Molecular Ecology Notes (2006) **6**, 998–1002 doi: 10.1111/j.1471-8286.2006.01428.x

TECHNICAL NOTE

An inexpensive, automation-friendly protocol for recovering high-quality DNA

NATALIA V. IVANOVA, JEREMY R. DEWAARD and PAUL D. N. HEBERT
Biodiversity Institute of Ontario, Department of Integrative Biology, University of Guelph, Guelph, Ontario, Canada N1G 2W1

Abstract

Although commercial kits are available for automated DNA extraction, 'artisanal' protocols are not. In this study, we present a silica-based method that is sensitive, inexpensive and compliant with automation. The effectiveness of this protocol has now been tested on more than 5000 animal specimens with highly positive results.

Keywords: DNA barcoding, DNA extraction, robotics, liquid handling

Received 7 March 2006; revision accepted 31 March 2006

With single capillary sequencers able to process 0.5 million samples a year, it is no surprise that large-scale sequencing projects are becoming common in molecular ecology. For example, major DNA barcoding facilities now process up to 100 000 specimens a year. Achieving these production goals requires vigorous optimization of protocols to lower costs and speed processing (Hajibabaei *et al*. 2005). Because DNA extraction is a critical and expensive first stage in all sequence analyses, it is an obvious target for optimization. Chelex-based protocols are one low-cost option (Walsh *et al*. 1991; Jaulhac *et al*. 1998; Ivanova *et al*. 2003), but DNA extracts produced by this method are unstable and the approach is poor at extracting DNA from museum specimens.

Silica-based methods have now largely replaced traditional phenol–chloroform DNA extractions in applications where high quality DNA is required. These approaches, which rely on the binding of DNA to silica in the presence of a high concentration of chaotropic salts (Boom *et al*. 1990), not only deliver a stable DNA extract, but they are also very sensitive (Hoff-Olsen *et al*. 1999). One downside to their use is cost; commercial kits average about $2.00 per specimen. An earlier study identified an inexpensive silica-based approach that performed nearly as well as commercial kits (Elphinstone *et al*. 2003; Hajibabaei *et al*. 2005), but it was prone to cross-contamination and was not compatible with automation (personal observation N.V. Ivanova). The present study seeks to overcome these constraints.

Correspondence: N. V. Ivanova, Fax: (519) 767-1656; E-mail: nivanova@uoguelph.ca

Our study evaluated the effectiveness of several commercial glass fibre filtration (GF) plates, the core functional component in all silica-based DNA extraction kits. We worked only with 96-well plates that appeared structurally compatible with robotic systems. Specifically, we compared the performance of seven GF plates — two manufactured by BioLynx (B1, B2), three by PALL (P1-3) and two by Whatman (W1, W2). The B1 plate (no. F2008) was a 0.7-μm glass fibre with 0.8 mL well; B2 (no. F2007) was a 1.0-μm glass fibre with 0.8 mL well; P1 (no. 5051) was a 1.0-μm glass fibre with 1 mL well; P2 (no. 5053) was a 3.0-μm glass fibre media/0.2 μm Bio-Inert membrane with 1 mL well; P3 (no. 5031) was a 1.0-μm glass fibre media with 350 μL well; W1 (no. 7505–0003) was a mini prep DNA binding plate with 0.8 mL well and W2 (no. 7700-7801) was a Unifilter plate with glass fibre media with 0.8 mL well.

We began by identifying both buffer systems and protocols that enabled the use of these GF plates for manual DNA extraction. We then tested their performance under automation with a Biomek NX liquid-handling station (Beckman-Coulter) equipped with a filtration manifold. To provide a solid test of performance, we compared these results with those from a high performance commercial kit — the NucleoSpin96 (Machery-Nagel), hereafter termed the MN kit (Hajibabaei *et al*. 2005).

Our performance comparisons employed DNA extracted from frozen tissues of six mammal species (*Glaucomys volans*, *Sorex fumeus*, *Clethrionomys gapperi*, *Blarina brevicauda*,

Tamias striatus, *Tamiasciurus hudsonicus*). A large volume DNA extract was prepared using 0.5 g of macerated tissue incubated overnight at 56 °C in 6 mL of vertebrate lysis buffer (VLB — see below). The lysate from each species was then diluted 2-, 5-, 10-, 20-, 100-, 200- and 1000-fold with VLB and 50 μL of the 48 lysates (6 species, 8 concentrations) were dispensed into wells in the odd rows of 96-well microplates (Eppendorf). In this way, wells containing lysate were separated by blank wells filled solely with VLB, allowing quantification of cross-contamination events.

Working solutions

Vertebrate lysis buffer (VLB) — 100 mM NaCl, 50 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0, 0.5% SDS.

