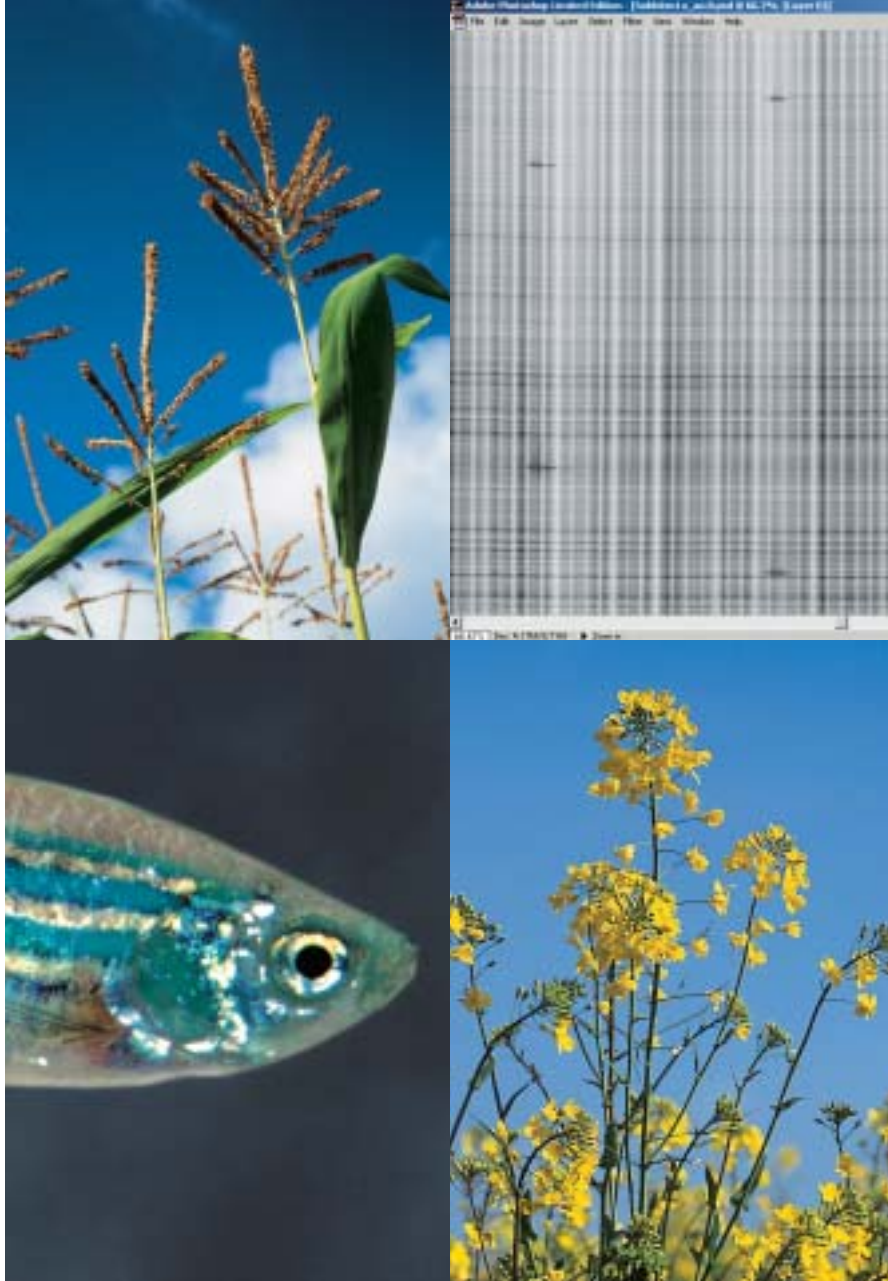
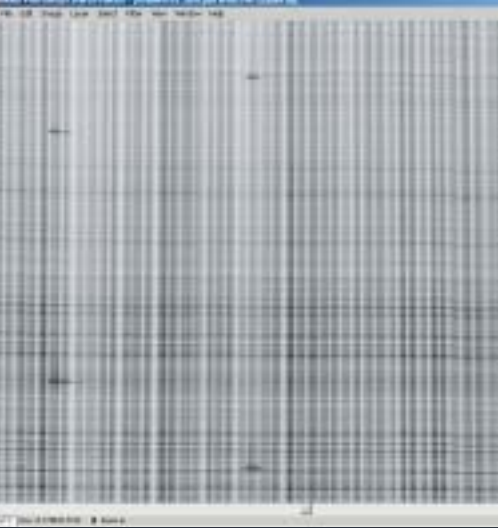


