ENU mutagenesis protocol for Medaka

1. Making neutralizing reagent

You should prepare two containers. One should be placed into a chemical hood. The other should be placed outside a chemical hood. Neutralizing reagent is made from mix of following components in each container.

Warm water	10 L
Sodium thiosulfate	2.5 kg
Sodium hydroxide	40 g (pH should be more than 10)

Sodium thiosulfate can hardly be dissolved in cold water. It is better to use warm or even hot water. This solution should make at first of experiment.

After this preparation, every procedure should be done in a chemical hood. Experimenters must wear a lab coat and disposable gloves. It is better to wear two pairs of gloves when handling ENU solution. After finish any procedure, glove wear outside should be thrown into the neutralizing solution.

2. Making ENU solution

Materials:

100 mL of 0.01 M sodium phosphate buffer (pH 6.3) in a beaker

1 g ENU powder in an isopack bottle (Sigma, Cat. No. N 3385)

50 mL syringe

20 G needle

Final solution: 1 g of ENU (one bottle) is solved in 80 ml of phosphate buffer.

Procedure: Keep negative pressure condition inside the bottle. Pick 40 mL buffer with a 50 mL syringe without a needle. Then, attach a 20 G needle to the syringe. Stick the needle into the bottle through the bottle rubber cap. Vacuum up 10–20 mL air from the bottle, and the buffer should go into the bottle automatically by the negative pressure of the bottle. After the buffer movement has stopped, same amount of air should be in the syringe. The air in the syringe can be contaminated by the ENU so that air must release into the neutralizing reagent within the container placed in the chemical hood. Do same procedure twice to pour 80 mL buffer into the bottle. To solve ENU completely, the bottle which rubber cap is wrapped with Parafilm must vigorously agitate for more than 1 hour with 26°C.

3. Preparation for ENU treatment solution

Materials:

Two water baths with a thermostat: the inside dimension of a container is 41 cm in width × 31 cm in depth × 15 cm in height.

A container for ENU treatment: the inside dimension of a container is 27.4 cm in width × 15.4 cm in depth × 15 cm in height.

ENU treatment solution:

Component	Volume	Final Conc.
0.03 % sea saltwater	2.8 L	
1 M Sodium phosphate (pH 6.3)	2.8 mL	1 mM
ENU solution	80 mL	3 mM

A container for washing: the inside dimension of a container is 33.5 cm in width × 19 cm in depth × 15.5 cm in height.

Washing solution: 4 L of 0.03 % sea saltwater

Procedure: The container for ENU treatment is filled with ENU treatment solution without ENU solution. The container for washing is filled with washing solution. Each container will put into a water bath to keep its temperature at 26°C (Figure 1). About 1 hour of duration is enough to equilibrate water temperature 26°C. It is good idea water bath start heat up when make ENU solution. After completely dissolved, ENU solution should move into the container for ENU treatment. Use same syringe with needle as used for making ENU solution. Pull back syringe until about 40 mL and then needle stick into the bottle through the bottle rubber cap. Turn the bottle upside down. Pull about 10–20 mL of syringe to remove ENU solution into the cylinder. Now pressure inside the bottle must be negative. Air can be automatically moved into the bottle. After 20 mL of replacement, pull syringe again to remove ENU solution. Total around 40 mL of replacement will be enough for one procedure. ENU solution must pour gently into the ENU treatment solution. Do same process twice.

Then, the bottle can be treated with filling of about 80 mL neutralizing solution. The way to fill is same as making ENU solution. If you can see the small bubbles when you inject the neutralizing solution into the bottle, it is working for

neutralization. Lastly, send the isopack bottle, syringe, needle to the bottom of the neutralizing solution.

