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An Efficient and Cost Effective Homogenisation Method for Isolating High Quality RNA from Fish Tissue Samples

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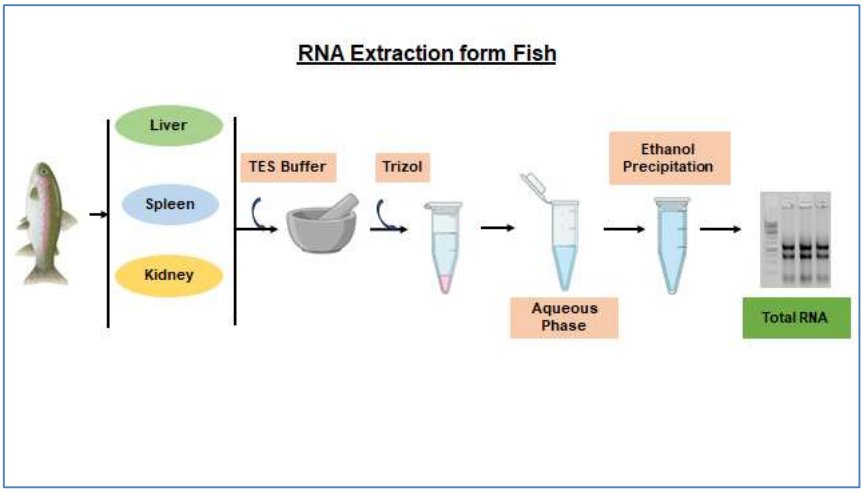
Abstract

Aquaculture sector has huge economic importance and provides jobs to millions of people besides providing nutritionally rich food to billions of people around the world. However, over the years the industry is challenged by various diseases that affect fish health and productivity, leading to economic losses and food security concerns. It is therefore important to understand the molecular basis of these diseases and develop new effective strategies. Transcriptomics is one of the powerful approaches in understanding the pathophysiology at molecular level. Extraction of the high quality RNA from the fish tissues has always remained a challenging task for transcriptomic analysis and tissue homogenisation is one of the initial and the most crucial step in RNA isolation. Here we report an easy, efficient and affordable method for homogenising fish tissues that yielded high quality RNA from fish tissues. The applicability of this method was evaluated for downstream applications including cDNA synthesis and RT-PCR analysis of housekeeping gene GAPDH. The experimental procedure consistently provided sufficient intact total RNA (n=30) and RIN number between 8.2-9.0. This homogenisation method would be highly useful for aquaculture researchers and help in understanding the transcriptomic changes in response to diseases or changed habitat.

