Chapter 19

Mutant Generation in Vertebrate Model Organisms by TILLING

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Abstract

TILLING (Targeting Induced Local Lesions IN Genomes) is a popular reverse genetic approach that has been successfully applied in several genetic model organisms such as zebrafish, rat, *Drosophila*, *Arabidopsis,* or medaka. In contrast to classical targeted knockout technologies that work in mice by directly targeting a gene of interest, TILLING follows an indirect strategy. The first step of the TILLING pipeline is the generation of a TILLING library that consists of large numbers of mutagenized individuals. In a second step, these individuals are screened for mutations in any gene of interest. Screening is performed by PCR amplification of specific exons from each individual of a library followed by mutation detection. This could be done, for example, by direct re-sequencing of PCR fragments or alternatively, by CEL1 endonuclease-mediated mutation discovery. Individuals carrying potentially deleterious point mutations are isolated from the library and mutant lines are established. TILLING allows the identification of a whole range of point mutations, covering nonsense, splice site, and missense mutations in only one screening round, because the generation of mutations by mutagenesis as well as the screening tools is not biased. Potential knockout mutations are initially the mutations of choice, but TILLING screens can also be used to isolate allelic series of point mutations ranging from complete null phenotypes to hypomorphic or even dominant-negative or conditional alleles. These allelic series can be helpful for a comprehensive functional analysis of a gene of interest. TILLING is applicable to any kind of genetically tractable model organism, as long as this model organism is amenable to chemical mutagenesis, and genomic sequence information for a gene of interest is available. This chapter describes the design and pipeline of a TILLING facility as we are currently operating it for zebrafish in Dresden. Protocols for mutation detection by direct re-sequencing are described in detail. However, alternatives to this pipeline do exist and will be mentioned briefly.

Key words: TILLING, reverse genetics, zebrafish, point mutations, amplicon re-sequencing.

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1. Introduction

Since 2001, when the outcome of the human genome sequence project was published, several other whole genome sequencing projects of various organisms were initiated. Currently, circa 110 (stand 1st of June 2011) different vertebrate species and their genomic sequence data are found on the NCBI Eukaryotic Genomic Sequencing Projects homepage.

With the recent development of the "next generation sequencing technologies" (NGS), whole genome sequencing has become much faster and affordable. Therefore, the number of sequenced and annotated genomes of organisms will increase further and will finally span the range of common as well as "exotic" genetic model systems. However, correlating the DNA sequence data with the actual function of the encoded gene product is lagging behind and is therefore a primary goal for current and future research.

Forward and reverse genetic tools applied to various genetic vertebrate model systems help to define these correlations. Forward genetic screens induce mutations in model organisms and analyze them for phenotypic effects followed by molecular cloning of the mutated gene. Reverse genetic technologies make use of the molecular information about a gene of interest and specifically target this gene, followed by phenotypic characterization of the resulting mutant. Targeting of genes could take place at the level of the mRNA expression or translation (e.g., antisense oligonucleotides, RNA interference, and alternative approaches). Alternatively, gene targeting could take place directly at the genomic locus. Recently, modified zinc finger nucleases have been used to target and hereditarily destroy genomic loci in several species (**Chapter 20** by Jasmine M. McCammon et al., this volume). Targeted inactivation or knockout of specific genes in embryonic stem cells and subsequent embryonic stem cell transfer back into the animal is so far limited to mice only (**Chapter 11** by Anne E. Griep et al., this volume). An alternative tool to induce mutations at the level of the genomic DNA is achieved by the TILLING (Targeting Induced Local Lesions In Genomes) technology. TILLING was first invented in *Arabidopsis* (1, 2) and later adapted to several vertebrate model systems, such as zebrafish (3, 4), rat (5), and medaka (6), reviewed in (7). TILLING screens for induced mutations in any gene of interest in libraries of mutagenized individuals. The targeted gene is amplified by PCR and mutations are detected by different technologies that are suitable for large-scale setups such as mutation detection by high-performance liquid chromatography (HPLC) (2), CEL1-endonuclease-mediated cleavage of DNA heteroduplexes (1, 8), and targeted re-sequencing of PCR fragments (4).

Zebrafish are an excellent vertebrate model system not only for human diseases but also for studying genetic networks controlling vertebrate development. Genomic sequence and annotation data are provided by the *Danio rerio* genome sequencing project performed at the Sanger Institute (http://www.sanger.ac.uk/Projects/D_rerio/). A systematic screen for point mutations in zebrafish was initiated in the context of the Sixth Framework Programme Project "Zebrafish models for Human Development and Disease, Zf Models." The targeted knockout project was the first collaborative project that took place in three different European Laboratories (Edwin Cuppen, Utrecht; Derek Stample, Hinxton; Michael Brand and Sylke Winkler, Dresden). Zebrafish TILLING libraries were established in the individual laboratories and shared among the partners. Point mutations were detected by large-scale re-sequencing of PCR fragments as shown in **Fig. 19.1**, which illustrates a general TILLING strategy for zebrafish. Over the course of 5 years (2004–2009), a

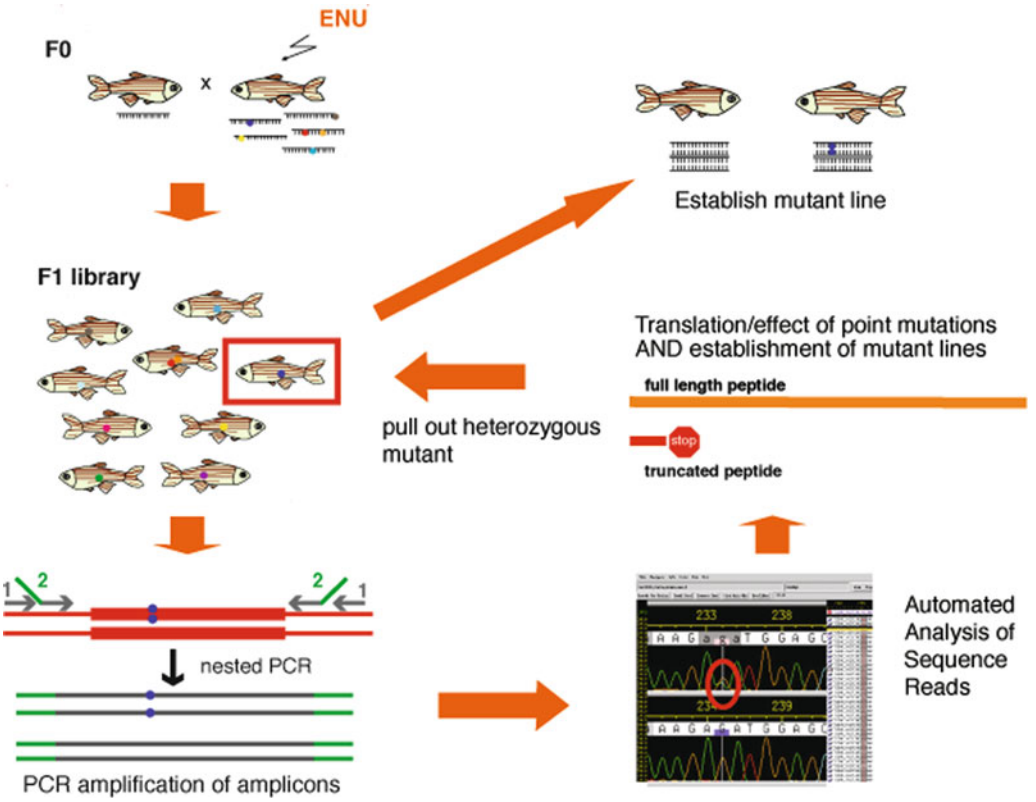

Fig. 19.1. TILLING strategy. Zebrafish males are treated with the chemical mutagen ENU to induce point mutations and subsequently crossed against wild-type zebrafish females. A TILLING library is generated from this F1 offspring, which consists of genomic DNA and living fish and sperm samples, respectively. The library is screened for a gene of interest by direct re-sequencing of PCR fragments. Sequence data are analyzed for point mutations and verified mutations are translated into peptides. Founders of potentially deleterious alleles are isolated from the TILLING library and propagated to establish a mutant line.

total of 251 potential knockout alleles covering 212 different genes have been identified from more than 20,000 mutagenized zebrafish. The targeted knockout project focussed on nonsense and splice site mutations that are expected to destroy the function of the resulting peptide. This extremely successful project is going to be continued at the Sanger Institute (http://www.sanger.ac.uk/Projects/D_rerio/mutres/) and Dresden TILLING facility (http://www.mpi-cbg.de/en/facilities/profiles/tilling.html). In addition to the European Initiatives, a consortium of three laboratories coordinates and performs TILLING screens in zebrafish in the USA (Cecilia Moens, Seattle; Lila Solnica-Krezel, Nashville; and John Postlethwait, Eugene) (9). Furthermore, the Australian Centre for Vertebrate Mutation Detection (ACVMD) has been initiated recently (Peter Curry and Joan Heath, Melbourne, and Graham Lieschke, Parkville). The actual screening strategies vary among these laboratories and new technologies for mutation detection that are based on "next generation sequencing" are being explored in individual laboratories that will finally increase screening efficiency and reduce screening costs.

