

Outline of Medaka TILLING

What is Medaka TILLING?

TILLING is a method used to acquire a mutant of a gene of interest. For this purpose, the National Institute for Basic Biology (NIBB) has managed the Medaka Library, which maintains mutants of various genes, as mentioned below. Researchers screen this library by themselves in the National BioResource Project (NBRP) to find a target mutant. Since point mutation is adopted, complete disruption by a premature termination codon or amino acid substitution may occur.

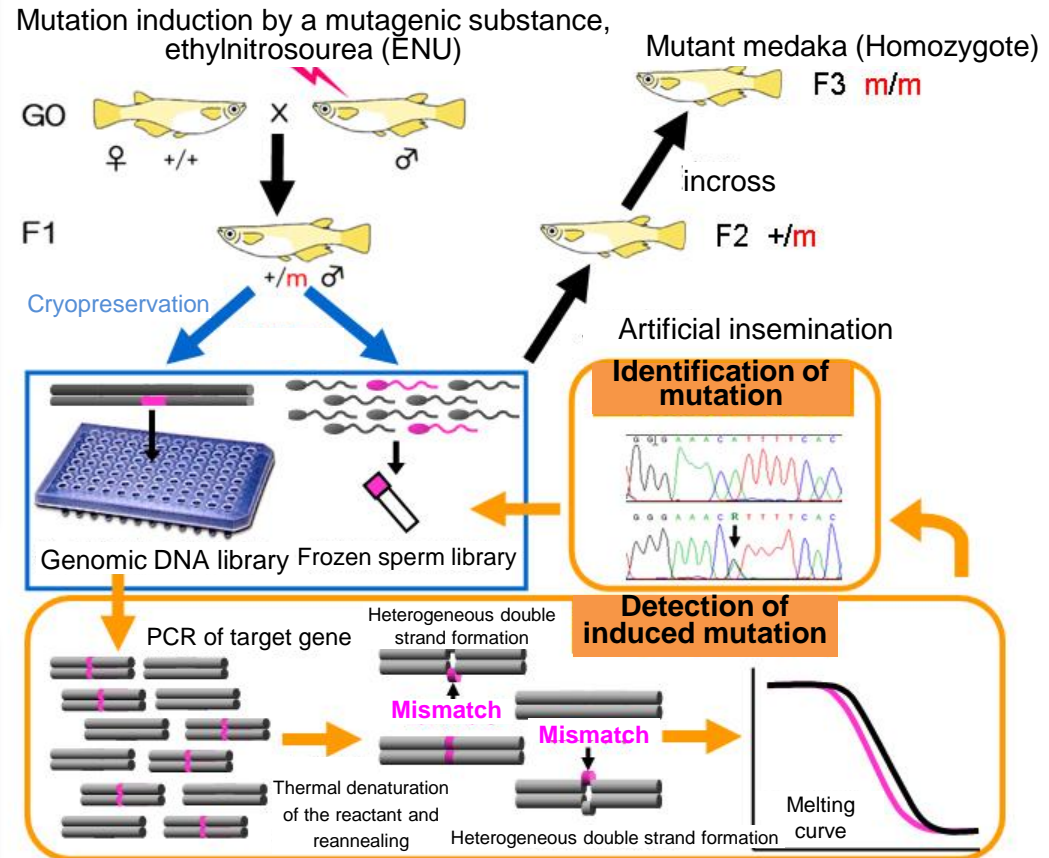
(The library and the screening method are explained in detail later.)

Expenses and expectation

If you are a first-time participant, two people form a group in principle, and five days at NIBB are required. The expenditure is 180,000 yen for consumable goods, in addition to the lodging and travel expenses. By performing a mutation search for a 500-bp region, a null mutant [knockout (KO) medaka, *Oryzias latipes*] can be acquired with 30% probability, in addition to approximately 5 amino acid substitution mutants.

Procedure for producing a mutant medaka using TILLING

Targeting Induced Local Lesion IN Genome (TILLING)

