

## SNP Discovery through TILLING®

### Using JelMarker™ Image Reader Software

Tamela Serensits, Yiqiang Jacie Wu, Zhang Xuanhe, Ni Shouyong, Jonathan Changsheng Liu

#### Introduction

Targeting Induced Local Lesions In Genomes - or TILLING® - was first introduced in 2000, through the Arabidopsis genome project (1). The TILLING technique uses chemical mutagenesis with ethylmethanesulfonate (EMS) to induce point mutations throughout an entire genome. The mutagenized founder population is crossbred and the resultant population is analyzed for gene specific point mutations. EcoTILLING takes regular TILLING one step further in that it allows for haplotyping groups or species based on natural allele variation identified through SNP discovery.

To start the TILLING procedure, the genes of interest are identified with gene specific primers and PCR amplified. The primers are labeled with two dye colors – the forward primer is labeled with a shorter wavelength fluorescent dye (LI-COR's IRDye® 700 - blue) and the reverse primer is labeled with a longer wavelength fluorescent dye (LI-COR's IRDye 800 - green). Fragments are then run through a thermal-cycler and heteroduplexes are formed at the point of the polymorphism between two hybridized DNA fragments. The hybridized fragments are then cleaved at the heteroduplex site by CEL I nuclease or Surveyor™ Nuclease. The DNA is denatured and the resulting shorter fragments are run through gel electrophoresis and a scanned image of the gel is produced (2, 3).

SNPs are detected by comparing the two dye color gel images (Figure 2). The IR Dye 700-labeled image will show a mutation fragment band just below the wildtype band. The counterpart band will be visible in a location exactly opposite of it on the IR Dye 800-labeled image. This fragment in the 800 dye-labeled image is the complementary cleavage product of the 700 dye-labeled image band. The sum of the length of these two fragments is equal to the original amplicon, which makes it easy to distinguish from PCR artifacts.

JelMarker automatically identifies lanes and bands using a highly accurate image reading algorithm. Once the image is processed, the user has full control to manipulate lane/band position and orientation, individual fragment markers and standard identification.