1.1. Suitability of Genetic Model Organisms

The decision if a vertebrate model organism is suitable for TILLING approaches should take the following considerations into account:

- The vertebrate species allows genetic approaches (prerequisites: short generation time, large number of offspring, and laboratory handling of many individuals).
- A chemical mutagenesis can be performed (either by exposing the whole animal or by intraperitoneal injections).
- DNA sequence data for a gene of interest are available.

1.2. TILLING Libraries

One of the basic requirements of the TILLING technology is the induction of mutations at random positions in genomes by chemical mutagens such as the alkylating agent *N*-ethyl-nitrosourea (ENU). As indicated before, TILLING is applicable to various vertebrate model systems that allow chemical mutagenesis. Certainly, the specific conditions to induce mutations have to be explored systematically, since the "ideal" TILLING library should contain as many mutations as possible leading to the best possible survival rates of the treated animals.

In zebrafish, ENU mutagenesis is well established (10–12). It is based on repeated treatments of males with ENU, which acts by transferring its ethyl group to nucleophilic nitrogen or oxygen sites on each of the four deoxyribonucleotides. This event is manifested in the affected cells during subsequent cell proliferation and DNA replication events and results in heritable mutations. For TILLING libraries, mutagenized males are outcrossed against wild-type females to manifest ENU-induced mutations in

spermatogonia. Mutations are found in heterozygous mutant F1 offspring, which actually represent the library for further screens. Zebrafish ENU mutagenesis protocols were optimized for TILLING libraries recently (13) and lead to high-frequency point mutations with only minor influences on survival and fertility of mutagenized individuals.

Living libraries of zebrafish offspring (F1 generation) and their corresponding genomic DNA derived from tail fins can be kept. These fish can be screened up to the age of about 18–24 months depending on their robustness and fertility. This strategy requires regular performance of ENU mutagenesis to continuously provide TILLING libraries. Alternatively, freezing sperm from 9- to 12-month-old F1 males allows the generation of permanent TILLING libraries (14). However, both ENU mutagenesis and sperm-freezing protocols have to be established and require experience and training. Sharing libraries among different laboratories in a collaborative project is therefore a way to distribute this effort among individual laboratories.

1.3. Success of TILLING Screens

The success of a TILLING screen, which is defined as the identification and establishment of a mutant line that, for example, carries a nonsense mutation, depends on several aspects:

A. The frequency of point mutations in a library is determined by the mutagenesis protocol and the robustness of mutagenized individuals. In zebrafish, a frequency of one point mutation in 100–200 kb coding sequence can be achieved under well-established and optimized conditions.

B. The size of the library, as more mutagenized individuals increase the probability to find an interesting mutation (see also calculation below). However, this number might be restricted by available space for keeping either living individuals or for storing sperm samples.

C. The amount of coding sequence that is screened in one screening round. This number depends on the amount of coding sequence (exon size) and is limited by the technology that is used to identify point mutations. Re-sequencing of PCR fragments restricts this length to 500–750 bp. Since zebrafish genes often exist of small exons surrounded by large introns, this number should be sufficient for the majority of all zebrafish genes (3).

D. The codon composition of a defined exon: It has been shown that ENU exposure of zebrafish genomes results mainly in C/G to T/A transitions (32–40%) and to a lesser extent by A/T to T/A transversions (21–27%) (**Fig. 19.2a**). However, the exact numbers are variable when comparing different assays (detection of point mutations by re-sequencing performed at the TILLING

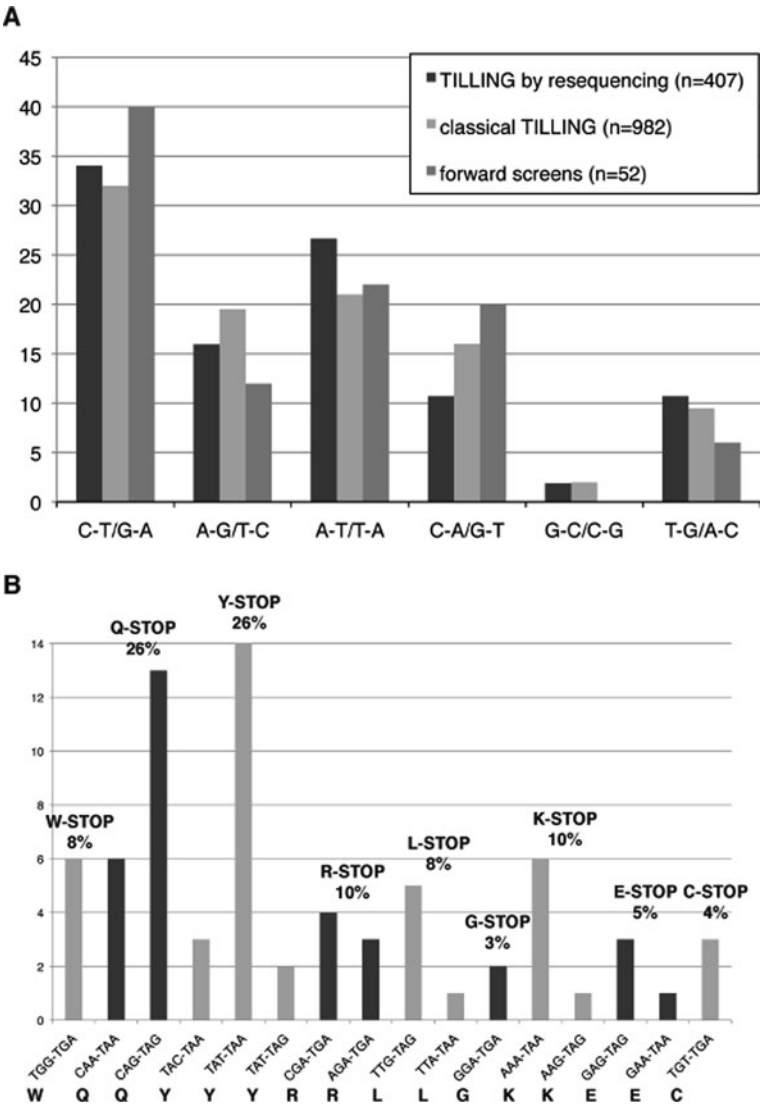

Fig. 19.2. (**a**) Distribution of six groups of nucleotide exchanges. Data were generated at the Dresden TILLING facility (mutation detection by re-sequencing of PCR fragments), in a classical TILLING approach (assaying by Cel-1 endonuclease cleavage) and based on forward screens after molecular cloning of the mutation (9). (**b**) Quantification of codons and their corresponding amino acids that were found to be mutated to stop codons ($n = 73$). Based on our data, mostly codons representing glutamate ($Q = 26\%$) and tyrosine ($Y = 26\%$) are mutated to stop codons, followed by arginine ($R = 10\%$), lysine ($K = 10\%$), tryptophane ($W = 8\%$), leucine ($L = 8\%$), glutamic acid ($E = 5\%$), cytosine ($C = 4\%$), and glycine ($G = 3\%$). Screens were performed at the Dresden TILLING facility.

facility in Dresden, by classical TILLING approaches, and positional cloning of point mutations resulting from forward genetic screens) (9). This might be due to a bias in the individual screening tools and should be experimentally determined for TILLING approaches on other vertebrate model systems.

The specific amino acids that can be mutated to nonsense codons are mostly represented by glutamate (Q) and tyrosine (Y) (52%) as shown in **Fig. 19.1b** (re-sequencing of PCR fragments of zebrafish TILLING libraries using ENU as mutagen). As a simplified rule, exons containing several codons that give rise to glutamate and tyrosine are good candidate exons to detect nonsense mutations.