Keywords: Tissue Homogenisation, RNA extraction, Fish tissue, Transcriptomics

Graphical Abstract



Introduction

Fish is the most important single source of high-quality protein containing about 16% of the animal protein consumed by the world's population. Globally, aquaculture practice has been developed as one of the cost effective and safest protein sources for human consumption. According to the 2024 edition of the *FAO's 'State of World Fisheries and Aquaculture'* (SOFIA) there has been a substantial growth in annual per capita consumption from 9.1 kg/capita in 1961 to 20.7 kg/capita in 2022. Global production of fish via capture and aquaculture reached about 178 million tons with a total value of USD 406 billion in 2020**1**. The growth of aquaculture industries is hampered by unpredictable mass mortalities due to sudden infectious disease outbreaks**2**.The worldwide economic loss due to disease outbreaks in aquaculture industries has been estimated as more than US $ 6.0 billion per annum**3.** Therefore despite its importance, the industry is challenged by various diseases that affect fish health and productivity, leading to economic losses and food security concerns. Pathogens, pollution and climate change cause challenging issues for the industry with negative impact**4.** Since, the effectiveness of immune defence mechanisms is strongly related to fish health **5,** the host-pathogen interactions concerning infectious diseases in fish are under intense investigation and it is anticipated that the systemic study of –omics datasets through a systems biology approach will enable scientists to describe the complexity and characteristics of interactions in the host-pathogen network ,leading to the identification of new biomarkers and drug targets for fish diseases**6**. Advances in transcriptomics have enabled researchers to gain deeper insights into the molecular mechanisms of fish diseases, thereby facilitating better management practices and therapeutic strategies**7.** Genes and pathways involved in the immune response, disease susceptibility and pathogen host interactions can be analysed through transcriptome analysis. This knowledge can be used to develop vaccines, selective breeding programs for disease resistant strains and new therapeutic strategies. RNA isolation is a critical initial step in transcriptomics as it involves extracting high quality RNA from biological samples. The quality and integrity of the isolated RNA directly affects the reliability of downstream processes such as cDNA synthesis quantitative PCR and RNA sequencing**8**. Accurate and efficient RNA isolation ensures the reliability of transcriptomic data and the validity of research findings. However, despite its importance, RNA isolation from fish tissues presents several challenges. The quality and quantity of RNA can be affected by factors such as sample degradation, contamination and the presence of inhibitors. Additionally, the extraction process can be complex and labour-intensive, requiring careful optimization to obtain high quality RNA. RNA, extraction methods, come with several limitations and the tissue homogenisation being one of them. Although a number of homogenization techniques exist, each presents unique challenges that can compromise RNA yield and quality. For example, the classic mortar and pestle technique, often yields insufficient RNA quantities, particularly from tough or lipid-rich tissues, and demands considerable time and effort; more sophisticated methods like ball mill homogenization require expensive equipment that may not be accessible in all laboratories and has the risk of RNA degradation due to the use of high mechanical force; ceramic bead-based approaches can give inconsistent results and can lead to RNA shearing, while liquid

nitrogen based pulverization, though effective for preserving RNA integrity, presents safety concerns and lacks practicality for processing large sample numbers **9,10.**
Through the present studies on fish tissues, we have found that homogenization using Tris, EDTA, and SDS (TES) buffer offers a remarkably effective alternative to conventional methods, providing excellent RNA yield while maintaining simplicity and cost-effectiveness. This method addresses many of the above limitations through its simplicity and affordability. The combination of Tris for pH stabilization, EDTA for nuclease inhibition, and SDS for protein denaturation creates an ideal environment for RNA preservation during tissue disruption. The method requires basic laboratory equipment, eliminating the need for high end and costly homogenization tools while still delivering high RNA yields. To further validate the quality of RNA isolated using this method, we also examined the expression of housekeeping gene (GAPDH) across various fish tissue samples through qPCR analysis.

Materials and Methods

Materials used

1. TES buffer (Tris, EDTA and 0.5% SDS)
2. Chloroform
3. Isopropanol
4. 70% DEPC ethanol
5. Pestle & Mortar
6. Centrifuge
7. Tissues (Liver, spleen and kidney) from *Schizothorax niger* (*n*=30, of random sex)

Steps in RNA isolation

1. Preparation of TES buffer (3ml)

Calculations:

- **Preparation of ImM Tris (working) from 1M stock**

1M×V1=10×103×3
V1=30µl

- **Preparation of 10Mm EDTA (working) from 500mM stock**

500 ×V1= 10×3
V1=60µL

- **Preparation of 0.5% SDS from 10% SDS**

10/100× V1= 0.5/100 ×3
V1= 10×0.5/100×3
= 150 µL

- **Total TES**

=30µl+60µL+150 µL= 240 µL/ 2.4 ml

Therefore, for 3ml TES buffer, 760 µL/7.6ml of DEPC treated water was used.

2. Modified Homogenisation: Take 50-70 mg of the fish tissue sample (liver, spleen or kidney) in DEPC water treated pestle & mortar. Add 0.2 ml of TES to the tissue and crushed until a fine homogenate is obtained.

Note: Homogenisation should be carried out on ice as shown in fig. 1

Fig. 1. 50-70 mg of fish tissue (spleen) to be homogenized in mortar using TES buffer

3. Trizol based RNA isolation

3.1 Collect the homogenate in 2 ml DEPC treated/autoclaved Eppendorf tube.
3.2 Add 0.6 ml chloroform and 0.4 ml trizol to the homogenate.
3.3 Incubate for 15 minutes at room temperature with gentle mixing.
3.4 After incubation centrifuge the mixture at 14000 rpm for 15 minutes at 4 degrees.

