
**Nanotechnologies — Assessment
of nanomaterial toxicity using
dechorionated zebrafish embryo**

*Nanotechnologies — Évaluation de la toxicité des nanomatériaux au
moyen d'embryons déchorionés de poisson zèbre*

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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT) see www.iso.org/iso/foreword.html.

The committee responsible for this document is ISO/TC 229, *Nanotechnologies*.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Introduction

Fish assays are important and widely used tools for evaluating the toxicity of chemicals in the aquatic environment. However, there are animal welfare concerns regarding the use of vertebrate animals for chemical testing, including fish. The use of early life stage embryos, instead of adult or juvenile fish, is considered an alternative assay because there are animal welfare benefits to testing fish embryos as an alternative to the clear distress caused by testing more developed juvenile fish, e.g. by using OECD TG 203.

Nanotechnology is positively affecting many commercial sectors, but there remain concerns regarding the potential adverse environmental effects from nano-enabled products. The OECD test guideline using fish embryos to evaluate acute toxicity (see OECD TG 236) states that some substances having a molecular weight ≥ 3 kDa, a very bulky molecular structure, and substances causing delayed hatching might be inappropriate for testing using that method. The presence of the chorion could also confound assessment of the nanomaterials biological activity. The chorion is the outmost acellular envelope of a fish embryo and it can serve as an exposure barrier for some chemicals or nanomaterials. It is currently not possible to predict which nanomaterials might be blocked by the chorion. Using dechorionated embryos for toxicity assessments may not provide direct ecotoxicological information, but may help to better identify potentially hazardous nanomaterials. Accordingly, many researchers around the world have developed a number of methods for removing a chorion from early life stage zebrafish embryos^[1]^[2]. There are two ways to remove chorion from embryos: by enzymatic or mechanical method. The enzymatic dechoriation method has some advantages over the mechanical dechoriation method (see [Annex A](#)), including time and labour efficiency by easy preparation for dechoriation, no mechanical embryonic damage, and the ability to simultaneously prepare a large number of dechorionated embryos for a high throughput-based approaches. On the other hand, there is a disadvantage of variability in pronase activity that could influence the success rate of chorion removal. Numerous groups have used dechorionated embryos for the assessment of chemical and nanomaterial toxicity^[3]^[4]^[5]^[6]. However, these methods have not yet been fully standardized^[7]^[8]^[9]^[10].

Dechorionated zebrafish embryos toxicity assay can serve as a surrogate system to detect potentially hazardous nanomaterials for other vertebrate systems. As the use of higher organism animal models for toxicity testing is being refined, there is an increasing need for alternative test methods. Early life stage zebrafish (up to independent feeding, e.g. 120 HPF) could be an excellent alternative model of *in vivo* toxicity^[22]^[23]^[24]^[25]^[26]^[27]. Compared with other animal models, zebrafish have a number of advantages for assessing toxicity, including the relative ease of rearing and breeding, high fecundity (external fertilization, 200 embryos to 300 embryos from a female), short generation time (approximately 3 months to adulthood), availability of genomic resources (complete zebrafish genome sequence), and genetic similarity to humans. About 70 % of human diseases have at least one zebrafish orthologue and 84 % of the human genes associated with disease have orthologues in zebrafish^[11]. Therefore, the use of zebrafish to assess chemical toxicity is increasing.

This document provides an optimized procedure to remove chorions along with recommendations on how to conduct toxicity assays using dechorionated zebrafish embryos. It also discusses the advantages of the fish toxicity assay using dechorionated embryos.