Binding buffer (BB) — 6 M GuSCN, 20 mM EDTA pH 8.0, 10 mM Tris-HCl pH 6.4 and 4% Triton X-100 (Myakishev *et al*. 1999; with minor modifications) was prewarmed at 56 °C to dissolve.

Binding mix (BM) — 50 mL of ethanol (96%) was thoroughly mixed with 50 mL of BB (stable at 20 °C for 1 week).

Protein wash buffer (PWB) — 70 mL of ethanol (96%) was thoroughly mixed with 26 mL of BB (stable at 20 °C for 1 week).

Wash buffer (WB) — ethanol (60%), 50 mM NaCl, 10 mM Tris-HCl pH 7.4 and 0.5 mM EDTA pH 8.0 (stored at –20 °C).

Detailed instructions on buffer assembly, the project and method files for the robotic protocol and a diagram of custom-made equipment are available at www.barcodeoflife.org/DNAextraction/.

Manual protocol

1 Mix 5 mL of VLB and 0.5 mL of proteinase K (20 mg/mL) in a sterile container and dispense 50 μL into each well of a 96-well skirted microplate. Add 1–2 mm^3 of vertebrate tissue to each well (flame-sterilize instruments between samples). Cover the plate with cap strips, incubate overnight at 56 °C and then centrifuge at 1000 *g* for 1 min.
2 Add 100 μL of BM to each well using a multichannel pipette. Cover the plate with cap strips, mix by rotation and centrifuge at 1000 *g* for 20 s.
3 Remove the cap strips and transfer 125 μL of each lysate into a well in a GF plate sitting on a 2-mL square-well block. Seal the GF plate with self-adhering foil and centrifuge at 5000 *g* for 5 min to bind DNA to the GF membrane.
4 For the first wash step, add 180 μL of PWB to each well of the GF plate before sealing it and centrifuging at 5000 *g* for 2 min.
5 For the second wash step, add 750 μL of WB to each well of the GF plate before sealing it and centrifuging at 5000 *g* for 5 min.
6 Remove the seal, place the GF plate on the lid of a tip box, and incubate at 56 °C for 30 min to evaporate residual ethanol. Position a collar (PALL Cat. no. 5225) on the collection microplate and place the GF plate on top. To release the DNA, add 60 μL of ddH_2O (at 56 °C) to each well of the GF plate before sealing it and incubate at room temperature for 1 min.
7 Place the assembled plates on a square well block to prevent cracking of the collection plate and centrifuge at 5000 *g* for 5 min to collect the DNA eluate. Remove the GF plate and discard it.

Automated protocol

1 Prepare lysates using the manual protocol.
2 Load the deck of liquid-handling unit with labware and reagents.
3 Add 100 μL of BM robotically to each of the 96 wells in the plate.
4 Mix each lysate by repeatedly (4×) withdrawing and re-injecting 50 μL of it. Transfer 125 μL of each lysate into a GF plate sitting on a 36-mm collar positioned on the vacuum manifold. Apply a vacuum of 23 In Hg for 4 min and discard the filtrate.
5 Add 180 μL of PWB to each well and place the plate under vacuum for 3 min.
6 For the first wash step, add 220 μL of WB to each well and apply vacuum for 2 min.
7 For the second wash step, add 660 μL of WB to each well and apply vacuum for 10 min to dry the plate.
8 Incubate all plates at 56 °C for 20–30 min to evaporate residual ethanol.
9 Use a custom-made frame to raise the collection plate close to the outlets of the GF plate to minimize eluate splashing. Place a 96-well skirted microplate (Eppendorf) on this frame inside the vacuum manifold and add 60 μL of sterile ddH_2O (at 56 °C) to each well of the GF plate. Incubate it for 2 min and then apply vacuum for 5 min to collect eluates into the receiving plate.

Overview of protocols and results

We began by establishing a standard DNA extraction protocol that was effective for all seven GF plates. We then evaluated the performance of these plates with robotic protocols in two ways. First, we processed 48 vertebrate lysates manually on a P1 plate to compare the efficiency of DNA extraction via manual protocols (which employ a centrifugation step) and their robotic counterparts (which employ filtration). Second, we extracted the 48 lysates with an MN kit using recommended ratios of their proprietary

buffer systems [100 µL of BQ buffer : ethanol (1:1) mix for the binding step, 180 µL of BW and 660 µL of B5 buffer for wash steps]. We then tested the seven GF plates for automated extraction, employing a uniform protocol except that the second wash step was reduced to 220 µL for the P3 plate (because of its smaller wells). After automated extraction, the bottom of each GF plate was examined for the accumulation of chaotropic salts and the surface of each recipient plate was checked for splashes of eluate.