Note: Mixture would be separated into three distinguished phases after centrifugation **(Fig. 2)**

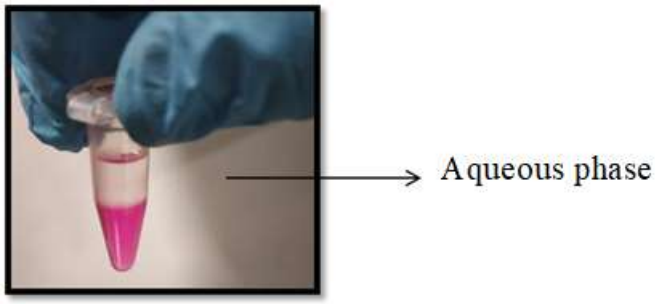


Fig 2. Three phases after centrifugation

3.5 Take the upper aqueous phase into 1.5 ml DEPC treated autoclaved eppendorf tube and add 0.5 ml chilled isopropanol to precipitate the RNA.
Note: To attain the better precipitation of the RNA, incubate the isopropanol added mixture at -20°C for 2 hours.
3.6 Incubate the mixture at RT for 15 minutes. It helps selectively precipitate RNA while leaving contaminants in the supernatant.
3.7 After incubation centrifuge the sample at 14000 rpm for 15 min.
(Note: A clear pellet will be seen at the bottom of tube).
3.8 Remove the supernatant without disturbing the pellet and wash the pellet twice with 70% DEPC treated ethanol by centrifuging at 14000 rpm for 10 minutes
3.9 Discard the supernatant and the air dry the pellet for 30 minutes.
3.10 Resuspend the pellet in 30-50 µl DEPC treated nuclease free water.
3.11 Check the quality and quantity of the sample using a nanodrop spectrophotometer**.**

cDNA synthesis &RT-PCR

After getting the RNA in desired quantity, the experiment was proceeded to synthesize cDNA using M-MuLV reverse transcriptase (Thermo scientific) with an oligo (dT)18 primer.

The expression of the house keeping gene (GAPDH)was analysed by real-time PCR (BIO-RAD, CFX96TM Real-Time System) using primers specific for GAPDH (forward: 5′ - GGACCCCTGTTGAAGGAACT-3′ and reverse: 5′- GACTGGCCGTGCAGAATATC -3′), and SYBR Green PCR Core Reagents (Pro-Mega) in 25 µL volumes which contained the following components: 12.5 µL of SYBR Green Mix (Pro-Mega), 4 µL cDNA (1 µg/µL), 1 µL of each primer (10 mM) and 6.5 µL nuclease free water, followed by 40 cycles of 95 °C for 10 min, 95 °C for 15 s, 60 °C for 1 min. Finally, a melt curve analysis detected the single product

(temperature from 65 °C to 95 °C). All samples were tested in triplicates. The 2−ΔΔCT method was used to analyze the expression level of the selected gene.

Agilent Bioanalyzer analysis

The Agilent 2100 Bioanalyzer with the RNA 6000 LabChip1 kit (Agilent Technologies) was used to analyze 200–300 ng of RNA and to give a RNA integrity number (RIN) for each sample.

Statistical Analysis

The difference in the total RNA yield, RIN, and A260/A280 was between the two homogenisation methods was calculated using non parametric Mann Whitney (U test; $p<0.05$)

