Detection of mitochondrial tDRs in killifish embryos and other non-model organisms

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Contents

Abstract

In recent years a diversity of small noncoding RNAs have been identified that originate from the mitochondrial genome. These mitosRNAs are often dominated by tRNA-derived small RNAs (mito-tDRs). Differential expression of mito-tDRs is associated with responses to stress. They also appear to be expressed differentially during development and their expression may be enriched in stress-tolerant animals. Very little is currently known about roles or modes of action of these sequences, although they are implicated in a diversity of processes such as cell cycle regulation, mRNA stability, regulation of ROS production, and import of proteins into the mitochondrion. To better understand the various roles these sequences may play, it is critical that we understand their diversity, cellular location, and the context for their expression. This protocol outlines the methodologies used to detect mitosRNAs, including mito-tDRs, in embryos and cells of the annual killifish *Austrofundulus limnaeus*. We highlight critical steps in the isolation of RNA, creation of sequencing libraries, bioinformatics processing of sequence data, and methods for validation of expression that support a robust discovery pipeline for mitosRNAs even from species with incomplete reference genome sequences.

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1. Introduction

Mitochondria are of critical importance to cellular function and disease. In addition to their role in oxidative ATP production, they are involved in sensing and responding to changes in the cellular environment, and to stresses both external and internal. Mitochondria are potent sensors and integrators of a cell's physiological state, and even slight changes in metabolic flux can lead to powerful responses such as epigenetic reprogramming of nuclear gene expression or triggering of programmed cell death. Mitochondria have their own genome that in mammals encodes for 13 polypeptide chains involved in oxidative phosphorylation, 2 ribosomal RNA molecules (rRNA), and a full complement of 22 transfer RNAs (tRNA) (Filograna et al., 2021). Mitochondrial DNA copy number may vary from 500–100,000 copies per cell (Filograna et al., 2021). Thus, while the genome is small and gene diversity is low, gene copy number can be several orders of magnitude greater in the mitochondrial compared to the nuclear genome, making it a potential rich source of noncoding RNAs, especially tRNA-derived small RNAs (tDRs). Despite the central importance of mitochondria to cellular function and their ability to respond quickly to changes in cell state, very little research has focused on small noncoding RNAs with a mitochondrial origin (mitosRNAs), including tRNA-derived small RNAs (mito-tDRs).

tDRs were first identified in the late 1970s, but have only been deeply explored and characterized in numerous model systems more recently due to technical advances in DNA sequencing (Di Fazio & Gullerova, 2023). Work has been focused almost exclusively on the tDRs derived from nuclear-encoded tRNAs (Akiyama & Ivanov, 2023; Bhatter et al., 2024; Muthukumar et al., 2024). MitosRNAs, including mito-tDRs, were first identified in the human mitochondrial transcriptome in 2011 (Mercer et al., 2011) and shortly thereafter in mouse and human mitochondrial genomes (Ro et al., 2013). Since then, mitosRNAs, including mito-tDRs, have been identified in diverse species including a variety of mammals, molluscs (Pozzi & Dowling, 2019; Pozzi & Dowling, 2021; Pozzi et al., 2017), and several anoxia-tolerant vertebrates including the annual killifish (Riggs & Podrabsky, 2017; Riggs et al., 2018; Riggs, Woll, et al., 2019), among others. Of the human mitosRNAs, the proportion of mito-tDRs ranges from ~38 % (Ro et al., 2013) to ~86 % (Mercer et al., 2011). Like tDRs of nuclear origin, mito-tDRs are most often identified in stress or disease contexts. For example, anoxia induces mitosRNA expression, with

a particular enrichment for mito-tDRs (Riggs & Podrabsky, 2017). Given the proposed roles of nuclear-encoded tDRs (Brogli et al., 2023; Ivanov et al., 2011; Saikia et al., 2014), it is likely that mito-tDRs play a key role in the cellular responses to stress and disease (Brogli et al., 2023; Ren et al., 2023; Shaukat et al., 2021).

