# A high-throughput method for identifying ENU-induced point mutations in zebrafish

Bruce W. Draper[1,2], Claire M. McCallum[3], Jennifer L. Stout[1,2], Ann J. Slade[3], and Cecilia B. Moens[1,2]*

[1]Howard Hughes Medical Institute

[2]Division of Basic Science, Fred Hutchinson Cancer Research Center, 1100 Fairview Ave. N., Seattle, WA 98109

[3]Anawah, Inc., 1102 Columbia St., Suite 600, Seattle, WA 98104

I. Introduction

II. Rationale for reverse genetics in zebrafish

III. Rationale for using the CEL1 assay to detect induced mutations

IV. Rationale for generating a cryopreserved mutant library

V. Method of ENU mutagenesis and rearing of $F_1$ founder fish

- A. Determining ENU concentration
- B. Producing $F_1$ mutagenized founder fish and testing specific allele frequency by *in vitro* fertilization.

VI. Preparing a cryopreserved mutant library

- A. Equipment
- B. Reagents, Buffers
- C. Method

VII. Isolating genomic DNA

- A. Equipment
- B. Reagents and Kits
- C. Method

VIII. Choosing fragments to screen.

IX. CEL1 endonuclease assay

- A. Equipment
- B. Reagents, Buffers and Kits
- C. CEL1 assay method
- D. Analysis of mutations

X. Method of mutant recovery and tracking

- A. *in vitro* fertilization with cryopreserved sperm
- B. Method of mutation tracking

XI. Materials cost estimate

References

## I. Introduction

The zebrafish has become an important model system for vertebrate biology. While forward genetic screens have uncovered the functions of many zebrafish genes, until recently no reliable, inexpensive and high-throughput technology for targeted gene disruptions has been developed. Here we outline an approach for identifying mutations in any gene of interest by TILLING (Targeted Induced Local Lesions IN Genomes), a sensitive method for detecting single nucleotide polymorphisms (SNP) in mutagenized genomes (McCallum *et al.*, 2000a,b; Colbert *et al.*, 2001). This method utilizes the CEL1 assay to detect mutations in DNA isolated from *N*-ethyl-*N*-nitrosourea (ENU) mutagenized $F_1$ individuals, which are heterozygous for randomly induced mutations (Fig. 1A). The CEL1 endonuclease specifically cleaves DNA 3' to any single base pair mismatches that are present in heteroduplexes between wild-type and mutant DNA (Olekowski et al., 1998). Genomic DNA isolated from mutagenized individuals is used as template for PCR-amplification with gene-specific, fluorescently labeled (IRDye) primers. Since both wild-type and mutant alleles are amplified from heterozygous fish, heteroduplex PCR fragments are formed by denaturing and slowly re-annealing the fragments. After CEL1 digestion, cleavage products are separated on a high resolution polyacrylamide sequencing gel to reveal the presence and approximate location of induced mutations in the target sequence. We have found that this method allows the detection of rare ENU-induced mutations in the background of pre-existing polymorphisms that are present even in inbred zebrafish strains.

To date, we have screened 25,303 kb from 5,050 mutagenized genomes using the approach described in this chapter, and we have identified 48 new mutations (1 mutation/527 kb screened). We anticipate, based on our data and data of others (Till et al. 2003; Weinholds et al., 2003) that about 5% of mutations in coding DNA identified by TILLING will result in loss-of-function alleles. We therefore project that with DNA and frozen sperm from 10,000 individuals it should be possible to generate an allelic series that consists, on average, of 20 mutations including at least 1 loss-of-function and several hypomorphic alleles, provided the gene has an open reading frame that is ≥1 kb in size. We estimate that a library prepared as outlined below can be screened at least 50,000 times before the initial DNA resource is depleted, at which time more DNA can be isolated from reserved tissue.

The screening approach is outlined in Fig. 1B. Briefly, sperm collected from ENU-mutagenized $F_1$ adult males is cryopreserved in liquid nitrogen using an efficient and rapid sperm cryopreservation protocol that archives two sperm samples per $F_1$ male. Importantly, our sperm cryopreservation protocol allows the recovery of an average of 109±84 (n=46) viable $F_2$ progeny (or 28%±18% fertility) when one of the two samples is used for *in vitro* fertilization (see below). Genomic DNA is then purified from euthanized sperm donors using a 96-well DNA isolation format. Next, mutants are identified with the CEL1 assay in a two-step screening process. For the primary screen, template DNA is pooled from 4-8 individuals, such that 384-768 mutagenized genomes are analyzed per 96 well plate of pooled DNA. PCR using IRDye-labeled primers and partial CEL1

digestion is performed in 96-well format. Because the forward and reverse primers are labeled with unique fluorescent tags, it is possible to confirm mutations identified in one channel with the presence of the corresponding cleavage product in the second channel (Fig. 2). After identifying positive pools, mutant individuals are identified in a secondary CEL1 assay, and the nature of the mutation is determined by sequencing. Finally, fish lines carrying mutations that are likely to be deleterious are recovered using the cognate cryopreserved sperm sample to fertilize eggs isolated from wild-type females (Fig. 1). The presence of the mutation in the $F_2$ generation is confirmed by PCR of tail-clipped DNA using allele-specific primers or RFLP detection.

Our method differs in several ways from that of Weinholds et al., (2003). 1) $F_1$ males are preserved as frozen sperm rather than as a living library. This eliminates the need for maintaining several thousand live fish during the screening process and provides a long-term (i.e., many year) resource for mutation detection; 2) genomic DNA is normalized and pooled prior to PCR amplification rather than after PCR. This approach has allowed us to detect mutations efficiently in 8-fold pooled samples rather than in 4-fold pooled samples, and thus decrease the expense of the mutation detection process (B. W. D., C. B. M., B. Till, L. Comai and S. Henikoff, unpublished); 3) a single-step PCR approach using gene-specific labelled primers is used rather than a nested PCR approach using universal labelled primers. While this is more expensive in terms of primer cost, it requires less liquid handling capacity and therefore may be better suited to the smaller laboratory.

An example of the data produced by the CEL1 assay is presented in Fig. 2. In this example, a 443 bp fragment was screened for induced mutations in 4-fold pools of template DNA. Following PCR amplification with IRDye labeled primers, heteroduplexes were formed, digested with the CEL1 endonuclease, and separated on a LI-COR sequencing gel. The gel images generated for the IRD700 and IRD800 primer channels are shown in Fig. 2A and 2B, respectively. Bands that are present in multiple lanes identify pre-existing polymorphisms in the target sequence, while unique bands that are present in one channel and that have the corresponding cleavage product in the second channel (boxed in Fig. 2) indicate an induced mutation- one of which is visible on this gel.