Several software tools such as LIMSTILL and CODDLE were specifically designed for TILLING approaches and make use of these statistics to select the best amplicons to screen. Criteria to choose a suitable exon in a gene of interest are defined by the nature of a mutation of interest. If the mutation should knockout the function of a gene completely, a target exon should be chosen that is located in the 5′-region of the gene and shows a high chance of generating a stop codon (stop probability). If one is looking for potential missense mutations that lead to a weaker phenotype compared to an already existing null phenotype, one can specifically target functional domains of the gene. Since TILLING screens on randomly mutagenized individuals are not biased toward null phenotypes, also hypomorphic or dominant-negative mutations could be identified that ideally lead to an allelic series of mutations for a given gene.

Based on the frequency of point mutations in a given library, the size of the exon, and the expected stop probability for an exon, the total amount of individuals that have to be screened to find one nonsense mutation can be calculated:

$$\frac{\left[\left(\frac{100}{x}\%\right) \times y \text{ kb}\right]}{z \text{ kb}} = \text{Individual to be screened}$$

where x is the stop probability for an exon of interest (based on the codon composition of each exon, see above); y is the frequency of point mutations in the TILLING library (has to be determined for each TILLING library/ENU mutagenesis); and z is the screened coding sequence (usually determined by the exons that are included in the chosen amplicon). For example

$$\frac{\left[\left(\frac{100}{6.5}\%\right) \times 150 \text{ kb}\right]}{0.5 \text{ kb}} = 4{,}615 \text{ individuals}$$

where $x = 6.5\%$ stop probability for an exon of interest; $y = 1$ in 150 kb is the frequency of point mutations; and $z = 0.5$ kb coding sequence per amplicon.

This calculation gives an idea about the number of individuals that have to be screened to identify one stop mutation with a probability near 1 for one gene of interest under almost ideal conditions (full sequence read is covered by coding sequence and a very high frequency of point mutations). However, exons in

the zebrafish genome are often significantly smaller than 500 bp. Based on our experience (78 screened exons), the average size of a zebrafish exon that is suitable for TILLING screens is 320 bp (early in the gene, stop probability about 6%). Taking this number into account, 7,211 individuals have to be screened on a TILLING library (frequency 1 point mutation in 150 kb) to identify one nonsense mutation with a probability near 1. If the frequency of point mutations decreases further, e.g., to 1 in 180 kb, this number increases to more than 8,600 individuals. These examples show that it is extremely important to optimize conditions for mutagenesis to achieve a high frequency of mutations since this significantly reduces screening effort and costs especially for small exons. Alternatively and if only libraries of a lower efficiency are available, one should consider to increase the amount of coding sequence to be sequenced and screened for the gene of interest.

1.4. Detection of Point Mutations

Different strategies to detect point mutations have been reported in the literature. These include the classical TILLING approach, where heteroduplexes resulting from re-annealed and fluorescently labeled PCR fragments are cleaved by the endonuclease CEL1 (1, 8, 15–17). Cleavage fragments can be separated on high-resolution PAGE sequencers such as the LiCor DNA analyzer or alternatively on other agarose gel or PAGE-based systems. Positive candidates are validated and further defined by Sanger sequencing. In an alternative approach, point mutations can be detected by direct re-sequencing of PCR fragments (Sanger sequencing) followed by sequence alignment (4). Heterozygous positions and effect on the resulting peptide sequence can be detected, for example, with Polyphred, a non-commercial base calling software tool for large sample numbers (18, 19).

With the development of massively parallel sequencing platforms that allow the simultaneous generation of millions of short DNA sequence reads, large-scale screens for point mutations from mutagenized individuals are going to be elaborated. Pooling and barcoding strategies can be used to identify individuals in combination with high-throughput sequencing of shorter DNA fragments of a size of 50 to 200 bp, or sequencing of captured DNA fragments by customized microarrays or in-solution strategies. Some of these strategies are currently explored (pers. communication Derek Stemple and Edwin Cuppen).

1.5. Establishment and Propagation of Mutant Animals

After identification and verification of mutations, the effect of a mutation has to be determined before a mutant line is established. Mutated DNA is translated *in silico* and the resulting peptides analyzed using bioinformatics approaches. Point mutations can create nonsense codons or affect splice sites leading to

premature stop codons, which finally result in truncated peptides. These truncated/mutated versions most likely interfere with the function of the generated peptide. Missense mutations could result in deleterious effects if they change the biochemical properties of the affected amino acid (non-conservative substitution). The probability of interfering with the function of the translated peptide is high if the substituted amino acid is found in a functional domain of the peptide and/or if the affected position is conserved among different species. Individuals carrying interesting and potentially deleterious point mutations are isolated from the actual library by re-sequencing of pooled individuals and

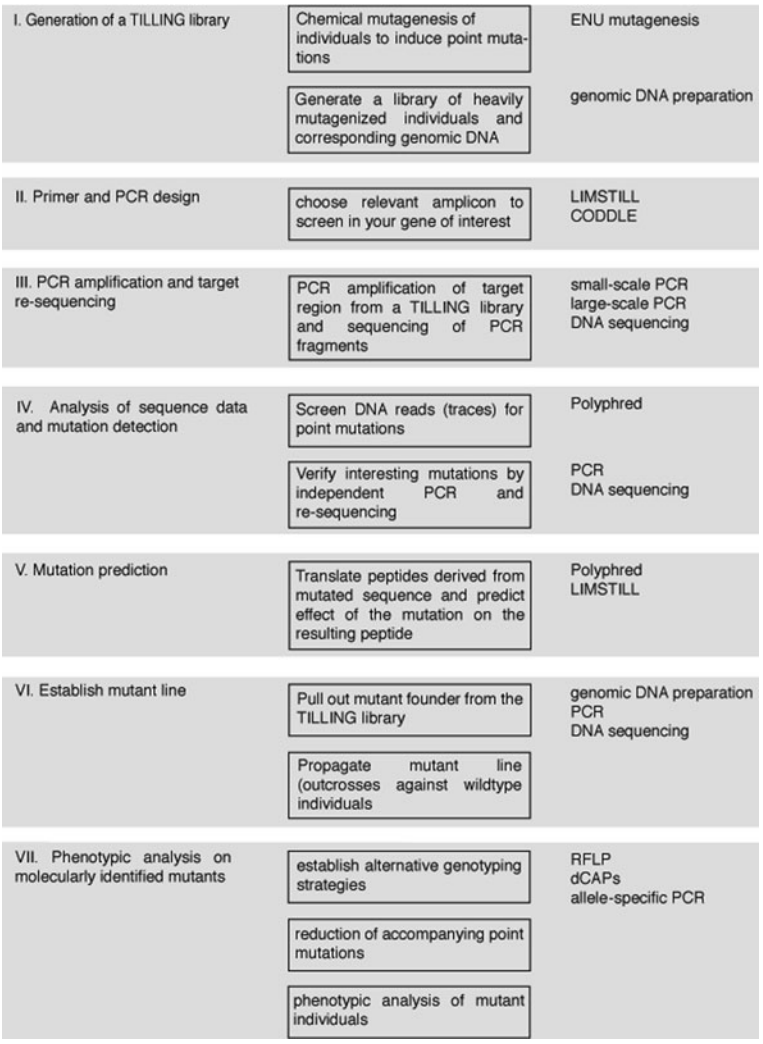

Fig. 19.3. Overview of the TILLING workflow for mutation detection by direct re-sequencing of PCR fragments.

subsequent breeding against wild-type individuals (outcross). If frozen sperm libraries exist, positive sperm samples are defrosted and used to fertilize wild-type oocytes. The resulting offspring are raised to fertility, genotyped for the identified point mutations, and individuals carrying this mutation are mated to each other (incross) to screen homozygous offspring for phenotypes.

In this chapter, we describe a TILLING setup as we are currently operating it for zebrafish libraries in Dresden. Our TILLING workflow of this pipeline is illustrated in **Fig. 19.3** and is based on direct re-sequencing of PCR fragments (Sanger sequencing) from libraries of mutagenized zebrafish. This pipeline can be established for any given genetic model in a laboratory with background in molecular biology and access to significant sequencing capacity or alternative mutation detection technologies, such as conventional TILLING by CEL1-based cleavage (for details, *see* **Note 1** and (16, 17, 20, 21)).

