

Application of TILLING®/EcoTILLING To Screen For Small Mutations in Mammals

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ABSTRACT

We demonstrate the use of TILLING®/EcoTILLING for rapid identification of mutations in mice. TILLING was tested to determine if a known point mutation, *albino* (*Tyr^c*), a G to C change in the tyrosinase gene, could be detected in genomic DNA. Successful results utilizing mung bean and Surveyor™ nucleases suggest that this technique could be used to uncover point mutations, even if the source is a small portion of a pooled set of mouse genomic DNA isolates.

The second application was to determine if TILLING can be used to rapidly identify an unknown point mutation from a set of candidate genes. An N-ethyl-N-nitrosourea (ENU) mutagenesis project at University of Nebraska-Lincoln was initiated to screen mice for a variety of abnormalities, including hair loss problems. A new semi-dominant mutant with progressive hair loss was identified and mapped to mouse Chromosome 11. Three candidate genes in a non-recombinant region were selected and analyzed for point mutations using TILLING. The results indicated a mutation, and sequencing identified a C to T change in a conserved part of the last exon. The same technique, used with another hair loss mutant, *Red^{den}*, that maps to the same region, resulted in detection of a mutation in a different part of the same exon. The speed at which this data was acquired by combining TILLING with the LI-COR DNA Analyzer makes this technique ideal for rapid candidate gene analysis of small mutations in mammalian genomic DNA.

INTRODUCTION

TILLING and EcoTILLING are closely related methods useful in rapid detection of small mutations or natural polymorphisms, respectively. These methods rely on the enzymatic cleavage of heteroduplexed DNA with a single strand specific nuclease (i.e., Cel I, mung bean nuclease, S1 nuclease, etc.). A thorough study by Till et al. (2004) concluded that many of these nucleases can be effective in polymorphism detection under the proper conditions.

To determine if TILLING could be used to detect point mutations in mice, the procedure was tested using genomic DNA from *albino* (*Tyr^c*) and pigmented mice. Details on optimization of the procedure, pooling DNA samples, types of single strand specific nuclease (Surveyor™ nuclease (Transgenomic) and mung bean nuclease (NEB)), and nuclease concentrations, are discussed.

In collaboration with the University of Nebraska-Lincoln, TILLING was used as part of an effort to identify unknown small or point mutations in mouse mutants. An N-ethyl-N-nitrosourea (ENU) mutagenesis project, initiated to screen mice for a variety of abnormalities, led to identification of a new dominant hair loss mutant. Genetic mapping of the

mutation using microsatellite markers narrowed the area containing the mutated gene to a 1.77 MB region on distal mouse Chromosome 11. Three candidate genes were selected in this non-recombinant region for analysis with TILLING. A mutation was found in one of the candidates, Gasdermin3. A second mouse mutant, *Red^{den}*, with a similar hair loss phenotype mapping to the same region, was also analyzed for mutations using TILLING. The results support the hypothesis that Gasdermin3 is important in hair growth and maintenance. Results of these studies suggest that TILLING can be a fast, reliable, inexpensive method of detecting point mutations in the mouse.

MATERIALS AND METHODS

IRDye™ 700-labeled forward and IRDye™ 800-labeled reverse primers were designed to amplify regions of approximately 1 to 1.5 kb spanning exons for all candidate genes. Primers were tested for effective target amplification prior to TILLING using ExTaq polymerase (Takara; RR001A). Initial PCR amplification of the target region was done using 12.5–100 ng genomic DNA from wild-type and mutant DNA samples and ExTaq polymerase (PCR conditions: 1 cycle of 94°C for 2 min; 30–40 cycles of 94°C for 30 sec, 64°C for 1 min, 72°C for 1.5 min; and 1 cycle of 72°C for 10 min; and hold at 4°C). Formation of heteroduplexes is performed by mixing 50:50 target regions from wild-type and mutant reactions and annealed using PCR conditions as follows: 1 cycle of 95°C for 10 min; 95°C to 85°C (-2°C/sec); 85°C to 25°C (-0.1°C/sec); hold at 4°C. When working with a heterozygous sample, there is no need to mix the sample with an equal portion of wild-type DNA, and the user can proceed with annealing. All reactions receiving mung bean nuclease (NEB; M0250L), used a BIS-TRIS buffer (50 mM MgSO₄, 1 mM ZnSO₄, 100 mM BIS-TRIS pH6.5, 0.01% Triton X-100, and 0.001 mg/mL BSA). Surveyor nuclease (Transgenomic, 706025) was used in accordance with kit recommendations. Nuclease incubation conditions are: 42°C for 20 min (Surveyor nuclease) and 60°C for 30 min (mung bean nuclease). Reactions were stopped by the addition of Stop Solution (Transgenomic kit) and 2 µL 0.2% SDS (sterile; mung bean nuclease). Electrophoresis conditions: 6.5% KB^{Plus} gel, 18- or 25-cm plate assembly, 0.25 mm spacers, and LI-COR DNA Analyzer with running conditions of 1500-2000 V, 30 mA, 50 W and 45°C for 1.5–5 hours, depending on plate assembly chosen and size of initial amplification products.