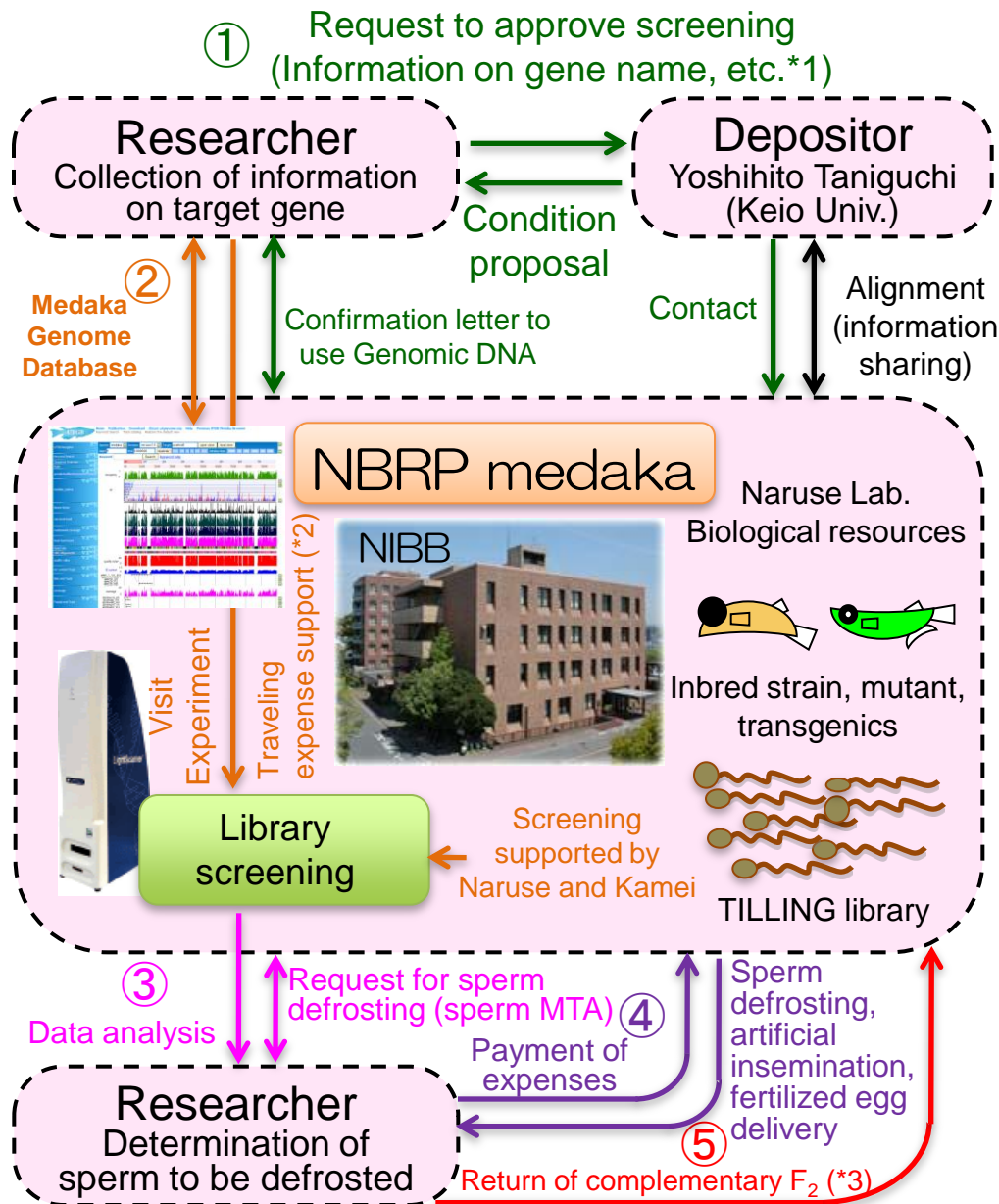


Modified from Dr. Deguchi's Home Page

Scale (quality) of the library

Frozen sperm and genomic DNA = 5760 F1 individuals (sixty 96-well plates)
Mutation rate = one mutation/60 bp (one individual 1/360 kb)
Equivalent to approx. 25 mutations in a 1.5-kb coding region
(nonsense is 1/25)

Procedure to acquire TILLING mutants



How to acquire a mutant?

Researchers screen by themselves at NIBB.

Step ① (green)

The TILLING Library was deposited by Dr. Yoshihito Taniguchi (Keio University). You should contact Dr. Taniguchi to obtain approval. At that time, please inform Dr. Taniguchi of the gene name (*1 the gene name is not disclosed to anybody other than Taniguchi, Kamei, and Naruse without your permission). After the approval, please inform Naruse of NIBB. We will prepare a confirmation letter regarding the use of genomic DNA of the medaka TILLING library.

Step ② (orange)

Please establish a screening plan with Kamei of NIBB (through e-mail, etc.). You need to obtain the intron-exon structure and sequences of the medaka genome. Following the plan, you perform the screening using a High Resolution DNA Melting curve analysis (HRM) method at NIBB. (If you have an "application for individual collaboration," your travel expenses may be provided. *2) The Naruse and Kamei laboratories will technically support you. Since the library, consumable goods, and the equipment are prepared at NIBB, you should bring only primers.

Step ③ (pink)

In the HRM method, candidates including a mutation are narrowed down, and the mutation itself is confirmed by sequencing. After identifying the mutation, if the mutation is predicted to cause a change in phenotype, a request will be made for sperm to be defrosted and a material transfer agreement (MTA) of sperm donation will be prepared.