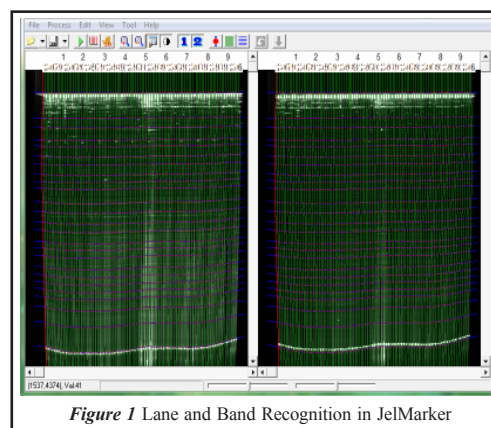


Figure 1 Lane and Band Recognition in JelMarker

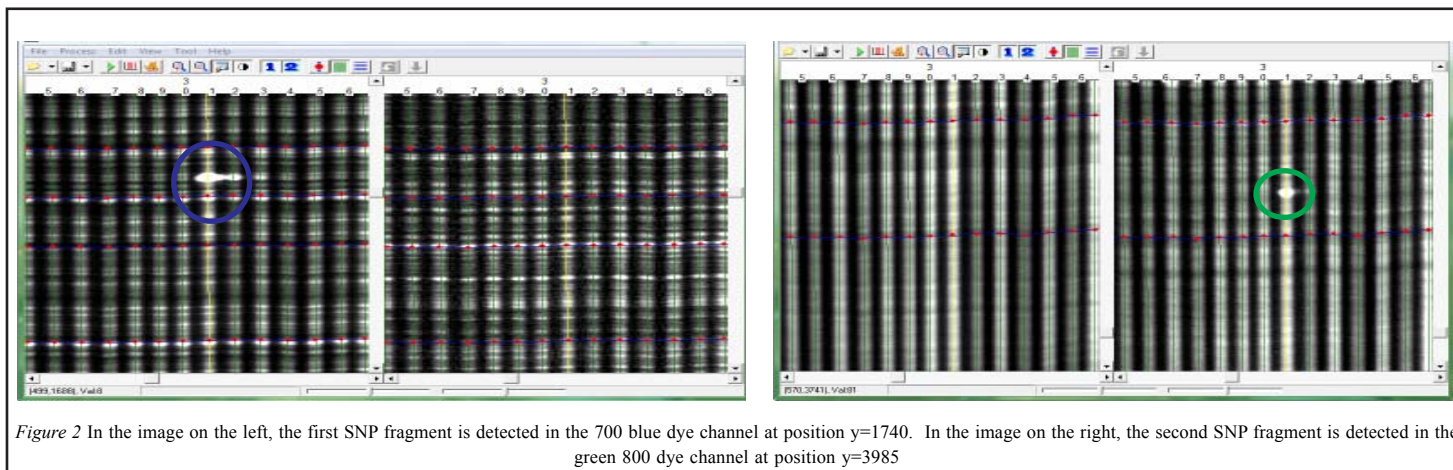


Figure 2 In the image on the left, the first SNP fragment is detected in the 700 blue dye channel at position y=1740. In the image on the right, the second SNP fragment is detected in the green 800 dye channel at position y=3985

JelMarker was developed in response to a growing demand for software that can analyze fluorescence, chemiluminescence and autoradiography gel image files – especially those from LI-COR®'s 4300 DNA Analyzer and KODAK®'s Image Station 4000R. It is an alternative software to GelBuddy®(4). JelMarker can import up to two TIFF, BIP, GEL, JPEG, BMP, and TXT files for comparison analysis. The software also exports trace files for easy upload to the fragment analysis software - GeneMarker®.

#### Procedure

##### Automatic Lane and Band Tracking

1. Launch JelMarker
2. Upload the 700 and 800 dye images into the appropriate fields in the *Open Files* dialog box
3. Select **Process** → **Options** from the menu bar
4. Set the *Image Type* to **Tilling** and enter the number of Lanes and Standards
5. Click the **Auto Lane Tracking** icon
6. The image now contains vertical Lanes and horizontal Bands (Figure 1)
7. Manually edit lanes and bands as required

##### Incorporating a Size Standard

8. Click the **Edit Size** icon, enter the lane number of the *Standard* lane, and select **Edit** → **AutoMatch**
9. The LI-COR size standard that most closely matches the Standard lane will be chosen
10. Add the last peak to the *Size Standard* by selecting **Insert Size** from the right-click menu in the trace
11. Right-click the newly added last peak and select **Extend Size** to fill in the missing sizes
12. **Save** and **Exit** the *Edit Size* module

##### Detect the Mutations

13. Click the **Show Mutations** icon
14. The *Show Mutations* window appears
15. Click the **Tools** icon to adjust settings (Figure 3)
16. Once the analysis settings are chosen, click first the **Alignment** icon then the **Normalization** icon
17. A new *Mutations* folder will appear in the file list
18. Expand the *Mutations* folder and select samples to be analyzed

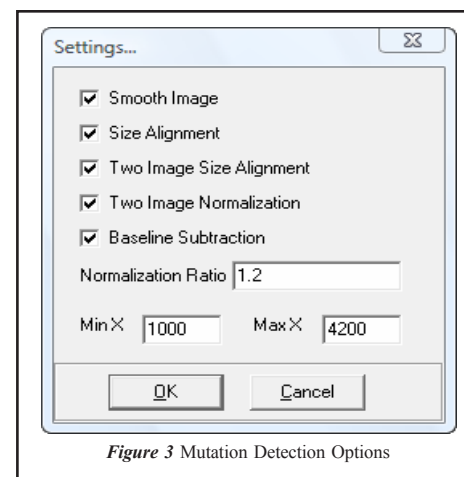


Figure 3 Mutation Detection Options

## Results

Once the two images have been analyzed for Lane and Band positions, JelMarker's *Show Mutations* module must be launched to detect differences between a wildtype *Reference* lane and the *Sample* lane in question. In the *Show Mutations* window *Settings* dialog box, the list of calculations that can be applied to the image are shown (**Figure 3**). Selecting **Smooth Image** applies a Fast Fourier Transform (FFT) calculation to reduce noise in the trace. **Size Alignment** shifts the trace left or right along the x-axis so that the frame (or pixel) positions of the Reference and Sample traces correspond to one another. To account for variations in signal intensity from the bottom of an image to the top of an image, a **Normalization** calculation is applied. Finally, the difference in signal intensity between the two images is removed with a **Baseline Subtraction** function.

