 10 min and shaken briefly before use. One-microliter samples were used directly for PCR in 15-µL reactions. A detailed protocol can be found in the Supplementary Material.

LiOAc-SDS gDNA extraction (12). Broth-grown microorganisms were centrifuged and the supernatant removed, or bacterial or yeast colonies were picked from culture plates and suspended in 100 µL 200 mM LiOAc, 1% SDS solution. After incubation at 70°C for 15 min and the addition of 300 µL 96% ethanol, the samples were mixed by brief vortexing and then centrifuged for 3 min at 16,060 × g. The supernatant was withdrawn, and the pellet was dissolved in 100 µL TE. Cell debris was spun down by centrifugation for 1 min at 16,060 × g, and 1 µL of the supernatant was used for PCR.

QIAamp DNA Mini Kit. The manufacturer's recommendations were followed for all organisms that included initial treatment in ATL buffer, either without pretreatment (Gram-negative bacteria) or after treatment with lysostaphin (Gram-positive bacteria) or lyticase (yeast).

ChargeSwitch gDNA Mini Kit. The kit was purchased from Life Technologies, (Burlington, Canada) and was used according to the manufacturer's recommendations, using a magnetic rack to manually separate magnetic beads.

Primers and probes. Part of the rRNA 16S or 23S gene from bacteria was amplified with primers 16SUni783F (5'-AGGATTAGATACCCTGGTAGTCCA-3'), and 16SUni1094R (5'-ACTTAACCCAACATCTCACGACAC-3') (13) or Uni23S1926F (5'-TAAGGTAGCGAAATTCCCTGTGG-3') and Uni23S2261R (5'-GGMGACC-GCCCCAGTYAAC-3'). Specific primers for *S. aureus* and *E. faecalis* were also used: EFNuc286F (5'-GCGATT-GATGGTGATACGGTT-3'), EFNuc564R (5'-AGCCAAGCCTTGACGAACATAAGC-3') for *S. aureus* and 16SEntco45F (5'-ACATG-CAAGTCGAACGCTTCT-3'), 16SEntco237R (5'-ACATGCAAGTCGAACGCTTCT-3') for *E. faecalis*.

The primers used to amplify the partial rRNA 28S gene from yeast were: 28SUni185F (5'-TGGGTGGTA-AATTCCAACCGCA-3'), 28SUni270F (5'-CAAAGTTCTTTCATCTTCC-WTCAC-3')(14) or 28Suni997F (5'-AGGATAG-CAGAAGCTCG-3') and 28Suni1154R (5'-CCACTAAAAGCTTCAATTCAACAGG-3'). *E. coli* and *C. albicans* were detected in the mix with probe 23Scoli2210R (5'-/ROX/ACGGGCCAAGGTTAGAACATCAAAC/BHQ1-3'), 28Salbic1092Rb (5'-/FAM/TTGTCACGTTCAATTAGCAACAAAGG/3IABkFQ/3'). Primers and probes were purchased from IDT (Coralville, IA).

Real-time PCR. Each extract was tested on the LC480 real-time PCR thermocycler (Roche Diagnostics, Indianapolis, IN). PCR was performed in a 15-µL final reaction

Table 1. Microbial DNA extraction using different concentrations of NaOH.

	NaOH concentration			
	0.1 M	0.2 M	0.25 M	0.3 M
<i>C. albicans</i>	23.6 ^a	19.3	18.9	21.6
<i>E. coli</i>	17.9	15.8	15.6	18.2
<i>S. epidermidis</i>	17.4	16.5	16.2	19.5

^aCq values obtained by PCR using 1-µL samples of DNA extracted from 100-µL samples containing 106 rRNA equivalents of *C. albicans*, 107 rRNA equivalents of *E. coli*, or *S. epidermidis* with e0.1 M, 0.2 M, 0.25 M, or 0.3 M NaOH in 61% ethanol.