Step ④ (purple)

Artificially fertilized eggs are delivered. After discussing the method of payment with Naruse of NIBB, please pay the accrued expenses.

Step ⑤ (red)

Since the frozen sperm library is finite, you are requested to return 5 F₂ males for complementation of the library. NBRP will freeze the sperm for backup (*3).

Details of Medaka TILLING 1: Library

About the Medaka TILLING Library

In the Exploratory Research for Advanced Technology/Solution-Oriented Research for Science and Technology (ERATO/SORST) Kondo Project (1998–2003 and 2003–2007), large-scale mutant screening of medaka using the forward genetics approach was performed, and many mutants were collected. From 2004 to 2006, the Takeda and Todo laboratories (Kyoto University) created the Medaka TILLING Library, which entails a reverse genetics approach, using the large-scale mutagenesis technology developed in the Kondo Project (Ref.1). At present, this library is deposited in the NBRP.

The library performed ENU treatment on 102 males of the “Cab” strain (once a week for three weeks), and obtained 87 males that survived for more than one month. The 87 males were then mated with wild-type females, and 26,224 F_1 eggs were obtained. Subsequently, the obtained eggs were hatched out, the larvae were sexually matured for 3–5 months, and 5760 F_1 males were obtained. From the F_1 males, F_1 sperm was obtained and frozen. The library consists of the frozen F_1 sperm and its genomic DNA from its body.

To verify the quality (mutation rate) of the library, a specific locus test was performed, and hypermutation was confirmed to be induced. Through actual screening using “direct sequencing,” (Ref.1) “temperature gradient capillary electrophoresis (TGCE),” and “high-resolution melting curve analysis (HRM)” (Ref. 2), the mutation rate was confirmed to be approximately 1/360 kb. This means that one point mutation was induced in one F_1 fish (360-kb genome); i.e., one mutation per approximately 60 bp in the entire library (5760 individuals). If the screening is performed on the entire library (5760 individuals) regarding a 1.5-kb coding region, 24 point mutations will be detected. In cases where 24 random mutations are detected, one nonsense mutation will have occurred stochastically.

About backup of the library

The Medaka TILLING Library stores 6 frozen sperm capillaries per F_1 male, and one tube consists of 3 capillaries. One set (5760 tubes) is deposited in NBRP, and another set is stored in the Todo Laboratory, Osaka University. The set in the Todo Laboratory will be used when the set in NBRP is lost due to either natural disasters or accidents (backup).

When frozen sperm is defrosted, 5 F_2 males obtained by artificial fertilization are stored for backup. The backup consisting of 5 F_2 males complements the mutations contained in the F_1 males at approximately 97%. Therefore, when you perform screening in NBRP, NBRP defrosts frozen sperm and you receive artificially fertilized eggs, and you are requested to breed each ID separately, and it is your responsibility to return 5 males (at least one male with a heterozygous genotype containing the corresponding gene mutation) to NBRP after raising to maturity. NBRP will freeze sperm obtained from the 5 males.

Although the actual screening uses genomic DNA, NBRP keeps it for only 10000 PCRs. Therefore, you should be particularly careful not to waste or contaminate it.

About the approval to use the library

Before starting to screen using the deposited library, you are requested to obtain approval from Dr. Yoshihito Taniguchi of the Department of Preventive Medicine and Public Health, School of Medicine, Keio University at Shinanomachi 35, Shinjuku-ku, Tokyo 160-8582. Tel: 03-5363-3758 and E-mail: taniguchi@a8.keio.jp

(For terms written in blue, refer to the page “Explanation of terms.”)

Details of Medaka TILLING 2: Screening

Actual screening

The high-resolution melting curve analysis (HRM) ([Ref.2](#)) is used for screening of the medaka TILLING Library in NIBB. PCR is performed on the genomic DNA library using primer sequences for target gene amplification, and the denaturation/reannealing reaction (heating/cooling) is finally performed. When a mutation is present, a heteroduplex is formed. Therefore, if the temperature is raised subsequently, an ID containing the mutation starts to dissociate prior to other IDs that do not contain a mutation. Using a fluorescent intercalator, melting temperatures are measured, and the obtained melting curve is analyzed. An ID, which starts to melt (at a lower temperature) prior to other IDs, is considered as a mutation-induced candidate, and the next step is performed.

For analysis of the melting curve, a device dedicated to HRM (Lightscanner) and exclusive software are used. The software is also used for primer design. Because of the fluorescence properties and PCR conditions, you are requested to use 96-well plates, polymerases, and other consumable goods, which have been examined by us. The screening system is composed of the abovementioned analytical device and thermal cyclers (7 cyclers are currently available), and researchers must perform PCR and melting curve analysis by themselves in NIBB (if you have a corresponding device in your laboratory, please consult us).