The final elution volume for all DNA extractions was 60 µL. Polymerase chain reactions (PCRs) had a total volume of 12.5 µL and contained 2 µL of DNA extract (see Hajibabaei *et al*. 2005 for details). We amplified a 658 bp segment of the cytochrome *c* oxidase I (COI) gene using a cocktail of three forward primers: VF1 5′-TTCTCAACCAACCACAAAGACATTGG-3′; VF1d 5′-TTCTCAACCAACCACAARGAYATYGG-3′; VF1i 5′-TTCTCAACCAACCAIAAIGAIATIGG-3′; and three reverse primers VR1 5′-TAGACTTCTGGGTGGCCAAAGAATCA-3′ (named Fish Reverse 1 in Ward *et al*. 2005); VR1d 5′-TAGACTTCTGGGTGGCCRAARAAYCA-3′; VR1i 5′-TAGACTTCTGGGTGICCIAAIAAICA-3′.

Primers with a concentration of 10 pmol/mL were mixed in a 1:1:2 ratio (VF1 : VF1d : VF1i for the forward cocktail; VR1 : VR1d : VR1i for reverse), and 0.625 µL of both the forward and reverse cocktails (C_VF1di, C_VR1di) were used in each PCR. PCR products were separated on a 2% agarose E-Gel96 gel (Invitrogen), visualized under UV light, photographed with a Alpha Imager 3400 imaging system (Alpha Inotech) and processed using Invitrogen E-EDITOR software.

Chaotropic salts accumulated on the bottom of four plates (B1, B2, W1, W2) following automated extraction and these same plates splashed eluates onto the surface of recipient plates. The occurrence of cross-contamination was further confirmed because PCR products were commonly generated from blank wells in both B1 and W1 plates (Fig. 1). The same problems (salt contamination, splashing, PCR products in control wells) were not encountered with either the NucleoSpin96 kit or with any of the PALL plates. Problems with contamination were also absent in manual processing of BioLynx and Whatman plates (data not shown).

High success was obtained with both automated and manual extractions of lysate diluted up to 20-fold (Figs 1 and 2). However, PCR products were recovered less often from higher dilutions and the manual extraction performed slightly better in these cases. The B2 plate showed excellent success under automation, but the accumulation of salts was a problem. All three PALL plates and the MN kit were also effective and they experienced no salt accumulation. Although this study only examined a small panel of vertebrate lysates, we have now employed both manual and robotic versions of our DNA extraction protocol on more than 5000 vertebrate and invertebrate specimens (> 500 species) with highly positive results. Protocols for all species were similar, barring the use of a different Lysis Buffer (700 mM GuSCN, 30 mM EDTA pH 8.0, 30 mM Tris-HCl pH 8.0 and 0.5% Triton X-100, 5% Tween-20) for arthropods. This work did reveal that the P2 plate delivered higher success for samples low in DNA, such as 3–5 mm segments of arthropod legs (results not shown).

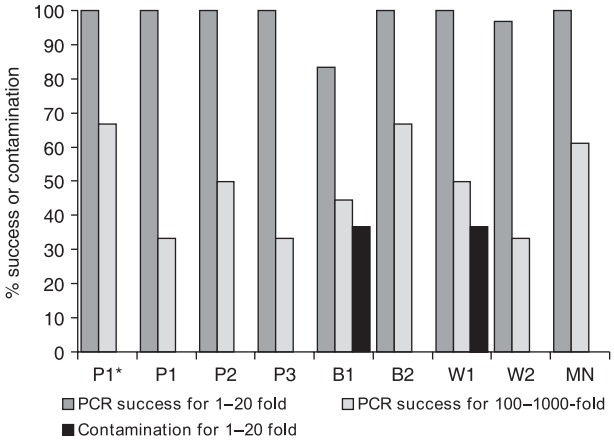

Fig. 1 Percentages of PCR success and contamination in a test of seven glass fibre plates (P1-3, B1-2, W1-2) and one commercial kit (MN). DNA was extracted from eight lysate concentrations from six mammal species. All plates were run using automation except P1*.

In summary, our work has identified a protocol for DNA extraction that matches high-performance commercial kits. Because it delivers these results for just $0.50/sample, its adoption can result in annual savings of $150 000 in a facility processing 100 000 specimens a year. We further note that this protocol can deal with different animal tissues and that it can be carried out either manually or through automation. Whereas robotic protocols speed analysis and reduce analytical error, very high production rates can be achieved through manual protocols aided by electronic multichannel pipettes. Aside from far lower capital and service costs, we have found that manual protocols are slightly more sensitive, an important factor to consider in work on small organisms.