Automated Reverse Genetics





TILLING: Automated Reverse Genetics



Rapid acquisition of genomic sequence data has elevated a new discipline, functional genomics, which focuses on determination of gene function. Reverse genetics methodologies are an important part of functional genomics.

Traditional reverse genetic methods, such as the use of transposons to “knock out” a specific gene, can accurately determine phenotype but require time consuming transgenic or sophisticated tissue culture methodologies (1). Such “knockout” methods are limiting because the entire gene is knocked out – the effects of partial loss of function of an active gene cannot be observed.



DETERMINING GENE FUNCTION THROUGH TILLING

To overcome the limitations of knocking out an entire gene and to expand knowledge of active gene mutations, researchers from Fred Hutchinson Cancer Research Center developed a process for Targeting Induced Local Lesions In Genomes, or TILLING (2).


Elegantly simple, yet highly efficient, TILLING uses chemical mutagenesis

to yield a traditional allelic series of point mutations for virtually all genes. The TILLING process is of particular value for essential genes where sublethal alleles are required for phenotypic analysis. The value of TILLING for genetic research is enhanced by its proven viability for a rapidly growing range of organisms.

- *Drosophila*
- *Arabidopsis*
- Zebrafish
- *Maize*
- *Medicago truncatula*
and more...

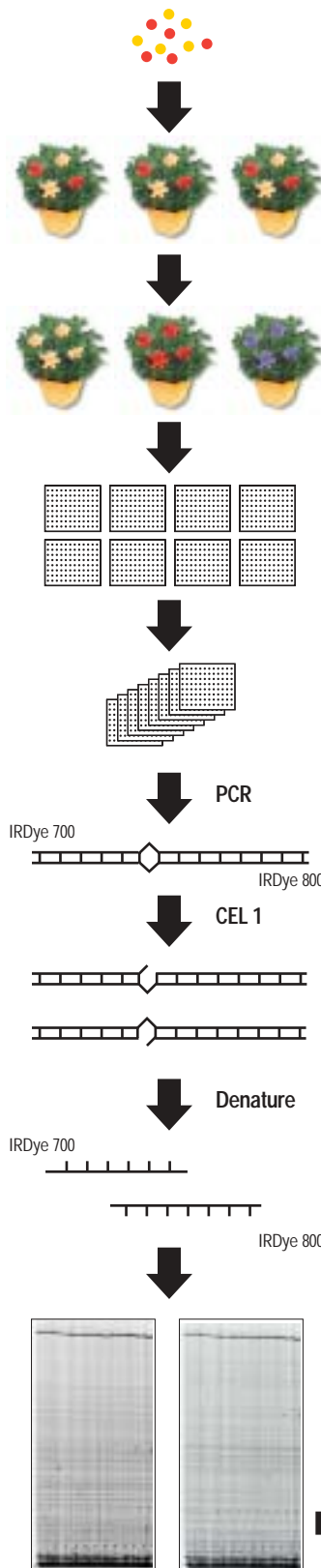
REQUIREMENTS FOR TILLING

Point mutations of a gene are relatively subtle, making discovery a challenge for the researcher and the detection instrument. Sensitivity of the instrument is critical for detection. The ability to distinguish false positives is of equal importance. Gel images from slab gel electrophoresis have emerged as a data format well suited for TILLING (1). On gel images produced by an instrument with high sensitivity, new bands resulting from mutations are easily identified.



Typical TILLING Workflow

(based on *Arabidopsis* as an example organism)



1) Seeds are mutagenized to induce point mutations throughout the genome.

2) A founder population is grown from mutagenized seeds.