2. Materials

2.1. Generation of a Genomic TILLING Library from Zebrafish Tail Fins

1. Tricaine anesthetic stock solution: 0.4% ethyl 3-aminobenzoate methanesulfate (MESAB, MS-222, Tricaine) in 70 mM Na_2HPO_4 solution. Keep the stock at 4°C. Dilute freshly 25× in fish water to anesthetize adult zebrafish for fin clipping.
2. Scalpel, forceps, and two beakers with 70% ethanol and ddH_2O each to rinse tools.
3. Two deep-well plates (96-well, well volume 2 mL), kept on dry ice. Alternatively, 2 mL Eppendorf tubes can be used if less individuals are clipped (*see* **Note 2**).
4. Lysis buffer: 100 mM Tris-HCl, pH 8.0; 200 mM NaCl; 5 mM ethylenediaminetetraacetic acid (EDTA); 2% sodium dodecyl sulfate (SDS); prepare 10× stock solutions, dilute in ddH_2O for each preparation and add 100 μg/mL proteinase K for each experiment. We keep the already diluted lysis buffer without proteinase K for ca. 1–2 weeks at room temperature.
5. Water bath or incubator at 55°C.
6. 100% isopropanol, 70% ethanol, 1× Tris-EDTA solution (TE, 10 mM Tris-HCl, 1 mM EDTA, pH 8.0).
7. 0.7% agarose gels and agarose gel setup.
8. *Hin*dIII-digested lambda DNA as DNA marker for quantification of genomic DNA and conventional DNA ladder.

9. ddH_2O, microplates (deep-well and PCR plates in 384-well or 96-well format), or Eppendorf tubes for dilution and aliquots of genomic DNA for PCR application.

2.2. Choice of TILLING Targets

1. CODDLE (http://www.proweb.org/coddle/).
2. LIMSTILL (http://limstill.niob.knaw.nl/index.html).

2.3. PCR Amplification and Targeting of Amplicons from a TILLING Library

1. PCR suitable multiwell plates (384 or 96 well) or other single tubes.
2. 5× PCR buffer: 125 mM tricine, 425 mM NH_4acetate, 40% glycerol (w/v) 10% dimethyl sulfoxide (DMSO) in ddH_2O (w/v), adjust to pH 8.7 with 25% ammonia, store in small aliquots at –80°C. The buffer does not like repeated freezing and thawing.
3. dNTPs mix: 10 mM each, store small aliquots at –80°C.
4. 25 mM $MgCl_2$ (molecular grade) in ddH_2O, store small aliquots at –80°C, the working solution can be kept at 4°C.
5. PCR-polymerase (e.g., Taq-polymerase, *see* **Note 3**).
6. PCR primer: *see* **Note 4** when working in small-scale format and *see* **Note 5** when working in large-scale format (≥384 samples).
7. Agarose gels (1%) and agarose gel setup for quality control steps.
8. Exonuclease I (20,000 units/mL, e.g., NEB).
9. rAPid enzyme mix and 10× rAPid buffer (rAPid, Roche).
10. Plate sealing material (adhesive foil or heat sealing foil, alternatively re-usable full plate covers, e.g., Life Technologies).

2.4. Sequencing of PCR Fragments

1. Big dye version 3.1 (Life Technologies/Applied Biosystems).
2. 5× sequencing reaction buffer (Life Technologies).
3. Sequencing primers (*see* **Note 6**).
4. DNA precipitation mix: 7.1 M ammonium acetate, 0.03 M EDTA (stable at room temperature).
5. 100% ethanol (ice cold).
6. 80% ethanol.
7. Formamide–ddH_2O mix (50% each, *see* **Note 7**).

2.5. Detection of Mutations

1. Open-source tool: Polyphred (http://droog.gs.washington.edu/polyphred/).
2. Required system: UNIX or LINUX operating system.

3. Methods

3.1. Generation of TILLING Libraries

3.1.1. Chemical Mutagenesis to Induce Point Mutations in Zebrafish

ENU is the agent of choice to induce point mutations that are randomly distributed all over the genome. Improved protocols to achieve high frequencies of point mutations in pre-meiotic germ cells of adult zebrafish males have been published recently ((13); **Chapter 7** by Sreelaja Nair and Francisco Pelegri, this volume) and will not be described in detail. The mutagenesis and subsequent breeding of about 100 males should result in 6,000–8,000 heavily mutagenized F1 individuals, which will form the TILLING library. These animals can either be kept alive or alternatively a permanent library of male sperm could be generated at this point (for details (14), *see* **Note 8** for the decision, which library format is optimal for zebrafish under defined laboratory conditions).

3.1.2. Mutagenesis of Other Vertebrate Model Systems Than Zebrafish

ENU-based mutagenesis protocols are available for mammals, such as mice (22), rat (5), and other fish species, such as medaka (6). Several of them are optimized for TILLING approaches. In general, TILLING or a systematic screen for point mutations can be adopted for every genetic vertebrate model system. However, mutagenesis conditions have to be developed and optimized for the individual cases.

3.1.3. Preparation of the Genomic DNA Library in Zebrafish

Since the preparation of genomic DNA from larger numbers of individuals is required for an adequate TILLING library all steps are described for handling 96-well plates (*see* **Note 9** for single tube preparations). Alternatively to this inexpensive protocol for DNA isolation, commercially available kits to prepare genomic DNA can be used (*see* **Note 10**):

1. Adult zebrafish (>6 months) are anesthetized for about 2 min in Tricaine working solution in batches up to five individuals. Cut some tissue of the tail fin of individual fish with a scalpel (ca 5 mm). Transfer fin tissue to a defined well in a deep-well plate that is kept on dry ice. Transfer affected fish immediately to the dedicated tank before getting the next fish. Rinse scalpel and forceps first in 70% ethanol followed by ddH_2O. Anesthetized zebrafish should wake up within a few minutes.
2. Warm deep-well plate to room temperature and immediately add 500 µL of lysis buffer. Incubate for 2 h to overnight at 55°C. Vortex plates regularly during the first 2 h.
3. Incubate samples for 10 min at 85°C to inactivate proteinase K.
4. Vortex deep-well plate extensively and immediately spin plates for 30 min at 3,761×*g* at room temperature. Carefully transfer the supernatant to a fresh plate (*see* **Note 11**).

5. To precipitate genomic DNA add 400 μL of isopropanol, seal plate, and mix by inverting the plate several times. Spin plates for 1 h at 3,761×*g* at room temperature and discard the supernatant.
6. Wash DNA pellet with 300 μL 70% ethanol, spin 20 min at 3,761×*g*, and discard supernatant.
7. Repeat washing step for long-term storage of the genomic DNA library (not necessary for genotyping experiments).
8. Dry DNA pellet for 25 min (first upside down, then turn plate upside up).
9. Carefully dissolve genomic DNA in 200 μL TE. This could be done for several hours on a shaker at room temperature.
10. Check an aliquot (ca. 2 μL) of genomic DNA for integrity and concentration on 0.7% agarose gels. An aliquot of Lambda DNA digested with *Hin*dIII can be used as reference DNA (load 5–10 μL at 50 ng/μL). The yield of the genomic DNA preparation can be estimated when comparing the intensity of the genomic DNA band with the largest Lambda band of 23 kb (concentration 24 ng/μL) (*see* **Note 12** for an example).
11. Based on the quantitation, the concentration of the genomic DNA can be normalized to 1–2 ng/μL (*see* **Note 13**).
12. The genomic DNA is stored as an undiluted stock at –20°C (*see* **Note 14**).
13. Dilute genomic stock DNA in water (e.g., 1–50) as template for PCR (required are about 2–20 ng of genomic DNA per PCR). The aliquoted and diluted DNA is kept frozen at –20°C (*see* **Note 15**).

3.2. Choice of TILLING Targets

Although the frequency of point mutations in genomes is increased by mutagenesis, the detection of nonsense mutations is still a relatively rare event. For example, one nonsense mutation should be found while screening 4,615 individuals at a given frequency of mutations of 1 in 150 kb and 500 bp coding sequence analyzed per individual (see calculation in **Section 1.3**). To keep the number of screened individuals, PCRs, and sequencing reactions as low as possible, one should determine carefully which part of a gene will be targeted.

Guidelines to choose the best region:

– Screen as much coding sequence as possible per individual per amplicon. This is actually very much influenced by the screening method that is available, e.g., direct re-sequencing of PCR fragments allows amplicon sizes up to 750 bp, whereas CEL1-based screens allow amplicons up to 1,500 bp in size.

