

AQU_002 Methods of Euthanasia in Zebrafish

I. OBJECTIVE

To promote the safe and humane euthanasia of Zebrafish within UQBR facilities, as per Clause 3.3.45 of the Australian Code for the care and use of animals for scientific purposes.

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)

III. COMMENTS / RECOMMENDATIONS

- Relative to animal ethics applications, when using this SOP, the following must be described in the individual ethics application: any intended variations.
- As per routine the conditions (see document footer) this procedure must be performed by, or under the direct supervision of, personnel who are competent to perform the procedure.

IV. EQUIPMENT

Ice Slurry

Equipment items

- Nally Bin (due to large numbers), OR 8L tank / esky (if handling small numbers)
- Temperature probe
- Nets or transfer baskets

Consumables

- Ice from machine (IMB)
- System Water

Administration

- Cull Logs
- Outbox Log
- Zebrafish Database
- Barcode Scanner

Anaesthetic Overdose

Equipment items

- 8L Fish Tank with water
- Pipette

Consumables

- Sedative Agent (Aqui-S or MS-222)

Administration

- Cull Logs
- Outbox Log
- Zebrafish Database
- Barcode Scanner

Conditions:

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V. PREPARATION

Ice Slurry

1. Collect Cull Log and Outbox Log from each Zebrafish Aquarium
 - i. Cull/Outbox data is entered at the Seddon Computer in the WebDB database (<https://webdb.imb.uq.edu.au/db/zfish/list.php>)
2. Collect Ice from IMB Ice Machine and fill into a Nally Bin trolley
 - i. Distribute Ice to each Zebrafish Aquarium into the designated Ice Slurry Box, ensure there is enough room within the ice slurry box to place the strainer containing cull fish later (Refer to Figures 1 and 2)



Figure 1- ice machine located at IMB Figure 2- Nally bin filled with Ice

3. Add system water to the Ice slurry box
 - i. Enough to just cover the ice; too much system water will overheat the slurry and the solution will either not fall below 1°C, or will begin to heat too quickly following the addition of fish. (Refer to Figure 3)



Figure 3- System water (left) being added into nally-Ice (right) to make slurry

4. Leave Ice slurry to cool to below 1°C (usually 5-10 minutes depending on the volume being used)
5. Push strainers into the ice slurry solution, ensuring a water depth of at least 2 cm within the strainer (for larger number of culls, aim for a water depth of around 5-8cm). – Refer to Figure 4

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Figure 4- Strainers located in Ice slurry

6. Use either the digital or mercury thermometer to monitor water temperature within the strainer
7. When ice slurry reaches below 1°C, proceed to fish euthanasia procedure

Note: The temperature of the water within the strainers must be monitored constantly. Depending on the numbers worked with, the addition of fish into the strainers will cause the water temperature within to rise above 1°C very quickly. In order to ensure safe, quick and ethical euthanasia throughout the procedure, the technician will need 1 – 3 strainers within the ice slurry at a time and will need to switch between them accordingly; waiting until the water temperature within a particular strainer is once again below 1°C before adding more fish.

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Anaesthetic Overdose using AQUI-S (isoeugenol)

1. Check manufacturer's instructions and recommendations before using the Anaesthetic product

Note: Specific gravity of AQUI-S® is 1.089

Isoeugenol (active ingredient) conc. is 50% of AQUI-S

2. Make up stock solution (100mg/ml)

- i. To make a standard stock solution; Add 1 mL of AQUI-S into 10ml of system water. Shake well and ensure that this solution is an even milky-looking mixture.

Note: Water used to make AQUI-S® stock solutions must be between 12°C and 25°C

Do not store AQUI-S® stock solutions for more than 6 hours

Shake vigorously before each use

3. Make up anaesthetic bath (300µg/ml)

- i. To make a working solution; Add 1.5 mL of standard stock solution per 100 mL of system water.
- ii. Stir well, ensuring that no white granules linger at the bottom of the tank.