## II. Rationale for reverse genetics in zebrafish

Zebrafish forward genetic screens have been, and continue to be exceptionally productive. However as the content of the zebrafish genome becomes available in the form of primary sequence information, it becomes increasingly evident that many essential genes have not been identified by this approach. There are several possible explanations for why mutations in certain zebrafish gene have so far not been identified. First, mutant phenotypes may be subtle or even undetectable in forward genetic screens because of the nature of the screen. For example, most genetic screens performed to date have focused on identifying phenotypes during the embryonic period while the embryo is still transparent, and therefore easy to screen for morphological defects in the light microscope, or following staining with tissue-specific markers. In contrast, genes

that function primarily in larvae and adults have remained largely inaccessible to genetic analysis. Furthermore, while existing screening tools may allow for an assessment of organ differentiation and shape, they rarely allow assessment of organ physiology or function.

Second, mutant phenotypes may also be subtle or undetectable because the functions of some genes are compensated for by gene duplicates or redundant pathways. Vertebrate genomes have undergone whole genome duplication events (Furlong and Holland, 2002), and it is common for multiple copies of individual ancestral genes to be present and have overlapping expression patterns. Thus mutations in single members of a duplicate pair can often lead to subtle phenotypes, while loss-of-function of both family members causes more severe phenotypes, as has been shown in numerous cases in the zebrafish (e.g. Feldman *et al.,* 1998; Waskiewicz *et al.* 2002; Draper *et al.*, 2003). Indeed, the problem of redundancy is compounded in the teleost lineage, where an additional genome duplication is thought to have taken place since its divergence from the tetrapod lineage (Amores, *et al.*, 1998; Prince, 2002). Therefore a reverse genetic approach that allows the identification of mutations in gene duplicates independent of phenotype would be beneficial, even necessary, to assess gene function in zebrafish in a comprehensive manner. By identifying mutations independent of phenotype, a reverse genetic approach will provide access to genes and biological processes that have thus far been beyond the reach of zebrafish genetics.

**III. Rationale for using the CEL1 assay to detect induced mutations**

There are currently several technologies available that allow the identification of single nucleotide polymorphisms (SNP), the most common class of mutations induced by ENU in zebrafish spermatogonia (Imai *et al*., 2000). These include denaturing high performance liquid chromatography (dHPLC), temperature gradient capillary electrophoresis (TGCE), and the gel-based CEL1 endonuclease assay. Among these different technologies, the gel-based CEL1 assay has several advantages that make it the ideal choice for reverse genetics in zebrafish. First, the CEL1 nuclease assay can detect induced SNP in the context of a highly polymorphic genome like that of the zebrafish. The CEL-1 TILLING methodology was originally developed for Arabidopsis (Colbert *et al.*, 2001), an organism that is highly inbreed and therefore does not have a high degree of heterozygosisity for pre-existing SNP. In contrast, commonly used zebrafish lines, such AB and Tübingen, are much less inbred and thus have a high degree of heterozygosity for pre-existing SNP (Nechiporuk *et al.*, 1999). Indeed, we have found an average of 1-3 pre-existing SNP per 500 bp fragment that we have analyzed. However, as exemplified in Fig. 3, the protocol outlined here allows for the identification of induced SNP in fragments containing as many as 11 pre-existing SNP. Thus, in contrast to an induced SNP detection method that utilizes dHPLC or TGCE (McCallum *et al.*, 2000a,b) (C.M.M. and A.J.S, unpublished), the presence of multiple pre-existing SNP appears to have little or no effect on the ability of the gel-based CEL1 assay to detect induced mutations.

A second advantage of the gel-based CEL1 assay is that it is high throughput. The CEL1 assay has been used to identify induced mutations in PCR fragments that range between 400-1,500 base-pairs in length and that are amplified from 4-8 fold pools of genomic DNA (our results; B. W. D., C. B. M., B. Till, L. Comai and S. Henikoff, unpublished). Thus in a single 96-well assay it is possible to screen over 1,000 kb of sequence for induced mutations. With a mutation frequency of 1 in 500 kb, as we have observed in our library, we can identify 1-2 mutations per gel for a 1 kb fragment, or 2 mutations per gel for a 1.5 kb fragment. Therefore, a relatively small laboratory with only two gel apparatuses can reasonable expect to identify 10-20 induced mutations per week. Because approximately 5% of mutations in coding DNA are expected to be deleterious (Till et al., 2003; Weinholds et al., 2003; our unpublished data), it is possible to identify useful mutations every 1-2 weeks of screening.

A final advantage of the CEL1 assay is that the primary screen not only identifies positive pools, but also indicates the position of the induced point mutation relative to the fluorescently labeled primers. The resolution of the LI-COR gel is such that mutations can be localized to within 10 bp in the primary screen. Thus for primers that amplify both exon and intron sequences, as exemplified in Fig. 3, only induced mutations that localize to coding DNA or sufficiently close to the canonical splice donor/acceptor sites need to be analyzed further.

**IV. Rationale for generating a cryopreserved mutant library**

Prior to screening for ENU-induced mutations in zebrafish, we chose first to generated a library consisting of cryopreserved sperm isolated from the $F_1$ progeny of ENU-mutagenized males. While it is possible to screen for mutations in fish that are kept alive, a cryopreserved library has several advantages. First, for some genes it may be necessary to screen as many as 10,000 mutagenized genomes to identify a useful mutation. For example, we anticipate that 5% of all induced mutations will be deleterious to gene function. Thus, with a mutation frequency of 1 in 500 kb, it will be necessary to screen 1 kb of coding DNA in 10,000 mutagenized genomes to identify a single deleterious mutation. For small- and medium-sized fish facilities, this could be a prohibitive number of live fish to maintain at any one time. Second, under even the best conditions, zebrafish are only fecund for 1.5-2 years of age. Thus it is necessary to generate a new living library on a yearly basis. In contrast, a cryopreserved library can be large enough to ensure the identification of mutations in almost any gene and once made is stable indefinitely and therefore can be a resource for many years.