The time required for PCR is 60 - 90 minutes. Under the conditions in which premix preparation, dispensing time, and analytical time are taken into account, 6 - 8 thermal cyclers are used, and two persons cooperatively perform the screening, the analysis of one amplicon consisting of sixty 96-well plates (5760 IDs) usually takes approximately 3 days. If you take into account the time to examine the screening conditions and partially analyze the sequence, the standard duration for screening is 5 days.

The flow of screening and supporting system

Generally, two persons come to NIBB and jointly perform the operation.

○ Primer design

You should email Kamei of Spectrography and Bioimaging Facility, NIBB Core Research Facilities beforehand to discuss the design. Each researcher will design and order their own primers.

○ Examination of PCR conditions

Each researcher will examine the PCR conditions to a certain extent. Since PCR is performed under special conditions at the facility (including an intercalator and oil), each researcher should first examine the PCR conditions during their visit. This examination usually takes half a day. Hara of Laboratory of Bioresources, NIBB will assist you.

○ PCR and HRM analysis (primary screening)

You perform the solution preparation, dispensing operation, HRM measurement, and analysis by yourself. For troubleshooting, Kamei and Hara will support you.

○ Secondary screening and sequencing

In general, sequencing of a candidate obtained in the primary screening is performed, and the induced mutation is identified. This can be done at NIBB. However, you are allowed to take the candidate to your laboratory and perform the sequencing there. If the obtained melting curve is complicated, a second screening may be performed.

Details of Medaka TILLING 3: Mutation identification and other matters

After mutant identification

After a mutation is identified in sequencing and the [useful mutation](#) is detected, the useful mutation is activated (Detailed flow chart on the next page).

Please perform artificial insemination using “[Application to TILLING strain](#)” on the NBRP medaka website. The library is finite and a precious community resource. Please avoid unnecessary requests. In cases where same mutation was obtained from several IDs, please inform Kamei if artificial insemination was conducted previously on the ID and you do not require F1 frozen sperm to be defrosted. Since you are obliged to return F₂ adult male fish, your request should be within the range where you can safely breed the fish by yourself. After completing artificial insemination, we will send you [F₂ fertilized eggs](#).

About expenses

Expenses for consumable goods and others

Each researcher is requested to pay “actual expenses” for screening. The ordinary screening expenses for one amplicon are approximately [180,000 yen](#). You are additionally required to pay sequencing expenses for mutation confirmation and those for artificial insemination (for the unit price of each item, refer to “Explanation of terms”).

Expenses for visiting NIBB (lodging and travel expenses)

Regarding expenses for visiting NIBB, if you apply in advance for [collaboration with NIBB \(individual collaboration\)](#) and your application is adopted, these expenses may be provided. Although this collaboration is offered to the public in December every year, the application is occasionally accepted during every fiscal year. Since the examination of the application takes two months, it is more preferable for you to submit the application earlier. Expenses for visiting NIBB can be paid for university and graduate students. (Now, this traveling support is adopted only for Japan domestic traffic.)