Massively parallel sequencing technologies have made it possible to profile gene expression in almost any organism in almost any condition and this has led to the discovery of small noncoding RNA expression in many new and exciting contexts. We are just beginning to understand the diversity of small noncoding RNA expression, and we still know very little about the function of most noncoding RNAs.

Identifying noncoding RNAs in any organism, but especially in non-model organisms, presents some unique challenges. First, having annotated mitochondrial and nuclear reference genomes for the species of interest is a must. Even slight changes in genome sequences across organisms can make clear identification of small noncoding RNAs difficult or even impossible. This is especially important when looking for mitochondrially-derived sequences due to the faster rates of sequence divergence compared to nuclear genomes, the frequency of genome rearrangements, and the link between genomic structure and transcriptional output that is driven by polycistronic transcription. There is also the potential issue of nuclear mitochondrial-like sequences (NUMTSs, mitochondrial pseudogenes) that vary widely in their number and distribution across species and must be evaluated as a potential source of what otherwise may appear as a mitochondrial-derived sequence (Bernt et al., 2013; Pozzi & Dowling, 2019). Second, many existing bioinformatics pipelines are designed to work on well-annotated genomes, or make assumptions about sequence features that may not apply broadly. Third, it is important to approach sequence discovery for small noncoding RNAs without assumptions of function or structure based on the limited knowledge of well-characterized small RNAs. For example, count data from highly similar sequences that differ only in sequence length should not be consolidated into a single longest sequence, rather each unique sequence should first be evaluated individually for biologically-relevant expression patterns. It is much better to have more data and filter for relevance at the end, rather than losing the ability to see potential patterns by making early assumptions that may filter out relevant sequences. The protocols and bioinformatics pipelines presented in this chapter represent our best attempt at dealing with the diversity of small RNA sequences and the challenges of working in a non-model organism with a reference genome that lacks the level of completeness found in major model organisms.

1.1 mito-tDR induction

While any unbiased small noncoding RNA experiment can be used to examine the presence and expression of mito-tDRs, certain experimental conditions or biological contexts may induce mitosRNA expression. In our experiments with the annual killifish *Austrofundulus limnaeus*, both developmental stage and environmental stress influence mitosRNA expression (Riggs & Podrabsky, 2017; Riggs et al., 2018; Riggs, Le, et al., 2019; Riggs, Woll, et al., 2019). We observed higher induction of mitosRNAs and specifically mito-tDRs during anoxia and aerobic recovery from anoxia in embryonic stages with extreme anoxia tolerance (Riggs & Podrabsky, 2017). Recent experiments have shown mito-tDR induction in response to arsenite and actinomycin treatment in a cell line derived from *A. limnaeus* embryos (WS40NE; (Riggs, Le, et al., 2019)), indicating mito-tDR induction may be a general stress response, as has been shown for nuclear-derived tDRs or tRNA-derived stress-induced fragments (tiRNAs) (Yamasaki et al., 2009). Thus, exposure to stress may be one way to experimentally induce mito-tDR expression. Stress tolerant organisms or developmental stages may also have higher baseline mito-tDR expression levels (Riggs & Podrabsky, 2017). For example, several mito-tDRs were identified in adult brain tissue from anoxia-tolerant vertebrate species, but a robust response to anoxia was not detected (Riggs et al., 2018). Further, mitosRNAs, including mito-tDRs, may be of critical importance to support normal development in the face of environmental stressors (Riggs & Podrabsky, 2017) and changes in their abundance have also been associated with several pathologies and diseases (Ren et al., 2023). Thus, for researchers interested in comprehensively assessing mito-tDRs in their model, we suggest including a range of developmental stages, stress, or disease conditions for analysis. Table 1 summarizes conditions shown to induce mito-tDRs, among other mitosRNAs, in *A. limnaeus* killifish embryos and cells. These conditions may be suitable for mitosRNA induction in other systems.

2. Methodology and protocols

The detection and characterization of the small noncoding RNA transcriptome requires specific methodologies that are unique from most bulk RNAseq analyses. Further, most bioinformatics pipelines must be adapted specifically for working with small RNA sequencing data and this sometimes requires using tools in non-standard ways. We outline these unique and critical methods in the following protocols.