## V. Method of ENU Mutagenesis and rearing of $F_1$ founder fish

A mutant library can efficiently be produced by randomly mutagenizing adult zebrafish spermatogonia with ENU following a standard protocol (Solinca-Krezel *et al.*, 1994). We have added the following modifications that ensure a consistent mutagenesis efficiency and to maximize the production of $F_1$ offspring with the minimum amount of effort.

### A. Determine ENU concentration

After re-suspension of approximately 1 gm of ENU (Sigma) in 85 ml of ENU dilution buffer (0.03% Instant Ocean, 10mM $NaPO_4$, pH 6.5) determine the concentration by measuring the $OD_{398}$ of a 1:20 dilution. A 1 mg ENU/ml solution has an $OD_{398}$=0.72 (Justice *et al.*, 2000).

**B. Producing $F_1$ mutagenized founder fish and testing specific allele frequency by *in vitro* fertilization.**

One month after the final mutagenesis, $F_1$ founder fish are most efficiently produced by *in vitro* fertilization using sperm squeezed from mutagenized males (Westerfield, 1995). Re-suspend squeezed sperm in 100 µl Hank's saline (see section VI.B.2), and use 10µl of this solution to fertilize eggs isolated from wild-type females (for more on *in vitro fertilization*, see Section IX.A or Westerfield, 1995). Using this strategy, 1,500-2,000 $F_1$ progeny per mutagenized male can routinely be produced in a single day. In order to prevent the isolation of multiple mutations that result from a single mutagenic event, it is important to keep track of all $F_1$ founders that are the progeny of a single ENU mutagenized male. To minimize this possibility, we limit to 1,000 the number of $F_1$ progeny a single mutagenized male can contribute to the mutant library .

An additional advantage of the *in vitro* fertilization strategy for generating $F_1$ founders is that a specific allele frequency can simultaneously be obtained using the excess sperm. For example, we routinely use excess sperm to fertilize eggs squeezed from females that are homozygous for the *nacre* mutation. *nacre* is an ideal tester locus because it is homozygous viable and mutant embryos lack body pigmentation, a phenotype that is easily scored two days post-

fertilization. In addition, the *nacre* gene is of average size (1.2 kb), and thus is a representative target (Lister *et al.*, 1999). We routinely identify new nacre alleles by non-complementation at a frequency that ranges from 1 in 500 to 1 in 1,200 haploid genomes screened.

## VI. Generating a cryopreserved mutant library

The sperm cryopreservation method we use to generate a cryopreserved mutant library is an adaptation of the Harvey method (Harvey *et al.*, 1982; Westerfield, 1995) that both streamlines the procedure and increases sample uniformity. First, after sperm is isolated from individual males, the volume is normalized using freezing medium that does not contain cryoprotectant, prior to adding freezing medium containing cryoprotectant. Second, cryopreserved sperm are stored in screw cap cryovials instead of capillary tubes. With these simple modifications, teams of 2 people can collect and cryopreserve the sperm from 100 males in 2 hrs. Importantly, using this method we routinely achieve an average of 28%±18% fertility, recovering 109±84 (n=46) viable $F_2$ progeny following *in vitro* fertilization.

### A. Equipment

1. 10µl disposable pipettes (Fisherbrand cat# 22-358697)
2. 250ml beakers with fish water containing Tricaine
3. Watch glasses (Pyrex cat# 9985-75)
4. P20 pipetman (or equivalent) and tips
5. Sponge with slit cut in top to hold male fish while squeezing
6. Plastic spoon

7. Dissecting microscope with above-stage lighting
8. 2.0ml screw cap cryogenic vials (Corning cat# 430488)
9. 2.0 ml microcentrifuge tubes for freezing and storing male fish after sperm isolation.
10. Cryogenic freezer [e.g. Taylor-Warton (Theodore, AL) 10K]
11. 10X10 cryoboxes for storing sperm in liquid nitrogen (Nalgene cat#03-337-7AA)
12. 15 ml conical tubes (Falcon # 352099)
13. Large styrofom cooler (8 in. X 12 in. inside dimensions) filled with at least 6 in. depth of finely pulverized dry ice for freezing sperm.
14. Ice bucket filled with dry ice for storing males.
15. Dry ice crusher (e.g. Clawson Ice Crusher, model RE-2)
16. Large Dewar flask (e.g. Nalgene 10 liter, #4150-9000) containing liquid nitrogen.
17. Long forceps (e.g. CMS Fisher Health Care cat# 10-316B)
18. Cryogloves

**B. Reagents and Buffers**

1. *Tricaine anesthetic:* (3-aminobenzoic acid ethyl ester) (Sigma #A5040) 4.2 ml tricaine solution mixed with 100 ml fish water.

   *a. Tricaine solution:* 400 mg tricaine dissolved in 97.9 ml $ddH_2O$. Adjust to pH 7.0 with approximately 2.1 ml 1 M Tris (pH 9).

2. *Hank's saline*. 0.137 M NaCl, 5.4 mM KCl, 0.25 mM $Na_2HPO_4$, 0.44 mM $KH_2PO_4$, 1.3 mM $CaCl_2$, 1.0 mM $MgSO_4$, 4.2 mM $NaHCO_3$.

*a. Hank's final:* 9.9 ml Hank's premix, 0.1 ml Stock #6

*b. Hank's premix:* Combine the following solutions in order: 10 ml stock #1, 1.0 ml stock #2, 1.0 ml stock #4, 86.0 ml dd$H_2O$, 1.0 ml stock #5

*c. Hank's Stock solutions:*

| Stock #1 | Stock #2 | Stock #4 | Stock #5 | Stock #6 |
|---|---|---|---|---|
| 8.0 g NaCl<br>0.4 g KCl<br>in 100 ml<br>dd$H_2O$ | 0.358g<br>$Na_2HPO_4$<br>Anhydrous<br>0.60 g<br>$K_2H_2PO_4$<br>in 100 ml<br>dd$H_2O$ | 0.7 g $CaCl_2$<br>in 50 ml<br>dd$H_2O$ | 1.23 g $MgSO_4$ X<br>7$H_2O$<br>in 50 ml dd$H_2O$ | 0.35 g<br>$NaHCO_3$<br>in 10 ml<br>dd$H_2O$ |

3. *Ginsburg's Fish Ringers:* For 500 ml final, add in order: 450 ml dd$H_2O$, 3.25 g NaCl, 0.125 g KCL, 0.175 g $CaCl_2$•$H_2O$, 0.10 g $NaHCO_3$. Adjust final volume to 500 ml with dd$H_2O$ and store at 4ºC. This solution should be made fresh every 3 days.
4. *Freezing Medium WITHOUT Methanol:* For 10 ml final volume, add in order 9 ml Ginsburg's Fish Ringers (room temperature) and 1.5 gm powdered skim milk. Adjust final volume to 10 ml with Ginsburg's Fish Ringers.
5. *Freezing Medium WITH Methanol:* For 10 ml final volume, add in order 8 ml Ginsburg's Fish Ringers (room temperature), 1 ml Methanol and 1.5 gm powdered skim milk. Adjust final volume to 10 ml with Ginsburg's Fish Ringers. After assembling freezing media, mix well for 20 min. on orbital shaker or rocker and aliquot into 1 ml microcentrifuge tubes.

