AQU_004 Microinjection of zebrafish embryos (Expiry: May 2029)

I. OBJECTIVE

To describe safe and consistent technique for genetically manipulating zebrafish embryos via microinjection within UQBR Aquatics facilities

II. DEFINITIONS

Competent - "the consistent application of knowledge and skill to the standard of performance required regarding the care and use of animals. It embodies the ability to transfer and apply knowledge and skill to new situations and environments." (as per, Australian code for the care and use of animals for scientific purposes, 2013)

E2 - "Embryo media. This is a buffered saline solution with anti-fungal treatment used to ensure isotonic state and healthy developmental conditions for zebrafish embryos" (See Reference Information for Recipe)

III. COMMENTS / RECOMMENDATIONS

- This procedure should not take longer than 3 hours in total (due to limited natural spawning time-frame of animals, and environmental conditions for human technicians leading to eye-strain / potential dehydration)
- Trainers must ensure that trainees have the opportunity to discuss the ethical and social issues, and legal responsibilities, involved in the care and use of animals for scientific purposes, at a level appropriate to their learning ability and comprehension, and before the use of animals commences.
- This SOP involves the use of genetic modification / cytotoxic substances, consideration is required relative to appropriate personnel, equipment and facilities
- This SOP involves the use of compressed air, it is a requirement that the Compressed Gas Safety (OHSB07) online module is completed prior to procedure
- The SOP presents the risk of needle-stick injury, completion of risk assessments relevant to materials/solutions being used must be completed prior to procedure

IV. EQUIPMENT

- Glass capillaries for creation of micromanipulation needles, and Micropipette Puller. Size may vary according to need, but usually 0.1mm OD capillary is required
- Unique injection mixture / agent required by project (e.g. Tol2 DNA construct with RNA transposase)
- Petri dish
- Disposable 0.3ml plastic pipettes
- 1.5% agarose or Sylgard Silicone Elastomer and a plastic mould with sloped grooves designed to organise embryos for injection
- Embryo strainer for collection (with mesh size no smaller than 500um)
- Stereomicroscope
- Pneumatic microinjector instrument

V. PREPARATION

1. Prepare needles to use for microinjection using an appropriate needle puller e.g. Sutter Instrument (Model P-97) Micropipette Puller. Pull a 1.0mm OD glass capillary (e.g. Harvard Apparatus Cat#: EC1-30-0038) into two needles using optimised settings on Micropipette puller.
    a. Custom-pulled needles are stored in a lidded Petri dish and stabilised on raised supports made from a secure, cushioning material (e.g. Blu Tack). This method is used as the custom needles are delicate and have no manufactured storage. The arrangement provides clear visual access while preventing rolling or uncontrolled movement. It also minimises the risk of needle-stick injury during selection and handling, while maintaining fully enclosed containment when the lid is in place.
2. The day before injections, set-up male and female zebrafish in breeding tanks (>2) with dividers, in a ratio of 2:3 respectively in favour of female bias.

    Note: this setup is essential to "prime" the fish by allowing them to enact the courtship behaviour that precedes a natural spawning session. Dividers are required to ensure fish can be time-mated. Mating setups should only be done with clean, fresh aquarium water, at least 1 hour after the animal's last feed. Zebrafish are agastric (stomachless) and if setup too soon after a feed, will release waste that will adversely affect water quality overnight.
3. Prepare chambers to hold embryos for microinjection. This is usually made using 1.5% agarose or Sylgard Silicone Elastomer and a plastic mould with sloped grooves designed to organise embryos for injection.

VI. PROCEDURE

Egg Production and Collection

1. In the morning, remove dividers from the breeding tanks and change water. Ideal mating conditions are within the first two hours of the lights turning on. Allow fish to mate undisturbed for ~20 minutes.

    Note: Zebrafish are dawn triggered, asynchronous batch spawners; meaning that females stagger their egg release into the water column shortly after "lights on". It is essential therefore to take note of the light cycle of the particular aquarium being worked in, and to remember that a single group may lay 2-3 clutches of eggs over the course of the morning. Zebrafish will only spawn once males and females physically interact, and embryos develop rapidly post fertilisation, so pulling dividers at staggered intervals allows the technician to time-mate smaller quantities of embryo's appropriate to the rate of collection and subsequent injection.
2. Collect eggs using a strainer, rinse with E2 solution and transfer to Petri dish.
3. Clean the dish of unfertilised embryos and debris using a transfer pipette.
4. Place embryos into pre-made agarose or Sylgard chambers for injection, aligning them in single columns.
5. Fish can be set-up again or re-grouped into larger tanks to produce additional eggs for injection.

Needle Loading & Set-up

6. In the morning, prepare injection mixture. This may be a e.g. Tol2 DNA construct with RNA transposase, sgRNAs with Cas9 or morpholinos. Keep prepared injection mix on ice until ready to load needle.

7. Ensure compressed air flow is open and adjusted to the desired pressure (e.g. 40psi). Turn on stereomicroscope and pneumatic microinjector instrument. Ensure microinjector unit has been adjusted to the desired pressure parameters and is optimised for either gated or time delivery (dependant on the type and volume of injection mixture being delivered).