Table 1 Treatments to induce mito-tDRs in the annual killifish *Austrofundulus limnaeus*. Similar conditions in other models may also stimulate mitosRNA expression.

Stress treatment	Concentration	Duration	Model	References
Anoxia	0 % O_2, note: hypoxia has not been tested	24–48 h	Embryos, WS40NE cell line	Riggs and Podrabsky (2017); Riggs, Woll, et al. (2019)
Aerobic recovery from anoxia	0 % O_2 (anoxia) followed by ambient O_2	2 and 24 h aerobic recovery from 24 h anoxia	Embryos	Riggs and Podrabsky (2017)
Actinomycin (nuclear transcription inhibitor)	10 μM	3 h, followed by 24 h recovery	WS40NE	Riggs, Woll, et al. (2019)
Arsenite (oxidative stress inducer)	250 μM	1 h	WS40NE	Unpublished data (Riggs)

2.1 Total RNA extraction

To efficiently detect small noncoding RNAs, it is essential to begin by recovering all RNA classes (messenger RNA, rRNA, tRNA, and many other noncoding RNAs). In addition, it is important not to set any limitations on the type of RNA isolated, including size fractionation of any sort. A total RNA extraction protocol that yields a high quantity and quality RNA sample that also preserves the fidelity of the transcriptome (e.g. protects from endogenous RNases) is essential to generate an RNA sequencing outcome that is accurate and repeatable. We find total RNA isolation by acid guanidinium isothiocyanate–phenol–chloroform extraction using TRIzol reagent (Invitrogen) is a rapid and reliable method for isolating total RNA of high quality and purity (Chomczynski & Sacchi, 2006). For fish embryos or other tissues that contain high quantities of polysaccharides, proteoglycan, or polyphenols, a modified protocol that uses a high salt precipitation of RNA is necessary to get pure RNA for downstream processes (Chomczynski & Mackey, 1995).

Endogenous RNases are particularly dangerous to noncoding RNA and can quickly destroy populations of smaller length sequences or cause nonspecific degradation products to show up in the small RNA fraction. It is therefore essential to exercise the maximum level of care when handling tissue or cell samples to avoid RNase contamination and to effectively block RNase action quickly.

2.1.1 Total RNA extraction solutions and supplies

1. **1 mM sodium citrate buffer, pH 6.5 (RNase-free)**
   1 L:
   0.2941 g sodium citrate (MW = 294.10 g/mol)
   900 mL nuclease-free water
   Adjust pH to 6.5 using 1 M NaOH
   Bring up to 1 L with nuclease-free water
   Sterilize the solution by filtration and store in RNase-free container
2. **0.8 M sodium citrate containing 1.2 M NaCl**
   1 L:
   206.38 g sodium citrate (MW = 257.973 g/mol)
   70.123 g sodium chloride (MW = 58.44 g/mol)
   Bring up to 1 L with nuclease-free water
   Sterilize the solution by filtration and store in RNase-free container
3. **TRIzol reagent** (Invitrogen #15596026)

4. **Phosphate buffered saline (1X), pH 7.4**
   1 L:
   8.0 g NaCl (MW = 58.44 g/mol)
   0.2 g KCl (MW = 74.55 g/mol)
   1.44 g Na_2HPO_4 (MW = 268.07 g/mol)
   KH_2PO_4 (MW = 136.09 g/mol)
   Dissolve in 900 mL of nuclease-free water
   Adjust pH if needed to 7.4 using HCl or NaOH
   Bring up to 1 L with nuclease-free water
   Sterilize the solution by filtration and store in RNase-free container
5. **100 % isopropanol**
6. **60 % ethanol**
   200 mL:
   120 mL 100 % ethanol (molecular biology grade)
   Bring up to 200 mL with nuclease-free water
7. **Chloroform**
8. **SYBR Green II RNA gel stain** (10,000X, Thermofisher # S7568)
9. **1.5 % agarose gel**
   200 mL:
   2.25 g agarose
   150 mL gel running buffer (such as TBE, below)
   Swirl gently to mix, then heat to boiling twice to ensure solubilized
   Allow the gel solution to cool a few minutes and add 20 μl SYBR Green II RNA gel stain (10,000X dilution)
   Swirl to mix completely
   Pour into gel casting tray and insert well combs
   Allow to sit at room temperature to polymerize
10. **1X tris-borate-EDTA (TBE) gel running buffer**
    1 L:
    10.8 g tris base (MW = 121.14 g/mol)
    5.5 g boric acid (MW = 61.83 g/mol)
    900 mL nuclease-free water
    4 mL 0.5 M EDTA solution (pH 8.0; Thermofisher #AM9260G)
    Adjust volume to 1 L with nuclease-free water
11. **RNA gel loading dye** (2X; Thermofisher R0641)
12. **GeneRuler 1 kb DNA ladder** (Thermofisher #SM0311)
13. **Special equipment**
    a. Motorized homogenizer with a generator probe that will fit into a 2 mL microcentrifuge tube