**C. Method**

1. *MARK CAPILLARY TUBES*: Prior to beginning, use a lab pen to place a mark 16.5 mm from the bottom of a10µl capillary tubes. This mark indicates the target sperm volume of 3.3 µl.
2. *ANESTHETIZE MALE:* Place male(s) in a 250 ml beaker containing 100 ml of tricaine anesthetic.
3. *DRY FISH:* Once anesthetized, remove male from beaker with plastic spoon and blot dry by gently rolling fish on paper towel, paying special attention to dry ventral side. Water activates sperm so it is important to dry thoroughly around the urogenital pore. It is important that no pressure be applied to the torso while drying as this may result in premature expulsion of milt.
4. *POSITION FISH ON SPONGE HOLDER:* Position the male in a sponge holder, ventral side up and place on the dissecting microscope stage.
5. *COLLECT SPERM:* Expose the urogenital pore by carefully spreading apart the anal fins using the end of the capillary tube. Expel sperm by gently squeezing the sides of the fish between your index finger and thumb, massaging in an anterior to posterior direction. Collect sperm in capillary tube as it is expelled using gentle suction, avoiding feces that may be expelled with sperm.
6. *FREEZE MALE ON DRY ICE:* While the sperm donor is still anesthetized, place him in a labeled 2.0 ml microcentrifuge tube and freeze on dry ice. Store males at –80ºC until time to isolate genomic DNA (see DNA isolation method below).

7*. NORMALIZE SPERM VOLUME TO 3.3µl:* If the volume of sperm isolated reaches, or exceeds, pen mark on capillary tube (i.e. 3.3 µl or greater), then proceed to step 8. If sperm volume is less than 3.3 µl, then normalize to 3.3 µl using room temperature *Freeze Medium WITHOUT Methanol*. Minimum amounts of sperm that are acceptable varies with the quality of sperm. Good sperm is white and opaque. Poor sperm looks watery. In general we accept as minimums: 1µl good sperm and 2 µl poor sperm .

8*. ADD CRYOPROTECTANT TO SPERM :* Gently aspirate room temperature *Freeze Medim WITH Methanol* to the orange band on capillary tube (total volume is now 20µl). Expel sperm and cryoprotectant mixture onto clean area of a watch glass, paying special attention to not introduce bubbles. Gently mix by pipetting.

9*. ALIQUOT 10µl SPERM INTO EACH OF 2 CRYOVIALS:* Pipette 10µl of the sperm solution into the bottom of two separate cryovials that have been labeled with relevant information. Cap vials and drop them into the bottom of *room temperature* 15 ml Falcon tubes- one cryovial/tube. Cap Falcon tube.

10*. FREEZE SPERM FOR 20 MIN. ON DRY ICE:* Immediately insert the pair of cryovial-containing Falcon tubes into crushed dry ice. The tubes should be inserted into the dry ice deep enough that only their caps show. To keep track of tubes in dry ice, number pairs of tubes from 1-20 (on caps) and record the time they go into the dry ice. *Speed is important!* Steps 6-9 should take no more than 30 sec.

11. *PLACE CRYOVIALS INTO LIQUID NITROGEN:* After 20 min., transfer cryovials to a liquid nitrogen-containing Dewar flask. Vials are stored here until time to place into liquid nitrogen freezer. When placing in the freezer boxes, place freezer box in a bath of liquid nitrogen to maintain temperature. Use long metal forceps to recover vials from Dewar flask and handle with cryogloves. *Store cryovials long term in a cryogenic liquid nitrogen freezer.* To maintain viability of sperm, it is important that vials are stored immersed in liquid nitrogen, not in the vapor phase.

## VII. Genomic DNA Isolation

High quality genomic DNA is an essential reagent for the TILLING methodology. To assure the highest quality, it is necessary to store fish tissue at –80ºC until time to isolate DNA. We use a 96-well format DNA isolation system to minimize sample handling and to increase throughput. While there are several equivalent options for preparing high quality DNA, our protocol below utilizes the QIAGEN 96-well format DNeasy Tissue kit (Qiagen, Inc., Valencia, CA). However, with appropriate modifications, other kits can be substituted. Using this method, we routinely recover 20-40 µg of high molecular weight genomic DNA per sample- enough to screen over 50,000 times.

### A. Equipment

1. High-speed tabletop centrifuge (e.g. Qiagen 4-15C) equipped with a 96-well plate compatible rotor (e.g. Qiagen plate rotor 2X96).
2. Dog nail clippers (scissor style; available at most pet stores).

3. Multichannel pipetters (8 or 12 channels)
4. Three medium sized styrofoam boxes filled with finely pulverized dry ice.
5. 96-position microcentrifuge tube rack (e.g. Fisher cat# 05-541-29)
6 Additional 96 X 1.4 ml plates [Micronic Systems (McMurray, PA) cat# M42000]

**B. Reagents and Kits**

1. DNeasy 96-tissue kit (Qiagen cat# 69582)

**C. Method**

1. Prior to tissue isolation, array the fish-containing microcentrifuge tubes into the appropriate positions of a 96-position microcentrifuge tube rack. To prevent tissue from prematurely thawing, the rack should be partially buried in crushed dry ice.
2. Remove fish from tube and, while still frozen, use dog nail clippers to clip off the head just behind the gills. Transfer the head to the appropriate DNA preparation tube (e.g. a 2 ml, 96-well format tube included with the DNA isolation kit). These tubes should also be partially buried in crushed dry ice. Place the remaining fish carcass back into its original microcentrifuge tube and place on dry ice. This remaining tissue is stored long term at –80ºC and, if necessary, can be used for isolating additional DNA.
3. Isolate the DNA from the fish tissue following the kit manufacturer's recommendation, with the following modification: because a large amount of insoluble material is present following tissue lysis (e.g. bone, cartilage and scales), it is necessary to clear this debris from the lysis by centrifugation

and to transfer the cleared lysate to a new set of 2 ml, 96-well format tubes. Following manufacturers recommendation, RNase-treat the DNA samples at this stage prior to proceeding with DNA isolation.