Note: This anaesthetic bath should be used within an hour of mixture to ensure maximum efficiency

4. Make up overdose bath (600µg/ml)

- i. If using one overdose bath, place additional 1.5mL stock solution into anaesthetic bath to create overdose bath, at least 1 minute after the addition of the last fish ,

OR

- ii. Make a second bath for overdosing (vs anaesthetic) by adding 3ml of stock solution into 100mL water to make overdose bath

5. Proceed to Anaesthetic overdose procedure

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Anaesthetic Overdose using MS-222 (Ethyl 3-Aminobenzoate methane-sulfonic acid salt)

1. Make up stock solution at x25 or x50 strength:
 - i. To make x25 stock solution (3mg/ml)
 - a. Add 1.5 g MS-222 powder to 491.6 mL of DI water
 - b. Add 8.4 mL of 1M tris at pH 9
 - c. Verify pH is neutral (7) using pH test strip
 - ii. To make x50 stock solution (6mg/ml)
 - a. Add 3 g MS-222 powder to 483.2 mL of DI water
 - b. Add 16.8 mL of 1M tris at pH 9
 - c. Verify pH is neutral (7) using pH test strip
2. Refrigerate stock solution until use. Extra stock solution may be refrigerated for further use for up to 2 months, which must always be shaken well before each use.
3. Light sedation bath (0.01mg/ml)
 - i. If using x25 stock; Add 0.5 mL of stock solution per 100 mL system water
 - ii. If using x50 stock; Add 0.25 mL of stock solution per 100 mL system water
4. Make up anaesthetic bath (0.15mg/ml)
 - i. If using x25 stock; Add 10 mL of stock solution per 100mL system water
 - ii. If using x50 stock; Add 5 mL of stock solution per 100mL system water
5. Make up overdose bath (0.3mg/ml)
 - i. To make one overdose bath
 - a. For x25; Add 10 mL of stock solution to anaesthetic bath,
 - b. For x50; Add 5 mL of stock solution to anaesthetic bath

Note: This extra stock solution should only be added 1 minute after the addition of the last fish into the standard anaesthetic bath
 - ii. To make separate overdose bath
 - a. For x25; add 20mL of stock solution per 100mL fish water
 - b. For x50; add 10mL of stock solution per 100mL fish water
6. Proceed to Anaesthetic overdose procedure

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VI. PROCEDURE

Ice Slurry

Note, during the procedure:

- If the temperature of the ice slurry rises above 1°C, mix the slurry and leave to cool down to below 1°C again before adding more fish
 - It helps to manually agitate the ice slurry in between additions of fish to ensure an even temperature throughout the solution and procedure
1. Use a net or strainer to capture fish
 2. Transfer the fish to be euthanized into a strainer located within the ice slurry (being mindful to only add fish to this solution and no additional system water) - Refer to Figure 5.



Figure 5- Fish placed within strainer of Ice Slurry

3. Within seconds, the fish should stop moving and sink to the bottom of the strainer

Note: Only transfer enough fish to cover the bottom of the strainer in a single layer at a time; adding too many fish at once will adversely affect the cull procedure as lingering, shared body heat between the fish will prolong the euthanasia process.
4. Repeat the transfer of fish into the strainer(s) until all fish have been processed, or all strainers have a single layer of fish lining their base
 - i. If all of the fish have been transferred, proceed to step 6
 - ii. If the base of all the strainers have a single layer of fish, but there are still more fish to process, proceed to step 5
5. After fish have been in the ice slurry for at least 5 minutes, combine the contents of all strainers into 1 holding strainer, and repeat steps 2 -5 with the newly empty strainers, until all fish have been processed
6. Leave fish in the ice slurry for at least 1 hour, before verifying death by checking for:
 - i. Lack of opercula/gill movement
 - ii. Lack of heartbeat

OR

 - iii. Decapitate the fish with a sharp instrument following step 5 to ensure death
7. Place euthanized fish into a clinical waste bag, and then into the freezer

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Anaesthetic Overdose

1. Use a net or strainer to capture fish
2. Add fish to the prepared anesthetic bath
3. Leave the fish undisturbed for at least 1 minute within the bath, to reach stage 3 anaesthesia

Note: Stage 3 anaesthesia is here defined as:

