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TRIzol-Based RNA Extraction in Adult Zebrafish

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Abstract

This protocol describes the extraction and quantification of total RNA from adult zebrafish (*Danio rerio*) whole brain tissue using an adapted TRIzol-based method. The procedure was optimised for pooled samples of six frozen brains (62.17 mg of tissue) to ensure sufficient RNA yield for downstream molecular analyses, including quantitative PCR. Following homogenisation in TRIzol, phase separation with chloroform, and precipitation with isopropanol, the RNA pellet is washed with 75% ethanol, briefly dried, and resuspended in RNase-free water. Quantification is performed using iQuant, permitting evaluation of RNA concentration and purity through A260/A230 and A260/A280 ratios. This protocol forms part of the project Targeting Protein Folding Pathways in Stress-Resilient Zebrafish: Insights into the Unfolded Protein Response, publicly available on the Open Science Framework (OSF: https://osf.io/z5nrm/overview), and was conducted under ethical approval from the Comissão de Ética no Uso de Animais of the Universidade Federal do Rio Grande do Sul (CEUA protocol nº 47092). The resulting workflow provides a reliable and efficient method for obtaining high-quality RNA from zebrafish neural tissue.

Guidelines

This protocol outlines an adapted TRIzol-based workflow for extracting and quantifying total RNA from adult zebrafish whole brain tissue. The method was optimised for pooled samples of six frozen brains (62.17 mg of tissue) to ensure adequate yield and consistency for downstream applications such as quantitative PCR. Because neural tissue is rich in lipids and endogenous RNases, careful handling, rapid processing, and strict RNase-free technique are essential for preserving RNA integrity. All steps should be performed using clean, RNase-free consumables, and tissues should remain frozen until homogenisation.

Materials

- RNase-free microcentrifuge tubes (1.5 mL and 2.0 mL)
- RNase-free pipette tips (filtered)
- Micropipettes (0.5–10 µL, 10–100 µL, 100–1000 µL)
- Homogenisation device suitable for small tissue samples (e.g., motorised pestle, bead mill, or handheld homogeniser)
- Hettich ROTINA 420R microcentrifuge capable of refrigeration (4 °C) and speeds up to 12,000 × g
- Vortex mixer
- Ice bucket
- Tube racks (compatible with 1.5 mL and 2.0 mL tubes)
- Graduated cylinder (100 mL)
- Disposable RNase-free gloves
- Protective laboratory coat and safety glasses
- Fume hood for handling TRIzol and chloroform
- Dry bath or heating block (55–60 °C)
- iQuant or equivalent microvolume spectrophotometer
- Lint-free tissues for cleaning iQuant pedestals
- Marker pens for tube identification
- Waste container appropriate for hazardous chemical disposal
- TRIzol Reagent Invitrogen catalog #1559628
- Chloroform (molecular biology grade) Merck catalog #650498
- Isopropanol Neon catalog #4843
- Ethanol Merck catalog #64-17-5
- RNase-free water Sigma-Aldrich #10977015
- RNase AWAY or equivalent surface decontaminant

Troubleshooting

Problem

RNA yield is low or inconsistent

Solution

a) Insufficient homogenisation: ensure the brain tissue is fully disrupted; incomplete homogenisation reduces RNA release and affects phase separation; b) Inadequate TRIzol-to-tissue ratio: maintain 1 mL TRIzol per 50–100 mg of tissue to guarantee efficient lysis; c) RNA loss during transfers: avoid aspirating the pellet or discarding residual aqueous phase; handle tubes at a slight angle to maximise recovery; d) Pellet overdried: limit drying to 5–10 minutes; overdrying reduces RNA solubility, lowering apparent yield.

Problem

Poor RNA purity (low A260/A230 or A260/A280 ratios)

Solution

a) Aqueous phase contaminated with interphase: avoid disturbing the protein/DNA interphase during transfer; aspirate only the clear upper phase; b) Carryover of organic solvents: ensure thorough removal of chloroform and isopropanol; residual solvents affect spectrophotometric ratios; c) Incomplete ethanol removal: discard all ethanol after centrifugation; traces can interfere with iQuant readings and downstream enzymatic reactions; d) Dirty or contaminated measurement pedestal: clean the iQuant surfaces before each reading with RNase-free lint-free tissues.

Problem

Pellet difficult to resuspend

Solution

a) Overdrying: limit drying time; overly dry pellets become insoluble; b) Insufficient heating: ensure incubation at 55–60 °C for at least 5 minutes; c) Inadequate RNase-free water volume: adjust between 20–50 µL depending on yield and pellet size.

Problem

Inaccurate iQuant readings

Solution

a) Residual solvent: traces of ethanol or isopropanol interfere with absorbance; ensure pellet is adequately dried; b) Improper blanking: perform a fresh blank measurement before each sample; c) Sample bubbles: avoid air bubbles in the 2 µL droplet; reload the sample if necessary; d) Contaminated pedestal: clean upper and lower measurement surfaces after every reading.