4. Once the DNA has been isolated, it is necessary to normalize their concentrations. The concentration of the DNA samples can be determined using any number of methods. For example, DNA concentrations can be rapidly determined in a 96-well format using the PicoGreen (MolecularProbes, Eugene, OR) fluorescent assay (Singer et al., 1997) and a 96-well fluorimeter (PerkinElmer Life Sciences Inc, Boston, MA). Alternatively, DNA concentrations can be estimated by comparing the intensity of genomic DNA bands to lambda DNA standards following brief (20 min. at 20 V/cm) electrophoresis through a 1% agarose gel (B. Till, L. Comai and S. Henikoff, pers. com.). Once the individual concentrations have been determined, all DNA samples are normalized to a standard concentration (e.g. 40 ng/μl) by addition of the appropriate amount of TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA). Prior to CEL1 analysis, DNA samples are pooled either 4 or 8-fold and diluted to a final concentration of 4 ng total DNA/μl in TE. In order to detect all possible mutations in pooled samples, it is essential that the individual DNA concentrations be accurately normalized prior to pooling (B. Till, L. Comai and S. Henikoff, pers. com).

## VIII. Choosing fragments to screen.

The ability to identify useful mutations in any gene of interest depends on three factors- mutation frequency, target size and size of library. PCR fragments between 0.4-1.5 kb can be screened using the CEL1 assay (our results; B. W. D., C. B. M., B. Till, L. Comai and S. Henikoff, unpublished), and in some cases, it may be possible to screen 1.0 kb of protein coding sequence in a single assay. If the frequency of mutations in the library is reasonably high (e.g 1 induced mutation/ 500 kb screened), then it should be possible to identify 15-20 mutations in a 1.0 kb fragment by screening 7,500-10,000 mutagenized genomes. However, most genes in zebrafish do not consist of large exons, but rather are composed of many small exons separated by large introns. Thus, for the majority of genes, it will be necessary to screen multiple smaller amplicons to assay a sufficient amount of protein coding DNA to assure the identification of useful mutations.

For genes that have several possible fragments that can be screened, gene structure/function models can be used to determine the optimal gene fragment(s) by considering the following three criteria: 1) the likelihood that a mutation within a particular gene fragment would disrupt a conserved functional domain, 2) the statistical likelihood that an ENU-induced mutation within a particular gene fragment would create a premature stop codon, and 3) exon size. To simplify this analysis, we employ the CODDLE web-based analysis program (http://www.proweb.org/coddle/)(McCallum *et al.*, 2000b). CODDLE builds gene structure/function models and identifies exons that have the highest proportion of codons that could mutate to either a non-conservative amino acid substitution or

a premature stop codon given the spectrum of mutations induced by ENU in zebrafish.

## IX. CEL1 endonuclease assay

Screening for ENU-induced point mutations with the CEL 1 assay consists of two basic steps: mutation detection using pooled DNA templates and mutation confirmation using individual DNA templates, as outlined below. While it is possible to detect unique SNP in 12-fold pools of individuals template DNA, the signal from 4-8-fold pools is more robust.

### A. Equipment

1. Thermal cycler with a 96-well block
2. LI-COR Global $IR^2$ gel scanner (LI-COR, Lincoln, NE)
3. Computer with internet access
4. Table top centrifuge with 96-well plate compatible rotor
5. 96-well PCR plates (e.g. ABgene, Rochester NY)
6. Multichannel pipettors (8 or 12 channels)
7. 100 tooth membrane combs (The Gel Company, San Francisco, CA)
8. Loading Tray for Membrane Combs (The Gel Company, San Francisco, CA), or Comb loading robot (MWG, Biotech)
9. IRDye700 50-700 Sizing Standard (LI-COR cat# 4200-60)
10. Image analysis software [e.g. Photoshop (Adobe, San Jose, CA) or equivalent]
11. Apricot 96-channel pipettor (Perkin Elmer, Boston, MA)(Optional)

12. 96-well heat blocks (Optional)

**B. Reagents and buffers**

1. *Ex Taq polymerase* (Takara Mirus Bio, Kyoto, Japan)
2. *Primer mix*: 45 μM IRD700-labled forward primer, 5 μM unlabeled forward primer, 45 μM IRD800-labled reverse primer, 5 μM unlabeled reverse primer. IRD-labeled primers are light sensitive so care should be taken to minimize exposure to light during the assay.
3. *CEL 1 digestion solution:* 10mM HEPES (pH 7.5), 10 mM $MgS0_4$, 0.002% (w/v) Triton X-100, 20 ng/ml bovine serum albumin, and 1:300-1:10,000 dilution of CEL 1. Because the activity of CEL1 varies from batch to batch, the exact concentration necessary to achieve optimum digestion must be determined empirically.
4. *CEL 1:* This enzyme is isolated from celery (Oleykowski *et al.*, 1998) and can be obtained commercially from Transgenomic, Ltd. (Omaha, NE) under the trade name Surveyor™.
5. *100 bp IRD-labeled lane marker:* To make it easier to determine lane numbers following electrophoresis, we add a 100bp IRD-labeled marker in every $12^{th}$ lane. This marker can easily be made by PCR using dye labeled primers designed to amplify any 100 bp product. The exact amount to add per lane must be determined empirically.
6. 6.5% $KB^{Plus}$ polyacrylamide (LI-COR, Lincoln, NE)
7. *Formamide load solution:* 1 mM EDTA (pH 8) and 200 μg/ml bromophenol blue in 33% deionized formamide.
8. *1% Ficol solution:* 1% Ficol (w/v) in $dH_2O$.

9. *Stop solution:* 75 mM EDTA (pH 8), 2.5 M NaCl.