3) Founder population is self fertilized to produce a crossed population.

4) Seeds from the crossed population are stored and DNA samples are collected in 96-well plates.

5) Up to eight 96-well plates are pooled into one and the samples (768) subjected to PCR with two gene-specific primers labeled with different IRDyes™.

6) Resulting amplicons are heated and cooled, resulting in heteroduplexes between wild type and mutant samples.

7) CEL I nuclease is used to cleave at base mismatches.

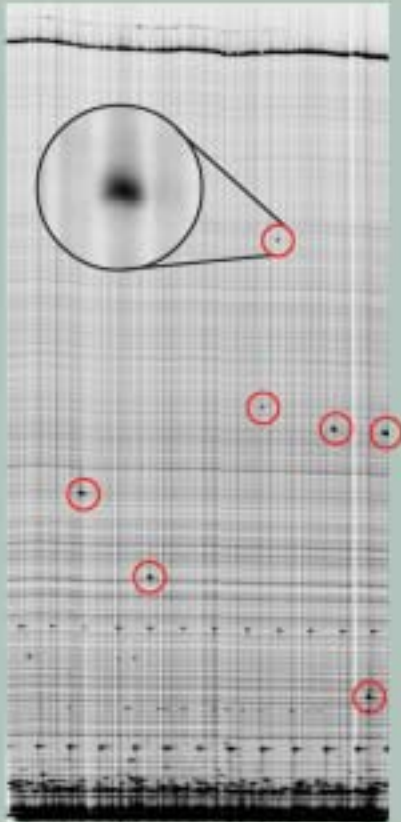
8) Samples are denatured and electrophoresed on a LI-COR 4300 DNA Analysis System.

9) In lanes that have a mutation in the pool, a band will be visible below the wild type band on the IRDye™ 700 image. A counterpart band will be visible in the same lane on the IRDye 800 image. This band is the cleavage product labeled with IRDye 800 from the complementary DNA strand. The sum of the length of the two counterpart bands is equal to the size of the amplicon, which makes it easy to distinguish mutations from amplification artifacts.

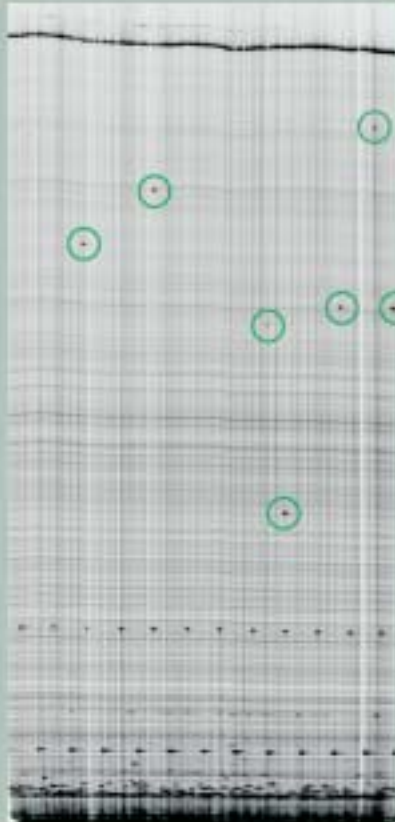


10) After detection of a mutation in a pool (lane), the individual DNA samples in the pool are screened again to find out which of the eight pooled samples from the crossed population has the mutation.

IRDye™ 700 Channel Image



IRDye™ 800 Channel Image



LI-COR TILLING IMAGES

Even on these highly reduced gel images, bands of mutations are clearly visible (circled red and green). When heteroduplexes created during PCR are cleaved at base mismatches (see TILLING Workflow), the result is two cleavage fragments labeled with different IRDyes. With two color imaging, a true mutation has two mutant bands below the wild type band in the same lane. One band is on the IRDye 700 image and the other on the IRDye 800 image. Additionally, the sum of the molecular weights of the mutant bands in a lane must equal the molecular weight of the wild type band in order for the mutation to be confirmed. Using this two-color detection method, the 4300 System virtually eliminates false positive mutation identifications.

