

## A Rapid DNA Mini-prep Method for Large-Scale Rice Mutant Screening

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**Abstract:** A high throughput rice DNA mini-preparation method was developed. The method is suitable for large-scale mutant bank screening as well as large mapping populations with characteristics of maintaining relatively high level of DNA purity and concentration. The extracted DNA was tested and suitable for regular PCR amplification (SSR) and for Targeting Induced Local Lesion in Genome (TILLING) analysis.

**Key words:** rice; targeting induced local lesion in genome; DNA extraction; polymerase chain reaction

With the completion of whole genome sequence, focus on rice research has been apparently shifted from sequence analysis (genomics) to identifying and understanding the functions (functional genomics) of nearly 40 000 genes presented in rice genome<sup>[1-3]</sup>.

Gene functional identification requires new materials and methods. Mutant is considered as one of the most important sources of starting materials. In mutant detection, a new reverse genetics strategy termed TILLING (Targeting Induced Local Lesion in Genome), has been developed by the Fred-Hutchinson Cancer Institute, Seattle and Washington University in a study on point mutation of *Arabidopsis*<sup>[4-5]</sup>. The original TILLING methodology was to use the denaturing HPLC (High Performance Liquid Chromatography) for single nucleotide mutation discovery. Subsequently, it has been adapted into a high-throughput screening method with high precision and low cost for detection of point mutation using Cel I enzyme and fluorescent labeling, which not only can detect point mutation but also small Indels (inserts and deletions) induced by the chemical mutagen ethyl methanesulfonate (EMS). In theory, the method can be applied to any point mutation detection presented in genomes of both animals and plants.

We introduced TILLING technique to a most important monocot model plant and set up a new TILLING technology system for rice in 2003. The TILLING requires a high quality of template DNA due to its high precision. The ordinary DNA isolated method with poor DNA quality hardly meets

the TILLING requirement, leading to unstable PCR reaction. Moreover, we have developed a new rapidly mini-prepared DNA extraction method, to isolate DNA, such as mutant DNA extraction.

## MATERIALS AND METHODS

### Leaf sampling and conservation

During the experiment 384 mutants from rice variety IR64 treated by EMS were planted in the greenhouse at the International Rice Research Institute. One leaf was sampled from each mutant and conserved in liquid nitrogen or a  $-80^{\circ}\text{C}$  refrigerator in coin-envelops punched with a number of small holes. Then the samples were lyophilized by using Labconco Freeze Dry System to freeze-dry at  $-21^{\circ}\text{C}$ ,  $33\times 10^{-3}$  Pa or less for 24 h, and stored at  $4^{\circ}\text{C}$ , which can be conserved for more than one year in the sealed envelops with silica gel.

### Mini-prep DNA extraction

The mini-prep DNA was extracted as follows:

1) 30 mg of freeze-dry leaves were cut into small pieces using scissors and transferred them into sterilized DNA-extraction plates (Axygene Scientific) with 96 wells (1.64 mL/well) through a funnel, with one sample in one well. Four 96-well plates can accommodate 384 samples.

2) After loading the steel beads with diameter 3 mm into the wells with the help of a home-made bead loader, the plates were sealed by covers (AxyMat) and placed in Genogrinder 2000 (SPEX CertiPrep Inc.) for 30 min, and then centrifugated at 3000 r/min for 30 s.

3) After removing the covers, the steel beads were removed

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by a home-made bead picker and then 660  $\mu\text{L}$  extraction buffer (100 mmol/L Tris-Cl pH 8.0, 500 mmol/L NaCl, 20 mmol/L EDTA) were added into each well. Then the DNA-extraction plates were sealed and shaken vigorously till the samples were mixed completely.

4) After centrifugation, the cover was removed and 40  $\mu\text{L}$  of 20% SDS solution were added. The solution was mixed gently, while the DNA-extraction plates were incubated at 65°C for 15 min.

5) Later 100  $\mu\text{L}$  of 5 mol/L NaCl solution and 90  $\mu\text{L}$  10 $\times$  CTAB (1% CTAB, 0.7 mol/L NaCl) were added into the plates and mixed to incubate at 65°C for 15 min.