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Nanotechnologies — Assessment of nanomaterial toxicity using dechorionated zebrafish embryo

1 Scope

This document specifies a method for rapidly assessing nanomaterial toxicity (fish early life stage, 0 HPF to 120 HPF). It includes information on the importance of acellular chorion removal, detailed chorion removal procedures, and a complete protocol for the toxicity assessment of nanomaterials using dechorionated zebrafish embryos. The focus of this document is on testing nanomaterial toxicity.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO/TS 12805, *Nanotechnologies — Materials specifications — Guidance on specifying nano-objects*

ISO/TR 13014, *Nanotechnologies — Guidance on physico-chemical characterization of engineered nanoscale materials for toxicologic assessment*

ISO/TS 17200, *Nanotechnology — Nanoparticles in powder form — Characteristics and measurements*

ISO/TR 18196, *Nanotechnologies — Measurement technique matrix for the characterization of nano-objects*

ISO 22412, *Particle size analysis — Dynamic light scattering (DLS)*

ISO/TS 80004-1, *Nanotechnologies — Vocabulary — Part 1: Core terms*

ISO/TS 80004-2, *Nanotechnologies — Vocabulary — Part 2: Nano-objects*

OECD. *Test No. 236: Fish Embryo Acute Toxicity (FET) Test*. OECD Guidelines for the Testing of Chemicals. Section 2. OECD Publishing, Paris, 2013

3 Terms and definitions

For the purposes of this document, the terms and definitions given in ISO/TS 12805, ISO/TR 13014, ISO/TS 17200, ISO/TS 80004-1, ISO/TS 80004-2, OECD TG 236 and the following apply.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

- IEC Electropedia: available at <http://www.electropedia.org/>
- ISO Online browsing platform: available at <https://www.iso.org/obp>

3.1

solubilizing agent

solvent or dispersant that can disperse and stabilize nanomaterials in solution

3.2

spawning

releasing the eggs into the water for fertilization

3.3

positive control

any well-characterized material or substance that, when evaluated by a specific test method, demonstrates the suitability of the test system to yield a reproducible, appropriately positive or reactive response in the test system

[SOURCE: ISO 10993-10:2010, 3.14]

3.4

range-finding test

abbreviated acute test that exposes test organisms to a broad range of nanomaterial testing solutions to establish the range of concentrations to be used in the definitive test

Note 1 to entry: The test includes at least five concentrations of the nanomaterial and untreated control.

[SOURCE: OECD TG 236:2013, modified]

3.5

LC₅₀

concentration of a toxic substance that is lethal to half of a group of test organisms (50 %)

Note 1 to entry: Usually, the exposure to the substance is continuous and the LC₅₀ is defined by reference to a specified exposure period.

[SOURCE: ISO 6107-3:1993, 39, modified]

4 Abbreviated terms

DPF	day post fertilization
EM	embryo media
HPF	hour post fertilization
NOAEL	no observed adverse effect level
DO	dissolved oxygen

5 Materials

5.1 Organism (zebrafish, *Danio rerio*).

Zebrafish (*Danio rerio*, see [Figure 1](#)) is a tropical freshwater teleost belonging to the Cyprinidae, naturally distributed throughout India, Pakistan, Bangladesh, Nepal and Burma. The average size of an adult is 2 cm to 3 cm and has blue stripes, similar to a zebra on the side of the body. The adult male zebrafish has a thinner body, with blue and gold hue stripes. The female has a whitish belly, larger than male and silver stripes instead of gold. This species can be kept and bred in aquaria. The lifespan of zebrafish is approximately 2 to 3 years. Organogenesis of zebrafish is completed within 5 DPF and it is an adult within approximately 3 months.