   User specification: each project and construct may require unique settings here. Note requirements below for operator reference and continue to step 8:

   - ☐ Injection mixture _____
   - ☐ Injection volume required _____
   - ☐ Gated / Timed. If timed, define value _____ and range 100ms/10s
   - ☐ Injection pressure _____psi
   - ☐ Hold pressure _____psi
   - ☐ Define location: yolk / cell injection

8. Using the stereomicroscope and sharp forceps, cut the injection needle on an angle to allow it to pierce the chorion more easily.
9. Load desired volume e.g. 4uL of prepared injection mix into needle near tip and shake gently until there are few, or no, bubbles.
10. Insert the loaded needle into the microinjector

    Note: Use of micromanipulator is user-based preference, and users may manually hold microinjector if preferred / previous training was to this manual technique

11. Place a drop of mineral oil on a 0.01mm micrometre calibration slide (e.g. ProSciTech Cat#: S81K) and lower needle into oil. Depress the foot pedal to inject the mix into the oil. Calibrate the settings on the microinjector instrument (refer to defined user specification in step 7) or cut the needle again until the desired diameter of the injected bolus is achieved (e.g. 0.12mm which equates to 1nL injected volume)

Injection

12. Ensure the embryos are still within the one-cell stage of development. Refer to defined user specification in step 7, and inject into either cell / yolk depending on solution and desired manipulation outcome
13. Lower the needle towards the embryos with dominant hand, holding the dish in place with non-dominant hand.
14. Insert the needle into the embryo in one quick and smooth action. Inject the mix into either the yolk near the dividing cell, or directly into the cell.
15. After injecting all the embryos in the injection chamber, use a stream of E2 solution to wash injected embryos into a clean petri dish. Repeat until at >200 embryos have been injected. Keep some non-injected embryos to evaluate the toxicity of the injections. Should unexpected toxicity effects be noted, embryos should be ethically euthanised and solutions adjusted accordingly

    Note: the actual number of embryos injected will be project specific and may depend on a variety of factors including, but not limited to : husbandry and underlying health issues for that line, expected prevalence of

manipulation success, animal usage numbers defined in AEC approval certificates unique to individual projects

16. Grow injected and non-injected embryos in E2 solution in incubator at 28°C.

VII. REFERENCE INFORMATION

EMBRYO MEDIA E2:
The 1 × E2 medium contains:
15.0 mM NaCl 0.05 mM Na2HPO4
0.5 mM KCl 1.0 mM CaCl2
1.0 mM MgSO4 0.7 mM NaHCO3
0.15 mM KH2PO4

Prepare three stock solutions, E2A, E2B and E2C, which are then used to make a large volume of the 0.5X E2.

E2A:
Make 100X E2A by dissolving the following ingredients in a final volume of 2000 ml:

➢ 175.0 g NaCl
➢ 7.5 g Kcl
➢ 24 g MgSO4
➢ 4.125 g KH2PO4
➢ 1.375 g Na2HPO4

- add Millipore/RO water to 2000 ml
- shake and stir to dissolve the reagents
- autoclave
- stir overnight to dissolve any precipitation that has formed during autoclaving
- store at 40°C

E2B:
Make 500X E2B by dissolving 55 g CaCl2 (or 73 g CaCl2 × 2H2O) in the final volume of 1000 ml

- add Millipore/RO water to 1000 ml
- shake to dissolve the reagent
- autoclave
- aliquot into 50 ml portions (in 50 ml Falcon tubes)
- store in −20°C

E2C:
Make 500X E2C by dissolving 30 g NaHCO3 in the final volume of 1000 ml

- add Millipore/RO water to 1000 ml
- shake to dissolve the reagent
- autoclave
- aliquot into 50 ml portions (in 50 ml Falcon tubes)

TO MAKE 50 LITERS 0.5X E2, mix:

➢ 250 ml 100X E2A
➢ 50 ml 500X E2B
➢ 50 ml 500X E2C

- add RO water to 40 litres
- adjust pH to 7.0–7.5 (with concentrated HCl or concentrated NaOH)
- add RO water to 50 litres
- store at room temperature

Note: This solution is available for general use within the BR Aquatics Facilities

This SOP deserves notable reference and acknowledgement of authorship by Gillian Lawrence –Aquatics Facility Manager, UQ Biological Resources, The University of Queensland.

Version #	Reviewing AEC	AEC Review Date	Outcome
2.1	Native/Exotic Wildlife and Marine Animals AEC	12/05/2026	Approved to 12/05/2029

Conditions:
- Investigators named in an animal ethics application, relative to this SOP, must be competent to implement the SOP
- Any variation to this SOP must be described in the relevant animal ethics application
- If this SOP has not been reviewed and approved by a UQ AEC within the last three years it is no longer valid and cannot be used in animal ethics applications until reapproved (see "AEC Reviewed/Approved" date in this document's header).