6) The 700  $\mu\text{L}$  chloroform (chloroform : isoamyl alcohol = 24 : 1) were added and thoroughly mixed, then the plates were incubated at room temperature for 10 min.

7) After spinning down at 3 000 r/min for 30 min at 4°C, the supernatants were transferred into sterilized plates.

8) The 1/2 volume of pre-cooled isopropanol was added and mixed gently, while the plates were incubated at room temperature for 5 min, centrifuged at 3000 r/min for 15 min at 4°C. Discarded the supernatant.

9) The 200  $\mu\text{L}$  of 70% ethanol was added to rinse the DNA pellet and centrifuged at 3 000 r/min for 2 min, then the ethanol was discarded gently.

10) The DNA pellet was air-dried or breeze-dried in Laminar Flow, dissolved in 50  $\mu\text{L}$  of 1 $\times$ TE with RNase at concentration 10  $\mu\text{g}/\text{mL}$ , and incubated in water-bath at 37°C for 1 h.

11) 2  $\mu\text{L}$  of DNA solution were used for quantity assay.

#### DNA quantification

To quantify DNA concentration, an electrophoresis was performed on 1% agarose gel by loading a mixture of 2  $\mu\text{L}$  DNA and 10  $\mu\text{L}$  of 1.2 $\times$  loading dye. Standard  $\lambda\text{DNA}$  with 20 ng, 40 ng, 60 ng, 100 ng, and 120 ng were loaded respectively. After running for about 2-3 h, the agarose gel was soaked in freshly prepared ethidium bromide (EB) solution with a working concentration of 0.5  $\mu\text{g}/\text{mL}$  for 15 min. Pictures were taken for analysis of the DNA concentration using AlphaImager 5.0 (Alpha Innotech Corporation).

#### PCR amplification

The 36 DNA samples and 12 pairs of primers were

randomly selected to validate the quality of DNA extraction for conventional PCR amplification and TILLING analysis. Among them six were SSR markers (RM17, RM19, RM85, RM190, RM208 and RM5414) and another six were TILLING primers (Table 1), to carry out PCR following the methods of Chen et al [6] and Till et al [5], respectively.