Results and Discussion

Quantity and quality of total RNA

Across the two experiments the homogenisation with TES buffer increased the recovery of RNA for liver (639.6±56.84), spleen (874.6±96.96) and head kidney (718±54.79) and demonstrated significant differences ($p<0.05$) compared to the homogenisation without TES buffer i.e. 439.4±89.32, 775±91.73 and 626.2±68.22 for liver, spleen and head kidney respectively. Homogenisation with TES buffer provided comparatively higher RIN for liver (7.92±0.52), spleen (8.54±0.51), head kidney (7.84±0.30) than without the buffer i.e. liver (7.92±0.52), spleen (8.54±0.51) and head kidney (7.84±0.30) implicating less RNA stability in homogenisation without the buffer, although the differences were statistically significant only in case of spleen ($p<0.01$). The purity of RNA, as determined by the A260/A280 ratio, in case of homogenisation with TES buffer was 1.984±0.02, 1.988±0.017, 1.980±0.02 for liver, spleen and head kidney respectively as compared to homogenisation without TES buffer i.e. 1.98±0.02, 1.98±0.02 and 1.97±0.01 for liver, spleen and head kidney respectively with no statistically significant difference ($p>0.05$) **(Table 1)**. The extracted total RNA using the TES based homogenisation method showed two distinct bright bands of 28S rRNA and 18S rRNA with a indicating intact RNA **(Fig.3)**. Though 28S rRNA and 18S rRNA were also obtained in the homogenisation without buffer the RNA bands were faint and distorted **(Fig. 4**), indicating reduced yield of total RNA.

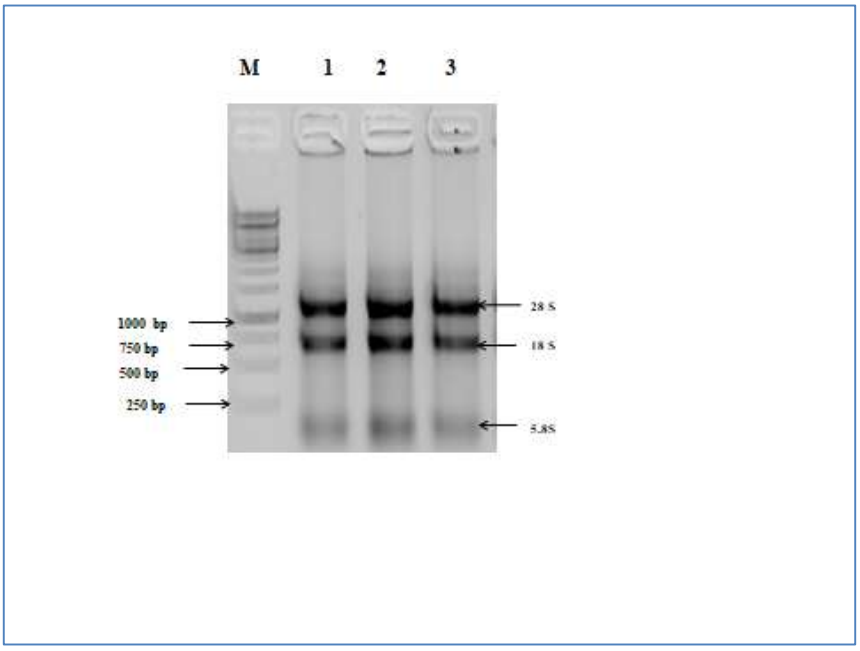


Fig. 3. Gel image showing RNA isolated from different fish tissues after homogenisation with TES buffer. M= 1kb ladder; Lanes 1-3= Isolated total RNA from *S. niger* Kidney, Liver and Spleen respectively

