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TECHNICAL ADVANCES

High throughput tissue preparation for large-scale genotyping experiments

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Abstract

A fast, efficient technique is described for the extraction of DNA from a large number of samples. The applications of this method include population genetics, plant breeding, and genetic screening. In the field, samples are collected in premeasured silica gel aliquots in polypropylene blocks, which are later used to grind the dried tissue. This permits naturalists, breeders, and collaborators to collect a large number of samples in a short amount of time and allows the samples to dry quickly during shipping. No phenol or chloroform steps are required to obtain high-quality DNA. Samples representing 12 plant families, three invertebrates, and a mammal were included. Quantities of DNA obtained were consistent with or better than other techniques. The quality of samples was tested by amplification of the internal transcribed spacer region. Test amplifications were successful, confirming the quality of extracted DNA.

Keywords: field collection, genetic screening, genotyping, silica gel

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Introduction

In breeding studies of plants and animals, numerous populations are sampled in order to categorize candidate genes for economically important traits (Chao *et al*. 2005; Schenkel *et al*. 2005). Similarly, in assessing genetic diversity of populations, multiple samples are collected from several populations (Slotta *et al*. 2005, 2006; Reusch 2006). A variety of preservation methods are used including drying at ambient temperature, cryopreservation with dry ice or liquid nitrogen, ethanol or isopropanol, or buffer solutions, which yield sufficient quantities of DNA for ecological, evolutionary and genetic studies (Kilpatrick 2002; Wehausen *et al*. 2004; Aime & Phillips-Mora 2005; Goolsby *et al*. 2006). However, preservation of samples can become an obstacle if dry ice, liquid nitrogen, or freeze-drying is unavailable. Transportation of samples stored in hazardous materials or flammable liquids may deter the inclusion of some isolated field sites and the representation of the genotypes therein. Silica gel is a feasible alternative in tissue preservation, is often used in collection of plant material (Chase & Hillis 1991; Arnaud-Haond *et al*. 2005; Reusch 2006), and has been used in faecal sample preservation (Roeder *et al*. 2004).

Once samples are obtained, the price of reagents and time to process thousands of samples can be staggering with kits costing $2–3 per sample (e.g. MP Biomedicals FastDNA and G-Nome, MoBio Laboratories UltraClean-DNA Extraction, Qiagen DNeasy) and standard extraction techniques requiring multiple days (e.g. Doyle & Doyle 1987). To expedite sample processing in plant breeding, population genetics and genetic screening studies, an efficient, large-scale, nontoxic method for collection and extraction of DNA was developed. Hazardous and toxic chemicals used in existing methods with CTAB (cetyltrimethyl ammonium bromide)/phenol/chloroform (Doyle & Doyle 1987) or DMSO (Kilpatrick 2002) and available kits were eliminated. Samples representing 12 plant families, three invertebrates, and a mammal were included. The quantity of DNA obtained was assessed and the quality examined by amplification of the internal transcribed spacer (ITS) region of the nuclear ribosomal DNA.

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Table 1 Summary of leaf samples extracted and amplified with two different weights of silica gel. Sixteen samples were included for each taxon with eight replicates for each quantity of silica gel. Tissue sample weights varied per replicate in order to determine optimal material quantity. The mean of tissue weight and corresponding DNA quantity mean of the extractions is reported. The results of ITS amplification varied per taxon. The mean product quantity and percentage of successfully amplified reactions with undiluted*, 1:20 diluted†, and 1:40 diluted‡ DNA samples is reported. The maximum threshold quantity of PCR able to be interpreted by the Fluor-S Imager was 100 ng/μL