b. Centrifuge that can generate 16,000 × *g*
c. Nanodrop or similar instrument that can read absorbance in the UV range
d. Gel casting tray
e. Agarose gel electrophoresis rig
f. Vacuum pump (for solution sterilization)

2.1.2 Total RNA extraction procedure from embryos or cells in culture

Isolate total RNA from whole embryos using TRIzol reagent. The following protocol includes modifications to the isolation procedure for tissues containing high amounts of polysaccharides. Note, use phenol-resistant plastics such as polypropylene for all steps up to RNA precipitation.

Extraction of total RNA from embryos

1. Collect embryos according to developmental stage using a dissecting microscope. Pooled samples of embryos in groups of 20–100 may be required to obtain a high enough yield of RNA to meet the input requirements of some library preparation methods.
2. Transfer the staged embryos onto a nylon mesh screen (~100 μm pore size), and blot dry with Kimwipes to remove excess culture media.
3. Transfer the embryos into a 2 mL microcentrifuge tube.
   a. Embryos can be used immediately for RNA extraction.
   b. Embryos can be flash-frozen by submersion in liquid nitrogen and stored at −80 °C until RNA extraction.
4. For killifish embryos that weigh approximately 2.5 mg, add 50 μl of **TRIzol reagent** per embryo. This is a 20x dilution of the tissue which is needed to help remove contaminating substances such as polysaccharides.
5. Immediately after addition of TRIzol, homogenize the embryos using a motorized homogenizer at room temperature until complete lysis is achieved - typically 15–30 s.
6. Incubate the samples for 5 min at room temperature to ensure dissociation of nucleoprotein complexes.
7. Centrifuge the homogenate at 16,000 × *g* for 5 min at 4 °C to remove cellular debris and lipids.
8. Transfer the supernatant into a fresh tube and add 0.2 mL of **chloroform** per mL of TRIzol reagent originally added.
9. Mix the samples by vortexing for 20–30 s.
10. Separate the aqueous and organic phases by centrifugation at 10,000 × *g* at 4 °C for 20 min.