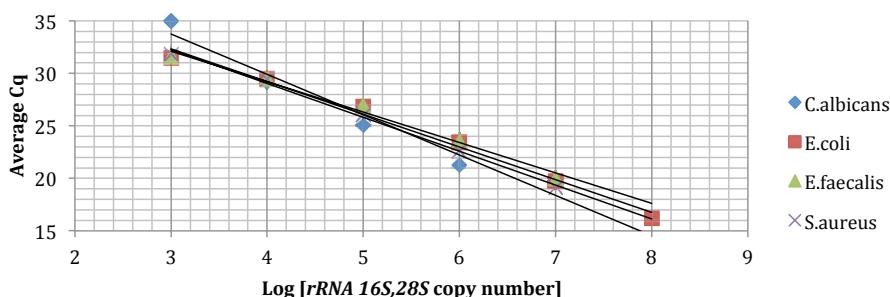


Figure 1. Extraction efficiency of microorganisms at different concentrations. Ten-fold dilutions in nutrient broth of overnight cultures of microorganisms were extracted with the EtNa crude procedure. One-microliter samples were subjected to PCR to obtain Cq values.

volume with 0.5 μ M of each primer, 1 μ L gDNA extract and 7.5 μ L LightCycler 480 DNA SYBR Green I Master kit (Roche Diagnostics). PCR cycling conditions were 1 cycle at 95°C for 5 min, 40 cycles of 95°C for 10 s, 55°C for 20 s, and 72°C for 45 s, followed by a melting curve of 95°C for 5 s, 65°C for 30 s, 55°C for 30 s, and then a gradual increase until 95°C was reached. A negative control containing all of the components of the reaction mixture without the DNA sample and a positive control containing 1 μ L of an *E. coli* DNA extract that had been amplified successfully previously were included in each run. This procedure was validated by amplifying a dilution series of *E. coli* DNA extracted

by EtNa without column purification. A PCR efficiency of 98.5% with an amplification factor of 1.98 was observed (calculated according to Thermo Scientific; www.thermoscientificbio.com/webtools/qpcrficiency/) (Supplementary Figure S1). When using probes, the DNA SYBR Green I Master Kit was replaced by LightCycler 480 Probes Master (Roche Diagnostics). Forward primer *rRNA 23S* (0.2 μ M), reverse primer (0.07 μ M), and the specific *E. coli* probe (0.1 μ M) were used. For yeast, 0.5 μ M of forward primer *rRNA 28S*, 0.1 μ M reverse primer, and 0.08 μ M specific yeast probe were used. PCR cycling conditions were 1 cycle at 95°C for 5 min, and 40 cycles of 95°C for 10 s, 67°C for 20 s, 55°C for 40 s, and 72°C for 40 s.

Table 2. gDNA extraction efficacy of EtNa for different types of microorganisms.

Microorganisms	rRNA 16S,28S copy number	Bacteria number CFU/mL	Number of rRNA copies/mL	Cq	Comparison with <i>E. coli</i>
<i>E. faecalis</i>	6	1.2×10^9	7.2×10^8	16.73	-2.5
<i>E. coli</i>	7	2.0×10^9	1.4×10^9	13.25	0
<i>P. aeruginosa</i>	4	1.5×10^9	6.0×10^8	15	-0.75
<i>S. agalactiae</i>	7	2.0×10^8	1.4×10^7	20.45	0
<i>P. mirabilis</i>	7	2.0×10^7	1.4×10^7	21.25	-1
<i>S. aureus</i>	5	1.0×10^8	5.0×10^7	19.68	-1.2
<i>C. albicans</i>	140	2.0×10^6	2.8×10^7	18.02	+1
<i>S. cerevisiae</i>	140	1.3×10^6	1.82×10^8	15	-0.5

Genomic DNA (gDNA) was extracted from various microorganisms with the EtNa pure method and Cq values were obtained. Using the Cq value from *E. coli* and the amplification efficiency of our PCR, a curve was plotted comparing Cq with the rRNA gene copy number. Using this curve, the expected Cq values of the other organisms were estimated based on their rRNA copy numbers and compared with the observed Cq values.