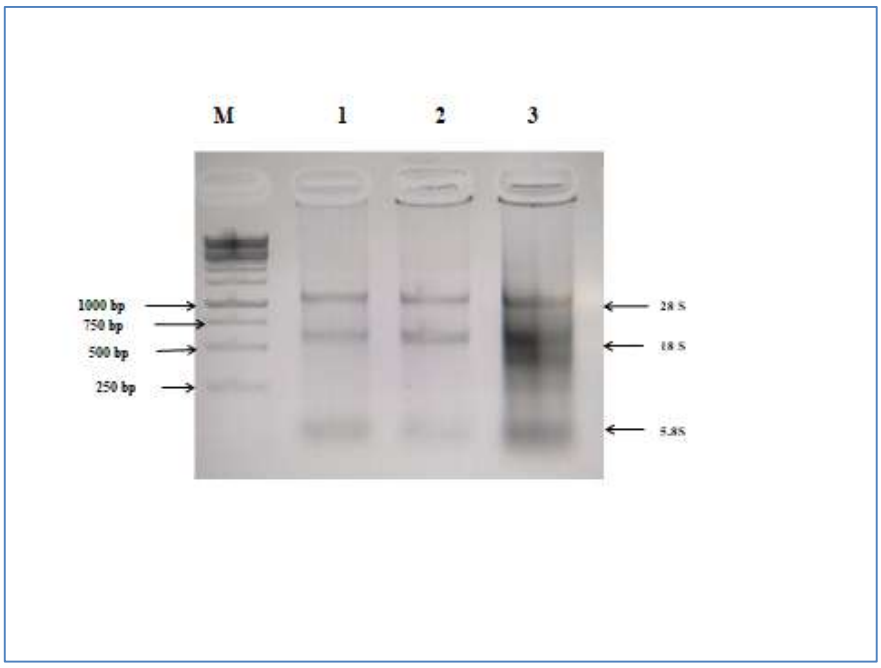


Fig. 4. Gel image showing RNA isolated from different fish tissues after homogenisation without TES buffer. M= 1kb ladder; Lanes 1-3= Isolated total RNA from *S.niger* Kidney, Liver and Spleen respectively

cDNA synthesis and RT PCR analysis

To further validate the effectiveness of isolated RNA for downstream applications, RT-qPCR was performed on the house keeping gene (GAPDH) known to be expressed in the studied tissues using gene specific primer. RT-PCR amplification yielded clear bands of around 190 bp for GAPDH fragment, on a 1.5% agarose- ethidium bromide gel for all tissue types (**Fig 5)** as compared to the GAPDH fragment in case of homogenisation without TES buffer where slightly faint bands were seen (**Fig 6)** .

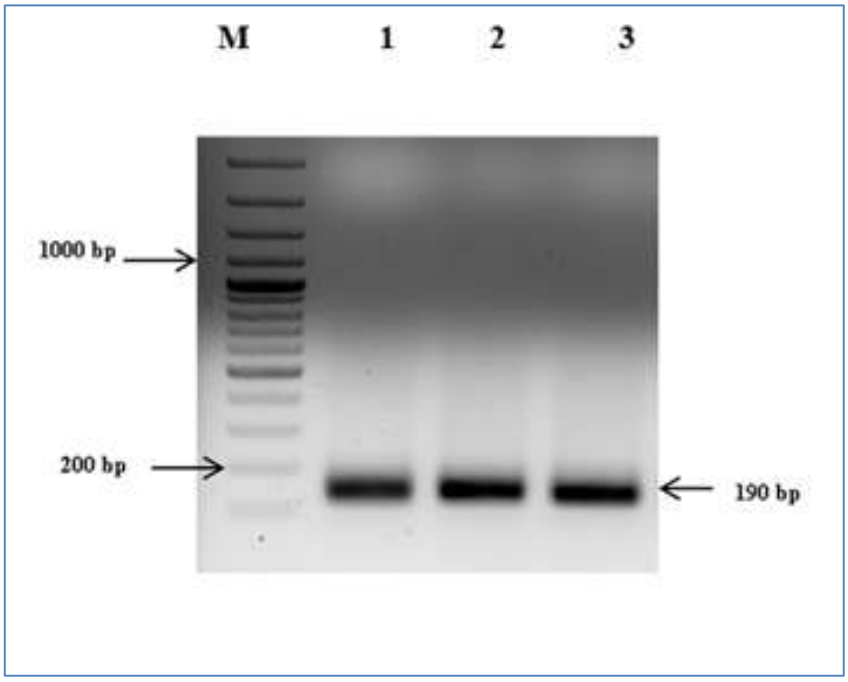


Fig. 5. RT-PCR of GAPDH in fish tissues after homogenization with TES buffer. Lane M= 100bp ladder; Lanes 1, 2, 3=Kidney, Liver and Spleen respectively

