GENOTYPING ASSAYS AT ZIRC

A. READ THIS FIRST - DISCLAIMER

Dear ZIRC user,

We now provide detailed genotyping protocols for a number of zebrafish lines distributed by ZIRC. These protocols were developed in-house at ZIRC, and they are now available to the research community as a courtesy.

The ZIRC protocols include general as well as fish line-specific information.

General Protocols: This generic section lists procedures, materials, and reagents that are used at ZIRC to genotype fish lines. The use of reagents or listing of products and/or companies represents no endorsement of these companies and is intended for reference purposes only. In addition, we reserve the right to change our protocols without further notice. Please visit our website to ensure that the protocols you are using are up to date. ZIRC does not guarantee that our results will be successfully reproduced in other laboratories. You can download individual sections, or all sections combined of the generic protocol (pdf) at http://zebrafish.org/zirc/documents/protocols.php?#Genotyping.

Fish Line-Specific Protocols: For lines genotyped at ZIRC, a PDF containing specific genotyping information is provided in a column on our Zebrafish Lines at ZIRC pages. You can search for lines and their corresponding genotyping protocols using our ZIRC search function. You can download the protocols by clicking on the PDF symbol.
NOTE: If there is no protocol linked to a fish line, this indicates that no such protocol currently exists.

Please keep in mind that ZIRC personnel and resources are limited, therefore, we **cannot** provide technical support for these protocols. We kindly ask that you consult with your colleagues for any assistance you may need with the protocols.

However, if you identify any mistakes or have recommendations for improvements, please send suggestions to: genotyping@zebrafish.org.

These protocols are provided "as is", and ZIRC's Terms of Use and Liability Limitations apply. We hope the information provided on these pages is helpful to you.

Your ZIRC staff

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B. OVERVIEW OF GENOTYPING ASSAYS AT ZIRC

A number of genotyping strategies have been employed to genotype zebrafish lines. The following types of genotyping assays have been used at ZIRC:

1) An **RFLP** genotyping assay (**R**estriction **F**ragment **L**ength **P**olymorphism) is used to detect a mutation that either creates or abolishes a site recognized by a specific restriction enzyme (Botstein *et al.*, Am. J. Hum. Genet. 32: 314-331, 1980). In the RFLP assay, a sequence of interest is first PCR-amplified and then the PCR product is subjected to restriction enzyme digestion. The presence or absence of the mutation is determined by the resulting restriction pattern.

2) A **dCAPS** genotyping assay (**d**erived **C**leaved **A**mplified **P**olymorphic **S**equence) is used to detect a mutation that, together with one or more mismatches introduced into a PCR primer, creates or abolishes a restriction enzyme site in the PCR amplification product (Neff *et al.*, The Plant Journal 14(3): 387-392, 1998). The PCR product is subjected to restriction enzyme digestion and the presence or absence of the mutation is determined by the resulting restriction pattern.

3) An **ASA** genotyping assay (**A**llele-**S**pecific **A**mplification) involves designing a PCR primer set in such a way that the 3'-terminal nucleotide of one primer corresponds to the point mutation with respect to its location in the DNA and base pairing. This nucleotide therefore represents a mismatch for the WT template. Because a single mismatch is typically insufficient to achieve a desired level of discrimination, additional mismatches are introduced to further increase this discrimination. If sufficient discrimination is achieved, PCR amplification is detected only for samples carrying the mutation (Newton et al., Nucleic Acids Research 17 (7): 2503-2516, 1989; Kwok et al., Nucleic Acids Research 18 (4): 999-1005, 1990).

4) **SSLP** markers (z-markers) are used at ZIRC to genotype mutations that have not been cloned yet. Typically, two closely linked SSLP markers that flank the mutation are used for genotyping. These markers display interstrain polymorphisms for a genetic background on which the mutation was induced and a background to which the mutation is outcrossed. An individual fish is considered to carry the mutation if no recombination is detected for both of these markers and the PCR products are specific for the genetic background on which the mutation was originally induced. Whenever possible, the fish identified by SSLP genotyping are verified by phenotyping or other identification procedures to confirm that they are indeed carriers of the mutation.

For sample preparation and other PCR related protocols, visit
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5) An **insertional mutation** is typically genotyped by a primer set designed in such a way that one primer anneals to the insert DNA causing the mutation, and the other primer hybridizes specifically to the genomic region flanking the mutation.

6) Transgenic lines for which genomic integration sites are unknown are typically genotyped with **transgene-specific primers**. It is possible that multiple copies of the transgene might have integrated into the genome during transgenesis and that some of these integrations are non-functional. Samples that contain only a non-functional transgene or its fragment will be identified falsely as positive in the genotyping assay. For this reason, it is recommended to use functional assays to verify individuals identified as positive in the genotyping assay.