- The stop chance calculated from the codon composition of exons. Since nonsense mutations are represented by only three different codons (TAA, TAG, and TGA) only a limited set of codons can be mutated in any of the three nucleotide positions to become a nonsense codon. Taking the mutagenic activity of ENU in a vertebrate background into account (e.g., in zebrafish 34% of nucleotide exchanges represent C to T/G to A transitions and 27% A–T, T–A transversions, numbers determined in the Dresden TILLING facility, **Fig. 19.2a**), it is possible to calculate the stop probability per codon for a given exon.
- The location of an interesting exon in the annotated gene: To isolate mutations that are expected to impair with the function of a protein, it is recommended to screen 5′ located exons. Alternatively, functional domains can be targeted, in which mutations are expected to impair the function of the mutated peptide.
- The size of an interesting exon: Ideally, large exons with a high stop probability should be screened.
- The existence of alternative translation initiation sites: This should be either determined experimentally or predicted bioinformatically. These alternative peptides can be functionally active and redundantly take over the action of the mutated version.

Available web-based tools to identify suitable amplicons:

1. CODDLE helps to identify regions in exons that have a high likelihood to harbor nonsense mutations and to identify regions that encode conserved domains where missense mutations might lead to a deleterious effect.
2. LIMSTILL allows gene annotation, primer design, and data management for several vertebrate and invertebrate species and was developed by Victor Guryev in Edwin Cuppens laboratory in Utrecht (*see* **Notes 16** and **17** and **Fig. 19.4**).

3.3. Targeting Amplicons from a TILLING Library by PCR

Several technologies have been established to screen for point mutations. Direct re-sequencing of PCR fragments amplified from a library of genomic DNA will be described in detail for two different scenarios. A small-scale protocol makes use of normalized genomic DNA as template and performs one single PCR step followed by direct re-sequencing of PCR fragments (**Section 3.3.1**). The large-scale protocol (**Section 3.3.2**) performs a nested PCR approach where normalization is achieved by an initial PCR step with gene-specific primers, followed by tailed inner primers (*see* also **Fig. 19.1**). This strategy allows usage of common sequencing primers. This large-scale approach requires liquid handling robotics and standardized PCR and sequencing

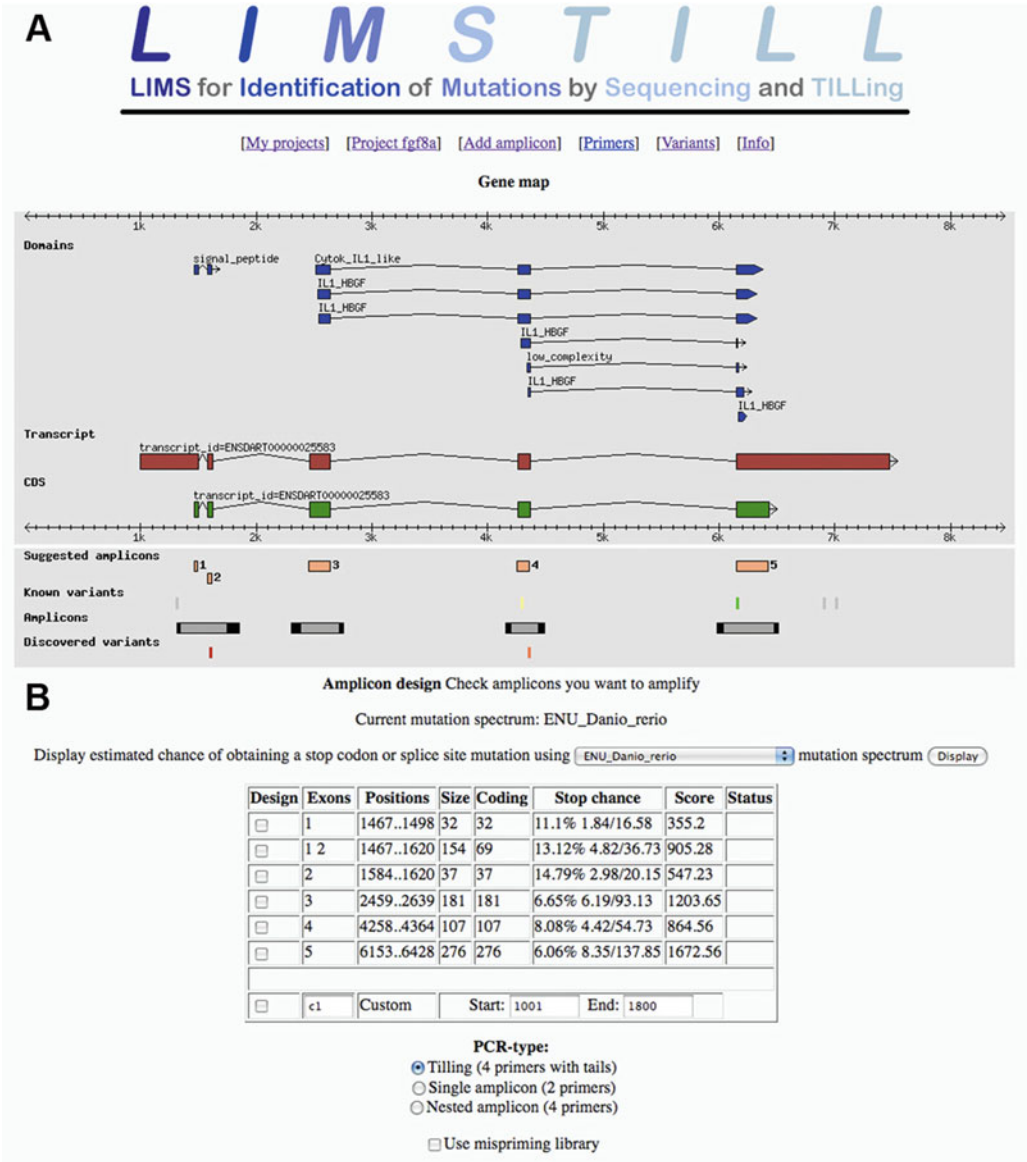

Fig. 19.4. LIMSTILL for project management, primer design, and mutant management (generated by Victor Guryev, Hubrecht Institute in Utrecht). (**a**) Overview of gene annotation, designed amplicons, known and discovered variants for an example derived from zebrafish, details are based on genome sequence and gene annotation as defined by Zebrafish Ensembl Zv 8 (Zebrafish genome sequencing project, Sanger Institute, Cambridge, November 2009). (**b**) Amplicon design tool, making use of statistics of ENU-based nucleotide exchanges in zebrafish (available also for other species).

conditions. An example of an automated setup as it is used by the Dresden TILLING facility is shown in **Fig. 19.5**.

3.3.1. Small-Scale/Single Tube TILLING PCR Protocol

PCR can be performed in single PCR tubes, PCR strips, or 96-well plates. Multichannel and multidispense pipettes should be used whenever it is possible to avoid pipetting errors:

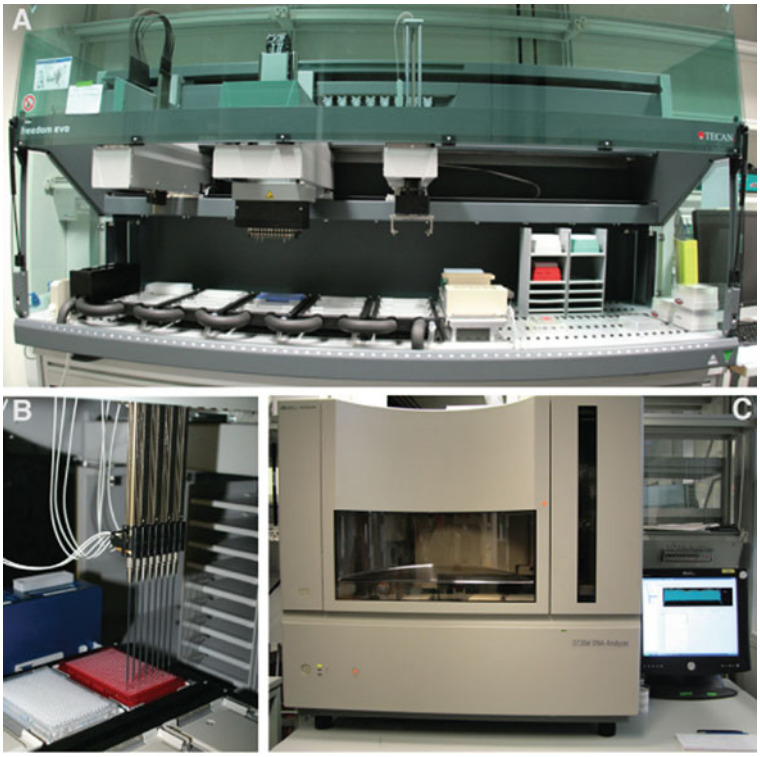

Fig. 19.5. Liquid handling setup of the Dresden TILLING facility. (**a**) The setup contains a liquid handling pipetting station (TECAN Freedom) that allows low volume (**b**) as well as multichannel pipetting (96-well liquid handling tool). All liquid handling, PCR amplification, and sequencing steps are performed in 384-well format (**b**). (**c**) 384-well plates are sequenced on an AB3730XL DNA sequencer.