11. Carefully remove only the top clear aqueous phase and transfer into a fresh tube. Take care to avoid disturbing the white interface which contains DNA (among other things). The sample should be kept on ice for the remainder of the protocol.
12. Precipitate the RNA by adding 0.25 mL of **0.8 M sodium citrate containing 1.2 M NaCl** and 0.25 mL of **100 % isopropanol** for each mL of TRIzol used initially.
13. Gently vortex the samples and incubate overnight at −20 °C to favor high levels of precipitation of even small RNAs.
14. On the following day, pellet the RNA by centrifugation at 16,000 × *g* for 30 min at 4 °C.
15. Carefully decant and discard the supernatant. Do not disturb the white RNA pellet.
16. Wash the RNA pellet by adding 1 mL of **60 % ethanol** . Re-pellet the RNA by centrifugation at 10,000 × *g* for 30 min at 4 °C.
17. Decant the supernatant and repeat the wash step.
18. After the final wash, remove and discard the supernatant. Centrifuge the pellet again at 10,000 × g for 1 min at 4 °C to collect any residual EtOH and carefully remove with a small volume pipettor (P10 or similar).
19. With the sample tube lid open, allow the RNA pellet to dry for 5–15 min. The pellet will turn clear as it dries. Do not over-dry the pellet, it makes it hard to resuspend.
20. Resuspend RNA pellets in **1 mM sodium citrate (pH 6.5)** to stabilize the RNA during storage. Pellets can also be resuspended in Nuclease-free H_2O. Adjust the volume to achieve sample concentrations appropriate for library kit input requirements (see below). For RNA isolated from a group of 20 embryos, we recommend resuspending in volumes between 20–50 μl.
21. Incubate samples for 5 min at 55 °C to facilitate RNA pellet resuspension.
22. Measure the concentration and purity of RNA using UV spectroscopy and determine the ratios of A_{260}/A_{280}, and A_{260}/A_{230}.
23. Assess the sample for possible RNA degradation using 1.5 % agarose gel electrophoresis. A sample of 0.5 μg of total RNA is sufficient for observing distinct banding for 18S and 28S rRNA subunits.
24. RNA samples can be used immediately or stored long-term at −80 °C.

Extraction of total RNA from cells in culture

1. Grow adherent cells in culture dishes to 70–80 % confluence.
2. Remove culture media from the plate.

3. Rinse twice with **1X phosphate buffered saline**.
4. Add 1 mL of **TRIzol reagent** directly to the plate.
5. Pipet the TRIzol over the plate repeatedly, ensuring TRIzol contacts the entire surface area of the plate.
6. Pipet lysate up and down several times to ensure cell lysis.
7. Transfer TRIzol to a 2.0 mL microcentrifuge tube.
8. Proceed with RNA isolation starting at "Step 6" above.

2.2 cDNA library preparation and RNA-sequencing

High-throughput RNA sequencing is still the prevailing method for the detection and quantification of small noncoding RNAs. Most importantly for applications in non-model organisms, it can be used for the discovery of novel RNA sequences with the detection of single base differences (Dard-Dascot et al., 2018).

The Illumina TruSeq small RNA protocol (v2, Illumina, San Diego, CA, USA) for generating complementary DNA (cDNA) libraries for sequencing was originally released in 2010 and has remained a reliable method for producing small cDNA libraries directly from total RNA. The Illumina TruSeq protocol captures small RNAs with a 3' hydroxyl and a 5' monophosphate, like those that result from the molecular processing from RNAse III enzymes, Dicer and Drosha (Tsuzuki & Watanabe, 2017). The cDNA libraries produced are enriched for small RNAs (22–30 nt) through gel electrophoresis-based size selection. This protocol is compatible with past and current Illumina sequencing platforms.

In recent years, biases introduced during the adaptor ligation step of library preparation have been discovered (Dard-Dascot et al., 2018; Hafner et al., 2011). Further, a few small noncoding RNA classes in insects, nematodes and mammals, and small interfering RNAs (siRNA) in insects and plants contain a 2'-O-methyl (2'-OMe) modification at their 3′ terminal nucleotide (Dard-Dascot et al., 2018) and will not ligate efficiently with the TruSeq kit. Studies have also found amplification bias can be introduced with the Phusion polymerase used in with the Illumina TruSeq kit (Quail et al., 2012; Van Nieuwerburgh et al., 2011). Other commercially available kits offer alternatives to the adaptor ligation step (e.g. NEBNext kit, New England Biolabs, Ipswich, MA, USA; Takara Bio SMARTer smRNA kit, Takara Bio, Kusatsu, Japan; Diagenode CATS kit, Diagenode, Liège, Belgium), but these ligation-free protocols are not yet the best performing methods (Herbert et al., 2020).

Generally, the best practices for analyzing RNA-sequencing projects rely on generating replicable and repeatable outcomes with the data. The number of biological replicates of libraries needed varies in size between 4 and 6 libraries per condition (developmental stage or anoxia treatment). Current standards recommend at least six libraries per condition and even more if the intention is to test for differential expression (Schurch et al., 2016).