**C. CEL1 Assay Method**

All PCR reactions are preformed in 96-well plates with a final reaction volume of 15µl. Because the same template DNAs are used repeatedly, it is convenient to aliquot the appropriate amount of pooled template DNA into many 96-well plates, seal and store at –20ºC until needed. Primers are designed with the aid of Primer3 (http://www.broad.mit.edu/cgi-bin/primer/primer3.cgi/primer3_www.cgi)(Rozen and Skaletsky, 2000) and to have a melting temperature between 65-72ºC and a length between 25-30 nucleotides. IRD700 and IRD800 dye-labeled primers can be obtained from MWG Biotech (Ebersberg, Germany). Re-suspended IRD-labeled primers to 100 µM in TE and stored at –20ºC. Because IRD-labeled primers are photosensitive, care should be taken to minimize light exposure when handling primer-containing solutions.

1. *Standard PCR reaction:* Aliquot 10 µl of the *PCR Master Mix* into each well of a 96-well plate that contains 5 µl of pooled template DNA (4 ng total DNA/well).

*PCR Master Mix:* Mix the following on ice:

| | |
|---|---|
| 124 µl | 10 X PCR buffer |
| 73µl | 25 mM $MgCl_2$ |
| 198 µl | 2.5 mM (each) dNTP mix |
| 6.6 µl | Primer mix |
| 11 µl | Ex Taq DNA polymerase |
| 687 µl | dd$H_2O$ |
| 1,100 µl | $V_t$ |

2. *Standard PCR cycling profile:* Our PCR cycling is carried out in an MJ Research (Waltham, MA) DNA Engine thermal cycler using the following parameters:

| Step | Temp. | Time |
|---|---|---|
| 1 | 95ºC | 2 min |
| 2 | 94ºC | 20 sec |
| 3 | $T_m$+3ºC | 30 sec (decrease 1ºC/cycle to $T_m$-4ºC) *ANNEALING* |
| 4 | ramp 0.5ºC/sec to 72ºC | |
| 5 | 72ºC | 1 min (ramp 0.5ºC/sec) *EXTENSION* |
| 6 | repeat steps 2-4 7 more times (8 cycles total) | |
| 7 | 94ºC | 20 sec |
| 8 | $T_m$-5ºC | 30 sec *ANNEALING* |
| 9 | ramp 0.5ºC/sec to 72ºC | |
| 10 | 72ºC | 1 min (for 600-1,000 base amplicon) *EXTENSION* |
| 11 | repeat steps 6-8 29-39 more times (30-40 cycles total) | |
| 12 | 72ºC | 5 min |
| 13 | 98ºC | 8 min *DENATURATION* |
| 14 | 80ºC | 20 sec |
| 15 | 80ºC | 7 sec (decrease 0.3ºC/cycle) *REANNEALING* |
| 16 | repeat step 15, 69 more times (70 cycles total) | |
| 17 | 8ºC | HOLD |

3*. CEL1 Digestions*

*a.* Place PCR plate on ice and add 30 µl CEL 1 digestion solution

*b.* Incubate plate at 45ºC for 30 minutes in PCR machine.

*c.* Stop digestion by adding 10µl Stop solution.

*d.* Add appropriate amount of 100 bp lane marker to wells that will be loaded every 12$^{th}$ lane on the gel (e.g. add to column 12 of the 96-well plate)

*e.* Precipitate digested DNA by adding 80µl Isopropanol per well, seal plate with plastic cover and incubate at room temperature overnight, protected from light.

*f.* Pellet DNA by centrifugation at 3220 RCF for 30 min. in a table top centrifuge.

*g.* Re-suspend pellet in 8 µl formamide load solution and incubate in a thermal cycler at 80ºC for 7 min, followed by 95ºC for 2 min.

4. *Running LI-COR gel:*

*a.* Membrane combs are loaded as follows: approximately 0.3 µl of each sample is spotted onto the middle 96 teeth of a 100 tooth membrane comb with an automated comb loading robot (MWG, Biotech). Alternatively, combs can be loaded manually with the aid of a comb loading tray (The Gel Company, San Francisco, CA) and a mutichannel pipettor that allows the tips to be variably spaced (e.g. Matrix Equalizer, Matrix Technologies, Hudson, NH).

*b.* After the samples have been loaded onto the comb, spot 0.3µl of the IRDye700 50-700 Sizing Standard (LI-COR) onto the empty combs that flank the samples.

*c.* Prepare a polyacrylamide gels using LI-COR 6.5% KB$^{Plus}$ polyacrylamide in 1X TBE, following manufacturers recommendations (LI-COR).

*d.* Once gel is positioned in the LI-COR apparatus, fill both upper and lower buffer chambers with 0.8X TBE and perform a pre-run focusing step for 20 min at 1,500-V, 40-W, and 40-mA limits at 50ºC.

*e.* After the pre-run, but prior to inserting the comb, remove as much of the buffer in the upper buffer chamber as necessary to expose the comb well. Then, with the aid of a syringe and strips of Whatman paper, remove as much of the buffer from the comb well as possible. Next, use a syringe to refill the comb well with a 1% Ficoll in $dH_2O$ solution, and carefully insert the comb until the teeth just touch the surface of the gel. Comb insertion is most easily accomplished by holding the comb at a 45º vertical angle to the gel. After the comb has been inserted, slowly and carefully refill the upper buffer chamber with 0.8X TBE.

*f.* Finally, run the gel for 3-4 hrs at 1,500-V, 40-W, and 40-mA limits at 50ºC. Gel images are stored on the LI-COR machine as TIFF files and can be retrieved and viewed using any computer with internet access and appropriate software (e.g. Photoshop) following manufacturers instructions.

5. *Secondary screen identifies individual mutant.*

After the primary CEL1 assay has identified DNA pools that contain mutations, re-screen the individual DNA samples that are contained within each positive pool using the same parameters outlined above. Finally, determine the nature of the mutation by sequencing the PCR fragment amplified from the positive individual using only unlabeled primers.

**D. Analysis of mutations**

Mutations that introduce premature stop codons 5' to conserved protein-coding domains are predicted to be null alleles since they cause protein

truncations. However, it is less straight forward to predict the effect of missense mutations on protein function. To aid in assessing the likelihood that a particular missense mutation could have deleterious effects on gene function we use the PARSESNP (Project Aligned Related Sequences and Evaluate SNPs) web-base program (Taylor and Greene, 2003; http://www.proweb.org/parsesnp/). PARSESNP builds intron/exon gene models of target fragments by comparing genomic and coding sequence information and then automatically identifies conserved domains. Once the mutation information is entered, PARSESNP uses sequence homology in the conserved domains to predict whether an amino acid substitution at a particular protein position will be tolerated or deleterious to protein function by calculating a position specific scoring matrix (PSSM) difference score for each altered residue. PSSM difference scores range from 0-30 and missense mutations that score above 10 are predicted to be deleterious to protein function.