After receiving F₂ fertilized eggs

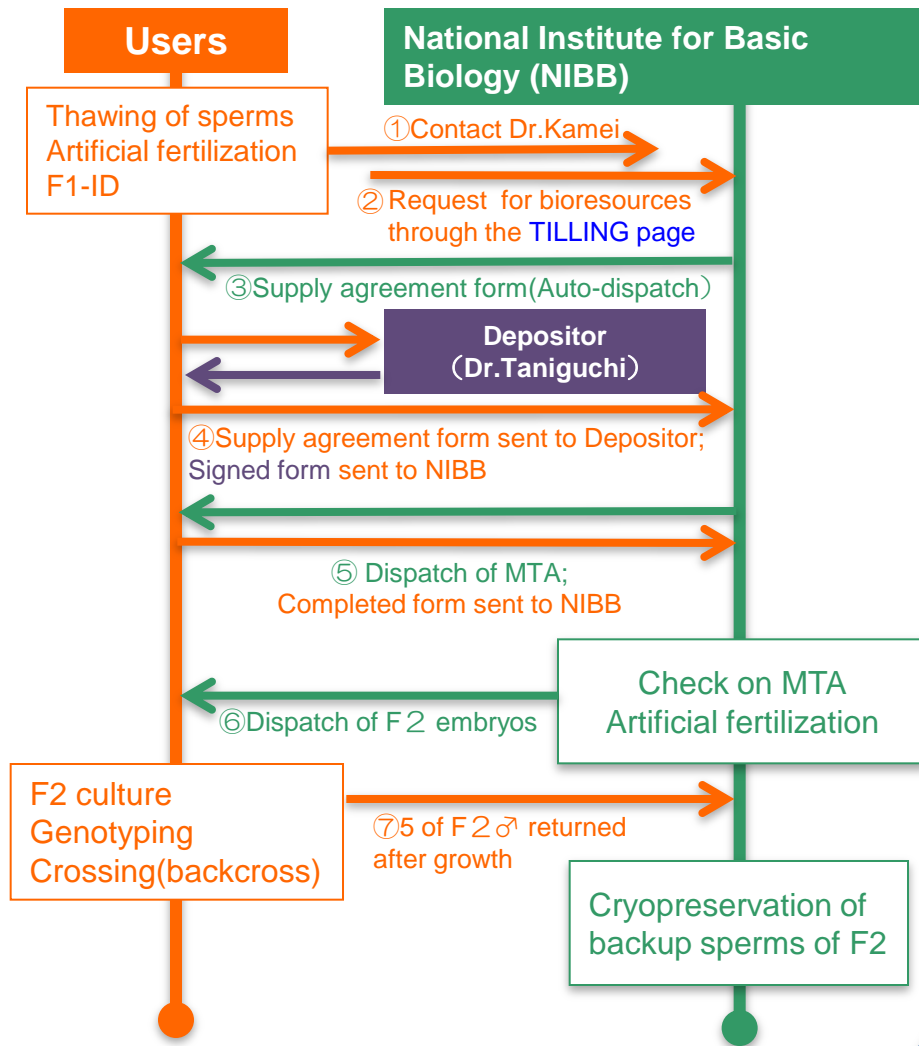
Since screening is performed on the library consisting of mutants obtained by random mutagenesis, a considerable number of [background mutations](#) (mutations other than those of the target gene) are included in the F₂ generation. A single hybridization with the wild-type can reduce the number of background mutations by half. Therefore, you are requested to perform mating with the wild-type (backcross) at least two times or more. The number of mutations other than those of the target gene will be reduced according to the probability. By replacing the sex chromosome with that of the wild-type, and by removing individuals thought to be embryo lethal mutants (although these have no lethal mutant genes), unnecessary mutations can be removed positively.

When the F₂ individuals are grown, you are requested to return 5 males to NBPR. Among these, please include at least one individual heterozygous for the target gene (in order to obtain a backup of chromosomes, including those of the heterozygous individual). Although you are allowed to return the 5 males after collecting the next generation (F₃), they must be able to copulate and fertilize. If you receive F₃ individuals, you are not required to return 5 males. Contact us at nbrc@nibb.ac.jp

In general, sequencing can chase mutation of a target gene. If the number of mutations is large, the mutations can be discriminated using the difference in the 3' terminal between primers at less expense (allele-specific PCR). Moreover, the existence of a restriction enzyme site may be used for the discrimination [restriction fragment length polymorphism (RFLP)]. Even if these methods are used, an individual to be used as the founder of the next generation should be confirmed using sequencing.

Details of Medaka TILLING 4: Method of requesting artificial fertilization

Flowchart of request for supply of TILLING mutants



How to request artificial fertilization

By using the F1 identification (ID; KT-No., Well-No.), a user can request thawing of sperms and artificial fertilization with the identified mutations by first contacting Dr. Kamei (ykamei@nibb.ac.jp) to check if there are multiple F1 with the same mutation ①. If the ID represents F1 that has already been used for artificial fertilization, then backup sperms of F2 will be used for artificial fertilization without thawing the cryopreserved F1, so as not to lose F1 sperms. The user then receives F3 that have been backcrossed. In such cases, the user is requested to identify individual medaka fish with the targeted mutation by genotyping F2 backup genomes prepared from 5 individuals.

② Except for the above case ①, please ensure “sperm condition” after inputting the F1 ID into the “[Application for TILLING lineage](#)” page linked to the homepage (HP) of NBRP Medaka. Up to 10 lineages can be ordered at one time. Please note that the artificial fertilization may not be successful if the sperm condition is not good. Please complete the NBRP user’s registration, which is necessary for your order, by following the browser’s instructions on data input and confirmation.

③, ④, ⑤ Please complete the procedures for sample supply agreement (SSA) and material transfer agreement (MTA) (please contact nbrc@nibb.ac.jp for detailed information).

⑥ After completion of artificial fertilization, F2 fertilized eggs, half of which are heterozygous mutants of the targeted gene, will be sent to the applicant. In cases where the backup sperms of F2 are used, ①, F3 fertilized eggs will be sent to the applicant.

⑦ Ensure that the eggs are cultured carefully for 3 months. If a male heterozygote is identified by genotyping, please preferably return it to National Institute for Basic Biology (NIBB). Backcrossing may be continued, but ensure that at least one male F2 heterozygote is returned. Regarding the procedure to return the male F2 heterozygote, please read the previous page carefully and follow the instructions properly.

