

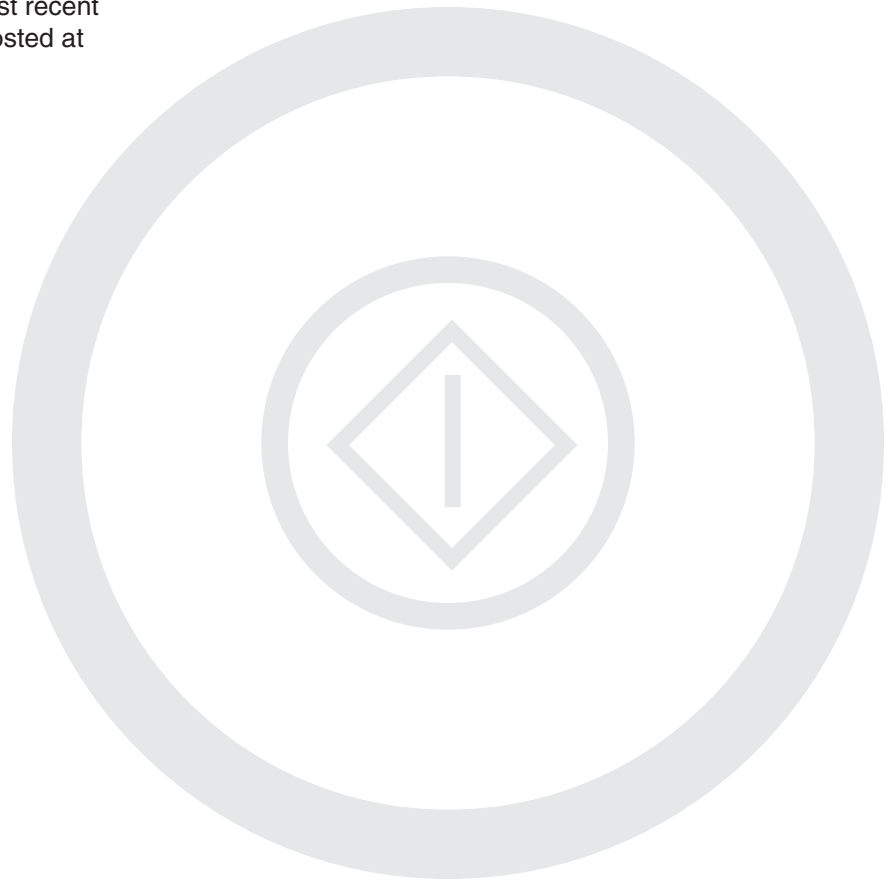
Model 4300

DNA Analysis System

Technical Note

TILLING[®] Example Utilizing Mung Bean Nuclease for Detection of a Point Mutation in the Mouse Tyrosinase Gene

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Contents

	Page
I. Required Reagents and Instrumentation.....	1
II. Overview.....	1
III. Amplification of Target Regions.....	2
IV. Heteroduplex Formation.....	3
V. Mung Bean Reaction.....	3
VI. Surveyor Nuclease Reactions.....	4
VII. Electrophoresis Conditions.....	4
VIII. Experimental Results.....	5
IX. Experimental Considerations.....	7
X. References.....	7

I. Required Reagents and Instrumentation

- DNA (100 ng/μL)
- Mung bean nuclease (New England Biolabs®; Cat# M0250L)
- MgSO₄ stock (100 mM)
- ZnSO₄ stock (100 mM)
- Bis Tris pH6.5 stock (0.5 M; BIS-TRIS Propane; Sigma® Cat# B-4679)
- dNTPs (2.5 mM)
- Ex Taq polymerase (Takara; Cat# RR001A)
- IRDye™ 700 and IRDye™ 800 labeled primers (one forward and one reverse bounding the gene target region)
- 6.5% KB^{Plus} Gel Matrix (LI-COR)
- 18- or 25-cm borofloat plates (LI-COR)
- 4300 DNA Analyzer (LI-COR)
- 0.25 mm spacers
- comb (user preference)
- Thermocycler

II. Overview

This example involves detection of a classic albino mutation in the tyrosinase gene in mice. The mutation involves a G to C change at nucleotide 308, located in the first exon of the gene. Primers were designed to amplify the 907 bp region around the mutation so cleavage would yield two unequal fragment sizes. The forward primer was labeled with IRDye™ 700 and the reverse with IRDye™ 800. In this example, the fragment sizes are 344 bp (IRDye™ 700 labeled) and 563 bp (IRDye™ 800 labeled).

III. Amplification of Target Regions

Two sources of DNA are needed. Equal concentrations of wild-type (no mutation) and mutant (containing the mutation) DNA are needed.