7) Mutations caused by a deletion for which breakpoints have been molecularly characterized are typically genotyped by **deletion-flanking primers**. In this assay, a short PCR amplification product is detected for a DNA template containing the deletion. The PCR product for WT template is larger and will typically not be generated in PCR reactions in which a short elongation time is set for the Taq DNA polymerase.

8) Mutations caused by a deletion for which breakpoints have not been characterized are genotyped with genomic markers covered by the deletion. Individual samples obtained from single haploid embryos are typically analyzed in this assay. The absence of marker-specific PCR products demonstrates that the sample contains the deletion. An additional primer set is used as an internal positive control in this assay.

9) Mutant samples for a number of alleles are genotyped at ZIRC by **sequencing**. In this assay, a set of primers is designed to PCR-amplify a genomic DNA region containing the mutation. PCR product is then column-purified, combined with either a forward or reverse primer and subjected to sequencing.

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C. DESIGNING AND HANDLING OF PCR PRIMERS

Primers for genotyping assays are designed with MacVector 7.2.2 (Accelrys) and Oligo 4.0-s (MBI) computer software. MacVector software recognizes NCBI accession numbers and imports sequences directly from GenBank. The accession numbers and the information about sequences are typically obtained from original publications or ZFIN, NCBI, and Ensemble websites. In addition to primer sequences, MacVector also provides calculations and predictions about the T_m (melting temperature) of primers and the optimal annealing temperature for each primer set. Typically, we design primer sets with primer melting temperatures within 2ºC of each other. The calculated annealing temperature is subsequently used in the PCR protocol. MacVector and Oligo 4.0-s programs are also used to review and, if necessary, optimize existing protocols that we have acquired from the provider of the line.

Primers are purchased in a lyophilized state. All primers are dissolved in water to a final concentration of 100 pmol/µl and stored at –20^{O}C.

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D. PCR SAMPLE PREPARATION

1) To obtain samples for genotyping, anesthetize fish in Tricaine/Mesab (MS-222; http://zfin.org/zf_info/zfbook/chapt10.html - wptohtml63) as described in the following sections of *The Zebrafish Book*:
Chapter 2.8, 2.9; http://zfin.org/zf_info/zfbook/chapt2/2.8.html#4,
10.63; http://zfin.org/zf_info/zfbook/chapt10.html#wptohtml63. Clip tail fins and transfer them into 50 µl aliquots of lysis buffer in a 96-well plate. The figure below shows a position within the tail fin where the fin clipping typically takes place (marked by a dotted line).

Lysis Buffer:

- 10 mM Tris-HCl (pH 8.0)
- 50 mM KCl,
- 0.3% Tween 20
- 0.3% NP40
- 1 mM EDTA

2) While the fins are being clipped, keep the plate on ice.

3) After clipping, cover the samples with 20 µl mineral oil and incubate at 98ºC for 10 minutes.

4) Add 5 µl Proteinase K solution (20 mg/ml) and incubate overnight at 55ºC. This incubation ends with 10 minutes at 98ºC to denature the Proteinase K.

5) Store the plate at –20ºC.

6) For PCR, dilute the samples in water (1:20) and use 2.5 µl of this dilution in the reaction.

7) Occasionally, samples are not prepared from fin clips but from embryos or juvenile fish. In these cases, an identical protocol is used to lyse the samples, except that the lysis buffer contains 4 mM EDTA. For PCR, 5 µl of 1:10 dilution (in water) is used.

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E. GENERAL PCR PROTOCOL

A PCR reaction is assembled on ice in a volume of 25 µl using the following reagents:

- DNA sample (2.5 µl of 1:20 dilution of the original tail fin sample)
- forward and reverse primers (the final concentration of each primer in the PCR reaction is 0.5µM)
- 2'-deoxynucleoside triphosphates (dNTPs) including dATP, dGTP, dCTP and dTTP (the final concentration of each dNTP in the PCR reaction is 0.2 mM)
- Taq DNA polymerase buffer (2.5 µl of 10 X buffer)
- Taq DNA polymerase (0.9 units in a 25-µl reaction)
- water

Mg^{2+} in the PCR reaction comes from the Taq DNA polymerase buffer. The standard Mg^{2+} concentration is 1.5 mM. The Mg^{2+} concentration is occasionally increased (up to 3.0 mM) when primers with mismatches are used (e.g. dCAPS protocols). This ensures better primer annealing and results in more reproducible PCR reactions.