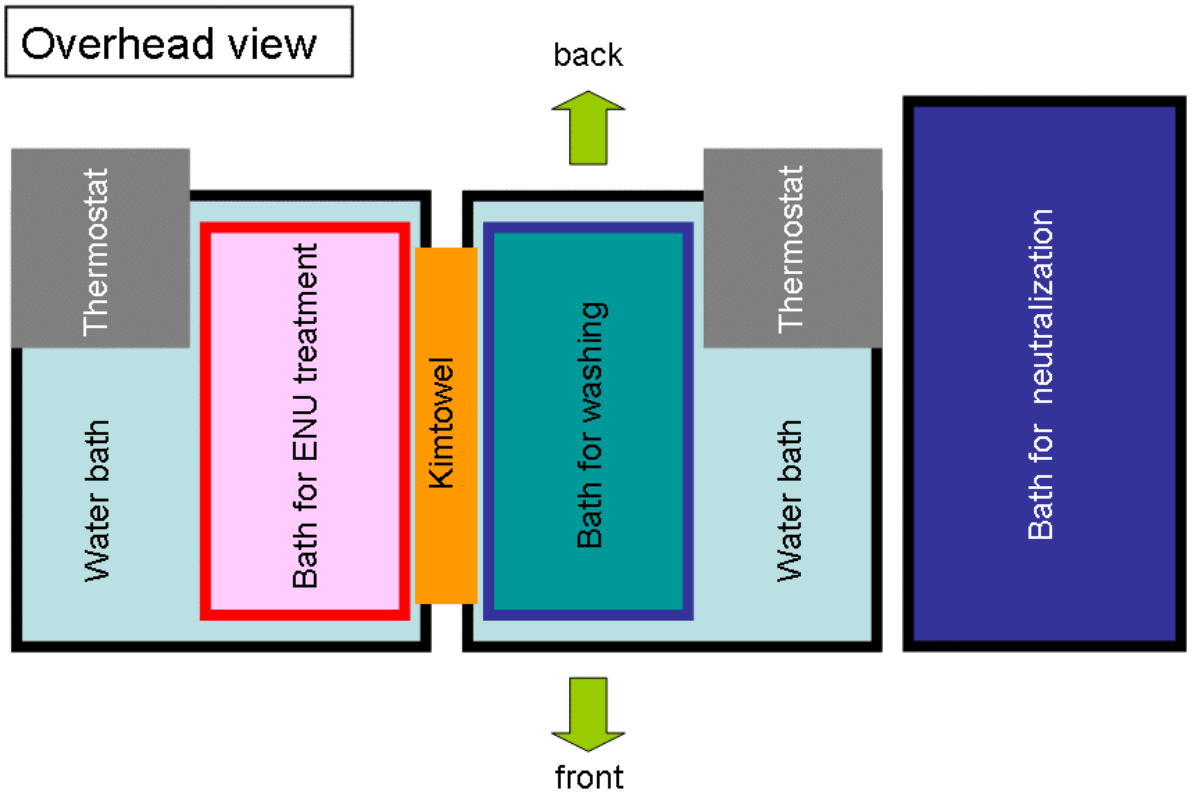


Figure 1 Arrangement in the chemical hood.

4. Fish treatment

Materials:

Fish: Medaka adult male fish. This system can treat at once up to 50 fish.

Two systems for transferring fish: the square tube, which outside dimension is 13.5 cm in width × 15.5 cm in depth × 20.5 cm in height, is attached a soft net (e.g., washing bag) to the bottom.

Two water tanks (9 L): filled with the water for breeding.

Procedure: The systems for transferring fish are set into the container for ENU treatment. Fish put into treatment solution at 26°C for 1 hour. The density is 25 fish of a system for transferring. Keep fish in the dark and quiet, and the survival rates will be improved. After 1 hour treatment, fish are transferred carefully from the treatment area to the washing area, because they must be washed out their excess of ENU on the body by the clean warm saltwater. During the transfer, cover the top of the system with Kimtowel to protect yourself from splash of ENU solution. After 10 minutes, fish move into 9-L tank for keeping overnight. 9-L tank must fill with the water for breeding. After stayed overnight, fish must be moved to a new water tank with fresh water. Fish back to normal breeding system and feed brine shrimp same as before.

5. Cleaning up

As describe in information about ENU, ENU solution is not stable in the solution unless it is acidic. Therefore, after at least 10 hours treatment by the basic neutralizing solution, ENU must be completely decomposed. Next day of experimental day, all solutions should be into the neutralizing solution tank.
Then you can throw all solutions into sink. Note: the neutralizing solution is a strong basic so that it is better to throw with a lot of amount tap water.

6. Frequency of ENU treatment

In zebrafish, ENU treatment should be repeated weekly two more times. In medaka, according to the result of specific locus tests, the frequency of treatment does not change into the result even twice and thrice either. Although medaka can be treated three times a week, we can get better results with one treatment per week.

7. Checking survival rate

After the last ENU treatment, set up the surviving males with female in pair mating. Then, collect eggs every day. Check survival rate of the collected eggs at 1, 4, and 8 day postfertilization (dpf). For a week from starting the egg

collection, the survival rate will show very low score (Figure 2). Two weeks later, the survival rate will show ascending suddenly. For the first 2 weeks after the mutagenic treatment, the collected eggs have fertilized by the sperm which the mutations were induced at postmeiotic stage. In is known that the type of mutations induced by ENU at postmeiotic stage is generally primarily chromosomal rearrangement and deletion. Time when the egg fertilized by the sperm that derives from the spermatogonia into which the mutation is introduced is obtained is after 4 weeks from mutagen treatment.