1. Set up PCR master mix on ice for *n* samples (*n* = number of samples plus excess volume = 1–2 reactions)

   1× PCR setup (master mix preparation, per sample):

   0.2 μL forward primer (10 μM)
   0.2 μL reverse primer (10 μM)
   0.2 μL dNTP (10 mM each)
   0.8 μL 25 mM $MgCl_2$
   2 μL 5× PCR buffer
   x μL PCR-polymerase (*see* **Note 3**)
   x μL ddH_2O (fill up to 5 μL total volume)

   1× PCR setup (reaction preparation, per sample):

   5 μL mastermix
   5 μL genomic DNA (normalized and diluted to 1–2 ng/μL)
   10 μL total PCR volume

2. PCR conditions
   Step 1 94°C 5 min
   Step 2 94°C 30 s
   Step 3 T_{anneal} 20 s
   Step 4 72°C 60 s perform steps 2–4 for 30 cycles
   Step 5 72°C 10 min
   15°C until collected
3. For quality control and quantification load 3 μL aliquots of PCR fragments on 1% agarose gels. The concentration of amplified fragment is estimated from these agarose gels that contain marker DNA of a defined concentration (e.g., conventional DNA ladders or specific ladders for quantification, *see* **Note 12**). The PCR should result in one discrete band only (*see* **Note 18**).
4. Dilute PCR fragments for sequencing reaction (*see* **Note 19**).

3.3.2. Large-Scale Approach

The large-scale setup is based on a nested PCR approach and subsequent direct re-sequencing of the PCR fragments. In this case, normalization of the final product is achieved during the nested PCRs and therefore the labor-intensive normalization step of the genomic DNA can be avoided. We perform all steps in 384-well format making use of liquid handling robotics that takes over all liquid handling steps such as dilutions and plate copies. All PCR conditions are standardized with regard to annealing temperature and dilution steps. This setup allows processing of up to 9,000 samples per week that are optimized for inexpensive PCR and sequencing conditions (one 96 capillary sequencer). Robotic pipetting is performed with a low volume system of reusable pipetting tips for all master mixes. Samples are transferred or copied to new target plates by disposable tips that are washed between the different pipetting steps (**Fig. 19.5**, please contact us directly for further details):

1. Set up outer PCR on ice (*see* **Note 20**):

   1× PCR with gene-specific outer primers (master mix preparation, per sample):

   0.02 μL forward outer primer (100 μM)
   0.02 μL reverse outer primer (100 μM)
   0.2 μL dNTP (10 mM each)
   0.8 μL 25 mM $MgCl_2$
   2 μL 5× PCR buffer
   x μL PCR-polymerase (*see* **Note 3**)
   x μL ddH_2O (fill up to 5 μL total volume)

1× PCR setup with gene-specific outer primers (reaction preparation, per sample):

5 μL master mix

5 μL diluted genomic DNA (corresponding to 2–20 ng)

10 μL total PCR volume

2. PCR conditions

Step 1	94°C 5 min	
Step 2	94°C 30 s	
Step 3	57°C 20 s (defined by LIMSTILL settings for primer design)	
Step 4	72°C 60 s	perform steps 2–4 for 25 cycles
Step 5	72°C 10 min 15°C until collected	

3. For quality control and quantitation take 3 μL of a subset of samples (e.g., one row or column) from each plate and run on 1% agarose gels (*see* **Note 12**).
4. Dilute PCR fragments for nested PCR setup 1:3 in ddH_2O (*see* **Note 21**).
5. Set up master mix for inner PCR on ice (*see* **Note 20**)

1× inner PCR with tailed gene-specific primers (master mix preparation, per sample):

0.006 μL forward inner primer (100 μM)

0.006 μL reverse inner primer (100 μM)

0.1 μL dNTP (10 mM each)

0.8 μL 25 mM $MgCl_2$

2 μL 5× PCR buffer

x μL PCR-polymerase (*see* **Note 3**)

x μL ddH_2O (fill up to 10 μL total volume)

1× inner PCR with tailed gene-specific primers (reaction preparation, per sample):

10 μL mastermix

1 μL of diluted PCR 1

11 μL total volume

6. PCR conditions: identical to PCR 1 (outer PCR) (**Section 3.3.2**).
7. For quality control and quantitation take 3 μL of a subset of samples (e.g., one row or column) from each plate and run on 1% agarose gels (*see* **Note 12** for quantitation and **Note 22** for quality adjustment).

8. Cleanup PCR fragments
   The removal of unused PCR primers and unincorporated dNTPs is performed in solution. This step is not absolutely essential but increases sequencing quality.

   Set up mastermix on ice (*see* **Note 20**)

   1× PCR cleanup reaction (sample mix preparation, per sample):

   0.25 μL rAPid enzyme mix

   0.05 μL exonuclease I

   1.5 μL 10× rAPid buffer

   3.2 μL ddH2O

   5 μL total

   1× PCR cleanup reaction (reaction preparation, per sample): add 5 μL rAPid/ExoI mix per well per plate.

9. Incubation:
   37°C 30 min
   75°C 2 min
   10°C until collected

3.4. Sequencing of PCR Fragments

The specific sequencing conditions have to be determined based on the DNA sequencing options that are available for the individual project. Herein, conditions for high-throughput sequencing of PCR fragments on a 96-capillary ABI DNA analyzer 3730XL are described.

3.4.1. Sequencing Reaction

1. Dilute PCR fragments for sequencing reaction (*see* **Note 23**)
2. Set up master mix for sequencing reaction on ice (*see* **Note 20**)

   1× sequencing reaction (master mix preparation, per sample)

   0.2 μL Big dye version 3.1

   0.4 μL 5× sequencing buffer (Applied Biosystems)

   0.05 μL sequencing primer (100 μM) (*see* **Note 6**)

   6.35 μL ddH_2O (to fill up to a final volume of 7 μL)

   1× sequencing reaction (reaction preparation, per sample):

   7 μL total sequencing mastermix per reaction

   1 μL diluted PCR fragment

   8 μL total volume sequencing reaction

3. Sequencing PCR
   Step 1 96°C 1 min
   Step 2 96°C 10 s

Step 3 52°C 5 s
Step 4 60°C 3 min perform 30 cycles of steps 2–4
4°C until collected

3.4.2. Purification of the Sequencing Reaction

1. Add to each sample 2 μL precipitation mix and 20 μL 100% ethanol (ice cold).
2. Spin at 4°C for 30 min at maximum speed (for multiwell plates, e.g., 3,761 × *g*).
3. Turn plates upside down on absorbent pad and spin for 1 min at 400 rpm to get rid of all ethanol.
4. Add 20 μL of 80% ethanol to each well to wash the DNA pellet.
5. Spin at room temperature for 10 min at maximum speed (for multiwell plates, e.g., 3,761 × *g*).
6. Turn plates upside down on absorbent pad and spin for 1 min at 400 rpm to get rid of all ethanol.
7. Dry DNA pellet for about 30 min at room temperature protected from light.
8. Dissolve pellet in 10 μL formamide-ddH_2O mix (1:2) (*see* **Note 7**).
9. Either sequence the plates directly, for example, on a DNA analyzer AB3730 XL or alternatively keep them frozen.

3.4.3. Load Plates Directly on the DNA Analyzer AB3730 XL

1. Load plates to DNA analyzer AB3730 XL according to the instructions of the manufacturer.
2. Injection conditions for sequencing of PCR fragments on 36 cm arrays (*see* **Note 24**): 1.5 V injection voltage, 5 s injection time, 7.5 V run voltage, run time 22–50 min for 300–750 bp fragments.
3. File format of trace files generated during the run: ab1 or scf files are generated and could be used for any further analysis (e.g., when using Polyphred for mutation discovery).