## RESULTS AND DISCUSSION

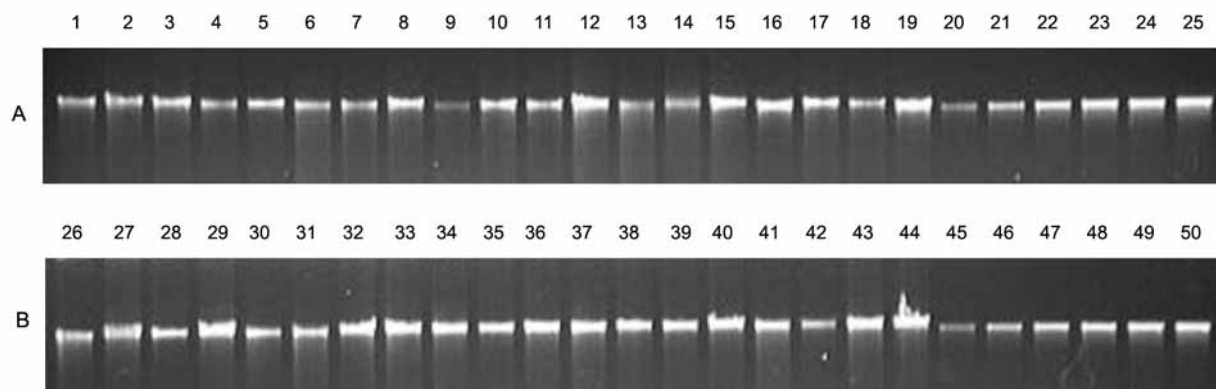
#### DNA quantity

Fig. 1 shows the DNA concentration of 38 mutants, of which mutant 9 (lane 9) was the lowest in concentration with 8.1 ng/ $\mu\text{L}$ , while mutant 38 (lane 44) was the highest with 69.9 ng/ $\mu\text{L}$ . The average DNA concentration was 39.8 ng/ $\mu\text{L}$ . It was clear from the picture that the molecular weights of mutant DNAs were higher than those of  $\lambda\text{DNA}$  standard, indicating the high integrity of the mutant DNA. Fig. 2 shows the concentration distribution of the 38 mutant DNA, from which we could note that the majority of mutant DNA concentration was between 20 and 60 ng/ $\mu\text{L}$ , indicating that 1-3  $\mu\text{g}$  DNA were retained from 30 mg of freeze-dry leaves. The amount of DNA harvested was similar to that reported by Chen and Ronald [7]. The method we reported here was likely to accommodate more samples in each round of extraction. The amount of DNA extracted by our method was enough for 100 to 300 SSR reactions using 10 ng/ $\mu\text{L}$  as template per reaction. Moreover, it has been noted that some of the mutants were relatively lower in DNA concentration, due to weak growth such as yellowish leaves and spotted leaves.

Previously, the DNA mini-prepared extraction methods have been reported [8-9], while most of them emphasized mainly on the quickness and simplicity of the methods. Thus, the quantity and quality of the DNA were usually only good applicable for ordinary PCR, but unstable amplification was frequently encountered. Furthermore, most rapid DNA mini-prep extraction methods were limited in throughput due to the application of centrifugal tubes [10-11]. Our method pays more attention to high throughput by using DNA extraction plate with 96 wells. It is especially useful for screening of a large number of samples, such as mapping population and mutants. In addition, the amount of DNA should be enough for  $F_2$  and  $M_2$  populations. The DNAs extracted by our method

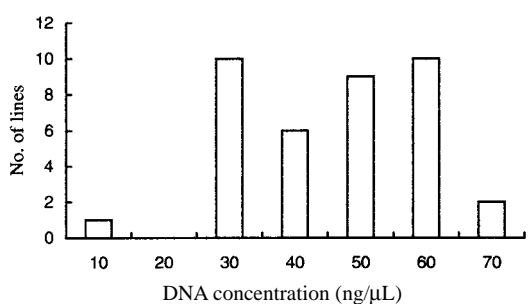
**Table 1. Primers used for TILLING.**

Primer	Primer sequence (5' - 3')	
Ox-7-1	GAACAAATCTTGACTAAACGCTCA	GCTAACAGTTGTGTTTATCGTTG
Ox-7-2	CTTCCAAATATACGTGGACTATCG	TGTTTGGATTAGATGTGACAAAGC
Ox-7-3	GCAGATCCCTGGACTTAATACT	AATATCAGTGGTAATGGTGGTGTG
Ox-8	GCTTAATTGACTTGTTCTCCATCA	AGTCCGTAAGCGGACAATATTTAG
Ox-9	AGAGAAGATAGCAGAAACCCAAAG	AGCTTGCAAGTATGCATAACAAGT
Ox-10	GCGGCTATATAAGCACATTAATCC	TGGAAGAACGGATAAGAAATACAA



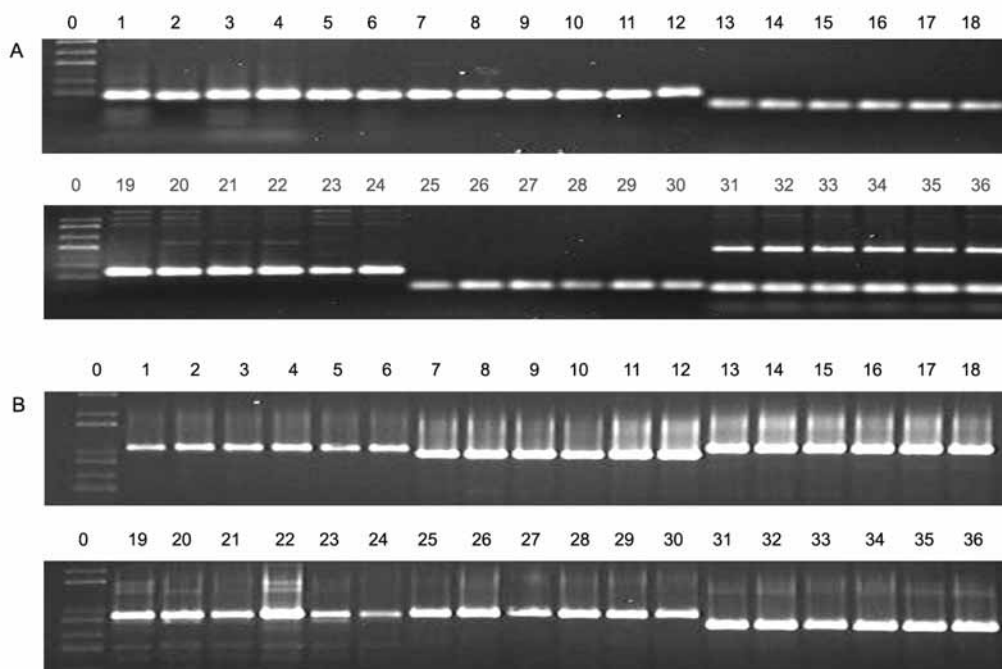
**Fig. 1. DNA concentrations of 38 mutant samples.**