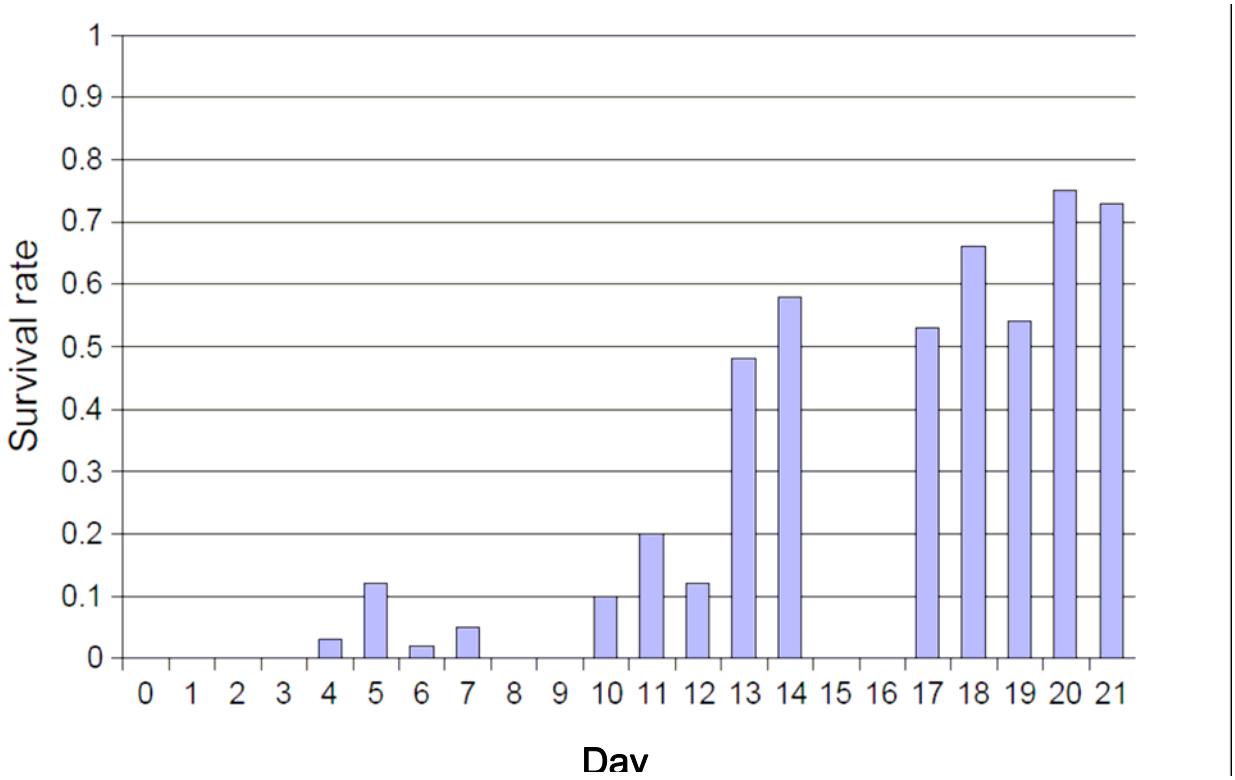


Figure 2 Survival rate of the collected eggs after the ENU treatment. Survival rate is survival eggs at 8 dpf per

fertilized eggs. Horizontal scale is day after mating. At day 1, 2, 8, 9, 15, and 16, eggs were not collected.

8. Specific-locus test

To estimate the mutational frequencies on your experiment, you must perform a specific-locus test. In this test, the males treated with ENU are crossed with females of the tester medaka (Shima and Shimada, 2001). First, cross at least ten males with homozygous Heino albino females in pair mating. Collect eggs every day for 1 month and culture that. Perhaps you can obtain 500–1,500 eggs. Observe the phenotype after 4 dpf under the stereoscopic microscope. Check the absence of melanotic melanophores. Under the described condition, the mutational frequencies of hitting the albino locus will be in the range of 10^{-2} order.