Statistical analysis

All experiments were performed at least in triplicate, and averages of values are recorded in the tables and figures. Statistical comparisons were performed using Student's *t*-test.

Results and discussion

To break yeasts, we increased the heating temperature in the original procedure from 70°C to 80°C and tested different concentrations of NaOH in 61% ethanol (Table 1); 0.2 or 0.25 M NaOH appeared to break yeasts as well as the Gram-positive and Gram-negative bacteria tested. In subsequent experiments, we used 0.2 M NaOH. To ensure that heating microorganisms for 10 min at 80°C in 0.2 M NaOH did not degrade the DNA or leave residues that would inhibit PCR, 10-fold dilutions of different microorganisms were extracted with the standard EtNa crude procedure (Figure 1). Even when 10^3 microorganisms per milliliter were extracted, no reduction in PCR efficiency was noted. The procedure extracted gDNA equally well from 10^9 bacteria per milliliter.

The EtNa extraction procedure would be much more useful if it could be combined with silica-based extraction and eventually automated. Because silica column wash buffers contain 70% ethanol (or more accurately 67% because reagent grade ethanol is only 96% ethanol), which is similar to the EtNa solution, and they do not elute DNA, we attempted to bind nucleic acids to the column by directly adding the EtNa extraction solution after heating the nucleic acids. Initial experiments showed that column extraction was much less efficient than manual extraction, but we observed that addition of EDTA to the extraction solution to a final concentration of 2.25 mM allowed efficient recovery on silica columns (Figure 2).

Cq values were obtained by real-time PCR for several different microorganisms representing Gram-negative bacteria, Gram-positive bacteria, and yeasts after EtNa pure extraction including silica column purification. Using the observed *E. coli* Cq value of 13.25 as a reference for 1.4×10^9 copies of the rRNA gene based on the number of bacteria and rRNA gene copy number (15), we plotted a Cq versus rRNA gene copy number curve based on this point and the amplification efficiency of our PCR reaction (see Supplementary Figure S1). Using this curve, we estimated the Cq values that

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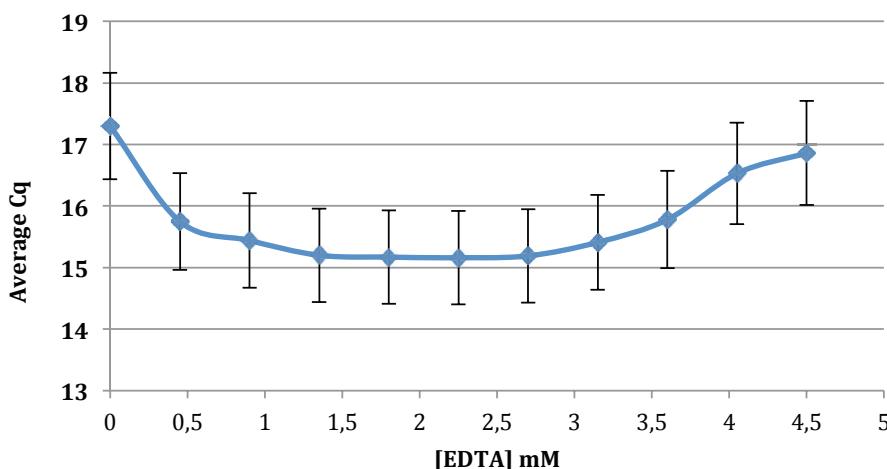


Figure 2. Effect of EDTA on silica column extraction. DNA was extracted from 100- μ L samples containing 10^7 rRNA equivalents of *E. coli* with EtNa containing 0–5.5 mM of EDTA. The sample was directly applied to a silica column (QIAamp DNA Mini Kit), washed according to the manufacturers recommendations, and DNA was eluted in the manufacturer's elution buffer. One-microliter samples were subjected to PCR to obtain Cq values.