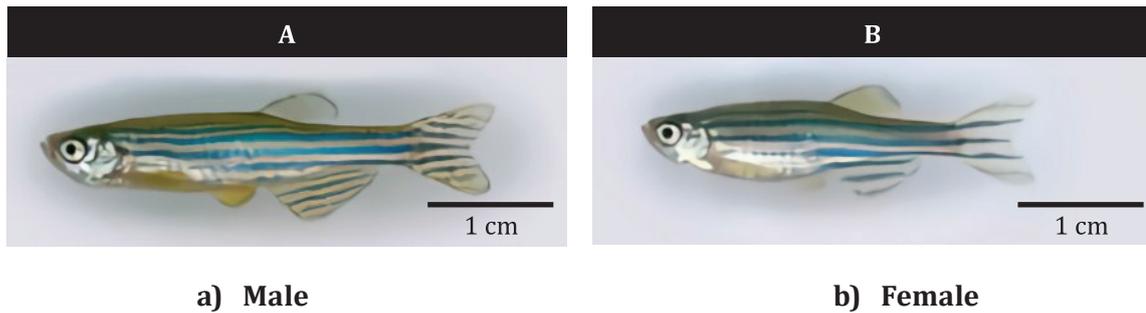


Figure 1 — Zebrafish

5.2 **Stock solution**, freshly prepared with purified water.

5.3 **Positive control**, 3,4-dichloroaniline (3,4-DCA) (CAS# 95-76-1).

6 Apparatus

6.1 Technical equipment

6.1.1 Vessels.

Multi-well plates (6 wells or 12 wells) can be used. To prevent evaporation during exposure, vessels should be sealed with a biocompatible substrate (i.e. parafilm or transparent self-adhesive foil).

6.1.2 **Incubator**, with controlled temperature at (27 ± 1) °C and photoperiod (10:14, light:dark).

6.1.3 **Microscope**, stereo- or inverted with a capacity of at least 80-fold magnification.

6.1.4 **Spawning tanks**, of any dimensions and design, typically consisting of a mating cage and a slightly larger container.

Mating cages with mesh bottom of grid size $(2 \pm 0,5)$ mm allow freshly released eggs to fall down to avoid being eaten by the parent fish.

6.1.5 Pipettes

A single channel pipette can be used.

6.2 Analytical instruments

Use the analytical instruments given in ISO/TR 18196 and the following.

6.2.1 Scanning electron microscopy (SEM).

Scanning electron microscope that produces magnified images of the nanomaterials by scanning the surface with an electron beam.

6.2.2 Transmission electron microscopy (TEM).

Transmission electron microscope that produces magnified images of the nanomaterials by an electron beam, which passes through the sample and interacts with it.

6.2.3 Dynamic light scattering (DLS).

DLS instrument to determine the size distribution profile of nanomaterials in liquids.

6.2.4 Static multiple light scattering (SMLS).

SMLS instrument to analyse particle size variation in concentrated dispersions.

6.2.5 Inductively coupled plasma – mass spectrometry (ICP-MS).

ICP-MS instrument for elemental analysis, used for the detection and quantification of metal ions at concentrations as low as one part in 10^{15} .

7 Procedures

7.1 Culture

7.1.1 Zebrafish strain

The strain of zebrafish shall be reported. Adults should be free of externally visible disease and infection, and not treated with any pharmaceutical treatment for 6 months. Adults should be kept under optimal conditions and density to maintain health.

7.1.2 Feeding regime

Adult zebrafish are fed twice daily with live brine shrimp nauplii (*Artemia* sp.) or commercially available dry diets. Overfeeding should be avoided. Any remaining food should be removed daily for optimal water quality. Larval and juvenile fish are fed thrice a day with dry diets. The diets used shall be reported.

7.1.3 Photoperiod

A lighting regime of 10 h of light and 14 h of dark is recommended. Deviations from this photoperiod shall be reported.

7.1.4 Temperature and pH

A water temperature of 26 °C to 28 °C is suggested for the maintenance of zebrafish and held near a neutral pH. The actual water temperature and pH shall be reported.

7.1.5 Tank material

Tanks used to hold zebrafish should be made of high-quality glass, polycarbonate or acrylic.

7.2 Spawning stimulation

Embryos are obtained by a spawning group aged between 3 and 12 months. Individual pair or group spawning of adult zebrafish can be used to generate embryos. Several commercially available apparatus or custom-made units are available. Adult fish are placed into the mating cage at a sex ratio of 2:1 or 1:1 (female:male). The full spectrum white light is turned on to stimulate spawning behaviour. Embryos are collected within 1 h and rinsed several times with EM. Unfertilized embryos (opaque and/or ruptured cells inside the chorion) are visually inspected and removed (see [Annex B](#)).