PCR reactions are assembled and performed either in 96-well plates or eppendorf tubes. 96-well plates are typically used when a large number of PCR reactions are carried out. To assemble PCR reactions, the forward and reverse primers are first pre-mixed with water to generate **a primer mix.** The primer mix is then combined with the dNTP stock solution, Taq buffer, water and Taq DNA polymerase to form **a master mix**. The master mix is added to the DNA samples and the entire solution is mixed thoroughly. PCR reactions are then covered with 7 µl of mineral oil and placed in the thermal cycler. To decrease non-specific amplification and therefore to increase sensitivity of the assay, PCR reactions are assembled on ice.

At ZIRC, PCR reactions are performed in PTC-100 and PTC-200 Peltier Thermal Cyclers (Bio-Rad). A typical PCR program entered into the thermal cycler is shown below:

1. 94ºC for 3 min
2. 94ºC for 30 sec
3. **55**ºC for **40** sec
4. 72ºC for **40** sec
5. Go to step 2 (above) for 34 cycles
6. 72ºC for 5 min
7. 8.0ºC hold
8. END

In this program, steps 1 and 2 are conducted to denature the DNA sample. In step 3, primers anneal specifically to the DNA template. The annealing temperature depends on

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the primer set and can vary for different primer sets. In step 4, the primers are extended by Taq DNA polymerase and new complementary DNA strands are synthesized. This process takes place at 72^{O}C. The timing of step 4 depends on the size of the synthesized product. Typically, 60 seconds are required to generate 1-kb-long products. To obtain a significant amount of a product, PCR cycles are reiterated a number of times. PCR products that are subjected to restriction enzyme digestion (e.g. RFLP or dCAPS genotyping assays) are typically synthesized in 40 cycles. For products that are not digested by restriction enzymes, 35 PCR cycles are used. PCR is finalized in step 6 by a 5-minute long primer extension. After this step, PCR reactions are cooled down and stored at 8.0ºC

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F. RESTRICTION ENZYME DIGESTION

Genotyping assays such as RFLP and dCAPS require restriction enzyme digestions. MacVector software is typically used to select restriction enzymes for RFLP assays. Selection of restriction enzymes for dCAPS genotyping procedures is based on the original computer application described by Neff et al. (Plant J. 1998 May; 14:387-92).

Restriction enzyme digestions are set up as 30 µl reactions. In each digestion, a 7 µl PCR reaction is used as a sample. The amount of restriction enzyme depends on whether the mutant or wild-type product is cleaved.

- If a mutant product is cleaved, 10 units of the restriction enzyme are used in each digestion reaction.
- If a wild-type product is cleaved, 20 units of the enzyme are used in each digestion reaction. An increased amount of restriction enzyme in these assays is to ensure that digestions are carried out to completion. Incompletely digested wild-type samples may be mistakenly regarded in these assays as heterozygous samples.

The volume of the restriction enzyme added to digestions should never exceed 10% of the total digestion volume (i.e. 3 µl). This is to keep glycerol concentration at less than 5% in a reaction. Restriction enzymes are typically supplied in 50% glycerol to prevent freezing at –20^{0}C. Some restriction enzymes show reduced specificity, or increased star activity, when the glycerol concentration in the final reaction is higher than 5%.

Some restriction enzymes require specific reagents such as BSA (Bovine Serum Albumin) or SAM (S-adenosylmethionine) in their digestion reactions:

- BSA is used to stabilize an enzyme. It can protect restriction enzymes from proteases, non-specific adsorption and harmful environmental factors such as heat, surface tension and interfering substances.

Restriction enzyme digestions are prepared in a PCR plate. After digestions are assembled, the plates are covered with adhesive and incubated for six hours at the temperature specified by the restriction enzyme. Typically, 37^{O}C and 55^{O}C digestions are conducted in the incubators. Digestions at temperatures higher than 55^{O}C are performed in thermal cyclers.

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G. GEL ELECTROPHORESIS

PCR and restriction enzyme digestion products are visualized by agarose gel electrophoresis and ethidium bromide fluorescence. Two types of agarose are used to prepare electrophoresis gels:

- agarose (Fisher Scientific, cat# BP1356)
- MetaPhor agarose (Fisher Scientific, cat# 50180)

Most gels are prepared with regular agarose, metaphor agarose is only used for gels with an agarose content of 2.5% or higher.

The content of agarose in the electrophoresis gel depends on **the size of PCR products** subjected to electrophoresis:

Size of PCR products	Agarose content
200 bp or larger	1% gel
between 150 bp and 200 bp	1.5% gel
smaller than 150 bp	2% or 2.5% gel

The content of agarose in the electrophoresis gel also depends on **size differences among restriction enzyme digestion products**:

Size differences of digestion products	Agarose content
150 bp or larger	2% gel
between 50 bp and 150 bp	2.5% gel
50 bp or smaller	3% gel

Note that 2.5% or 3% gels are prepared with both agarose and MetaPhor agarose combined in equal proportions. Gels whose agarose content is lower than 2.5% are solely made with agarose.