would be obtained from the rRNA gene copy numbers of the organisms that we extracted. We observed concordance of Cq values with a variation of about 1 Cq, which would indicate a variation in relative titers between 0.5 and 2 times the expected values (Table 2). Mixtures of different microorganisms representing Gram-negative bacteria, Gram-positive bacteria, and yeasts at different concentrations were extracted with the EtNa pure extraction protocol including silica column purification, and the extracted DNA was amplified using PCR reactions specific for the different microorganisms. We observed that the presence of other microorganisms did not affect the extraction efficiency of bacteria or yeasts, over a 100-fold variation in concentration (Table 3).

EtNa crude and EtNa pure were compared with various DNA extraction methods including the commercial QiaAmp DNA Mini Kit and ChargeSwitch gDNA Mini Kit that are based on guanidinium thiocyanate breakage and silica column purification, as well as the LiOAc-SDS and alkaline PEG procedures. All of the methods extracted

bacterial DNA successfully, but only EtNa and LiOAc-SDS successfully extracted gDNA from yeast (Table 4). The slightly higher Cq values observed for EtNa pure in comparison with EtNa crude are probably the result of the expected recovery suggested by the manufacturer of QiaAmp DNA Mini Kit.

Over the past 25 years, most protocols for breaking microorganisms and purifying their nucleic acids have relied on treatment with guanidinium thiocyanate after enzymatic or mechanical weakening of Gram-positive or yeast cell walls (but without pretreatment for Gram-negative bacteria) and then purification on silica columns (2). Replacing one of these two elements would thus require significant improvements. Here we describe such a microbe breaking strategy, called EtNa, that is efficient for all types of organisms and cells, does not require hazardous chemicals, is rapid, and can be integrated with silica column purification or used as a rapid crude extract for DNA amplification tests. Although this procedure yields ssDNA rather than double-stranded DNA (dsDNA) as obtained with guanidinium thiocyanate

Table 3. Extraction of mixtures of microorganisms.

	1	2	3	4	5	6	7	8	9	10
<i>E. coli</i>	20.47				20.8	20.08	21.03	20.75	21.27	20.64
<i>S. aureus</i>		24.18			21.7	22.4	22.95	26.58 ^a	30.45 ^b	30.36 ^b
<i>Enterococcus</i>			24.36		23.36	23.26	24.01	23.89	24.5	28.05 ^a
<i>C. albicans</i>				26.43	26.92	28.42 ^a	33.34 ^b	25.24	26.34	25.36

Twenty-five-microliter samples of overnight cultures of microorganisms or 1/10 (^a) or 1/100 (^b) dilutions of these cultures were mixed, and their genomic DNA (gDNA) was extracted with the EtNa procedure, including column purification. Efficacy of extraction was evaluated by amplification by real-time PCR specific for each microorganism and compared with the average Cq value obtained when the microbial DNA was extracted without mixing with other microorganisms (columns 1–4). Results are shown as average Cq values from three experiments. Ten-fold dilutions of DNA would be expected to increase Cq values by about 3.3 and 100-fold dilutions by about 6.7.

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Table 4. Comparison of gDNA extraction methods by real-time PCR.

	Cq		
	<i>E. coli</i>	<i>S. epidermidis</i>	<i>C. albicans</i>
EtNa	13.92	14.78	18.77
EtNa pure	15.34	15.71	18.84
LiOAc-SDS	14.80	14.75	19.96
Alkaline PEG	20.22	18.2	32.83
Modified PEG	14.47	15.75	28.4
QIAamp DNA Mini Kit	13.02	17.8	34.55
ChargeSwitch	12.88	17.27	33.2

Several genomic DNA (gDNA) extraction methods were compared with EtNa crude and EtNa pure for 100- μ L samples containing 10^7 rRNA equivalents of *C. albicans*, or 10^8 rRNA equivalents of *E. coli* or *S. epidermidis*.

extraction, this should be an advantage for PCR and should not prevent standard or next-generation sequencing, as protocols exist for preparing sequencing libraries from single-stranded nucleic acids. On the other hand, it would preclude RNA extraction and protocols requiring restriction endonuclease digestion.