## X. Recovery of Mutations from Cryopreserved Sperm

Live fish lines that are heterozygous for interesting mutations are recovered from cryopreserved sperm stocks by in vitro fertilization. Because this is a very important step, extreme care should be taken to fertilize only high quality eggs that have a uniform, yellowish appearance.

### A. Equipment

1. 33ºC water bath
2. Dewar flask containing liquid nitrogen

## B. Reagents and Buffers

1. *Tricane anesthetic:* See section VI.B.1
2. *Hank's saline:* See section VI.B.2

## C. *In vitro* fertilization with cryopreserved sperm

1. Isolate eggs from anesthetized females by gentle squeezing, and collect in a 35mm plastic culture dish (Westerfield, 1995). To maximize recovery of fertile eggs, it is possible to combine clutches of eggs isolated from multiple females prior to fertilization.
2. Thaw cryopreserved sperm by removing the cryovial cap and immersing the cryovial half way into a 33ºC water bath for 8-10 sec.
3. Add 70 µl of room temperature Hank's solution to cryovial and gently mix with sperm.
4. Quickly add the re-suspended sperm solution to the eggs and gently mix with the pipett tip.
5. Activate eggs and sperm by adding 750µl of room temperature fish water.
6. Following incubation at room temperature for 5 min, add an additional 5 ml of water to the dish and place in a 28ºC incubator.
7. After incubating 4 hr at 28ºC, sort fertile eggs into 100mm culture dishes (70 embryos/dish) containing fish water and raise fry using standard conditions.

### D. Tracking recovered mutations

Once fish carrying interesting mutations have been recovered, it is useful to develop a genotyping assay that is both easy and reliable. An ideal method is to design a PCR-based assay to screen DNA isolated from caudal fin-clips. For cases where the identified mutation either creates or eliminates a restriction enzyme cleavage site in the genomic DNA, PCR primers can be designed that amplify the polymorphic sequence and a simple restriction digest of the PCR fragment will reveal the genotype. However, for mutations that do not affect a restriction site, it is possible to design mismatched PCR primers that will create an allele-specific restriction site in the amplified fragment using a technique called dCAPS (derived cleavage amplified polymorphic sequence)(Neff et al., 1998). dCAPS primers can be designed for nearly any sequence and primer design can be facilitated by using the web-base program dCAPS Finder 2.0 (http://helix.wustl.edu/dcaps/dcaps.html).

## XI. Materials cost estimate

We have presented an efficient method for identifying ENU-induced mutations in specific genes of interest in the zebrafish. We have observed a mutation detection frequency of approximately 1 mutation per 500 kb screened. Since only about 5% of ENU-induced mutations in protein coding DNA are expected to be deleterious, it is necessary to screen, on average, 10,000 kb of coding target gene sequence to identify one or two deleterious mutations. Thus with DNA and frozen sperm from 10,000 $F_1$ individuals it will be possible to

generate an allelic series including loss-of-function mutations in any target ≥ 1 kb in size. The estimated cost of TILLING is summarized in Table 1, and depends on the target to be screened: targets consisting of a single, long contiguous open reading frame can be screened as a single fragment and thus will be less expensive than targets that must be screened as multiple small fragments. We estimate that the per-plate materials cost of TILLING is $60.00 including enzymes, plasticware, ladder and chemicals. IRDye labeled primers cost approximately $130.00 each. Thus the cost of screening a single 1 kb fragment in 10,000 8-fold-pooled individuals is $60.00 x 13 + $260.00 = $1040.00. The primary screen is expected to identify, on average, 20 mutations, so a secondary screen of a further two 96-well plates will add $120.00 to the total cost. Finally, sequencing 20 mutations will add an additional $140.00. Thus the total cost of TILLING a single 1 kb fragment in 10,000 individuals is approximately $1,300.00; larger fragments will cost less since they can be screened in fewer individuals, while multiple, smaller fragments will cost more (Table 1). Thus, the CEL1 assay is an efficient and cost-effective method for reverse genetics in zebrafish.

Acknowledgments

We thank Brad Till and the University of Washington Arabidopsis Tilling Project for sharing information on mutation detection frequencies, costs estimates of reagents, and for critical reading of this manuscript. C.B.M is an Assistant Investigator with the Howard Hughes Medical Institute.

**References**

Amores, A., Force, A., Yan, Y. L., Joly, L., Amemiya, C., Fritz, A., Ho, R. K., Langeland, J., Prince, V., Wang, Y. L., Westerfield, M., Ekker, M., and Postlethwait, J.H. (1998) Zebrafish hox clusters and vertebrate genome evolution. *Science* **282**, 1711-1714.

Colbert, T., Till, B.J., Tompa, R., Reynolds, S., Steine, M.N., Yeung, A.T., McCallum, C.M., Comai, L., and Henikoff, S. (2001) High-throughput screening for induced point mutations. *Plant Physiol.* **126**, 480-484.

Draper, B. W., Stock, D. W., and Kimmel, C. B. (2003) Zebrafish fgf24 functions with fgf8 to promote posterior mesoderm development. *Development* **130**, 4639-4654.

Feldman, B., Gates, M.A., Egan, E.S., Dougan, S.T., Rennebeck, G., Sirotkin, H.I., Schier, A.F. & Talbot, W.S. (1998) Zebrafish organizer development and germ-layer formation require nodal-related signals. *Nature* **395**, 181-185.

Furlong, R.F. & Holland, P.W. (2002) Were vertebrates octoploid? *Philos Trans R Soc Lond B. Biol Sci.* **357**, 531-544.

Harvey, B., Kelley, R. N., and Ashwood-Smith, M. J. (1982) Cryopreservation of zebra fish spermatozoa using methanol. *Can. J. Zoology*. **60**, 1867-1870.

Imai, Y., Feldman, B., Schier, A. F., and Talbot, W. S. (2000) Analysis of chromosomal rearrangements induced by postmeiotic mutagenesis with ethylnitrosourea in zebrafish. *Genetics* **155**, 261-272.

Justice, M. J., Carpenter, D. A., Favor, J., Neuhauser-Klaus, A., Hrabe de Angelis, M., Soewarto, D., Moser, A,. Cordes, S., Miller, D., Chapman, V., Weber, J. S., Rinchik, E. M., Hunsicker, P. R., Russell, W. L., and Bode, V. C. (2000) Effects of ENU dosage on mouse strains. *Mam. Genome* **11**, 484-488.

