



An investigation of a rapid DNA extraction method for routine MAS in the S.A. Barley Improvement Program

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Abstract

A crucial, but limiting step in any MAS program is the reliable and efficient isolation of DNA. While there are numerous DNA extraction protocols and commercial DNA isolation kits available, their cost in barley breeding programs is not economically feasible. Breeders need high-through-put DNA isolation which can be performed on thousands of individuals. An alkaline method was tested for its suitability and it was found that the DNA samples are suitable for only PCR based procedures. Another limitation is that the extracted DNA needs to be processed immediately, and is not suitable for long-term storage.

Introduction

Many traits of importance to barley improvement programs have been mapped allowing plant breeders to apply MAS to a range of agronomic and quality traits. MAS gives breeders an opportunity to complement phenotypic selection by providing faster backcrossing strategies, allele enrichment in complex crosses and double haploid production and ultimately facilitates the early release of superior malting lines. The South Australian Barley Improvement Program is currently screening 3,500 early generation samples with markers, which could potentially increase to 20,000 lines in the future. Initially RFLP markers were used for marker-assisted selection, however limitations in marker throughput, assay cost and levels of polymorphism have made RFLP markers somewhat redundant. Therefore PCR based markers such as SSRs were adopted which are more suitable for large-scale application. However, the rate limiting factor at this stage is still the DNA sampling and extraction. A rapid NaOH extraction procedure according to Schiemann and Backes (2000) using a 96-well Elisa-plate was assessed for its suitability across a range of markers routinely used in the S.A. Barley Improvement Program.

Materials and Methods

The steps in the NaOH DNA extraction were as follows:

1. Collect 5 pieces from a 2-3 week old seedling of each sample (approximately 3mm) and place into the well of a 96 well Elisa plate
2. Aliquot 50ul of 250mM NaOH to each sample then transfer to a boiling water bath for 60 secs
3. Crush the tissue with the aid of 48 pin stainless steel plant crusher (6cm long and 3mm in diameter) which fits the Elisa plate format
4. Aliquot 120ul of 100mM Tris-HCL pH 8.0 to each well using a multi-pipette
5. Plates are then briefly centrifuged and stored at 4°C.

This compares to the current DNA extraction, which has the following steps:

1. Collect a 15cm long piece of leaf tissue from a 2-3 week old seedling, fold in four and place in the bottom of a 10ml round bottom screw cap collection tube
2. Add 4 small (3mm) and 1 large (9mm) ball bearing, to each collection tube and freeze samples in liquid nitrogen for 5 minutes
3. Grind the tissue to a fine powder with the aid of a flask shaker
4. Once all samples are ground the ball bearings are retrieved (for re-use) with a magnet
5. Allow samples to slightly thaw for 5mins then add 700ul of DNA extraction buffer
6. Briefly vortex each tube and then add 700ul of phenol/chloroform/isoamylalcohol (25:24:1) and mix for a further 15 minutes on a shaking platform
7. Add .5ml of silica gel matrix to each tube and centrifuge at 4000rpm for 10 minutes
8. Pour off the upper aqueous phase into a 1.5ml eppendorf tube and add 600ul isopropanol and 60ul Na Ac pH 4.8 and precipitate by inversion using an orbital mixer for 5 minutes
9. Centrifuge samples for 5 minutes and pour off the supernatant without dislodging the pellet. Add 1ml 70% ethanol, discard supernatant then centrifuge tubes again for 2 minutes and pipette any remaining ethanol
10. Leave the samples at room temperature for 15-20 minutes to ensure that all the ethanol is removed
11. Resuspend pellet in 60ul of R40 and store at 4°C.

PCR amplification

DNA was extracted from Arapiles and Sloop samples using the NaOH method and were directly used for PCR using a very robust SSR marker following the method of A. Karakousis *et al.* (2000). A period of 10 days was allowed to elapse and the

samples were screened with the same marker. After 4 weeks of storage at 4°C again the samples were used for PCR. A 4.5 µl aliquot of the samples were loaded on 8% denaturing polyacrylamide gels and run for 3 hours at a constant voltage of 300V. Gels were stained with ethidium bromide and visualized under a UV transluminator.

Results and Discussion

The current modified DNA extraction protocol (Steiner *et al.*, 1995) relies on a multi-step procedure where samples are treated individually. This method allows one person to extract a maximum of 144 per day. With the projected scope and scale of the molecular marker program such a method would not be feasible for MAS because of the length of time required to process the samples and the difficulty in handling such large numbers in a short period of time.