Taxon	Sample weight (mg)	Silica gel weight (g)	DNA quantity obtained (ng/μL)	PCR quantity (ng/μL) and percentage amplified
Barley	56.4	0.25	56.8	71.5 (100%)*†
(*Hordeum vulgare*)	100.3	0.5	99.4	69.5 (100%)*†
Canada Thistle	56.3	0.25	16.9	78.0 (100%)*†‡
(*Cirsium arvense*)	103.3	0.5	51.4	60.5 (75%)*†‡
Daylily	55.6	0.25	28.5	64.4 (87.5%)*†
(*Hemerocallis* sp.)	95.9	0.5	43.8	53.7 (62.5%)*†
Leafy spurge	55.5	0.25	50.3	54 (75%)*†
(*Euphorbia esula*)	103.7	0.5	84.5	50.6 (100%)*†
Rice	56.0	0.25	53.5	33.2 (87.5%)*†
(*Oryza sativa*)	105.4	0.5	66.5	65.3 (100%)*†‡
Tomato	56.0	0.25	55.5	34.6 (75%)*†
(*Solanum lycopersicum*)	103.7	0.5	74.0	47.6 (87.5%)*†

Table 2 Summary of extraction and amplification of DNA from various taxa dried and ground in 0.5 g silica gel. The number of replicates varied per taxon and tissue-type based on sample availability. For several taxon, treatments varied to emulate field preparation available (e.g. removal of wings or seed coat). The mean tissue weight and DNA quantity obtained is reported. Product quantity in amplification of ITS varied per tissue type. The mean quantity and percentage of successfully amplified reactions with undiluted* and 1:20 diluted† DNA samples is reported. The maximum threshold quantity of PCR able to be interpreted by the Fluor-S Imager was 100 ng/μL

Taxon	Tissue type	Sample weight (mg) (replicates)	DNA quantity obtained (ng/μL)	PCR quantity (ng/μL) and percentage of samples amplified
Lady bug beetle (*Coccinella novemnotata*)	Insect with elytra removed	50 (2)	73.8	34.0 (100%)*
Lady bug beetle (*Coccinella novemnotata*)	Insect with elytra attached	50 (1)	31.9	31.2 (100%)*
Potato beetle (*Leptinotarsa decemlineata*)	Insect with elytra removed	100 (11)	17.4	20 (90.9%)*†
Spurge flea beetle (*Aphthona lacertosa*)	Insect body (1–4 beetles)	50 (4)	10.9	40 (100%)*
Weevil (*Rhinocyllus* sp.)	Insect body (1 weevil)	15 (1)	3.80	36.5 (100%)*
Mouse (*Mus musculus*)	Tail	8.2 (1)	4.67	62.0 (100%)*
Mouse	Liver	90 (1)	42.8	76.0 (100%)*
Mouse	Kidney	124.8 (1)	23.3	96.0 (100%)*
Mouse	Heart	164.2 (1)	6.82	55.0 (100%)*
Mouse	Muscle (leg)	95.6 (1)	2.93	25.0 (100%)*
Mushroom (*Agaricus bisporus*)	Cap tissue	65.2 (8)	2.73	80.9 (87.5%)*
Apple (*Malus domestica*)	Fruit	75 (8)	0.68	88.1 (75%)*
Grape (*Vitis vinifera*)	Fruit	65.7 (8)	1.06	65.5 (87.5%)*
Cassava (*Manihot esculenta*)	Root (tuber)	53.7 (8)	6.78	74.6 (75%)*
Carrot (*Daucus carota*)	Root	54.3 (8)	4.89	87.4 (87.5%)*
Spruce (*Picea* sp.)	Needles	84.6 (8)	10.7	100 (62.5%)*
Sugar beet (*Beta vulgaris*)	Leaf	56.0 (8)	24.8	63.5 (100%)*
Sunflower(*Helianthus annuus*)	Leaf	59.4 (8)	7.59	98.4 (100%)*
Sunflower	Seed (shell on)	162 (seed did not grind) (4)	2.92	20.0 (25%)*
Sunflower	Seed (shell off)	40.1 (4)	4.88	96.0 (100%)*

Materials and methods

One sample block (Phenix or Innovative Micro Plate 96-well 2.2 mL blocks with square wells) was prepared to study desiccation efficiency of differing tissue quantities with two replicates in alternating rows of 0.25 g (500 μL by volume) or 0.5 g (1000 μL) silica gel per well (Table 1). A second block was prepared to determine feasibility of the method for insects, mouse, and additional plant taxa (Table 2). In the second block, 0.25 g (500 μL) silica gel per well was

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used. Fresh material added to the blocks consisted of a range of weights (11–120 mg tissue) corresponding to number of needles (spruce), leaves, or insects or to equivalent tissue weights (e.g. fruits, roots, mushroom). After 2 weeks at room temperature, samples were thoroughly desiccated, most tissue samples (excluding mouse) were dried within 2 days.