Details of Medaka TILLING 5: Method of backcrossing (Reference 1)

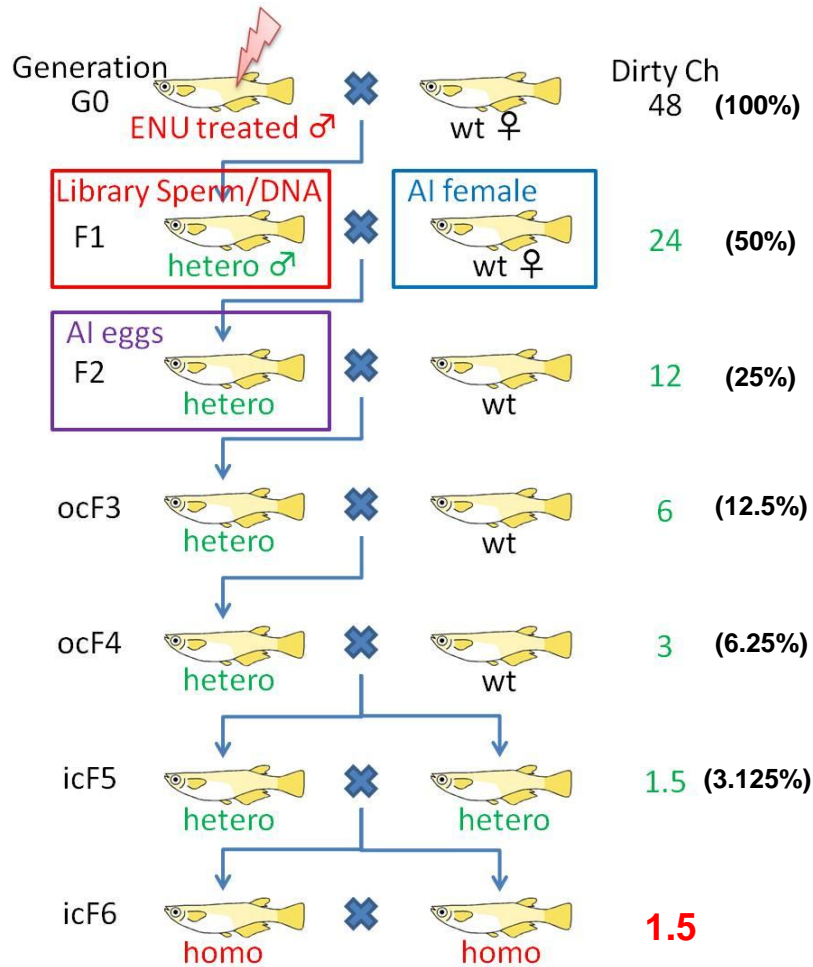


Figure. Cleaning of back ground mutations.

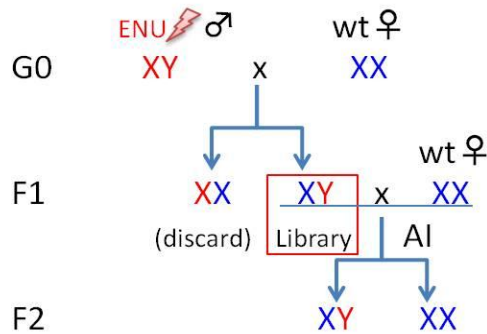
Backcrossing (Backcross)

Because when the library was constructed, TILLING method was performed by random mutagenesis with N-ethyl-N-nitrosourea (ENU); thus, individual organisms carrying mutations of the targeted gene (F1) may also include a large number of background mutations on different chromosomes. Hence, it is necessary to backcross with wild type (wt) species that have not been treated with ENU.

Once backcrossing is performed, the incidence of background mutation stochastically reduced by half. When backcrossing is repeated until the F5 heterozygotes are obtained, background mutation will decrease to 3.1% (see the Figure on the left). Hence, we are able to obtain data on homozygotes, which are phenotypically homozygous and little influenced by background mutations by crossing between male (♂) and female (♀) F5 heterozygotes.

The incidence of background mutation stochastically reduces to 3.1% in F5 even if backcrossing is simply repeated. There are, however, several methods to eliminate unwanted mutations positively: (1) cleaning of sex chromosome (Y chromosome) and (2) cleaning of detrimental mutations by use of carriers with detrimental mutations. The details are shown in the following page.