In comparison, the NaoH method is only a two step procedure where tissue is ground and then neutralised. One person (Schiemann and Backes 2000) is able to collect and process in the order of 400 samples per day. Both the modified DNA extraction procedure (Steiner *et al.*, 1995) and NaoH method were applied to a number of crosses pre-screened for double haploid production for a number of traits. To ensure identical gel and running conditions the samples for the two extraction methods were loaded on the same gel. The PCR results from the DNA extracted using the two methods were similar. (Data not shown).

One concern was the stability of the DNA during storage, so the NaoH method was tested on Sloop and Arapiles lines using a microsatellite marker HVMIO1A which is linked to MLO and produces a very clear intense banding pattern. Using the NaoH method, an almost perfect amplification rate with 96% amplification was achieved. However, only 83% of the samples would amplify after 10 days of storage at 4°C. It is quite evident that the samples were very degraded after 4 weeks of storage at 4°C with <30% showing a banding pattern which varied in intensity between samples (Figure 1).

A possible solution could be to store samples at -20°C or -80°C. This will need further investigation but even at these lower temperatures degradation could still be occurring possibly at a slower rate. The HVMIO1A marker is one of the more robust and reliable SSR markers currently in routine application, and the remaining 12 SSR markers exhibit a range in sensitivity to template purity and tendency for preferential amplification.

Additives such as DMSO and Betaine were also added to the PCR reaction, since they were reported by Henke, *et.al.*, (1997) to greatly improve PCR amplification. Our data supports these findings and such additives could be added to PCR reactions to attain better amplification of the PCR products.

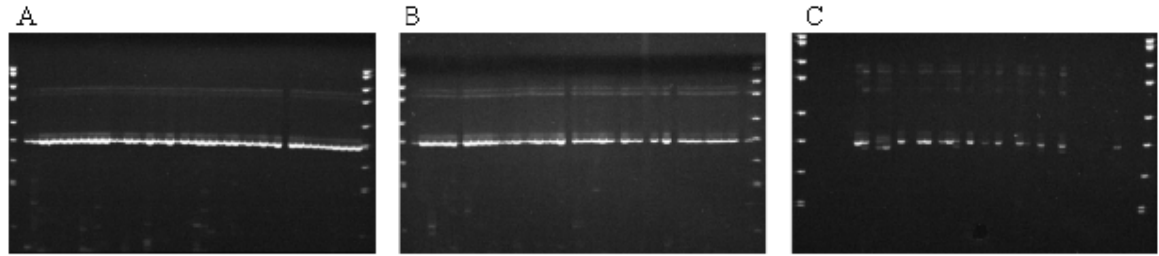


Figure 1: PCR amplification of HVML01A SSR marker on NaOH extracted barley lines (lanes 1-24: Sloop, lanes 24-48: represent Arapiles). (A) Day 1 extracted DNA (B) After 10 days storage at 4°C (C) After 4 weeks storage at 4°C).

The main advantage of the NaoH method is the speed and cost of this method. A cost analysis was undertaken for the two DNA extraction protocols, which included consumables and a labour component. The cost of the NaoH method was calculated at approximately \$0.54 per sample, one-third of the price for the current method which was estimated at \$1.60 per sample. Commercial kits are also available and the price per sample is approximately \$4.00 per sample which does not include a labour component.

There are obvious advantages in adopting the NaoH method in the S.A. Barley Improvement Programme because it allows one person to process approximately 400 per day as opposed to the current method which allows a total of only 144 samples per day. However, one serious drawback with the NaoH method was that the DNA could not be used for other marker systems such as RFLP, and AFLPs. Other concerns relate to the time required to harvest the material into the ELISA plates, estimated at 45mins -1 hour to harvest one Elisa plate. In the S.A. Barley Improvement Program a significant proportion of the breeding material is screened for multiple traits, and if the NaoH method was employed it would require careful sample management and planning to ensure assays were completed prior to DNA degradation.

A reliable cost-effective method that yields DNA that could be stored for periods of up to 3 months or longer and could be used for other marker systems for both wheat and barley implementation would be ideal. We are currently assessing alternative methods where plant tissue is lypholized and then undergoes a rapid phenol-chloroform extraction. The increasing demands for marker assisted selection in barley and other crops requires logistic and technical solutions to provide cost-effective high-throughput screening. The rate limiting step of DNA extraction will continue to be a focus for R & D activities within marker implementation programs.

Acknowledgments

The authors wish to acknowledge the financial support of the GRDC through the NBMMP UA424.

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