Buffer contains MgCl₂:

<u>Component</u>	
DNA (100 ng/μL)	2 μL
10X Buffer + MgCl ₂	2 μL
dNTPs (2.5 mM)	2 μL
Forward primer (5 μM)	2 μL
Reverse primer (5 μM)	2 μL
ExTaq (Takara)	0.3 μL
dH ₂ O	9.7 μL
<hr/>	
FINAL VOLUME	20.0 μL

Buffer does not contain MgCl₂:

<u>Component</u>	
DNA (100 ng/μL)	2 μL
10X Buffer (- MgCl ₂)	2 μL
dNTPs (2.5 mM)	2 μL
MgCl ₂ (25 mM)	1.6 μL (2mM Final Concn.)
Forward primer (5 μM)	2 μL
Reverse primer (5 μM)	2 μL
ExTaq (Takara)	0.3 μL
dH ₂ O	8.1 μL
<hr/>	
FINAL VOLUME	20.0 μL

PCR Conditions:

Cycles	Temperature (°C)	Time
1	94	2 minutes
40	94	30 seconds
	64	1 minutes
	72	1.5 minutes
1	72	10 minutes
1	4	hold

Notes:

- Optimization of the initial PCR amplification for a particular template and target region should be done with respect to primer concentration and PCR annealing temperature, as these conditions will vary.
- Choice of a high fidelity polymerase is up to the User; however, some modification or optimization in terms of quantity may be required.

Mung Bean Nuclease Reaction Set-up:

2.	Add ingredients in the following order:												
	<table> <tr> <td>dH₂O</td> <td>7.5 μL</td> </tr> <tr> <td>5X Bis-Tris pH6.5</td> <td>4 μL</td> </tr> <tr> <td>PCR amplified product, annealed</td> <td>7.5 μL</td> </tr> <tr> <td>Mung bean nuclease (10U/ μL)</td> <td>1.0 μL</td> </tr> <tr> <td><hr/></td> <td><hr/></td> </tr> <tr> <td>FINAL VOLUME</td> <td>20.0 μL</td> </tr> </table>	dH ₂ O	7.5 μ L	5X Bis-Tris pH6.5	4 μ L	PCR amplified product, annealed	7.5 μ L	Mung bean nuclease (10U/ μ L)	1.0 μ L	<hr/>	<hr/>	FINAL VOLUME	20.0 μ L
dH ₂ O	7.5 μ L												
5X Bis-Tris pH6.5	4 μ L												
PCR amplified product, annealed	7.5 μ L												
Mung bean nuclease (10U/ μ L)	1.0 μ L												
<hr/>	<hr/>												
FINAL VOLUME	20.0 μ L												
3.	Incubate at 60 °C for 30 min. Notes: <ul style="list-style-type: none"> Do not allow the reactions to sit in the thermocycler for an extended period of time after completion. 												
4.	Add 2 μ L 0.2% SDS (sterilized) and mix gently.												
5.	Add 2 μ L blue stop solution (LI-COR) to 5 μ L reaction and denature.												
6.	Load approximately 0.8 μ l per well. Volume will vary depending on comb selection.												

VI. Surveyor™ Nuclease Reactions

To compare results acquired from mung bean nuclease digestion, a Surveyor™ Kit (Transgenomic; Cat# 706010 PAGE Kit or Cat# 706025 Standard Agarose Kit) containing a nuclease from the Cel family of plant nucleases was purchased. Initial PCR amplification of the target region was done as described for mung bean nuclease reactions. Kit directions were followed for the Surveyor nuclease digestions and subsequent incubation at 42 °C for 20 min.

VII. Electrophoresis Conditions

- 18- or 25-cm plate assembly
- 6.5% KB^{Plus}
- 0.25 mm spacers
- Comb of choice
- 1500-2000 V
- 40 mA
- 40W
- 45 °C

Run time for 18-cm gel is approximately 1h 45min. (depends on length of target region).
Run time for 25-cm gel is approximately 3h 30min. (depends on length of target region).

VIII. Experimental Results

Figure 1 illustrates experimental results when F2 Normal (wild type) and F2 Albino (mutant) mouse DNA samples were plugged into the TILLING procedure. Two bands (one 800- and one 700-labeled) were generated after mung bean nuclease cleavage and electrophoresis. The 344 b product in the 700 channel is clearly visible along with the confirmation band of 563 b present in the 800 channel. The full length product is shown as the stronger 907 b product in both images.

