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A simple method of preparing plant samples for PCR

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The polymerase chain reaction (PCR) (1) is being increasingly used for detecting specific DNA sequences in plants (e.g. 2, 3). The time and effort required for DNA sample preparation is often the limiting step. Although several protocols are available for this purpose (4, 5, 6, 7, 8), all involve multiple steps. Since PCR requires only a minute quantity of template DNA for successful amplification and has good tolerance toward crude DNA preparations, it might be possible to extract sufficient DNA in an appropriate buffer and use it directly for PCR. The requirements for such a buffer would be that it allows sufficient DNA extraction while at the same time not inhibiting the amplification reaction.

Initial protocol developed for *Arabidopsis thaliana*

To explore such a possibility, a cold- and ABA-induced gene from *Arobidopsis thaliana* was chosen as the sequence to be amplified (9). PCR using two primers (left, 5′-TACTCGTGGC-ACCACACTCC-3′; right, 5′-TGCAGCATCCTTGGCC-TTGT-3′) amplifies a 395 bp fragment from purified genomic DNA. Initial extractions were performed with TE buffer modified by addition of substances such as non-ionic detergent NP-40, proteinase K or EDTA. The results were unsatisfactory (Figure 1, lane 2 and 3). Subsequently, the standard *Taq* PCR buffer (1×, consisting of 10 mM Tris, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin and 3 mM DTT (dithiothreitol) with pH 8.3), was investigated. Good results were obtained by extraction using concentrated PCR buffer in the presence of NP-40. In all the tests duplicate samples were prepared for the same treatment.

The procedure was as follows: *A.thaliana* leaves from greenhouse-grown plants were placed into a 1.5 ml tube and, for every mg of leaf tissue, 2 μl of 6×concentration of PCR buffer (1× to 10× were tested) containing 0.5% NP-40 was added. The leaf tissue was ground with a disposable pestle. The suspension was briefly centrifuged, then used directly for PCR.

PCRs were performed in a total volume of 40 μl consisting of 1× PCR buffer, 200 μM each of dNTPs, 0.25 μM each of the two primers, 1 U Taq DNA polymerase and 0.5 μl of the leaf extract. The amplification profile consisted of 1 min at 95°C, 30 cycles of 50s at 94°C, 50s at 57°C and 60s at 72°C followed by 3 min at 72°C for final extension. An aliquot of 7 μl was used for electrophoresis. Figure 1 shows some of the results. Amplification improved dramatically with increased PCR buffer concentration up to at least 6× (lanes 4–13 of Figure 1).

Identification of alkaline condition as the main factor for improved DNA extraction

When the above protocol was applied to several other plant species the results ranged from good (e.g. flax) to poor (e.g. tobacco). We therefore decided to identify the critical factor(s) which caused the improved sample preparation with concentrated PCR buffers, in order to modify the protocol to be more widely useful. Samples were prepared using a series of solutions, consisting of standard PCR buffer with elevated concentrations (10× relative to the standard PCR buffer) of individual components: Tris (100 mm, pH 8.3) or KCI (500 mM) or $MgCl_2$ (15 mM) or DTT (30 mM). In addition to *A.thaliana*, transgenic plants of *Brassica napus* were also used. A left primer 5′-GTGGAGAGGCTATTCGGCTA-3′ and right primer 5′-CC-ACCATGATATTCGGCAAG-3′ were used to amplify a 553 bp fragment from the NPTII (neomycin phosphotransferase II) coding sequence (10) in the transgenic plants. The results are shown in Figure 2A. In both species, 100 mM Tris produced the best result (Figure 2A: lanes 2 and 7). Increased concentration of KCl (500 mM) (Figure 2A: lane 8) or DTT (30 mM) (Figure 2A: lane 5) had some benficial effects at low Tris concentration (10 mM). Subsequently, KCl and DTT were further tested at higher concentration of Tris (100 mM, pH 8.3). Neither of these two factors at any of the concentrations tested (KCl, 0–500 mM and DTT, 0–100 mM) had a clear effect on already improved amplification (data not shown). Therefore, Tris was identified as the critical factor.

To further confirm the above results and to evaluate the optimal pH range, extracts were prepared using a set of buffers consisting of 1× PCR buffer but containing 100 mM Tris at pH 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, 10.0, 10.5, 11.0 or 12.0. Results using 0.5 μl extract are shown in Figure 2B. Good amplification was obtained only when the pH of the buffer was equal to or greater than 8.0. These results imply that better DNA extraction was achieved at alkaline conditions.

A general protocol for many plant species: NaOH extraction method

Since alkaline pH was identified as the most important factor, it was further reasoned that nuclear DNA might be optimally extracted with NaOH, which at the same time might inactivate nucleases during extraction. Indeed, positive results were obtained with extracts prepared using 0.1–1.0 N NaOH. Subsequent experiments were performed to optimize the conditions. Effects of some additives (salt-KCl, detergent-NP40, DTT and β-mercaptoethanol) were examined but none of them clearly improved the results. These experiments thus lead to the following protocol:

(1) Place a few milligrams of young leaf (callus or cotyledon) into a 1.5 ml tube and, to every mg of tissue, add 10 μl 0.5 N NaOH. Note: Sampling could be conveniently done by punching a leaf disc and adding the same amout of solution to each disc sample.