7.3 Dechoriation of embryos

7.3.1 Methods

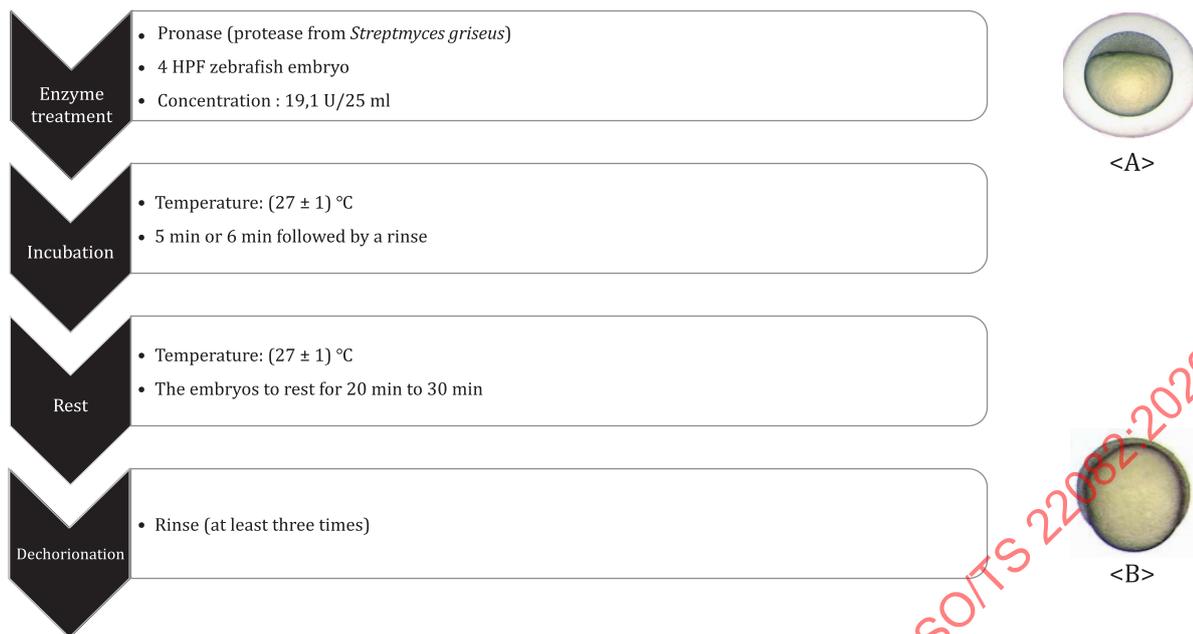
There are two types of dechorionating methods commonly used: mechanical and enzymatic dechoriation. Embryos can be mechanically dechorionated using two forceps under a stereomicroscope. In general, mechanical dechoriation requires only short preparation time and it does not require the use of enzymes. However, it is both time and labour intensive, which substantially limits the number of embryos that can be dechorionated at one time. It is particularly challenging to remove chorion of early life stage embryos (0 HPF to 10 HPF) as manual manipulations can cause unforeseen damage leading to defects later in life. Enzymatic dechoriation generally uses a protease extracted from bacteria species and allows for simultaneous dechorionating large numbers of embryos.

7.3.2 Enzymatic dechoriation method

Preparation of chemicals, supplies and equipment.