A 500 μL 96-well round bottom plate (Phenix) was used to premeasure and transfer silica gel to the 2.2 mL 96-well blocks by inverting the round bottom plate filled with silica gel onto the 2.2 mL block. Silica gel used here included an approximate 1:10 ratio of Type III indicating type (Sigma-Aldrich, catalogue no. S7625) and Type II granular (catalogue no. S7500). Blocks were sealed with a plate mat (Spex CertiPrep) to prevent cross-contamination and moisture entering wells.

Blocks sealed with plate mats and ground at 1500 strokes for 4 min in a Geno/Grinder (Spex CertiPrep). The silica gel served as the grinding agent for the dried tissue; no additional glass or metal bead was required. The blocks were centrifuged briefly to relocate powdered tissue and silica gel to well bottoms. The DNA extraction followed a modified SDS-based protocol that required no phenol-chloroform steps (Pallotta *et al*. 2003). Preheated (65 °C) extraction buffer (600 μL; 0.1 M Tris-HCl, pH 7.5, 0.05 M EDTA, pH 8.0, 1.25% SDS) was added to each well using a multichannel pipette. A crackling noise would occasionally occur as the remnant silica gel rapidly hydrated with buffer but this had no effect on the extraction process. Blocks were heated at 65 °C for 15 min then placed at 4 °C for 15 min. to cool before adding 300 μL chilled 6 M ammonium acetate (4 °C). A new plate mat was used to seal the block, preventing cross-contamination. Samples were mixed by slowly inverting the block several times then returned to 4 °C for 15 min. To pellet proteins and cell debris, blocks were centrifuged for 20 min at 2250 *g* at 4 °C. The supernatant was transferred to new blocks containing 400 μL 100% isopropanol (–20 °C) per well and covered with a new plate mat. The supernatant and isopropanol were mixed by slowly inverting the block and the block was placed at –20 °C for 15 min to aid in DNA precipitation. Samples were centrifuged (20 min, 2250 *g*, 4 °C) to pellet DNA. Blocks were quickly inverted and tapped on paper towels to remove isopropanol (< 1 min) while retaining pellets. Pellets were washed twice with 500 μL 70% ethanol (–20 °C) followed by 10 min centrifugation at 2250 *g* and inverted as above to remove excess alcohol. To facilitate drying of DNA pellets, blocks were placed in a chemical flow hood for 1–4 h at room temperature or until no evidence of alcohol remained. Pellets were resuspended overnight at 4 °C in 300 μL of sterile ddH_2O and by gentle agitation on a rotary flatbed shaker on low speed to expedite resuspension.

Quality of DNA was estimated by gel electrophoresis and quantification of DNA obtained using a NanoDrop Spectrophotometer (NanoDrop Technologies). DNA quality was further assayed by polymerase chain reaction (PCR) amplification of the ITS region (Baldwin 1992) using primers ITS1 and ITS4 (White *et al*. 1990). Products were amplified using Buffer E (Epicentre) in 25 μL reactions with undiluted DNA and DNA dilutions in sterile water of 1:20 and 1:40. Amplification cycling followed Baldwin (1992). Products were quantified by agarose gel electrophoresis with a standard 100 bp ladder (New England Biolabs) on a Fluor-S Imager (Bio-Rad).

Results

In the field, samples can be collected in polypropylene blocks containing premeasured portions of silica gel for sample drying and grinding used during the extraction process. Silica gel at 0.25 g was sufficient to dry most samples and produce sufficient DNA for PCR amplification (Tables 1 and 2). This permits naturalists, breeders, and collaborators to collect a large number of samples in a short amount of time and allows samples to dry quickly, within 2 days, thus preserving the samples. The mouse organ tissue (e.g. liver, kidney and heart) did not dry within 2 days, and additional silica gel (0.25 g) was added to expedite desiccation.