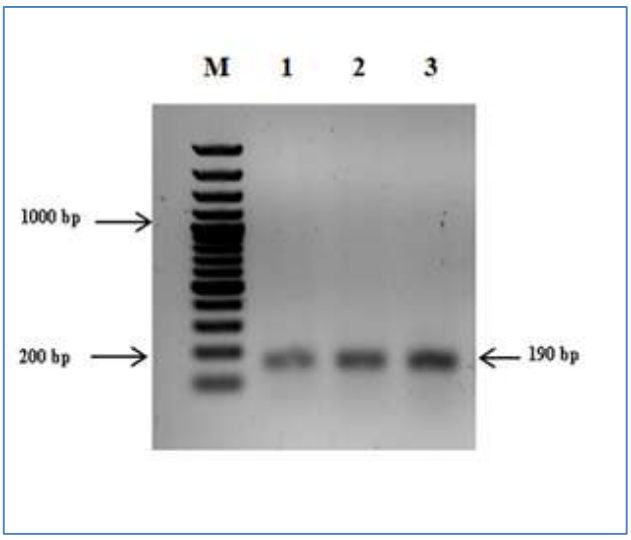


Fig. 6. RT-PCR of GAPDH in fish tissues after homogenization without TES buffer. Lane M= 100 bp ladder; Lanes 1, 2, 3=Kidney, Liver and Spleen respectively.

Table 1. RNA quality metrics of RNA isolated from various fish tissues

Tissue samples	Parameters tested	With TES (Mean ±SD)	Without TES (Mean ±SD)	Mann-Whitney U	P-value
Liver	Concentration (ng/µl)	639.6±56.84	439.4±89.32	1.5	<0.05
	A260/A280	1.984±0.02	1.98±0.02	10.5	>0.05
	RIN	8.28±0.25	7.92±0.52	7.5	>0.06
Spleen	Concentration (ng/µl)	874.6±96.96	775±91.73	0	<0.01
	A260/A280	1.988±0.017	1.98±0.02	5	>0.05
	RIN	9±0.07	8.54±0.51	0	<0.01
Kidney	Concentration (ng/µl)	718±54.79	626.2±68.22	7	>0.05
	A260/A280	1.980±0.02	1.97±0.01	11.5	>0.05
	RIN	8.36±0.25	7.84±0.30	9	>0.05

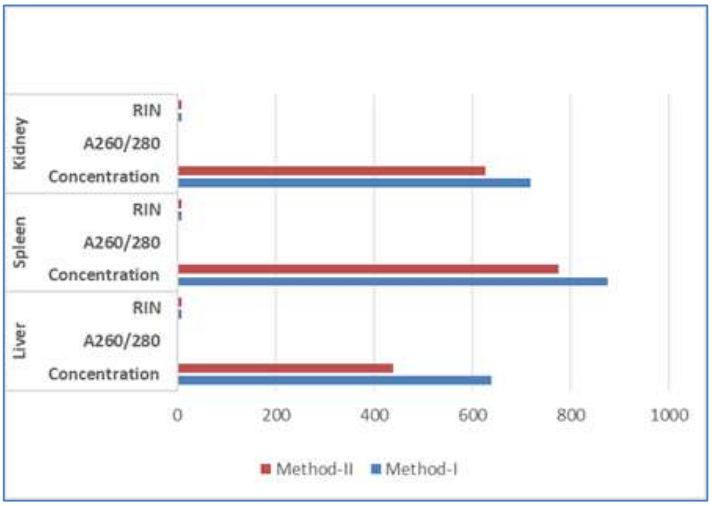


Fig. 7 Comparison of RNA yield, A260/A280 ratio and RIN between two tissue homogenization methods viz. method I: homogenization with TES buffer (bar graph in blue) and method II: homogenization without TES buffer (bar graph in red) and for I, N=15; For II, N=15. The p values were calculated using non parametric Mann Whitney test. The data is expressed as mean ±SD.

Discussion

The disruption of cells by homogenisation is very crucial step in RNA isolation. Establishing an optimal and cost effective homogenisation method that leads to the destruction of cell membrane without considerable

deterioration of intra-cellular components was the main concern of this study. This newly developed tissue homogenisation process was added to the already existing TriZol based RNA isolation protocol with slight modifications and was applied to a range of fish tissues (spleen, liver, and kidney). The isolated RNA was assessed for yield, purity and integrity using spectrophotometric analysis, gel electrophoresis and RT-qPCR to validate its suitability for downstream transcriptomic applications.