- Loss of equilibrium and lack of movement
 - Lack of response to surrounding stimuli (noises, vibrations to the tank etc.)
 - Lack of response to physical stimuli (no response to tail pinch)
4. Once the fish are anaesthetized, add the additional anaesthetic to create an overdose bath OR, if still using the anaesthetic bath, transfer the fish to a second, prepared overdose bath.
 5. Leave fish in the bath for at least 1 hour, before verifying death by checking for:
 - i. Lack of opercula/gill movement
 - i. Lack of heartbeatOR
 - ii. Decapitate the fish with a sharp instrument following step 5 to ensure death
 6. Place euthanized fish into a clinical waste bag, and then into the freezer

VII. FURTHER CONSIDERATIONS

1. For any Adverse events, refer to the UQBR SOP 22: UQBR Veterinary Care Protocol
2. When performing euthanasia via the ice slurry method, remember that the water within the strainer should be constantly monitored. A rise in water temperature above 1°C (due to animal body warmth, accidental system water addition during transfer, or high room temperatures) will adversely affect the procedure and prolong the euthanasia process for the animals.
3. When performing euthanasia via the ice slurry method, use appropriate strainers to ensure that fish do not come into contact with the ice directly as this will cause ice-burns to the animal(s) and cause unnecessary stress
4. Monitor fish when netting/straining and placing into either ice slurry or anaesthetic bath as they may jump out of the tank, into ice, onto the bench or onto the floor.
5. Do not use out-of-date anesthetics to prepare any of the solutions described.
6. If in doubt of the efficacy of a prepared anaesthetic solution, remake a fresh batch before proceeding.

VIII. SAFETY

- PPE use is essential when completing this task – consult with a biosafety adviser for specifics, as required.
- All accidents, injury or near misses are to be reported immediately to the Facility Manager and recorded on a UQ OHS Incident Report Form.

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IX. APPENDIX

Recipes:

AQUI-S Stock Concentration (100mg/ml)

- 1ml AQUI-S concentrate
- 10ml RO Water

Tricaine 25x Stock (3mg/ml)

- 1.5 g MS-222 powder
- 491.6 mL of DI water
- 8.4 mL of 1M Tris (pH 9)
- Verify pH is neutral (7) using pH test strip

Tricaine 50x Stock (6mg/ml)

- 3 g MS-222 powder
- 483.2 mL of DI water
- 16.8 mL of 1M Tris (pH 9)
- Verify pH is neutral (7) using pH test strip

X. REFERENCES:

1. AQUI-S Website : <http://www.aqui-s.com/aqui-s-products/aqui-s>
2. UQ OHS Incident Report Form: <http://www.uq.edu.au/ohs/index.html?page=141331>
3. UQBR SOPs: <V:UQBR/SOPs/Common/UQBR SOPs> and <http://biological-resources.uq.edu.au/secure/uqbrsops>
4. Australian code for the care and use of animals for scientific purposes (8th Edition, NHMRC 2013): <https://www.nhmrc.gov.au/guidelines/publications/ea28>
5. Department of Agriculture and Fisheries (DAF): <http://www.daf.qld.gov.au/>
6. Guidelines to promote the wellbeing of animals used for scientific purposes (NHMRC, 2008): https://www.nhmrc.gov.au/_files_nhmrc/publications/attachments/ea18.pdf
7. Matthews & Varga (2012) Anesthesia and Euthanasia in Zebrafish, ILAR, 192-204
8. OGTR PC2 work requirements and regulations: <http://www.ogtr.gov.au>
9. QLD WH&S Act 2011: <https://www.worksafe.qld.gov.au/laws-and-compliance/workplace-health-and-safetylaws/laws-and-legislation/work-health-and-safety-act-2011>
10. UQ Animal Ethics Unit SOPs: <http://www.uq.edu.au/research/integrity-compliance/standard-operating-procedures-sops>
11. UQ OHS Unit: <http://www.uq.edu.au/ohs/> & <http://www.uq.edu.au/ohs/index.html?page=141331>
12. UQBR SOPs: <V:UQBR/SOPs/Common/UQBR SOPs> & <http://biological-resources.uq.edu.au/secure/uqbrsops>

Version #	Reviewing AEC (note: all other relevant AECs ratify the approval)	AEC Review Date	Approval To Date
2	MBS	04/08/2021	04/08/2024

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