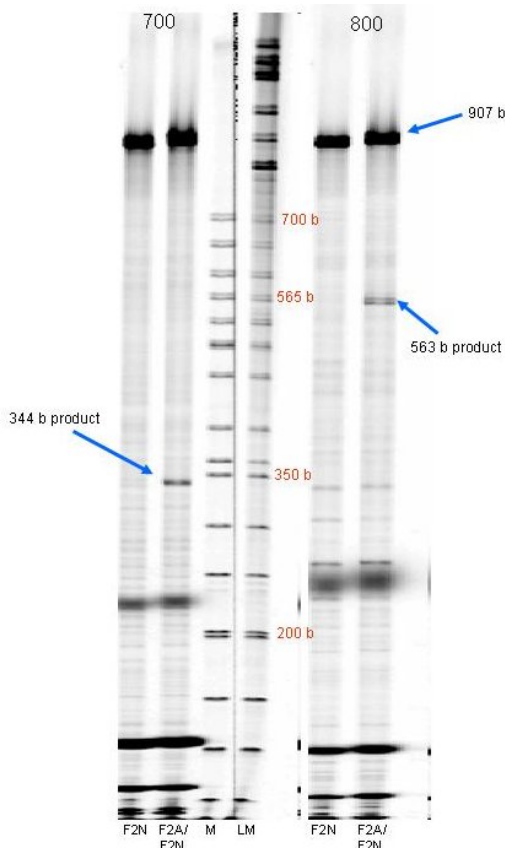


Figure 1. F2 Normal (F2N) and F2 Albino (F2A) mouse DNA was used to test for the point mutation causing the phenotype . A 344 b product is visible in the 800 channel and the 563 b complementary product is visible in the 800 channel. LM = long marker set; M = LI-COR 50-700b marker set.

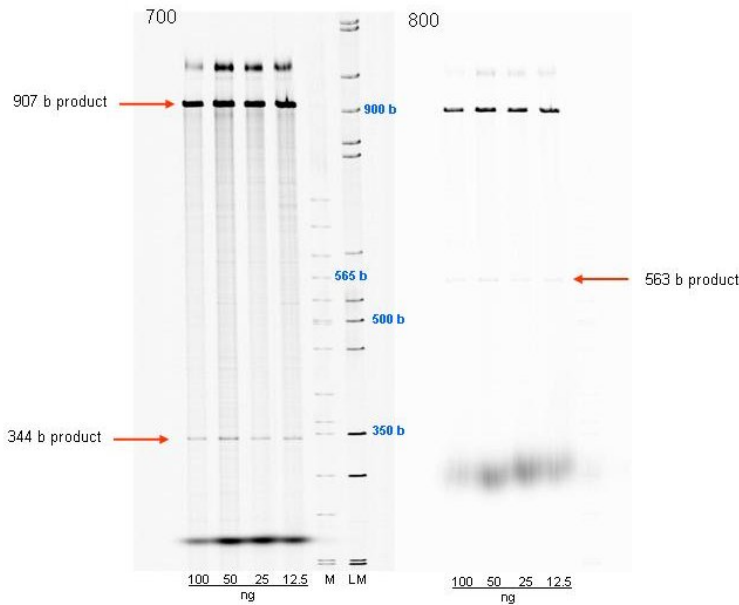


Figure 2. Surveyor Kit reactions show the detection of the tyrosinase gene mutation over a range of DNA concentrations used in the initial PCR reactions. 100 to 12.5 ng genomic DNA was used in the initial PCR amplification and when the full protocol was completed with Surveyor nuclease, 12.5 ng was sufficient to visualize a positive response in this case.

Due to a higher concentration of nuclease per microliter in the standard Surveyor kit, a test of different levels of the enzyme per reaction was tested for effective concentration. As illustrated in Figure 3 the concentration of the Surveyor nuclease from the standard agarose kit was approximately 4X stronger when compared to the nuclease from the PAGE kit. Two additional reactions testing two levels of mung bean nuclease showed little difference in the final results.