Here we highlight the general steps for the Illumina TruSeq small RNA library protocol and RNA sequencing and address the optimal modifications that were successful for the detection of mitosRNAs in embryos and cells of annual killifish.

2.3 cDNA library reagents

Illumina TruSeq small RNA library kit, or equivalent kit as discussed above.

2.3.1 cDNA library preparation and RNA-sequencing protocol using the TruSeq kit

1. Use an input of 1 μg total RNA as starting material for all libraries sequenced.
2. During the amplification step, product yield can vary based on RNA input amount, tissue type, and species. It is recommended that if the gel image does not include clear and distinct bands at the 22–30 nt range, increase the number of PCR cycles up to 15. For our purposes, 11 cycles produced distinct bands that are detectable for excision during the gel purification step.
3. To confirm quality libraries have been produced, purified cDNA libraries can be quantified by qPCR and their quality confirmed on a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) using a DNA 1000 chip.
4. Small noncoding RNA libraries should be sequenced using an Illumina platform sequencer for 30–50 cycles (single-end reads). The level of multiplexing can be adjusted to the sequencing platform aiming for 5–10 million reads per library.

2.4 Bioinformatics

Running a small RNA sequencing pipeline requires several types of key input data and associated files and generates several intermediate files. We suggest saving all intermediate files until the workflow is successfully completed, just in case you need to trouble-shoot any specific step. Our

workflow was performed at the High-Performance Computing (HPC) facility at Portland State University on the CentOS 7 Linux platform and is available on GitHub at https://jpod-lab.github.io/srnadocs/. This workflow is focused on small reads that are less than 50 nt long to facilitate finding small RNAs. The resulting output provides unique sequence-based counts, sequence annotation via established databases such as mirRBase, Rfam, and piRBase, sequence location corresponding to the reference genome, and various genomic features of the reference genome.

2.4.1 Input data

1. Raw Sequencing Data: These files are in fastq format (.fastq,.fq,.fastq.gz,.fq.gz) and contain the demultiplexed raw sequence reads for each sample from your small RNA sequencing experiment.
2. Reference Data: A reference genome sequence in fasta format (.fasta,.fa,.fna) with sequence available for the mitochondrial genome to find potential mitosRNAs.
3. Annotation Files: Genomic feature annotations (.gtf,.gff,.gff3) for the reference genome including features for the mitochondria are required.
4. Repository sequence files for small RNAs: specific microRNA (miRNA) sequence files (.fasta,.fa, fna) can be downloaded from databases like miRBase for alignments and identifying similarity of sample sequences against known sequences.
5. Adapter Sequences: A file that lists the sequences of any adapters used in the sequencing process that will be used for trimming the raw reads (.fasta,.fa).

2.4.2 Metadata

Sample Information: Metadata about the samples, such as sample names, treatment conditions, and replicates are needed for interpreting the experiment in downstream statistical analyses. FAIR guiding principles for data management should always be followed (Wilkinson et al., 2016).

2.4.3 Bioinformatics tools and virtual environments

Tools used in our pipeline are detailed in Table 2 and a step-by-step procedure is described below and outlined in Fig. 1. Variation in software versions and working on different platforms can lead to minor inconsistencies in results and we use virtual environments like conda and singularity to ensure the portability of the pipeline and reduce technical variability to a minimum. An exemplary pipeline is nf-core/smrnaseq, which provides reproducible and automated analyses focused on miRNA

Table 2 Bioinformatics tools, processes, and file types used in the processing of small RNA sequencing data.

Tools	Process	Input (I)	Output (O)	References
fastQC v0.12.1	Quality check for raw reads and trimmed reads	FASTQ reads	HTML report for each FASTQ file	(Andrews, 2010)
multiQC v1.23	Summarization of various QC reports	HTML reports and additional log files	Single HTML report	(Ewels, Magnusson, Lundin, & Käller, 2016)
miRTrace v1.0.1	Small RNA Quality check	FASTQ reads	HTML report	(Kang et al., 2018)
Trimmomatic v0.39	Trimming & filtering reads	FASTQ reads	FASTQ trimmed & filtered reads	(Bolger, Lohse, & Usadel, 2014)
Bowtie v1.3.1	Alignment with the reference genome for short reads	FASTQ trimmed & filtered reads FASTA genome reference	SAM alignments, FASTQ aligned reads	(Langmead, 2010)
Samtools v1.20	Handling alignment files, converting file formats, and counting alignments	SAM alignments	BAM alignments, BAI indexes, TXT counts, TXT statistics, TXT summary	(Danecek et al., 2021)
Picard	Quality check for alignments CollectAlignmentSummaryMetrics v3.2.0	BAM alignments, FASTA genome	TXT statistics	https://github.com/broadinstitute/picard