The results of this study demonstrate that homogenization with TES buffer significantly improves both the quantity and quality of total RNA extracted from liver, spleen, and head kidney tissues compared to homogenization without TES buffer. The increased RNA yield in TES buffer-treated samples suggests that the buffer enhances tissue disruption and RNA stabilization, thereby minimizing degradation during extraction. This is particularly evident in liver tissue, where RNA recovery nearly doubled with TES buffer, reinforcing its role in optimizing RNA isolation from tissues prone to high RNase activity.

Moreover, the RNA Integrity Number (RIN) values were consistently higher in samples homogenized with TES buffer, indicating better RNA stability. While the differences in RIN were statistically significant only for the spleen ($p < 0.01$), the trend across all tissues suggests that TES buffer contributes in preserving RNA integrity. The lack of significance in liver and head kidney may be attributed to tissue-specific variations in RNase activity or differences in homogenization efficiency. Nevertheless, the overall improvement in RIN values supports the use of TES buffer for maintaining RNA quality.

The A260/A280 ratios, indicative of RNA purity, were comparable between the two homogenization methods, with no statistically significant differences ($p > 0.05$). This suggests that while TES buffer enhances RNA yield and integrity, it does not significantly affect the purity of RNA. This demonstrates that while TES buffer optimizes RNA recovery and quality, the final purity depends primarily on post-homogenization purification rather than the homogenization method itself.

Gel electrophoresis confirmed the presence of intact ribosomal RNA bands (28S and 18S), corroborating the high-quality RNA obtained with TES buffer homogenization. The absence of smearing or degraded RNA in these samples further validates the effectiveness of TES buffer in preventing RNA degradation. The RNA quality was verified by RT-PCR analysis of the housekeeping gene GAPDH using the gene specific primers. The PCR product separated on 1% agarose ethidium bromide gel produced single fine band at 190 bp without any smearing against 100 bp ladder indicating that the RNA is intact for downstream processes. Therefore the TES based homogenisation was found to be suitable for isolating RNA from fish tissues, since it yielded a sufficient amount of RNA with good purity and integrity within a very short period of time from a small amount of tissue, which could be used in downstream application, compared to the other method.

Conclusion

The findings of this study show the effectiveness of the newly developed homogenisation step in RNA isolation protocol for small fish tissues which have been challenging for RNA extraction. This crucial step of the incorporation of a specialised homogenisation step, where the tissue is homogenised in the presence of DEPC treated TES buffer designed to rapidly lyse cells and inhibit RNAse activity simultaneously. The validation of the integrity and functionality of RNA was further done by the expression of GAPDH. The increased RNA yield and purity combined with high RNA integrity make this protocol valuable for transcriptomic studies.

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No funding was received for this work

Author contribution
Uzma Naseer: Data curation, Draft writing; **Ulfat Syed Mir** Conceptualization and Critical review; **Mohammad Altaf**: Review and Supervision, **Fayaz Ahmad**: Review, **Ibraq Khurshid**: Review

Declaration of Competing Interest
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethics Approval
All applicable international, national, and/or institutional guidelines for the care and use of animals were followed by the authors.

Data availability Statement
N/A

Informed Consent
Informed consent was obtained from all the authors.

Highlights
- Extraction of the high quality RNA from small fish tissues is a challenging task for transcriptomic analysis.
- We have developed and an efficient and affordable method for isolating high quality RNA from the fish tissue samples.
- By streamlining the process into a simple two step procedure, we have reduced the need for complex procedures and ensuring a more efficient and cost effective solution.

Abbreviations
cDNA: Complimentary DNA
DNA: Deoxyribonucleic acid
RNA: Ribonucleic acid
EDTA: Ethlenediaminetetraacetic acid
SDS: Sodium dodecyl sulfate
TES: Tris, EDTA, SDS
ng= Nanogram

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