Acknowledgements

This work was supported by the Gordon and Betty Moore Foundation, the Canada Foundation for Innovation, the Ontario Innovation Trust, the Canada Research Chairs Program, NSERC and by Genome Canada through the Ontario Genomics Institute. R. Russell, T. Christmas, J. MacLean-Gamsby and M. Chow provided technical advice, while T. Zemlak aided evaluation of protocols. We thank R. Hanner and M. Hajibabaei for comments on the manuscript.

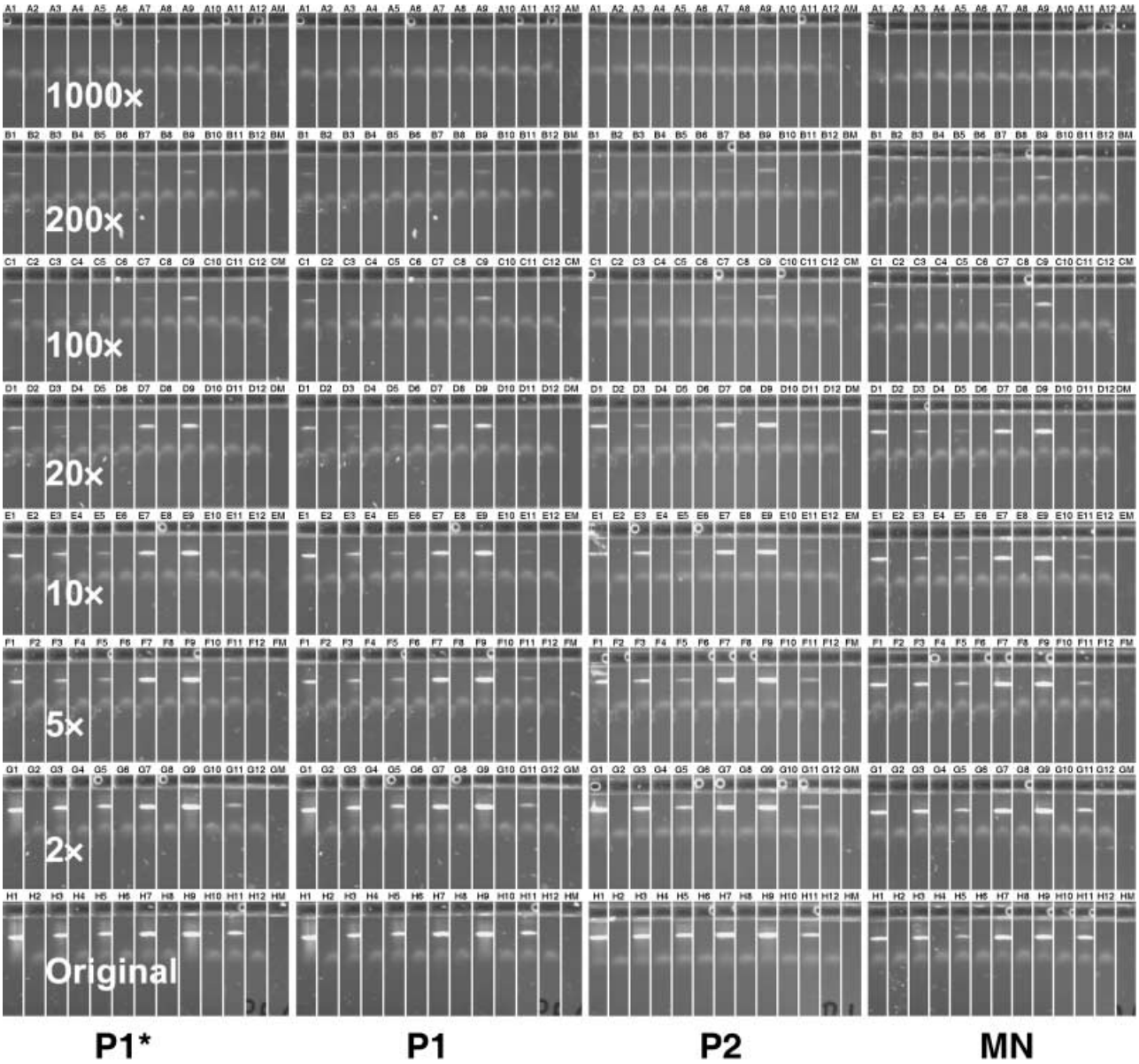

Fig. 2 PCR products (658 bp) for the COI (cytochrome *c* oxidase I) barcode region from DNA isolated using two glass fibre plates (P1, P2) and one commercial kit (MN) for 1–1000-fold dilutions of lysates from six mammal species. Except for P1*, results were obtained using automation. Some faint products are not visible in this agarose gel image. Even numbered lanes are negative controls.

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