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To prepare gels, agarose is dissolved in the 0.5 X solution of Tris borate with EDTA (TBE). The 0.5 X TBE working solution contains 44.5 mM Tris base, 44.5 mM boric acid and 1 mM EDTA. Typically, TBE is prepared as a 10 X stock solution that is subsequently diluted with water to obtain a 0.5 X working solution. To dissolve agarose in 0.5 X TBE, the solution is heated up and boiled for several minutes. After agarose has dissolved, the entire solution is cooled down and ethidium bromide is added. The final concentration of ethidium bromide in the gel is 0.5 µg/ml.

At ZIRC, gel electrophoresis is performed in a horiziontal electrophoresis system supplied by Fisher Scientific (cat# FB-SB-2025). The gels are typically run at the constant voltage of 100V. This voltage is decreased to 75V or more for gels with higher agarose content (i.e. 2%, 2.5% and 3% gels).

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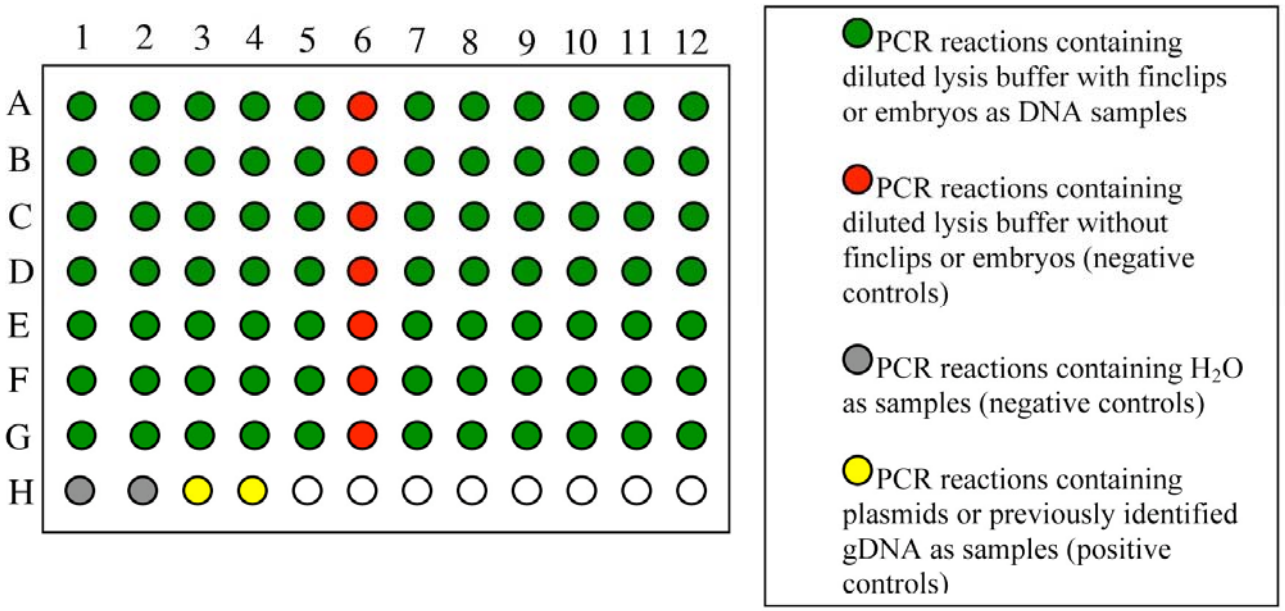
H. CONTROLS

Our greatest concern is to eliminate false positives. Because PCR is a very sensitive technique and even the slightest contamination may lead to false positives, we use several controls to monitor possible contamination. First, the wells in row 6 of all PCR plates are filled with lysis buffer, proteinase K and mineral oil, but contain no fin or other embryo material and should therefore produce no PCR amplification products. This serves as a control for the handling of the PCR plate between the time of the fin clip and the PCR reaction.
We use an additional negative control to monitor the PCR reaction. In this case, we add water instead of samples to designated wells containing the PCR master mix and primer sets. For positive controls, we use previously isolated genomic DNA samples. The setup of a typical PCR plate is shown below:



We use additional wild-type controls in RFLP and dCAPS genotyping assays to monitor restriction enzyme digestions. These controls are particularly important for assays in which wild-type DNA is cleaved as they provide crucial information with regard to whether digestions were carried out to completion. If digestions are not complete, partially digested wild-type samples may be mistakenly identified in these assays as heterozygous samples.

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