(2) Grind until no large pieces of tissue are left.

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(3) Transfer 5 μl quickly to a new tube containing 495 μl 100 mM Tris pH 8.0, mix well and use 1 μl directly in 30–40 μl PCR run for 35 cycles. Note: This will give a 1/100 dilution from the original extract. Centrifuge is not necessary if pipetting is not blocked. The sample should be stored at −20°C if it is not used right away.

Using this protocol specific DNA sequences were detected in seven diverse plant species and so far no species has proved recalcitrant. Results of six species, *A.thaliana*, *B.napus*, tobacco, flax, pea, *B.olerecea*, are shown in Figure 3. For *A.thaliana* the primers and target gene were as described above. For all the other species, transgenic plants containing the NPTII gene were used to reduce the number of primers required for different species.

This 'grinding and use' protocol does not require organic solvent or other treatments (e.g. heating, proteinase treatment etc.). It should save considerable time and reduce cross-contamination problem relative to most previous protocols involving partial purification. The method employing very small leaf pieces directly in PCR (11) produced poor results in our hands. Using tissues from the same sources as in Figure 3, results were all negative except for *A.thaliana* (data not shown). A more recent protocol (12) uses extract directly but still requires liquid nitrogen treatment, boiling etc. The present method will be particularly useful in screening large numbers of plants for defined DNA sequences. Since only a tiny amount of material is used, screening can be performed at early stages of plant regeneration or on small callus pieces.

The success of this protocol is probably due to the improved nuclear DNA extraction using alkaline (NaOH) solution, which in turn allows sufficient dilution of the extract to eliminate or significantly reduce the effect of potential inhibitors on PCR. It is noted that among the species tested *A.thaliana* was the most flexible material to produce PCR amplifiable extracts. Reproducible results were obtained using either NaOH extracts or extracts with a Tris buffer of alkaline pH. In the latter case either the original extracts or their 1/100 dilutions worked equally well. In addition, different types of tissues were also used in the study. Younger tissues (*in vitro* plantlets, callus, young leaves) in general gave better amplification. When applied to other plant species or new materials, a simple testing may be needed to determine the optimal dilution (at Step 3 in the protocol), which is usually around 1/100 and between 1/10 or 1/1,000 of the original extract.

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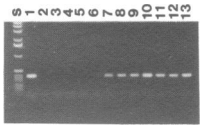

Figure 1. PCR results using samples prepared from *A.thaliana* leaves in different extraction buffers. To each PCR of 40 μl, 0.5 μl sample was added directly. Lane S, 1 kb DNA ladder standard (BRL). Lane 1, positive control using 50 ng of purified genomic DNA (in 0.5 μl volume) from leaves. Lanes 2 and 3 are samples prepared using TE buffer: lane 2, TE; lane 3, TE + 0.5% NP-40. Lanes 4 to 13 are samples prepared using 1× to 10× concentrations of PCR buffer all containing 0.5% NP-40: lane 4, 1×; lane 5, 2×; lane 6, 3×; lane 7, 4×; lane 8, 5×; lane 9, 6×; lane 10, 7×; lane 11, 8×; lane 12, 9×; lane 13, 10×.

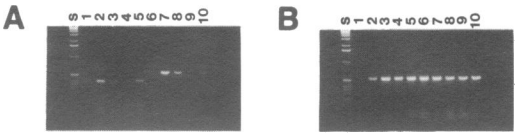

Figure 2. A Effect of buffer composition on preparing DNA extracts directly used for PCR. *A.thaliana* (lanes 1–5) and *B.napus* (lanes 6–10) leaves were extracted using standard 1× PCR buffer or modified with 10× concentration of individual components (all containing 0.5% NP-40): lanes 1 and 6, 1× PCR buffer alone; lanes 2 and 7, Tris increased to 100 mM (pH 8.4); lanes 3 and 8, KCl increased to 500 mM; lanes 4 and 9, $MgCl_2$ increased to 15 mM; lanes 5 and 10, DTT increased to 30 mM. PCR was run for 30 cycles for *A.thaliana* or 35 cycles for *B.napus* using 0.5 μl extract. **B** Effect of buffer pH on preparing DNA extracts directly used for PCR. Extracts were prepared from *B.napus* leaves and 0.5 μl extract was used in PCR. All buffers consisted of 1× PCR (except Tris increased to 100 mM) and 0.5% NP-40 with pH adjusted to (from lane 1 to 10) 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, 10.0, 10.5, 11.0 or 12.0.

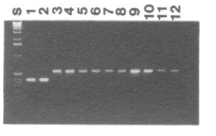

Figure 3. Results of PCR using DNA extracts of different species prepared by NaOH extraction method. Extracts (two from each species) were prepared according to the protocol and 1 μl of the 1/100 dilutions from the original extracts were used in PCR run for 35 cycles. The lane numbers and corresponding extracts are as follows: Lane S, 1 kb DNA standard; lanes 1–2, *A.thaliana* (greenhouse plants); lanes 3–4, *B.napus* (*in vitro* propagated plants); lanes 5–6, tobacco (greenhouse plants); lanes 7–8, flax (seedlings); lanes 9–10, pea (greenhouse plants); lanes 11–12, *B.olerecea* (*in vitro* plants).