- For 1 l of EM, dissolve 5,0 mM NaCl (0,292 g), 0,17 mM KCl (0,013 g), 0,33 mM CaCl (0,044 g), 0,33 mM MgSO₄ (0,081 g) in distilled water and adjust pH to ~7,4 using 0,01 N NaOH or 0,01 N HCl.
- Collect fertilized embryos and separate as many as needed (include 20 % more than needed) all at the same 4 HPF developmental stage.
- Prepare the enzyme [Pronase (EC 3.4.24.4; CAS# 9036-06-0), which is a mixture enzyme of several non-specific endo proteases and exoproteases that digest proteins down to single amino acids, from *Streptomyces griseus*] stock solution on ice using distilled water and into single-use microtubes in small volumes (~100 µl) and store in -80 °C until ready for use.
- Treat chorionated embryos (~1 000) with the enzyme at 19,1 U into 25 ml (0,764 U/ml) of EM in a 90 mm glass petri dish.
- Gently agitate for 5 min or 6 min followed by 6 min rinse with at least a total of 500 ml of EM, then a final mix (without pronase) for 3 min.
- After the rinse, allow the embryos to rest for 20 min to 30 min at (27 ± 1) °C. Following the rest, the weakened chorions left in a petri dish are removed by another rinse cycle (for 6 min) with EM.
- Do not let the embryo contact air or plastic during the process of dechoriation. The use of glass wide bore polish pipettes is recommended.

The method is shown in [Figure 2](#).



Key

- A normal embryo
- B dechorionated embryo

Figure 2 — Enzymatic dechoriation method

7.4 Preparation of nanomaterials stock solution

Stock solutions are freshly prepared with purified water. If necessary for the dispersion of nanomaterial in media, a stock solution may be prepared by ultra-sonication and/or stirring following the dispersion methods described in Reference [12]. Solubilizing agents (solvents or dispersants) may be used for the dispersion of nanomaterials when necessary. The stability of nanomaterials in media during the test should be confirmed using appropriate analyses for the material type. The concentrations of the solubilizing agent should not exceed a volume fraction of 0,01 % and should be the same in all test vessels. If the solubilizing agent is used, an additional solubilizing agent control is required. The solubilizing agent should have no effects on the embryo (e.g. survival, malformation, any other adverse effects). If appropriate, acid digestion followed by ICP-MS should be used to confirm the metal ion concentration of the stock solution.

7.5 Testing concentrations

The testing solution of the selected concentrations is prepared by serial dilution of stock solution. Solubilizing agents (solvents or dispersants) may be used for the dispersion of nanomaterials when necessary.

7.6 Dispersion of nanomaterials

The dispersion states of nanomaterials in accordance with ISO 22412.

7.7 Positive chemical: 3,4-dichloroaniline

3,4-dichloroaniline (3,4-DCA) is a non-specific membrane irritant or metabolic inhibitor that is exclusively used as an intermediate in the chemical industry for the synthesis of 3,4-dichlorophenyl isocyanate, the herbicide propanil and an azo dye for polyester fabrics. The toxicity of 3,4-DCA has been evaluated by many studies [13][14][15]. 3,4-DCA is proposed as a positive control in OECD TG 236 and it has successfully been used as a positive control in many studies [15][16][17]. In this document, 3,4-DCA

(4,0 mg/l for zebrafish) was selected as a positive control for test validation. At this test concentration, mortality for this positive control should be at least 30 % by 120 HPF.

7.8 Test methods

7.8.1 Water quality measurement

At the beginning and end of the test, the water quality of the testing solution is measured, recorded and reported. Measurements include pH, DO content, and conductivity, etc.

7.8.2 Preparation of dechorionated embryos

See [7.3](#).

7.8.3 Exposure

- Healthy and morphologically normal developing 4 HPF zebrafish embryos are selected and dechorionated using 0,764 U/ml pronase (see [7.3](#)).
- Analytically verify actual particle concentration at the beginning of the experiment.
- The newly dechorionated embryos are kept in the incubator until the start of the test. Ten healthy dechorionated embryos at 6 HPF are individually placed into a well micro titre plate containing testing solution (4 ml/well for 6 well plate; 2 ml/well for 12 well plate).
- The plates are covered with parafilm or transparent self-adhesive foil and placed in a temperature-controlled incubator.
- In the case of the semi-static system to maintain the stability of concentrations, the test media is renewed thoroughly every 24 h until the end of the test. Nanomaterial toxicity testing is based on exposures during 6 HPF to 120 HPF.
- The test concentrations for definitive test should have a minimum of five test concentrations spaced by a constant factor not exceeding 2,2. If the solubilizing agent is used, its concentration should not be greater than a volume fraction of 0,01 % and should be the same in all test vessels. The treatments are carried out in triplicate.
- Analytically verify the actual particle concentration after 24 h of exposure.
- Embryos are observed daily under a stereomicroscope during exposure. Dead embryos are recorded and removed from each well. Mortality is determined by examining visible endpoints (see [7.8.4](#)). The number of dead embryos is recorded for the production of a mortality response curve.
- To calculate an accurate LC_{50} , definitive test concentrations should include a NOAEL concentration and a higher concentration that exhibits 100 % lethality.