Details of Medaka TILLING 5: Method of backcrossing (Reference 2)

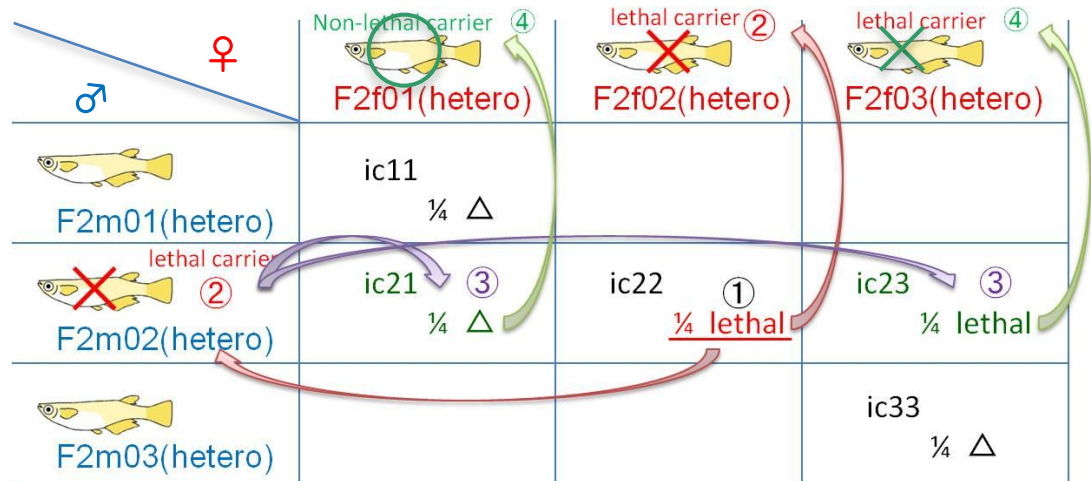


Cleaning of Y chromosomes

After crossing between ENU-treated ♂ (G0) and wt ♀, only ♂ (XY) of the obtained F1 hybrids are used to construct the library. Hence, Y chromosomes of F1 ♂ are “dirty” chromosomes as they contain plenty of other mutations because of the ENU treatment. Meanwhile, X chromosomes are “clean,” because they are obtained from wt ♀. To eliminate the “dirty” Y chromosomes, include a step involving crossing between heterozygous ♀ and wt ♂ anywhere during the backcrossing process.

Cleaning of detrimental mutations

During backcrossing, the initial step is crossing between multiple heterozygotes and observe whether “expected phenotypes” appear. Other than these expected phenotypes, phenotypes as a result of detrimental mutations are also observed. Given 3 pairs of heterozygous ♂ and ♀, as shown in the Figure on the right, only if the crossing between 2♂ and 2♀ result in the 1/4 lethal phenotype after crossing between heterozygous ♂ and ♀ (in-cross ①), such as 1♂ × 1♀, 2♂ × 2♀, and 3♂ × 3♀, then we can conclude that the phenotype (lethal) is not derived from mutation of the targeted gene, but from detrimental background mutations. This mutation should not be inherited to the next generation (backcross lineage).



By analyzing the backcrosses, we have established that both 2♂ and 2♀ heterozygously carry the detrimental mutation (②×). By using these carriers as the test fish, we then conducted further crossing, for example, crossings such as 2♂ × 1♀ and 2♂ × 3♀ in ③. If 1/4 lethal phenotype appears in the 2♂ × 3♀, then it can be considered that 3♀ also carries the detrimental mutation (④×), which means that it is not suitable as a parent for the next generation. Further, we can conclude that 1♀ is a suitable parent for the next generation because 1♀ does not carry the detrimental mutation with no lethal phenotype being emerged by 2♂ × 1♀ (④○) (1♂ and 3♂ can be assayed by conducting 1♂ × 2♀ and 3♂ × 2♀, respectively). These preliminary crossings are rather troublesome, but it is recommended to perform them in the early stages to facilitate successful completion of the experiment.

Present situation of Medaka TILLING: accomplishments and others

NBRP (NIBB) has already started pilot TILLING screening. As of the end of 2010, 6 research groups had completed screening of 8 genes at NIBB. Although the names of the groups and genes are not

mentioned here, the actual number of bases screened, mutations detected, and days of lodging at NIBB are listed in the following table.

Accomplishments between July and November, 2010 (totalized on February 2, 2011)

Table: Results of Medaka TILLING screening performed by research groups in NBRP (NIBB)

Names of universities and institutes		S university	K university	N university	T university	N university		Average
Name of representative researcher (num. of researchers)		S (2)	T (3)	F (3)	O (1)	H (3)		
Target	Target gene	<i>Gene A</i>	<i>Gene B</i>	<i>Gene C</i>	<i>Gene D</i>	<i>Gene E</i>		-
	Number of amplicons	1	1	1	1	2		-
	Base length (bp)	495	400	192	396	253	227	327.2
Result	Number of plates	60	60	60	60	60	60	-
	Number on analyzed bases (bp)	29700	24000	11520	23760	28800		19630.0
	Number of detected mutations	15	7	5	12	3	2	7.3
	Mutation rate (1/bp)	33.0	57.1	38.4	33.0	84.3		44.6 Theoretical value: 60
Days of lodging (total)		15	13	5	4	10		7.8