After applying the SNP detection algorithms, a second folder in the file list appears labeled *Mutations*. This folder contains the results of SNP detection analysis. Click on any sample in the *Mutations* folder and use the **Arrow Up/Down** keys to scroll through the list. The SNP detection results will appear in the window to the right of the file list (**Figure 4**). The top trace is a representation of all lanes averaged together to create a wildtype or *Reference* trace. The middle trace is the aligned *Sample Lane* trace. The bottom trace is the result of a trace-to-trace subtraction of the *Sample* from the *Reference*.

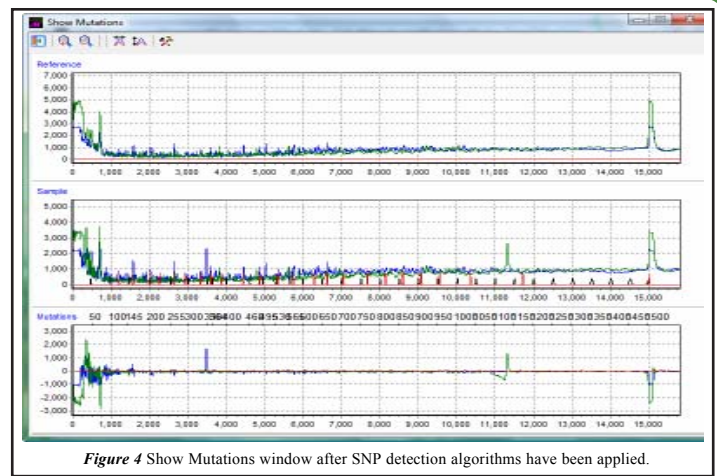


Figure 4 Show Mutations window after SNP detection algorithms have been applied.

Notice the basepair size ladder added to the top of the *Mutation* trace. This ladder contains the original basepair sizing from LI-COR's IRDye 700 Ladder up to size 700bp. From 700bp to 1500bp, JelMarker's unique **Extend Size** feature in the *Edit Size* module interpolates the mobility shift of the large peaks in TILLING data. From those interpolated sizes, an approximate size can be deduced with a resolution of less than 30 basepairs.

Even small fragments, though undetectable by the human eye, can easily be recognized and sized with JelMarker's SNP detection algorithms (**Figure 5**). In addition, JelMarker's unique sizing algorithm enables the researcher to quickly weed out false positives.



Figure 5 Two small peaks are easily seen in the Mutation trace in the image on the left. The image on the right is a zoomed-in view of the same peaks. Notice how the basepair size of the blue and green peaks add up to 1500 basepairs (with a resolution of approximately 30 basepairs) as expected. These SNP fragments were missed with standard image analysis.

## Discussion

SNP detection by the TILLING technique is highly accurate but time consuming. Converting the gel image to a trace view and doing an automatic trace difference comparison significantly reduces the amount of time an analyst has to spend identifying small fragments on a gel image. Converting to traces also allows for detection of less intense bands that the human eye may miss resulting in a false negative call. This is especially important when analyzing EcoTILLING data which contains one mutated portion out of 12 pooled samples. The SNP fragment in this case would be extremely faint.

In addition to saving time and increasing the number of SNP calls, a researcher must be cognizant of false positive SNP calls in a TILLING image. One such false positive result occurs when the fragment appears in multiple lanes in a single channel. JelMarker avoids this error by averaging all of the lanes together first and then individually comparing each lane to the *Reference* trace. If a peak appears in the *Mutation* trace and its counterpart fragment in the second dye color is present, then it can be considered a true SNP for that lane. A second false positive result is when a fragment appears in the same position in a lane in both channels. This is easily detectable in the JelMarker trace view as two peaks at the same x-axis position. It is thought that when bands appear in the same position in both channels, it is a double-stranded fragment with both of the dye-labeled primers being expressed.

## Acknowledgement

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## References

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**SoftGenetics LLC** 200 Innovation Blvd. Suite 235 State College, PA 16803 USA

Phone: 814/237/9340 Fax 814/237/9343

[www.softgenetics.com](http://www.softgenetics.com) email: [info@softgenetics.com](mailto:info@softgenetics.com)