Lanes 1 to 19 in A and lanes 26-44 in B are samples, lane 20 to 25 in A and 45 to 50 in B are  $\lambda$ DNA standards for 20, 40, 60, 80, 100 and 120 ng, respectively.



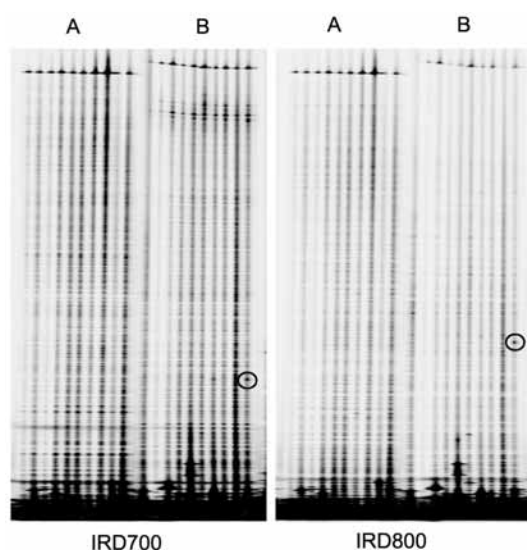
**Fig. 2. Concentration distribution of mutant DNA.**

were confirmed both in high throughput and quantity. The key steps in our method were to cut leaves into small pieces and transfer them into the plate wells. The time from adding extraction buffer to DNA precipitation is the same as in ordinary DNA extraction method. Generally using the method, a technician would complete extraction of 384 samples easily within one day, therefore a mutant bank with a size of 20000 lines can be completed within two and half months by one person in 20 working days per month.



**Fig. 3. PCR amplification of 36 DNA samples detected on 1.2% agarose gel.**

A, Using 6 SSR primers. Lane 0 is marker; Lanes 1 to 6 from RM208; Lanes 7 to 12 from RM17; Lanes 13 to 18 from RM5414; Lanes 19 to 24 from RM19; Lanes 25 to 30 from RM190 and Lanes 31 to 36 from RM85. B, Using 6 primers for TILLING. Lane 0 is marker; Lanes 1 to 6 from primer Ox-8; Lanes 7 to 12 from primer Ox-9; Lanes 13 to 18 from primer Ox-10; Lanes 19 to 24 from primer Ox-7-1; Lanes 25 to 30 from primer Ox-7-2 and Lanes 31 to 36 from primer Ox-7-3.



**Fig. 4. Detection of PCR products on 5%PAGE for discovery of point mutation by TILLING.**

A, Ten samples amplified from primer Ox-8. B, The same ten samples amplified by primer Ox-7-3, and one mutation was detected in sample 10 indicated by a circle in channel IRD700 and confirmed in channel IRD800.

#### PCR analysis

The DNA extracted by our method was applied to PCR analysis. The results showed distinct products amplified both using 6 pairs of SSR primers (Fig. 3-A) and 6 pairs of TILLING primers (Fig. 3-B). We detected a point mutation in gene *Ox-7-3* on chromosome 8 after screening a large number of mutants (Fig. 4). We further detected other 4 point mutations in 2 genes (*pp2a* and *cal7*)<sup>[12]</sup>. These results suggested that the DNA extraction method with high DNA quality was suitable for TILLING analysis, as well as general PCR. We believe that this method also can be applied to other plants and crops.

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