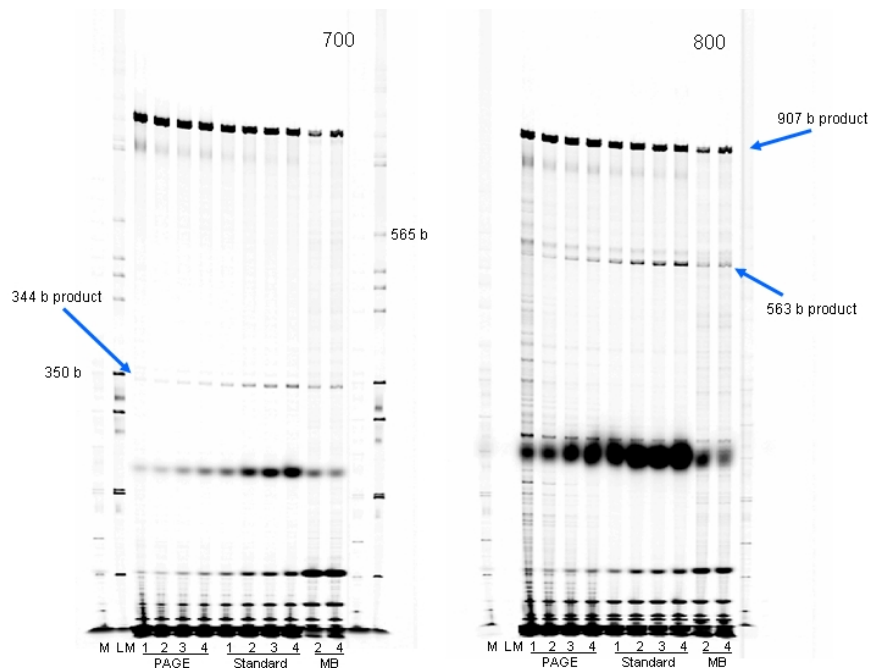


Figure 3. Comparison of nuclease dilutions from PAGE kit, Standard kit, and mung bean (MB; NEB). 1= 0.25 μ L, 2=0.5 μ L, 3= 0.75 μ L, and 4=1.0 μ L Surveyor nuclease or mung bean nuclease.

DNA pools of 1:2, 1:5, 1:10, 1:15, 1:20, and 1:25 were made where one albino aliquot (100 ng) was mixed with respective C57 (non-albino) individuals (100 ng/individual). These DNA pools were carried through the complete mung bean and Surveyor procedures as described above, and results shown in Figure 4.

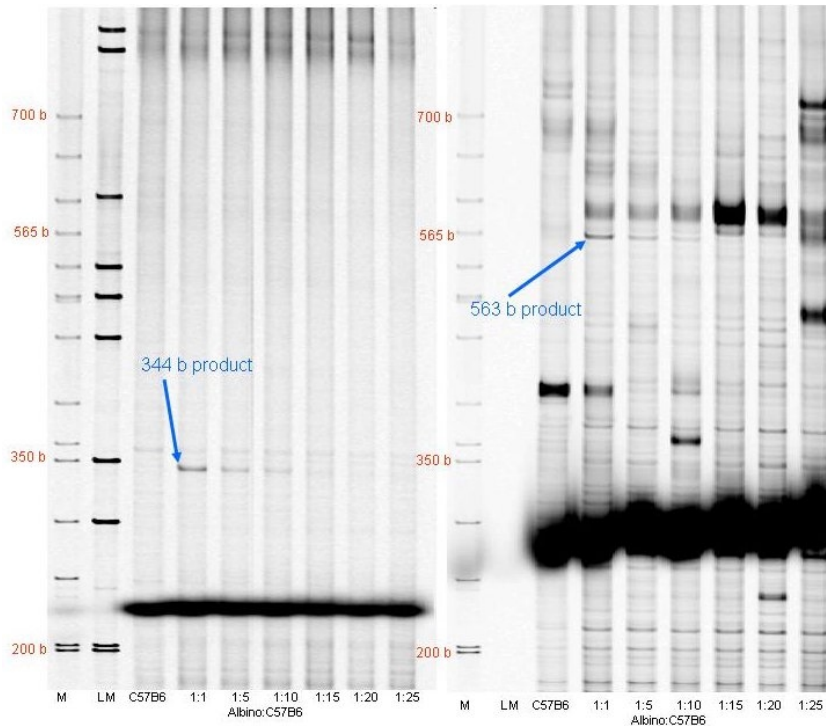


Figure 4. Target bands (344 and 563 b) are visible out to the 1:10 dilution after Surveyor nuclease treatment. 0.5 µL of a 2:5 mix of sample:loading buffer was loaded in 32-well comb and run on 18-cm 6.5% KB+ gel.

IX. Experimental Considerations

- **Ethanol precipitation:** Blobs can be seen in reactions at ~250 (700 channel) and 300 b (800 channel) due to the reaction mix. To eliminate these blobs, an ethanol precipitation can be done. If your bands of interest fall outside these ranges you should be fine without the precipitation.
- **3M sodium acetate:** Add 1/10th of this volume from 3M NaOAc stock.
- **200% Absolute ethanol:** Add 2.5-3X the volume of DNA/salt mixture. Mix well before freezing. Freeze in dry ice/ethanol bath for 20 min, a -70°C freezer for minimum of 40 min, or a -20°C freezer overnight.

X. References

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4308 Progressive Avenue • P.O. Box 4000 • Lincoln, Nebraska 68504 USA

Technical Support: 800-645-4260

North America: 800-645-4267

International: 402-467-0700 • 402-467-0819

LI-COR GmbH (Germany, Austria, Switzerland): +49 (0) 6172 17 17 771

LI-COR UK Ltd.: +44 (0) 1223 422104

www.licor.com

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