Data courtesy of the Arabidopsis TILLING project, Seattle, Washington.

GENERATING HIGH QUALITY TILLING IMAGES

The LI-COR 4300 DNA Analysis System is uniquely suited for TILLING because it uses two-color infrared fluorescence detection to generate two true gel images during electrophoresis. Unprocessed image data are critical for TILLING because systems that highly process fluorescence data during detection will likely filter out most, if not all, mutations.

TWO-COLOR IMAGING ELIMINATES FALSE POSITIVES

Mutation identification on the 4300 System is very accurate. False positives are virtually eliminated by two-

color imaging since lanes with mutations have a new mutation band in one image and a counterpart band in the other image (see LI-COR TILLING images above).

EASY MUTATION LOCALIZATION

After mutation identification, imaging programs like Adobe® Photoshop® can be used to determine the lane number where the mutation occurred and the approximate molecular weights of the bands, which confirms the mutation. This technique localizes the mutation within ± 10 base pairs, making it easy to identify by DNA sequencing. Localization of the mutation, particularly for heterozygotes, is a clear advantage over methods such as DHPLC and TGCE,

which do not indicate where the mutation occurred (3).

INFRARED DETECTION FOR HIGHEST SENSITIVITY

The high sensitivity of infrared detection in LI-COR's 4300 System is critical because exonuclease activity in TILLING results in some lost signal. The inherent low background of infrared detection compared to visible detection, combined with LI-COR's IRDyes™, results in another key advantage – wide dynamic range. Wide dynamic range is critical for resolving weak mutation bands along with strong wild type bands. Low infrared background from amplification artifacts makes it easy to resolve mutations with the 4300 System.

EFFICIENT MUTATION SCREENING

TILLING can be used as a high throughput screening technique. With the 4300 System as the enabling technology, the following TILLING performance results can be achieved with a single instrument*:

- Up to 750,000 base pairs screened per run.
- Up to 2000 samples screened per day.
- Up to 2 million base pairs screened per day.
- 1000 base pairs per sample.

* Results are dependent on species and other factors.

In the lab, the 4300 System is fast and efficient:

- 96-well membrane combs load in just a few minutes.
- 2-4 hour run time (dependent on the size of the amplicon).
- Pre-mixed KB^{Plus} gel formulations.
- Gels can be reloaded several times.