3.5. Analysis of Sequence Data and Mutation Detection

Several tools to screen sequence trace files for point mutations have been developed recently. Suitable open-source tools are, for example, Polyphred ((18, 19), see **Note 25**), SeqDoC (23), novoSNP (24), or SNPdetector (25). Furthermore, several commercial suppliers developed software tools to screen for point mutations.

3.6. Verification of Primary Hits

Primary hits carrying point mutations are verified by an additional and independent PCR amplification and sequencing reaction to rule out PCR-polymerase mistakes. This step becomes important if a non-proofreading enzyme is used and is performed in the same way as it was described for small-scale applications (**Sections 3.3.1** and **3.4** if a sequencing setup is available).

3.7. Translation of Verified Point Mutations and Effect Prediction

Translation of the affected DNA sequence can be done during the screening process, which immediately defines the effect on the resulting peptide (nonsense, splice site, missense, or silent mutation). The LIMSTILL software tool helps to predict the potential effect of the discovered point mutations and manages the identified mutations. LIMSTILL includes a matrix that defines the nature of an amino acid exchange (non-conservative versus conservative amino acid substitution) and includes a BLAST of the affected peptide region (uses a mutated amino acid with the adjacent 20 amino acids) against various peptide databases to see the conservation of this position (**Fig. 19.4a**) (*see* **Note 26**).

3.8. Establishment of Mutant Lines

If a living zebrafish library is screened, the individual has to be pulled out from the small number of F1 individuals pooled in one aquarium and verified by re-sequencing. Propagation of the line is achieved by breeding the identified F1 founder against wild-type zebrafish. When screening permanent libraries of sperm samples, the corresponding sperm sample is used for in vitro fertilization of female oocytes.

3.9. Molecular and Phenotypic Analysis of Mutants

Screened mutant founders of the F1 generation after mutagenesis carry a high number of accompanying point mutations. These mutations are typically unlinked to the identified mutation in a gene of interest and therefore distinguishable. However, accompanying mutations could also affect the expected mutant phenotype by either potentiating or reducing the expressivity of a phenotype when synergistic or interacting partners are affected by a point mutation.

Therefore is extremely important

(a) to reduce the number of genetically unlinked point mutations (*see* **Note 27** for an example) and

(b) to show that an observed phenotype is linked to the identified allele. It is recommended to work with identified carrier individuals only, which have been genotyped before. This can be either done by a molecular approach (see below) or genetically by complementation tests against alternative and phenotypically defined alleles of a gene of interest if these are available.

Fast and inexpensive genotyping strategies are required to explicitly identify mutant individuals, especially if direct re-sequencing strategies are not available. An unambiguous molecular identification of a carrier individual becomes extremely important if accompanying mutations influence an observed phenotype. Several alternative strategies to direct re-sequencing PCR fragments have been published recently that could be easily implemented into any molecular biology laboratory, and the actual strategy often depends on the individual point mutation (*see* **Note 28**).

4. Notes

1. The TILLING protocol described focuses on zebrafish as a genetic model system in combination with automated pipetting devices and access to DNA sequencing tools (an overview of the equipment is shown in **Fig. 19.5**). However, this pipeline is not limited to zebrafish but does also work for other vertebrate and invertebrate species. In addition, it is possible to screen lower sample numbers making use of multichannel pipettes, replicators, and dispensing tools for microplates.
2. Keep deep-well plate or Eppendorf tubes on dry ice during the clipping procedure to avoid any tissue and DNA degradation.
3. The required activity of the chosen PCR-polymerase has to be determined experimentally. The goal especially for large-scale screens is a careful titration of the polymerase to keep the screening costs as low as possible.
4. PCR primer small-scale format: Gene-specific forward and reverse PCR primers are designed with LIMSTILL or any other primer design software that is available. Ideally, the splice sites should be included in the chosen amplicon.
5. Primer large-scale format: Gene-specific forward and reverse PCR primers designed with LIMSTILL ("four primers for TILLING") or alternative software. The non-tailed primers are used in the first PCR. Tailed nested primers: the inner parts of these primers are gene-specific with universal tails: M13forward (TGTAAAACGACG GCCAGT linked to gene-specific 5′ primer) and M13reverse (AGGAAACAGCTATGACCAT linked to gene-specific 3′ primer) sequences. It is very important to test primer pairs, PCR conditions, and sequencing conditions on small numbers of wild-type DNA before the large-scale screen is initiated.
6. Choice of sequencing primers: For small-scale application use either forward or reverse gene-specific PCR primer as a sequencing primer. To calculate a suitable annealing temperature, please follow this rule:
   T_{melting}: 4(G+C) + 2(A+T)
   $T_{\text{annealing}}$: $T_{\text{melting}} - 5°C$
   Sequencing primers for large-scale setup are either M13forward (TGTAAAACGACGGCCAGT) or M13reverse (AGGAAACAGCTATGACCAT), which correspond to one of the tails of inner primer pairs. In addition, custom sequencing primers that are located internally to the

universal tales can be designed in case heterozygous insertions or deletions are present in the PCR fragments that result in a parallel and non-resolvable sequence read. The actual sequencing primer that is finally chosen depends on the initial tests of primer pairs on wild-type individuals and should cover as much coding information as possible.

7. Based on our experience, the fluorescently labeled dNTPs (Applied Biosystems Big Dye sequencing mix) used in the sequencing reaction are more stable in formamide–ddH_2O mix compared to ddH_2O alone.
8. The decision whether the library should be transient (living) or permanent (frozen sperm) depends on several factors:
   (A) The capacity of the fish facility: With zebrafish, 24–48 individuals can be kept pooled in a 15 L aquarium. More individuals in one tank would require more genotyping to identify and isolate the mutant of choice from your pooled population. Under optimal conditions in the fish facility, a healthy library of mutagenized F1 zebrafish can be successfully screened up to the age of 18–24 months.
   (B) The sperm-freezing capacity (pipeline and storage capacity): Ideally sperm should be frozen from males at the age of 9–12 months after these individuals had been successfully mated at least once. Therefore, a defined pipeline taking these steps into account is absolutely required. Since only half of a population (males) of mutagenized zebrafish is used, females can be kept as a living library in parallel.
   (C) The screening capacity for point mutations: This is defined by the PCR and sequencing capacity of the screening laboratory.

   Since both ENU mutagenesis in zebrafish and sperm freezing require some training and expertise, it is recommended to start with pilot experiments on smaller numbers of males. It is important to improve these procedures taking into account individual laboratory conditions as well as robustness of the chosen zebrafish background.
9. Genomic DNA preparation in single tube format: Alternative to multiwell plates, Eppendorf tubes of 2 mL volume can be used. In this case, all volumes used for DNA precipitation, ethanol washes, and final dilution of the pellet stay the same. All centrifugation steps can be performed in a suitable centrifuge and speed and duration can be adjusted:

- removal cell debris: 10 min at 15,700×*g*
- DNA precipitation: 20 min at 15,700×*g*
- salt removal from DNA pellet: 10 min at 15,700×*g*.

10. Commercially available genomic DNA preparation kits could provide optimized cell lysis conditions and might result in faster and cleaner DNA extractions, often depending on the genetic model organism.
11. This step removes cell debris and is recommended for long-term storage of genomic DNA stocks (not necessary for genotyping experiments).
12. Example of quantitation of DNA bands on agarose gels: When loading 2 μL of isolated genomic DNA (>23 kb when comparing to the largest *Hin*dIII-digested lambda marker band), a band appears double as wide compared to the largest reference band (23 kb, at 24 ng/μL) of the genomic DNA ladder. This corresponds to 48 ng/μL of loaded genomic DNA. Since 2 μL of genomic DNA was loaded, the concentration is about 24 ng/μL. Based on our experience, the overall yield of genomic DNA prepared from one fin clip is about 2–30 μg.

    For quantitation of PCR fragments choose a conventional DNA ladder spanning 100 bp to 5 kb. Please take care that at least for one band in the ladder the amount of loaded DNA is defined.