Safety warnings

- TRIzol and chloroform are hazardous chemicals that must be handled exclusively in a certified fume hood while wearing appropriate personal protective equipment, including a laboratory coat, nitrile gloves, and safety glasses. Both reagents are toxic, volatile, and may cause severe irritation to the skin, eyes, and respiratory tract. Avoid inhalation and direct contact at all times.
- All materials, work surfaces, and equipment should be treated with RNase-decontaminating agents prior to use, as RNA is highly susceptible to degradation. Use only RNase-free consumables and avoid touching tube rims or inner surfaces.
- Dispose of TRIzol, chloroform, and isopropanol waste in accordance with institutional and environmental regulations. Organic waste must never be discarded down the drain. Handle all samples and reagents with caution and maintain strict separation between clean and contaminated work areas.

Ethics statement

The work described in this protocol was performed under the approval of the *Comissão de Ética no Uso de Animais* (CEUA) of the Universidade Federal do Rio Grande do Sul, Brazil, protocol number **47092**, titled *Exploração da resposta a proteínas mal enoveladas em peixes-zebra resilientes ao estresse crônico imprevisível*. All zebrafish handling, tissue collection, and experimental procedures complied fully with institutional and national regulations governing animal welfare.

Before start

- Ensure that all work surfaces, instruments, and consumables are treated with RNase-decontaminating agents prior to beginning the procedure. RNA is highly labile, and even minimal RNase contamination can compromise sample integrity. Prepare an ice bucket and pre-chill all tubes and reagents that require cold handling. Set the refrigerated microcentrifuge to 4 °C and confirm that the rotor is compatible with 1.5 mL and 2.0 mL tubes.
- Retrieve the frozen pooled brain samples only when all materials and reagents are ready; tissues should remain at –80 °C until the moment of homogenisation to minimise RNA degradation.
- Verify that all hazardous reagents—including TRIzol, chloroform, and isopropanol—are available in sufficient quantities and will be handled inside a certified fume hood. Prepare RNase-free water and ensure that the heating block is pre-set to 55–60 °C for the final resuspension step. Label all tubes clearly before starting to avoid handling delays while working with volatile or toxic reagents.

PREPARING THE REAGENTS

1 The first step is to prepare all reagents required for the TRIzol-based RNA extraction procedure and to organise the workflow within the chemical fume hood. All solutions must be prepared using RNase-free consumables and handled with strict adherence to laboratory biosafety and chemical safety recommendations.

1.1 Preparing the Workspace and Transferring Materials to the Fume Hood

1.1.1 Decontaminate all work surfaces with an RNase-removal agent (e.g., RNase AWAY);

1.1.2 Place inside the fume hood all required materials, including TRIzol, chloroform, isopropanol, RNase-free tubes, pipette tips, and tube racks;

1.1.3 Ensure that an ice bucket, RNase-free water, and waste containers for organic solvents are available;

1.1.4 Label all RNase-free tubes in advance to avoid delays once TRIzol handling begins.

1.2 Preparation of [M] 75 % (v/v) Ethanol. To prepare 100 mL of [M] 75 % (v/v) ethanol:

1.2.1 Measure 75 mL of absolute ethanol using a graduated cylinder;

1.2.2 Add 25 mL of RNase-free distilled water to reach a final volume of 100 mL ;

1.2.3 Mix gently to ensure complete homogenisation;

1.2.4 Transfer to an RNase-free container and store at room temperature or 4 °C as preferred.

1.3 Preparation of TRIzol and Chloroform for Use

1.3.1 Bring TRIzol reagent into the fume hood and ensure that sufficient volume is available (1 mL per 50 mg – 100 mg of tissue);

1.3.2 Aliquot chloroform into smaller RNase-free tubes if necessary to minimise repeated opening of the stock bottle;

1.3.3 Keep both reagents inside the fume hood throughout the procedure due to their volatility and toxicity.

1.4 Preparation of Isopropanol

1.4.1 Ensure that molecular biology–grade isopropanol is available in the fume hood;

1.4.2 No dilution is required; isopropanol is used at [M] 100 % (v/v) for RNA precipitation;

1.4.3 Prepare enough volume for 500 µL per 1 mL of TRIzol used in homogenisation.

1.5 Preparation of RNase-Free Water for RNA Resuspension

1.5.1 Place RNase-free water (autoclaved or commercially certified) on the benchtop;

1.5.2 Pre-warm a small aliquot (20 µL – 50 µL per sample) in a heating block set to 55 °C – 60 °C for the final resuspension step;

1.5.3 Keep additional RNase-free water available on ice for intermediate steps if needed.

1.6 Preparation of Cleaning Supplies for iQuant Measurement

1.6.1 Set aside lint-free tissues exclusively for cleaning the measurement pedestals;

1.6.2 Confirm that the iQuant system is functioning properly and has been calibrated;

1.6.3 Ensure buffer or blanking solution is available for initial baseline measurements.

STEP-BY-STEP PROCEDURE

53m 15s

2 This section provides a concise sequence of steps for extracting and quantifying total RNA from pooled adult zebrafish brains using an adapted TRIzol method. Each stage must be performed promptly and with RNase-free technique to ensure high-quality, intact RNA for downstream analyses.