PROVEN PERFORMANCE, IMPRESSIVE RESULTS

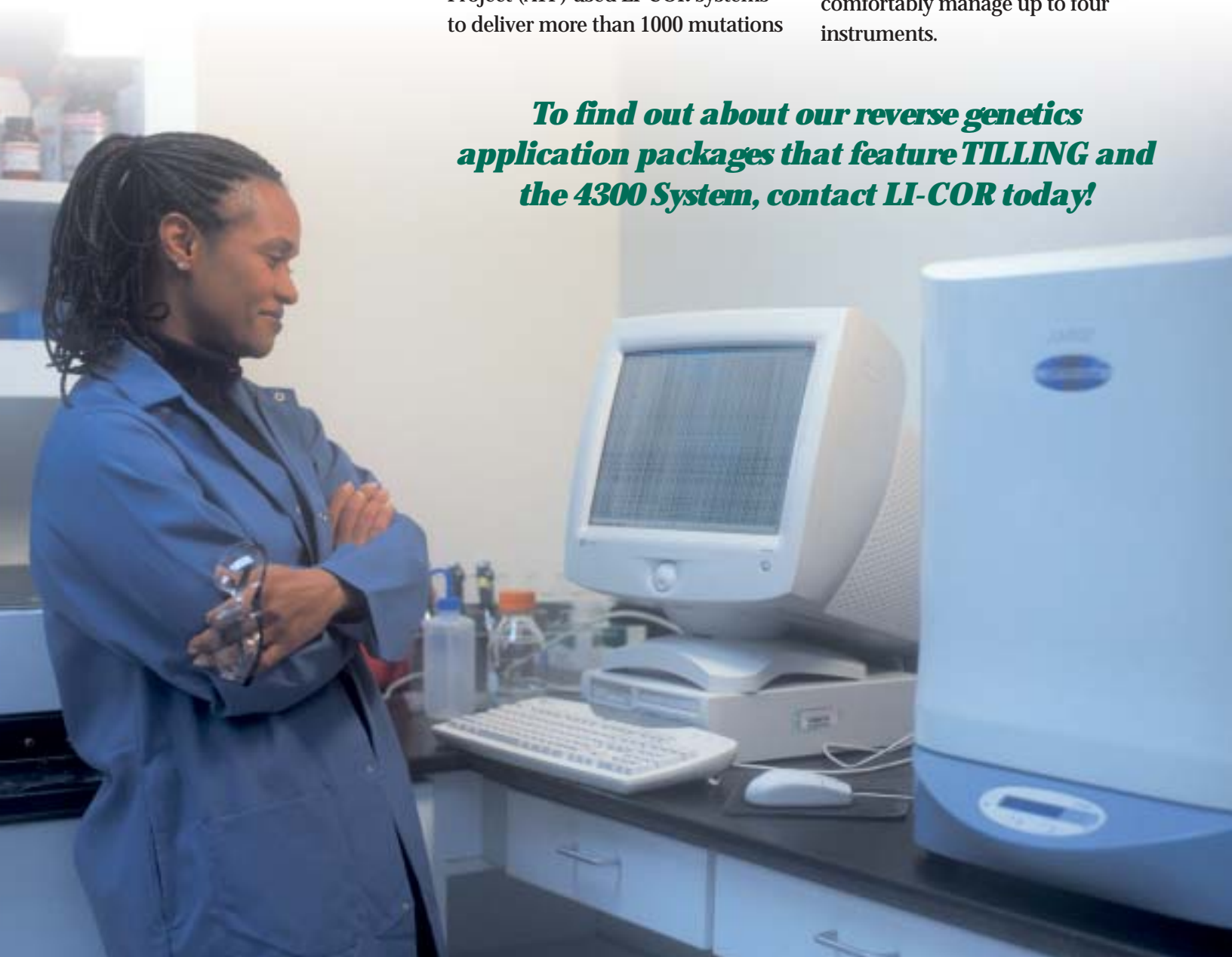
The strength of the 4300 System for TILLING is being proven every day in multiple labs around the world. For example, in the first year of operation, the *Arabidopsis* TILLING Project (ATP) used LI-COR systems to deliver more than 1000 mutations

in over 100 genes to *Arabidopsis* researchers (4).

EXPANSION WHEN YOU NEED IT

With competition for research funds at an all time high, it is often necessary to demonstrate proof of concept prior to the acquisition of full funding. A single 4300 System with its combination of high productivity and moderate initial investment is ideal for early phase research. After full funding, the modular design allows unlimited expansion with no modification in experimental technique. A single technician can comfortably manage up to four instruments.

To find out about our reverse genetics application packages that feature TILLING and the 4300 System, contact LI-COR today!



References

1. Colbert, T., Till, B.J., Tompa, R., Reynolds, S., Steine, M.N., Yeung, A.T., McCallum, C.M., Greene, E.A., Comai, L., and Henikoff, S. 2001. High Throughput Screening for Induced Point Mutations. *Plant Physiology* 126: 480-484.
2. McCallum, C.M., Comai, L., Greene, E.A., and Henikoff, S. 2000. Target Induced Local Lesions In Genomes (TILLING) for Plant Functional Genomics. *Plant Physiology* 123:439-442.
3. Henikoff, S., and Comai, L. 2003. Single-Nucleotide Mutations for Plant Functional Genomics. *Annual Review of Plant Biology*. 54:15.1-15.27.
4. Till, B.J., Reynolds, S.H., Greene, E.A., Codomo, C.A., Enns, L.C., Johnson, J.E., Burtner, C., Odden, A.R., Young K., Taylor, N.E., Henikoff, J.G., Comai, L., and Henikoff, S. 2003. Large-Scale Discovery of Induced Point Mutations With High-Throughput TILLING. *Genome Research* 13:524-530.

LI-COR[®]

Biosciences

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