The nanomaterials are tested in two rounds to identify the concentration that is lethal to 50 % of the individuals (LC_{50}). To compute this value, the nanomaterial will be tested in range-finding experiment where the results will inform the concentrations to use for a definitive test. For a range-finding test, the test concentrations should be widely spaced (e.g. 0,1 mg/l, 1,0 mg/l, 10 mg/l, 100 mg/l).

7.8.4 Endpoints observation

7.8.4.1 Mortality

Dead embryos (or larvae) can be determined by examining embryo coagulation, non-detachment of the tail-bud from the yolk, heartbeat and lack of somite formation under a stereomicroscope. Embryos (or larvae) are observed daily under a stereomicroscope during the test.

7.8.4.2 Coagulation of embryos

The coagulation is a marked loss of translucency and change in coloration and opaque appearance.

7.8.4.3 Failure of tail-budding

An embryo that fails to detach the tail from the yolk-sac is considered dead.

7.8.4.4 Heart beating

Heart beating is easily visible at the 48 HPF time point. An embryo (or larvae) showing the absence of respiratory movement and/or the absence of a heartbeat is considered dead. Irregular (erratic) heart beating is not considered dead.

7.8.4.5 Lack of somite formation

A normal embryo shows the formation of somites after 24 HPF and should be developed at least after 48 HPF.

7.9 Data analysis

Toxicity evaluation for mortality is determined by calculating 120 HPF-LC₅₀ with 95 % confidence limits. It is calculated using a probit analysis and nonlinear logistic regression models.

8 Test report

8.1 Test procedure

The test report shall be in accordance with the test procedures used.

8.2 Information to be included in the report

8.2.1 Test nanomaterials

- Test substance: manufacturer's code, catalogue or formulation number, batch number or date of manufacture, trade name, etc.
- Physico-chemical properties of nanomaterials: type, shape, size, coating materials, purity, etc.
- Stability of nanomaterials in EM and purified water.

8.2.2 Test species

- The origin of zebrafish strain (including species name and developmental stage).
- The number of zebrafish embryos at 4 HPF used for dechoriation.
- The number of zebrafish embryos at 6 HPF used for exposures.

8.2.3 Materials and apparatus

- Description of test vessels used: tank, beakers, spawning tanks, etc.
- Description of analytical equipment: DLS, SEM, TEM, ICP-MS, SMLS, etc.
- Description of nanomaterial preparation: stock and testing solutions preparation, solubilizing agent (if applicable), any solvent preparation, etc.

- Description of positive control preparation: stock and testing solutions preparation.

8.2.4 Maintenance

- Description of embryo collection: preparation of spawning tanks.
- Description of dechoriation (see [7.3](#)).

8.2.5 Test design

- Description of treatments: number of animals used, number of replications, etc.

8.2.6 Test conditions

- Description of exposure: source and water quality (pH, DO, conductivity, etc.) of dilution water, exposing conditions (temperature, light/dark condition, etc.).
- Measurement of nanomaterials' behaviour: stability (sizes of the aggregates and zeta potentials at initiating time and ending time, etc.), actual concentrations of nanomaterials (if possible) at the beginning and after 24 h, released ion concentration from metal-based nanomaterials (i.e. AgNPs).