RESULTS AND DISCUSSION

Optimization of the tyrosinase gene mutation was developed as a control template for future analyses with unknown mutation detection in the Hairloss and *Red^{den}* mutants. We utilized a known point mutation in the *albino* mouse tyrosinase gene, *tyr^c*; results are illustrated in Figure 1. IRDye™

700-labeled forward (5'-C TATTG GTG CAG ATT TGTAT G-3') and IRDye™ 800-labeled reverse (5'-GTG GCT GCT GAA GTA CCA G-3') primers amplified a 907 bp region containing a G to C base change at nucleotide 308. After cleavage with a single strand specific nuclease, two alternately labeled bands are produced (IRDye™ 700 344 b and IRDye™ 800 563 b).

Levels of nuclease addition required for a successful reaction for Surveyor (PAGE and Standard kits), and mung bean nucleases were evaluated. Four levels of nuclease were tested for either the PAGE or Standard Surveyor kits and two levels for mung bean. When band intensities were compared (Figure 2), those reactions containing Surveyor nuclease additions of 0.25 µL (Standard), 1 µL (PAGE) and either level of mung bean nuclease, produced similar results.

Pooling of DNA samples to reduce the screening time for a particular project and/or reduce costs associated with the assay may be desired. 100 ng DNA per animal was used in the following pools: 1:2 (mutant:wild-type) 1:5, 1:10, 1:15, 1:20, and 1:25. Analysis of the pool sets in Figure 3 suggests that a mutation can still be identified in a pool of 1:10.

To further challenge the system, a collaboration was set up with the University of Nebraska-Lincoln to identify an unknown ENU-induced point-mutation in a mouse with a hair loss phenotype. Preliminary mapping and microsatellite analysis had narrowed the target area to a 1.77 MB region on Chromosome 11. Tanaka (2003) suggested that three genes in the Gasdermin gene family were likely candidates in other mice with phenotypes very similar to the UNL-generated hair loss mutant. Since these genes mapped to the nonrecombinant region, they were selected for analysis with TILLING.

Primer pairs were selected to amplify 1–1.5 kb regions spanning the exons for candidate genes. In exon 12 for a paralog of Gasdermin, only those bands representing the cleaved products are visible in the mutant DNA lane (F2H), while no cleaved products are present in the lanes containing negative controls (Figure 4). Sequencing identified a C to T change in a conserved part of the last exon for this candidate gene.

A second mouse hair loss mutant, *Red^{den}*, mapping to the same region, was included in the TILLING screens. The same primer set was used on unaffected and *Red^{den}* mutant DNA. Results shown in Figure 5 confirm a mutation in the same exon for these two hair loss mutants. The *Red^{den}* mutation appears to be a 6 bp insertion resulting in duplication of adjacent amino acids, glutamic acid and alanine.

CONCLUSIONS

We demonstrate the use of TILLING with Surveyor nuclease (Transgenomic), mung bean nuclease (NEB), and pooling to identify mutations and provide a simple protocol for their implementation. This approach can be scaled to analyze many different genes and conserved regions for point mutations simultaneously in multiple mice. Side-by-side comparison of three different mutations was done with Surveyor and mung bean nucleases to evaluate their effectiveness. In all cases, mutations would have been detected irrespective of nuclease procedure used. This application appears to reduce time, labor and costs associated with sequencing all regions.