Probably the greatest advantage of EtNa is that the same protocol can be used for all types of microorganisms at any concentration with similar efficiency. This is particularly important for screening tests for human or other infections where

the responsible organism is not known and may be a Gram-negative or Gram-positive bacteria or yeast. It will also be increasingly important to characterize the microbiota in numerous sites where, again, a variety of microorganisms coexist and where it is important to extract them with equivalent efficiency so that their relative abundance can be reflected by subsequent next-generation sequencing of *rRNA 16S* or other genes. Indeed, we observed that EtNa worked with similar efficiency when extracting small (10^3 /mL) or large (10^9 /mL) numbers of microorganisms.

The EtNa procedure does not require hazardous chemicals such as guanidinium thiocyanate or phenol. It does not require ionic detergents that may interfere with enzymes necessary for subsequent tests such as PCR. It yields denatured ssDNA and degrades RNA. Although inappropriate when downstream tests require RNA or dsDNA, this is advantageous for PCR-based procedures because it reduces the importance of the initial heating step necessary to denature DNA prior to PCR.

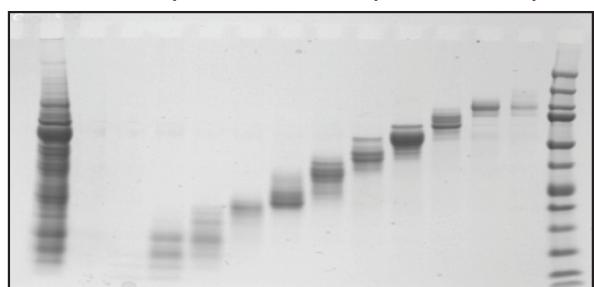
It has long been known that heat (16) or alkali (17) can be used to break down *E. coli* cell walls and extract plasmid DNA. We postulated that combining heat and alkali together with 61% ethanol to disrupt lipid membranes would enhance cell wall breakage and simultaneously precipitate the released DNA, allowing very rapid preparation of crude DNA extracts. This 61% ethanol would also create a chaotropic environment that would allow DNA to bind to silica. It was noted that the addition of supplementary EDTA enhanced binding to silica. This discovery was indeed fortuitous because a microbial breakage procedure that could not be efficiently coupled with

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purification on silica would have only limited usefulness.

In conclusion, we have developed a quick, low-cost, and reliable method for breaking yeast and bacterial cells prior to gDNA extraction. The EtNa reagent can be used to process a wide range of biological samples for PCR assay in clinical diagnostic and biomedical research in <25 min. ssDNA from this procedure can be used directly or further purified by directly binding it to silica columns from the EtNa solution. The EtNa method should be compatible with automation in sample processing robots.

Author contributions

L.V. contributed to the conception of the study, acquisition of data, analysis and interpretation of data, drafting of the article, and the critical revision. E.H.F. contributed to the conception of the study, analysis and interpretation of data, drafting of the article, critical revision, and general supervision.

Acknowledgments

The authors thank Sylvie Deslandes for her role in developing the initial gDNA extraction used in the clinical microbiology laboratory of the Centre Hospitalier Universitaire de Sherbrooke and members of the clinical microbiology laboratory of Centre Hospitalier Universitaire de Sherbrooke for bacteria strains. Geneviève Giroux, Stéphanie Mauleur, Karine Bourgade, Evelyne Benoit, and Patrick Dextras-Paquette are thanked for helpful discussions and experimental support.

Competing interests

The authors declare no competing interests.

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Received 15 September 2014; accepted 08 December 2014.

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