Explanation of terms

Useful mutation: Although all mutations obtained by TILLING are point mutations, there are three types of mutations in which the gene function varies. The first type is caused by the substitution of a base to form a termination codon; the second type is a splice-site mutation (this includes mutation of GT in a splice donor and that of AG in a splice acceptor); and the third type is an amino acid substitution mutation. With the first and second types, the possibility of a loss of protein function is high. With the third type, the amino acid is revealed to be important for protein function. Moreover, the third type is known to cause diseases in other species, including *Homo sapiens*. If the third type is highly conserved between species, since some type of functional change may occur, the highly conserved mutation becomes the object of artificial insemination. When a phenotype due to the quantity of synthesized proteins is considered to be severe, base substitution to rare codon usage may cause a novel phenotype.

F₂ fertilized eggs: Sperm cryopreserved in NBRP is the offspring of the G₀ generation (F₁ generation). Artificial insemination of these offspring results in F₂ fertilized eggs. The number of generations relates to backcrossing, which is explained below. In general, the F₂ generation is provided. However, if an individual has undergone sperm defrosting and artificial insemination in its past, F₃, the generation of which is advanced by one, may be provided. In this case, the number of backcrosses can be reduced.

180,000 yen: As of January 2011, the following are the consumable goods required for screening one amplicon consisting of 60 96-well plates. Depending on usage, the price will be reduced for enzyme. The billing price might differ according to the supply price of the consumable goods.:

SYTO9:	5,000 yen	Sequencing cost (reference)	
KOD plus:	105,000 yen	A unit consists of 16 samples	
Tips:	25,000 yen	Reagents:	1,600 yen
Plates:	33,000 yen	Run:	2,800 yen
Total:	168,000 yen	Total:	4,400 yen

At Present (April 2012)

Background mutation: Since the medaka genome consists of 700 Mb and the mutation frequency of the library is once per 350 kb, 2000 mutation points due to ENU are considered to exist in F₂ individuals. To reduce this number, i.e., to confirm the phenotype change following target gene mutation, backcross (mating with a wild-type) is required. Finally, the results obtained by rescue due to transgenics are also required.

References

- 1: Taniguchi et al. Genome Biology 7, R116, (2006)
- 2: Ishikawa et al. MBC Mol. Biol. 11, 70, (2010)

Please contact Kamei of Spectrography and Bioimaging Facility, NIBB if you have any questions (ykamei@nibb.ac.jp).

Collaborators of Medaka TILLING

Many researchers and technical support engineers have collaborated in the preparation and management of the library, the establishment of the screening method, the library dispensing, and from the establishment to the assistance of the screening system in NIBB (titles are omitted, positions are those at that time this page was prepared in April 2012).

1. Preparation of the library

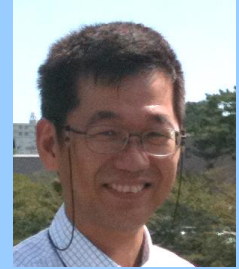
- Management and operation
Takeshi Todo, Shunichi Takeda (Kyoto University), and Hisato Kondo (E)
- ENU treatment
Yoshihito Taniguchi, Yasuhiro Kamei, Takeshi Todo (Kyoto University), and Tomonori Deguchi (E)
- Breeding and technical support
Makoto Furutani-Seiki and those who were engaged in the ERATO/SORST Kondo Project (E)
- Sperm freezing and technical support
Yoshihito Taniguchi, Yasuhiro Kamei (Kyoto University), Noriko Matsuo, and Takao Sasado (E)
- Genomic DNA extraction
Tomoko Ishikawa, Yoshihito Taniguchi, and those who were engaged in the Todo and Takeda laboratories (Kyoto University)

2. Establishment of the screening method (HRM) and technical support

Tomoko Ishikawa, Yasuhiro Kamei, Takeshi Todo (Osaka University), and Yasutoshi Yoshiura (Fisheries Research Agency)

3. Startup and operation of Medaka TILLING in NIBB

- System setup
Yasuhiro Kamei (NIBB); Masakazu Suzuki (Shizuoka University); Yasuhiro Tonoyama (Keio University); Ikoyo Hara, Kiyoshi Naruse, Tetsuaki Kimura, Yusuke Takehana, and those who were engaged in the Naruse Laboratory (NIBB); and Yoshihito Taniguchi (Keio University)
- Operation and visitor support
Kiyoshi Naruse, Yasuhiro Kamei, Ikuyo Hara, Yuta Kanie, Hiroe Ishikawa and those who were engaged in the Naruse Laboratory (NIBB)
- Artificial insemination and sperm freezing
Takao Sasado (NIBB)



Kiyoshi Naruse



Yasuhiro Kamei



Yoshihito Taniguchi