Lister, J. A., Robertson, C. P., Lepage, T., Johnson, S. L. and Raible, D. W. (1999) nacre enconds a zebrafish microphthalmia-related protein that regulates neural-crest-derived pigment cell fate. *Development* **126**, 3757-3767

McCallum, C. M., Comai, L., Green, E. A., and Henikoff, S. (2000a) Targeted screening for induced mutations. *Nat. Biotech.* **18**, 455-457

McCallum, C. M., Comai, L., and Henikoff, S. (2000b) Targeting induced local lesions IN genomes (TILLING) for plant functional genomics. *Plant Physiol.* **123**, 439-442.

Nechiporuk, A., Finney, J.E., Keating, M.T., and Johnson, S.L. (1999) Assessment of polymorphism in zebrafish mapping strains. *Genome Res.* **9**, 1231-1238.

Neff M. M., Neff, J. D., Chory, J., and Pepper, A.E. (1998). dCAPS, a simple technique for the genetic analysis of single nucleotide polymorphisms: experimental applications in Arabidopsis thaliana genetics. *Plant J.* **14,** 387-392.

Neff, M. M., Turk, E., and Kalishman, M. (2002) Web-based primer design for single nucleotide polymorphism analysis. *Trends Gen.* **18**, 613-615.

Oleykowski, C. A., Bronson Mullins, C. R., Godwin, A. K., and Yeung, A. T. (1998) Mutation detection using a novel plant endonuclease. *Nucleic Acids Res.* **26**, 4597-4602.

Prince, V.E. (2002) The hox paradox: More complex(es) than imagined. *Dev. Biol.* **249**, 1-15.

Rozen, S., and Skaletsky, H. J. (2000) Primer3 on the WWW for general users and for biologist programmers. *In* "Bioinformatics methods and protocols: methods in molecular biology" (S. Krawetz and S. Misener, Eds.) pp. 365-386.

Singer, V. L., Jones, L. J., Yue, S. T., and Haugland, R. P. (1997) Characterization of PicoGreen reagent and development of a fluorescence-based solution assay for double stranded DNA quantitation. *Anal. Biochem.* **249**, 228-238.

Solnica-Krezel, L., Schier, A. F., and Driever, W. (1994) Efficient recovery of ENU-induced mutations from the zebrafish germline. *Genetics* **136,** 1401-1420.

Taylor N. E., and Greene E. A. (2003) PARSESNP: A tool for the analysis of nucleotide polymorphisms. Nucleic Acids Res. **31**, 3808-3811.

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 Res.* **13**, 524-530.

Waskiewicz, A.J, Rikhof, H.A., and Moens, C.B. (2002) Eliminating zebrafish pbx proteins reveals a hindbrain ground state. *Dev. Cell* **3**, 723-733.

Westerfield, M. (1994) The Zebrafish Book. University of Oregon, Eugene, OR.

## Tables

Table 1. Estimated cost of materials for TILLING

| | 1 fragment | 2 fragments | 3 fragments | 4 fragments |
|---|---|---|---|---|
| primary screen | $780 | $1,560 | $2,340 | $3,120 |
| primers | $260 | $520 | $780 | $1,040 |
| secondary screen | $120 | $120 | $120 | $120 |
| sequencing | $140 | $140 | $140 | $140 |
| **total** | $1,300.00 | $2,340 | $3,380 | $4,420 |

Estimated cost of screening 10,000 mutagenized genomes for mutations in 1.0 kb of coding DNA. For some genes, 1.0 kb of coding DNA can be screened in a single fragment, while other genes will require analysis of 2-4 fragments. The above estimates assume that 8-fold pools of DNA are analyzed in the primary screen.

## Figure Legends

**Fig. 1. The CEL1 assay and a Pipeline for zebrafish TILLING.** The CEL1 endonuclease cleaves DNA 3' to single base-pair mismatches that are present in heterodupexed DNA formed between wild-type and mutant PCR fragments as outlined in (A). First, genomic DNA from fish heterozygous for induced mutations is used as template for PCR amplification with gene specific primers. Heteroduplexes are then formed by denaturing and slowly reannealing the PCR fragments. After CEL1 treatment, the digestion products are denatured and run on a LI-COR polyacrylamide sequencing gel. Unique bands indicate the presence and approximate location of induced point mutations within the analized fragment. (B) Pipeline for TILLING in zebrafish. ENU-mutagenized males are mated with wild-type females to produce $F_1$ progeny that are heterozygous for induced mutations. Sperm from adult $F_1$ males are cryopreserved and DNA is prepared from euthanized sperm donors in a 96-well format for screening. DNA templates are then pooled 4-8 fold prior to screening. The CEL1 assay identifies ENU-induced mutations and sequence analysis determines if these mutations are likely to be deleterious. Finally, lines of fish carrying interesting mutations are recovered from cryopreserved sperm by *in vitro* fertilization.

**Fig. 2. Mutation detection by CEL1 in zebrafish.** The CEL1 assay identifies an induced point mutations in a zebrafish gene. Typical images of a single LI-COR gel as visualized in the IRDye 700 (A) and IRDye 800 (B) channels. This gel represents a screen of 384 $F_1$ individuals (96 4-fold pools) for induced mutations in this fragment. Low molecular weight markers are included in every $12^{th}$ lane to aid in determining lane numbers. A unique band in lane 22 (square) indicates the presence of a ENU-induced polymorphism detected following CEL1 digestion. Note that both digestion products can be visualized by viewing separately the IRD700 and IRD800 gel images. Bands that appear in multiple lanes result from pre-existing polymorphism in the population, while unique bands that appear in the same position on both gel images are PCR artifacts (arrows point to two examples).

**Fig. 3. CEL1 can detect ENU-induced mutations in a background of pre-existing poymorphisms.** Circles indicate ENU-induced mutations in this 1,032 bp fragement that are unique to single F1 fish  Pre-existing polymorphisms appear as a band present in all lanes and were confirmed to be bona fide polymorphisms by sequencing this fragment from multiple F1 fish (sequence shown on left). Note that since the 3' primer is labeled (i.e. at the bottom of the gel), the A->C change in lane 5 is being detected in spite of the presence of eight pre-existing polymorphisms between it and the labeled primer.