8.2.7 Results

- The number of dead zebrafish embryos or larvae (see [7.8](#)) in control and nanomaterial-exposed groups.
- The calculated 120-HPF LC₅₀ for mortality (see [7.8.4](#)).
- Confirmation of the result validation (see [Annex C](#)).

Annex A (informative)

Mechanical dechoriation method

A.1 Operating procedures

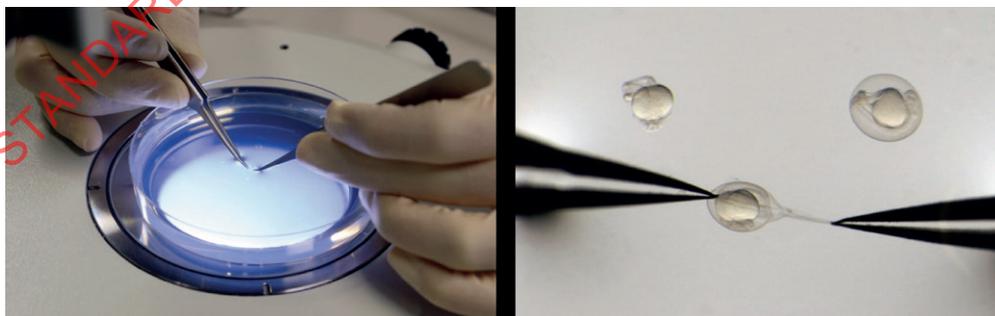
- a) Prepare a pair of sterilized fine forceps.
- b) Collect embryos fertilized on the same day and use 4 HPF embryos.
- c) With a pair of forceps in each hand, grasp the chorion at two close positions on the side of embryo under a stereomicroscope.
- d) Pull the forceps apart, creating a tear in the chorion.
- e) Observe the embryos for any sign of damage such as torn cell layers or yolk puncture. Do not use the damaged embryos for testing.

A.2 Advantages

- Simple to perform.
- Fast acquisition of a small number of dechorionated embryos.

A.3 Disadvantages

- Requires substantial practice to gain proficiency.
- Time- and labour-consuming to obtain a large number of dechorionated embryos.
- The high rate of mechanical damage to embryos.
- Not suitable for the high-throughput based approach.



NOTE Source: Reference [18].

Figure A.1 — Mechanical dechoriation method

Annex B (informative)

Zebrafish embryo spawning procedures

Operating procedures:

- prepare a mating cage (see [Figure B.1](#));
- embryos are obtained by a spawning group aged between 3 and 12 months;
- put females and males (sex ratio 2:1 or 1:1) into a mating tank separated by a tank divider;
- leave the tank in the dark;
- turn on the white light to stimulate spawning behaviour, remove the tank divider and let the fish spawn for about 1 h;
- collect the embryos using a mesh strainer;
- carefully wash the embryos with EM several times to remove debris;
- transfer the cleaned embryos into a petri dish with EM until it is time for the dechoriation procedure.

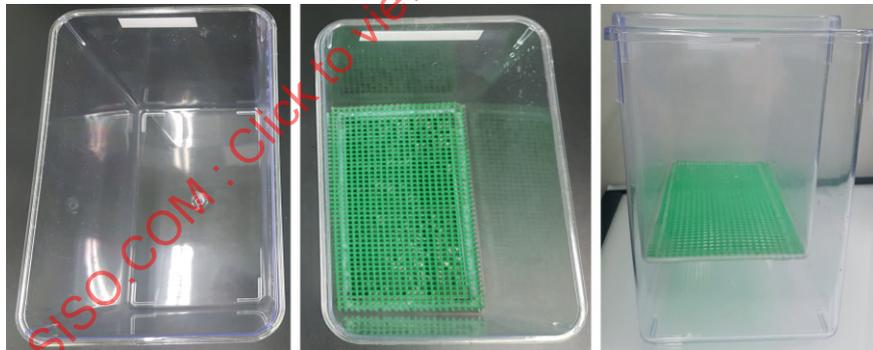


Figure B.1 — Zebrafish mating tank