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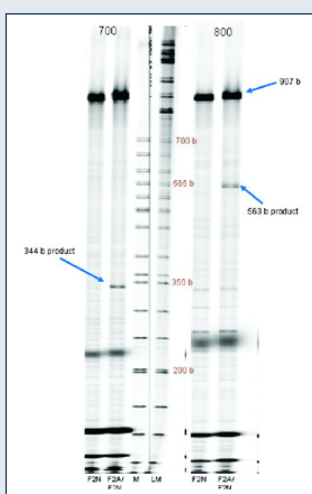


Figure 1. Images of tyrosinase positive TILLING reactions for F2N (wild-type) and F2A/F2H (albino:wild-type). Blue arrows show the 907 b full length product, 344 b (700-labeled) and 563 b (800-labeled) bands.

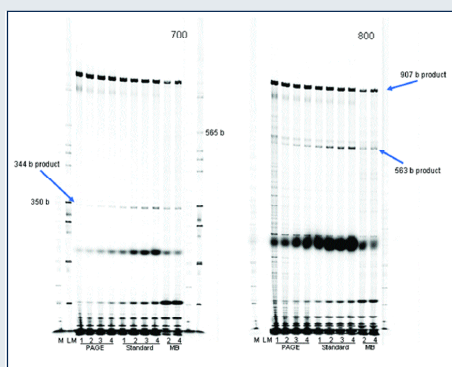


Figure 2. Nuclease concentration dilutions were done for the Surveyor nuclease in either the PAGE or Standard agarose kits and mung bean nuclease (NEB). The template used was the tyrosinase positive control. 1 = 0.25 µL; 2 = 0.50 µL; 3 = 0.75 µL; 4 = 1.0 µL.

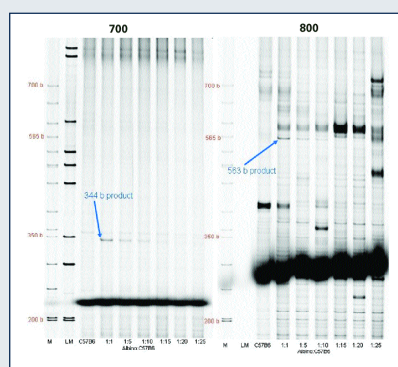


Figure 3. Pools containing 100 ng from each individual were prepared. M = 50–700 markers, LM = Long range markers, C57B6 = negative control. Six ratios of Albino:Normal mouse DNA were prepared (1:1, 1:5, 1:10, 1:15, 1:20, and 1:25).

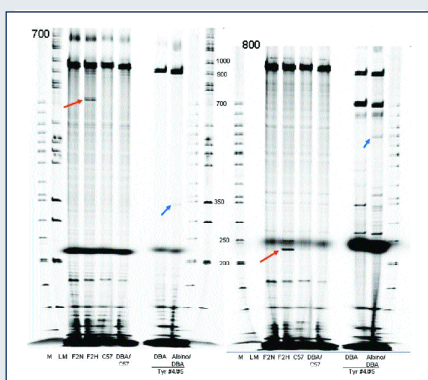


Figure 4. Confirmation gel run for positive TILLING hit on Normal (F2N), hair loss mutant (F2H), parental DNA (C57), and littermate (DBA/C57; negative for mutant phenotype). The tyrosinase control system was also included as a positive control.

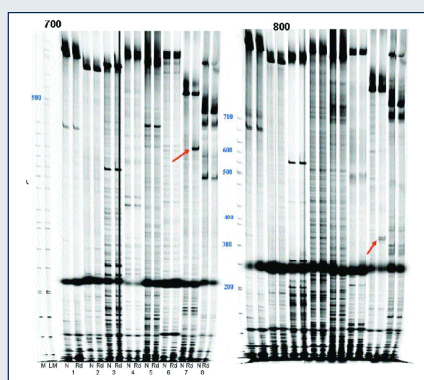


Figure 5. Eight primer pairs amplifying regions of paralogue 2 gene were run on Normal (N) and *Red^{den}* (Rd) DNA samples. Primer pair 7 was positive, showing band sizes of 943 bp target region, ~320/324 bp (800 channel), and ~623 bp (700 channel).