2.1 Tissue Handling and Homogenisation 5m

2.1.1 Retrieve the pooled sample of six adult zebrafish whole brains from -80 °C only when all reagents, tubes, and equipment are prepared;

2.1.2 Place the frozen tissue in an RNase-free 1.5 mL microcentrifuge tube kept on ice;

2.1.3 Add 1 mL of TRIzol for every 50 mg – 100 mg of brain tissue;

2.1.4 Homogenise thoroughly using a motorised pestle or homogeniser until the solution becomes uniformly lysed;

2.1.5 Incubate the homogenate at room temperature for 00:05:00 to permit complete dissociation of nucleoprotein complexes.

2.2 2.2 Phase Separation 18m 15s

2.2.1 Add 200 µL of chloroform per 1 mL of TRIzol used in the homogenisation;

2.2.2 Cap the tube securely and mix by vigorous shaking for 00:00:15 ;

2.2.3 Incubate the mixture at room temperature for 00:03:00 ;

2.2.4 Centrifuge the sample at 12000 x g, 4°C, 00:15:00 ;

2.2.5 Following centrifugation, three phases will be visible. Carefully tilt the tube to ~45°, and transfer only the clear aqueous phase (containing RNA) into a new RNase-free tube.

2.3 RNA Precipitation

20m

2.3.1 Add 500 µL of isopropanol to the aqueous phase for every 1 mL of TRIzol initially used;

2.3.2 Mix gently by inversion and incubate at room temperature (15 °C – 30 °C) for 00:10:00 ;

2.3.3 Centrifuge at 12000 x g, 4°C, 00:10:00 ;

2.3.4 Discard the supernatant carefully without disturbing the white gel-like RNA pellet.

2.4 RNA Washing

10m

2.4.1 Add 1 mL of [M] 75 % (v/v) ethanol per 1 mL of TRIzol used;

2.4.2 Vortex briefly to dislodge the pellet;

2.4.3 Centrifuge at 7000 x g, 4°C, 00:05:00 ;

2.4.4 Carefully remove and discard the ethanol supernatant.

2.4.5 Air-dry the pellet for 00:05:00 – 00:10:00 .

2.5 Resuspension of RNA

2.5.1 Resuspend the pellet in 20 µL – 50 µL of RNase-free water, depending on the expected yield;

2.5.3 Incubate the tube in a dry bath or heating block at 55 °C – 60 °C for 5 minutes to facilitate dissolution;

2.5.4 Briefly vortex and centrifuge to collect the solution at the bottom of the tube.

2.6 RNA Quantification Using iQuant

2.6.1 Clean the upper and lower pedestals with a lint-free RNase-free tissue.

2.6.2 Apply 2 µL of blanking solution (e.g., RNase-free water) to the lower pedestal and select “Blank” on the device;

2.6.3 Remove the blanking solution with a clean tissue;

2.6.4 Pipette up to 2 µL of the RNA sample onto the pedestal and select “Measure”;

2.6.5 Record RNA concentration, A260/A230, and A260/A280 ratios;

2.6.6 Clean the pedestals thoroughly and proceed with subsequent samples.

CRITICAL NOTES

3

- Maintain strict RNase-free technique throughout. Even minimal RNase contamination can irreversibly degrade RNA. Always wear clean gloves, use filtered RNase-free tips, and avoid touching tube rims or inner surfaces.
- Careful handling of the aqueous phase is crucial. Avoid aspirating interphase material, as contamination with DNA or proteins will affect purity ratios and downstream reactions.
- Do not overdry the RNA pellet. Excessive drying markedly reduces pellet solubility; a soft, translucent pellet is ideal. Limit drying to 00:05:00 – 00:10:00 .
- Ensure complete dissolution of RNA. Incubation at 55 °C – 60 °C improves solubility, especially for neural tissues that may contain residual lipids or membrane fragments.
- Monitor purity ratios critically. A260/A280 values around 1.9–2.1 and high A260/A230 ratios indicate good-quality RNA suitable for qPCR and other downstream enzymatic reactions.
- Store RNA at -80 °C for long-term preservation. Avoid repeated freeze–thaw cycles. RNA should be stored in aliquots to preserve stability and prevent degradation over time.