By placing samples directly in the 96-well blocks with desiccant, only a small amount of tissue for DNA isolation is used, and the need for a liquid nitrogen source and storage containers and the use of large amounts of silica gel are eliminated. As little as 11 mg of leaf or insect tissue was sufficient to obtain DNA, although not all samples yielded measurable amounts (> 1 ng/μL). No extra equipment was necessary for storage or shipping of silica gel-desiccated samples. Populations of rare organisms and experimental genetic lines are preserved since a small amount of tissue (< 1 g) is required.

Amplification of ITS indicated that samples with high DNA yields needed to be further diluted to facilitate amplification. Each taxon resulted in different optimal tissue quantities and it is recommended that prior to large-scale collection and extraction, parameters should be tested and optimized for the study taxon. Amplification of microsatellites, single nucleotide polymorphisms, or other techniques used in genotyping would need to be optimized for DNA quantity obtained using this technique.

Discussion

The amount of DNA obtained and successful amplification from samples did not correspond to the amount of silica gel used (Table 1). Efficient drying of larger tissue amounts with the greater quantity of silica gel, however, resulted in increased yields of DNA. In general, the ratio of 50 mg leaf or insect tissue to 0.25 g of silica gel was found to be

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adequate. For work with small insects, combining multiple individuals is necessary in order to obtain sufficient yield of DNA for detection and amplification. This could provide interference in fine-scale genetic analyses of insect populations. For larger insects, the removal of wings (elytra) and humane termination (e.g. refrigeration) are recommended for sufficient tissue grinding using silica gel. For plant material, fresh, young leaf tissue yields the greatest quantity DNA with high quality. If seeds are to be used that have a thick outer seed coat, it is recommended that the coat be removed to facilitate tissue grinding using silica gel. Fruit and mushroom tissue did not grind well, as the tissue became too hard when dried and is not recommended for this technique; rather a standard CTAB protocol (Doyle & Doyle 1987) or storing samples in CTAB (Aime & Phillips-Mora 2005) may work best. For animal tissue, a minimum of 0.5 g of silica gel is recommended in order to dry samples within 24 h and prevent spoilage.

In comparison to other high throughput DNA extraction methods, the presented protocol reduces costs relative to extraction kits, successfully produces DNA in a wide variety of organisms and tissue types for PCR amplification, and offers an alternative in sample storage and grinding technologies. Unlike single-sample methods, the use of 96-well blocks and the option of robotics increases efficiency in downstream applications such as genetic screening. Using 0.5 g of silica gel per sample resulted in a cost of approximately $0.05 per sample, compared to $2–3 per sample for single-tube kits and $1–2 per sample for plate-format kits. The success across the taxonomic range tested here in DNA quantity and PCR amplification is comparable to other high throughput methodologies using fresh-leaf samples (Dilworth & Frey 2000), laboratory-grown seedlings (Mace *et al*. 2003), and fish larvae (Richardson *et al*. 2007). The application of silica gel to both dry and grind tissue samples limits cross-contamination risks and eliminates the need for stainless steel grinding balls (Mace *et al*. 2003), glass beads (Dilworth & Frey 2000), and magnetic beads used in several kits (e.g. Qiagen, Promega, etc.).

This technique has been used in preparation of plant tissue in assessing population genetics and gene flow in Canada thistle (Slotta *et al*. 2006). Plant material was collected in the field in blocks as described. Additionally, samples were collected from smaller populations by collaborators in small zip-top bags containing silica gel then transferred to blocks for grinding and extraction. For optimal results, a single, young leaf (< 50 mg fresh weight) and a threefold dilution of the DNA prior to amplification yielded reproducible products in the analysis of microsatellites.

This technique has been used in the Small Grains Genotyping Laboratory to process wheat and barley samples for marker-assisted breeding projects (Brady and Chao unpublished data). Typically, one piece of one-and-a-half inch leaf blade at the three-leaf seedling stage was collected, folded and inserted in the block with 0.25 g silica gel. The DNA extraction process has been automated by using robotics to add and transfer buffers in multiple blocks at one time in order to further expedite output. A total yield of approximately 20 μg DNA can be obtained from approximately 25 mg of fresh weight tissue for both wheat and barley using this protocol. Caution must be taken to avoid collecting more tissue than the amount tested and optimized, as this can often lead to slow drying and sample degradation or mould growth during shipping and drying.

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