    Do not determine the concentration of genomic DNA in a spectrophotometer because residual RNA is not removed from these samples and interferes with photometric measurements.
13. Normalization of genomic DNA is required for direct resequencing of PCR fragments without a second nested PCR in small-scale TILLING screens.
14. For long-term storage of libraries, a copy of the genomic DNA to a master plate should be considered.
15. It is recommended to aliquot the diluted DNA in larger batches to avoid multiple freezing and thawing steps of the DNA stock as well as the diluted aliquots. When using a large-scale setup in 384-well format, the genomic DNA can be diluted and arrayed in 384-well deep-well plates making use of liquid handling robotics. In this case, 4 × 96-well plates are arrayed to a 384-well format. Dilutions are done in 384 deep-well plates (well volume, e.g., 100 μL) and 5 μL PCR template. DNA aliquots are transferred to 384-well PCR plates and kept frozen.
16. LIMSTILL is linked to several genomic databases (currently 24 different including zebrafish, human, mouse,

chick, rat, fugu) and these can be searched for potential target genes. Alternatively, Ensembl IDs or manually annotated genes can be uploaded. The genomic annotation and functional domains are visualized (**Fig. 19.4a**). Amplicons can be chosen directly taking exon size and the zebrafish ENU mutation spectrum into account (**Fig. 19.4b**). Primer design for interesting exons and TILLING projects could be done directly making use of primer3 algorithms. In addition, LIMSTILL allows management of identified mutations later on in the screening process (**Fig. 19.4b**).

17. LIMSTILL and TILLING screens in vertebrate model systems other than zebrafish: Whereas the number of sequenced vertebrate genomes is growing daily, annotation of gene structures is often missing. Especially for non-common vertebrate model organisms this requires manual annotation of genes by comparing cDNA and genomic sequences. Manually annotated genes or only defined exons can be uploaded into LIMSTILL according to its formatting options. In general, LIMSTILL databases can be easily adapted to "non-established" vertebrate model systems as long as an annotated genomic sequence of a gene of interest can be defined.
18. Smeared or multiple bands in a PCR should *not* be used for subsequent sequencing. Instead a nested PCR approach could be applied to increase PCR specificity.
19. The dilution of PCR fragments for Sanger sequencing depends on the chosen sequencing chemistry and DNA sequencer and has to be determined in collaboration with a provider of DNA sequencing.
20. Prepare master mix for all samples that should be processed in one batch; please take the dead volume of reservoirs used with your liquid handling setup into account. This should not exceed 5–15% of the sample volume to avoid extra costs.
21. For 384-well plates, add 20 μL water to each well. Take care: a total volume of 30–35 μL is the maximum volume that fits in 384-well plates.
22. The nested PCR should result in one discrete band. If not, optimize the nested approach or choose different primers. An unspecific PCR (manifested by multiple bands after PCR) should *not* be used for subsequent sequencing. The concentration of amplified fragments is estimated from agarose gels that contain marker DNA of defined concentration (e.g., conventional DNA ladders or specific ladders for quantification).

23. 20 µL of ddH_2O (molecular biology grade) is added to each nested PCR in a 384-well plate (1:3 dilution of the nested PCR product). This volume is limited by the maximum well capacity in a 384-well plate (30–35 µL). If single tubes or 96-well plates are used, the dilution volume could be increased up to 50–100 µL (1:5–1:11 dilution of PCR fragment).
24. In case a longer array (50 cm) is available for the AB3730 XL, these conditions have to be defined experimentally to achieve the optimal resolution.
25. PolyPhred is a program that compares fluorescence-based sequences across traces obtained from different individuals to identify sites heterozygous for single nucleotide substitutions. The recent version runs under LINUX/UNIX operating systems and is free to academic researchers (http://droog.gs.washington.edu/polyphred/). Our Polyphred settings allow the alignment and analysis of 768 traces in one step. Presumptive heterozygous as well as homozygous mutations are labeled in the alignment. The individual sequence read is directly connected to the chromatogram, where the sequence quality of the mutated context could be reviewed online (**Fig. 19.6**). All three open readings frames per strand can be translated and shown. Already during the screening process "interesting" mutations such as nonsense, splice site, or missense mutations can be kept and processed further whereas silent mutations can be ignored.
26. Whereas nonsense and splice site mutations result in truncated peptides that are expected to show a functional

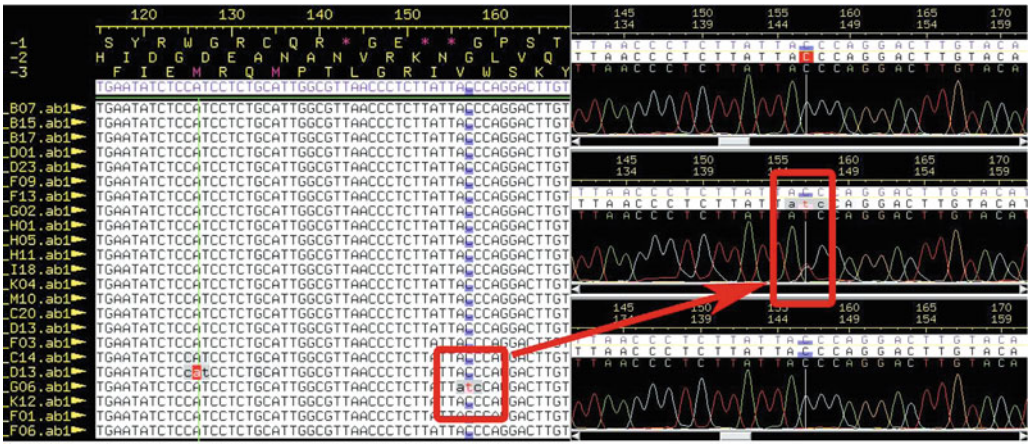

Fig. 19.6. Polyphred sequence alignment tool. Trace files are aligned and a consensus sequence as well as translation of three different frames is provided. Point mutations (homo- and heterozygous) are labeled (*squares* in alignment and chromatogram). Chromatograms of primary hits are directly linked, with "online" quality control.

defect, missense mutations might result in more subtle effects. Missense mutations that change biochemical features such as charge, polarity, or steric configurations of an affected amino acid (= non-conservative substitutions) might impair the function of the peptide. If furthermore amino acids are affected that are located in functional domains within the peptide and if they are conserved among different species, the chance of resulting in a deleterious effect is high. Only these "interesting" mutations are worth keeping and propagating; therefore, primary hits should be verified and analyzed carefully before a mutant line is established. If both criteria "non-conservative substitution and conserved position within a peptide" are fulfilled, the individual that carries the mutation should be propagated to establish a mutant line.

27. The following example illustrates the strategy for removing accompanying mutations: The size of the zebrafish genome is about 2×10^6 kb and the frequency of mutations of a given TILLING library is 1 in 150 kb. In this case, the genome of one F1 zebrafish founder of this library carries more than 13,000 different point mutations. To get rid of these, molecularly identified carriers are crossed against wild-type individuals. In each mating round about 50% of accompanying point mutations are lost, which results in only 3% accompanying point mutations (430 of 13,000) in the sixth generation after mutagenesis.
28. Since the molecular nature of a mutated allele is known, strategies such as restriction fragment polymorphisms (RFLP CAPs) that are generated by a point mutation can be applied. If no restrictions sites are directly affected, it is possible to design an artificial restriction site (derived cleaved amplified polymorphisms (dCAPs)) close to the mutation by mismatched PCR primers (26, 27). Alternatively, allele-specific PCR primers can be designed that discriminate directly between wild-type- and mutant-specific PCR products (28). The ideal genotyping strategy is defined by the molecular nature of the mutation and the available and easily accessible tools.

5. Useful Web-Based Links and Tools

Vertebrate resources for mutations:

Zebrafish: Mutation Resource Project at the Sanger Institute, England (http://www.sanger.ac.uk/Projects/D_rerio/

mutres/); MPI CBG TILLING facility (http://www.mpi-cbg.de/en/facilities/profiles/tilling.html)

Zebrafish genome sequencing (http://www.sanger.ac.uk/Projects/D_rerio/)

Mouse: Genome-wide mutant mouse library at the Riken Institute, Japan (http://www.brc.riken.go.jp/lab/mutants/RGDMSavailability.htm)

TILLING tools for primer design and organization of screens

LIMSTILL: http://limstill.niob.knaw.nl/index.html
CODDLE: http://www.proweb.org/coddle/
dCAPS: http://helix.wustl.edu/dcaps/dcaps.html
Polyphred: http://droog.gs.washington.edu/polyphred/

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