

≡ TILLING-U KitTM

Complete reagents For TILLING with unlabeled primers



TILLING and EcoTILLING applications were originally designed to be used on the LICOR DNA Analyzer, but have moved to numerous other platforms which do not require the use of dye labeled primers. The benefits are an inexpensive platform for reverse genetics and rapid SNP discovery, which still allows pooling of samples to increase throughput and reduce discovery bias. TILLING-U kits contain SNIperase-U, which was specifically developed for optimal cleavage of unlabeled PCR fragments

TILLING studies need to survey target PCR fragments in thousands, tens of thousands or hundreds of thousands of individuals to identify induced mutations correlated with a desired phenotype.

EcoTILLING studies are primarily concerned with identifying informative SNPs for population genetics, forensics, conservation and resource management work. Inclusion of too few individuals in the discovery panel can introduce ascertainment bias. EcoTILLING allows hundreds or thousands of individuals to be included in the discovery panel by pooling, which reduces ascertainment bias, and allows for the discovery of the most informative SNPs for the study at hand.

The TILLING-U kit includes reagents for PCR amplification of the target PCR fragment, cleavage of the heteroduplexes, halting of the reaction, and heterozygous and homozygous controls to troubleshoot.

Contents:

- SNIperase-U
- 2x Reaction Buffer
- SharkaTAQTM
- 10mM dNTPs
- 50mM MgCl₂
- Stop Buffer
- Heterozygous and Homozygous controls

Step 1. PCR amplify your target fragment.

For robust results at low cost we recommend using the SharkaTAQ™ supplied with the kit and performing at least 33 cycles of PCR. This typically generates sufficient product to be visible on an agarose gel. Primers should be optimized prior to the TILLING-U procedure to determine the ideal annealing temperature and to eliminate non-specific bands or duplicated loci. To optimize the PCR reaction, we suggest performing 12 reactions per primer pair using DNA from one individual of the target species using a gradient thermocycler and varying the annealing temperature from 50°C to 70°C. Standard DNA purification processes using silica columns or ethanol precipitation provide good quality DNA for this procedure. It is imperative that a single PCR product be produced for efficient TILLING.

Pooling several individuals in each PCR versus individual sample amplification

Pooling of samples is advantageous for several reasons:

- (1) More potential heteroduplexes may be seen
- (2) the number of individuals that can be surveyed at a time is increased
- (3) pooling can give an indication of the frequency of the SNP site in various populations prior to investing time and money in high-throughput genotyping.

How many samples can be pooled?

Up to 5 individual samples (~50ng/ul), 1ul each, can be pooled into a PCR reaction. Based on standard agarose systems, if more than 5 individuals per reaction are used, resolution may decline, but pooling number should be optimized for the instrument used to read the samples. A standard 96 well plate will allow one to screen 480 samples (see figure, right).

For a single 25uL control PCR reaction, add:

10X PCR Buffer 2.5 uL

50mM MgCl₂ 1.5 uL

10mM dNTPs 1.3 uL

SharkaTAQ 1.0 uL

H₂O 18.7 uL

Total: 25.0 uL

Cycling:

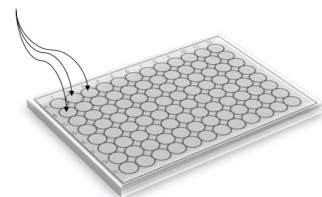
- 95°C for 3 minutes
- 33 cycles of:
 - 95°C for 30 seconds
 - 57°C for 30 seconds *
 - 72°C for 2 minutes**
- 15°C soak

*Annealing temperatures need to be determined for your PCR product empirically. The control primers anneal at 57°C.

**We recommend an extension time of 1 minute per kb. The control PCR product is 1,600 base pairs.

Use the SharkaTAQ™ Master Mix Calculator to determine how much of each reagent to add in order to create your personalized PCR Master Mix: <http://www.frontiergenomics.com/ssheets/SharkaTAQcalculator.xls>

Pool DNA from 5 individuals into each well



Five individuals per well x ninety-six wells = 480 individuals screened

Step 2. Create heteroduplexes of the PCR products:

PLEASE NOTE: WE HIGHLY RECOMMEND KEEPING SEPARATE AREAS (separate rooms if possible) FOR PCR SETUP AND PCR PRODUCT ANALYSIS. IF POSSIBLE, CARRY OUT ALL FURTHER STEPS IN A FUME HOOD EQUIPPED WITH A UV LIGHT. AT THE VERY LEAST, USE A DIFFERENT SET OF PIPETTES FOR PCR SETUP AND PCR ANALYSIS. CONTAMINATION OF PCR PRODUCTS IN PIPETTES AND ON BENCHTOPS CAN CREATE MANY PROBLEMS DOWNSTREAM. WIPE DOWN ALL BENCHTOPS WITH BLEACH AND UV IRRADIATE PIPETTES ON A REGULAR BASIS TO REDUCE RISK OF PCR CONTAMINATION.

At the end of the PCR reaction, create heteroduplexes by creating a 2-step cycling program on your thermocycler:

- 99°C for 5 minutes
- 70 Cycles of:
 - 70°C for 20 seconds (reduce by 0.6°C each cycle)
 - 69.7°C for 20 seconds (reduce by 0.6°C each cycle)
- Soak at 4°C or place on ice.

Step 3. Cleave heteroduplexes:

Prepare TILLING-U cleavage mix. For one 96 well plate, add 10µl of SNIperase-U to 1.25mL of 2X Reaction Buffer. Vortex and spin in microcentrifuge briefly. Remove PCR plate from thermocycler and remove caps or film. Using a multichannel pipette, or a repeat pipetter, pipette 10µl of the PCR reaction to a new plate and then pipette 10µl of the 2X cleavage mix into each well of this plate (this is the 10µL of SNIperase and the 1.25mL 2X Reaction Buffer). We suggest including both the homozygous and heterozygous control PCR products supplied with the kit to monitor amplification and cleavage reactions. Reseal or recap the plate, vortex, and spin in a plate centrifuge. Place in a thermocycler and heat samples to 45°C for 60 minutes followed by a 4°C soak. Remove plate from thermocycler and add 5µl of Stop Buffer to each well. This will inhibit further nuclease activity.

Step 4. Separate cleavage products:

Samples can be separated on various platforms. For standard agarose, we recommend loading the entire TILLING reaction into a well. Use of clarifying agents such as Synergel™ can improve resolution. Elchrom Scientific's Spreadex™ precast gel systems have excellent resolving power, and can be used as a high-throughput screening method. Advanced Analytical Technologies, Inc, sells the AdvanCE FS96™, which is a 96-well capillary system that can separate fragments up to 12kb in length.