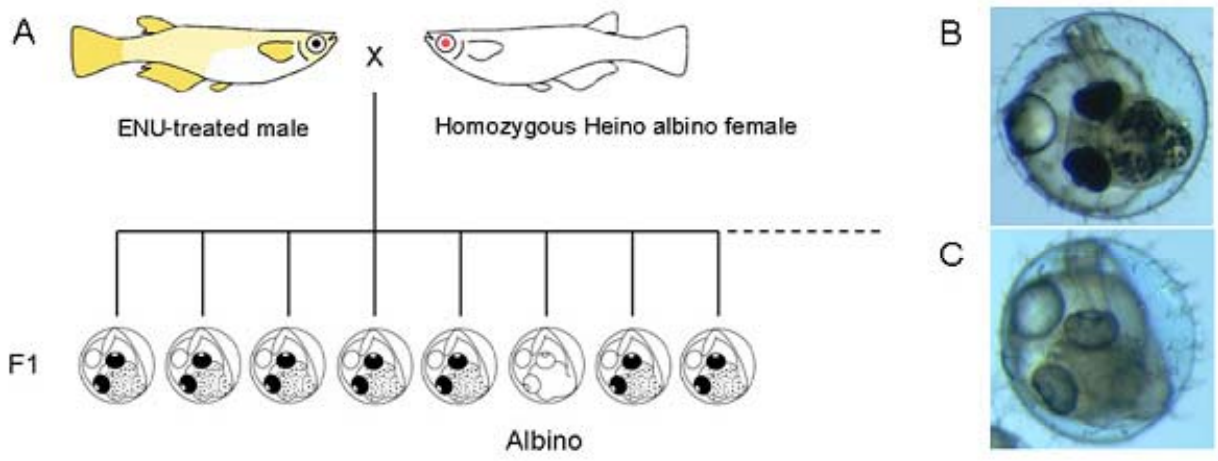


Figure 3 Specific-locus test. (A) The males treated with ENU are crossed with females of Heino. (B) Wild type. C) Albino phenotype.

9. Growing F1 fish

Collect eggs every day, and culture the collected eggs and grow the resulting F1 fish to adulthood.

10. Schedule

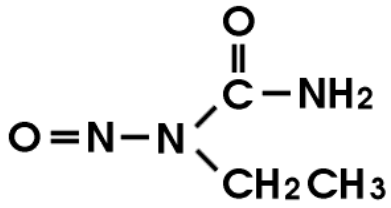
Example schedule – whole procedures -

1st week	Mon.	First ENU treatment.
	Tue.	Get back fish to the water system and clean up.
2nd week	Mon.	Second ENU treatment.
	Tue.	Get back fish to the water system and clean up.
3rd week	Mon.	Third ENU treatment.
	Tue.	Get back fish to the water system and clean up.
	Fri.	Cross with female in pair mating.
4th week	Mon.	Start collecting eggs every day.
5th week ~ 8th week	Everyday	Check survival rate of the collected eggs.
9th week ~	Everyday	Start growing F1 fish at the same time as a specific-locus test.

Example schedule – the treatment day -

First day	10:00	Making the neutralizing solution. Making sodium phosphate solution for ENU solution.
	10:20	Making ENU solution and start shaking. Set up water baths in the chemical hood. Start warming up water to 26 °C
	11:30	Pick ENU solution and make treatment solution.
	11:40	Start fish treatment.
	12:40	Stop fish treatment. Transfer fish into washing solution.
	12:50	Transfer fish into first 9-L tank.
Next day	13:00	Transfer fish into the water system. Clean up the solutions and containers.

Appendices A: Information of ENU



Product Name: *N*-Ethyl-*N*-nitrosourea, ENU

Synonyms: *N*-Nitroso-*N*-ethylurea

Molecular Formula: $C_3H_7N_3O_2$

Molecular Weight: 117.1

CAS: 759-73-9

MDL Number: MFCD00053635

Comments: Dissolving the contents in 100 mL of solvent yields a 1% solution. Injecting any compatible solvent permits preparation of any desired strength solution without exposure.

Storage Temp: Store below 0°C

Chemical Character

ENU decomposes to diazoethane in alkaline solution. Stability in aqueous solution is pH dependent.

Sensitive to humidity & light, highly reactive

pH	Half-life (hours at 20°C)
4	190
6	31
7	1.5
8	0.1
9	0.05

Ref.

IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Man.

Geneva: World Health Organization, International Agency for Research on Cancer,

1972-PRESENT. (Multivolume work). V17, p192 (1978).

Appendices B: 1M Sodium phosphate buffer (pH 6.3)

Distilled water	100 mL
NaH2PO4.2H2O	11.85 g
Na_2HPO_4	3.41 g