(continued)

Table 2 Bioinformatics tools, processes, and file types used in the processing of small RNA sequencing data. (*cont'd*)

Tools	Process	Input (I)	Output (O)	References
Sports v1.1	Small RNA annotations	FASTQ aligned reads	TXT annotations	(Shi, Ko, Sanders, Chen, & Zhou, 2018)
Bedtools v2.31.1	Overlap genomic intervals from GFF and BAM files	BAM alignments GFF genome feature file	BED alignment coordinates and genome features	Quinlan (2014)
Seqkit v2.8.2	Removing duplicated sequences from aligned reads	FASTQ aligned reads	FASTQ deduplicated reads	(Shen, Sipos, & Zhao, 2024)
R v4.4.0	Aggregation via in-house scripts	TXT outputs	TXT counts TXT annotations TXT exp. design	https://www.r-project.org/
DESeq2 v1.44	Downstream processing	TXT counts TXT annotations TXT exp. design	Differential expression statistical analysis	(Love, Huber, & Anders, 2014)
Geneious Prime JBrowse	Visualization (GUI required)	GFF genome feature file FASTA genome reference BAM alignments	Figures	(Diesh et al., 2023)

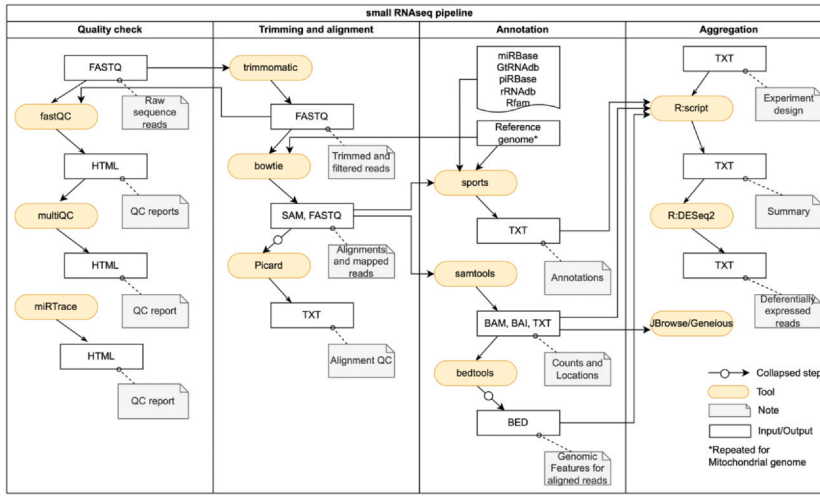

Fig. 1 Bioinformatics workflow for small RNA sequence detection. This pipeline involves sequence-based read counting for both reference and mitochondrial genome alignments. The resulting text files, prepared for downstream processing, are aggregated to include unique sequence reads, their respective counts, corresponding genomic locations, and annotations based on established databases.

identification. These environments are configurable and sharable, allowing installation of tools and software independent of platform. In our workflow, several conda environments were created to isolate most of the tools and prevent installation-dependent discrepancies. This pipeline is tested in an academic HPC environment and most tools are open-source and/or free to use for academic purposes. A graphical user interface (GUI) is only required at the final steps when using genome browser software to visualize small RNA alignments (see Table 2).

2.4.4 Protocol for in-silico small RNA identification

1. Create a **conda** environment named "condafastqc" that includes the following quality control (QC) tools: **fastqc**, **R**, **gcc (v12.1.0)**, **fastqcr**, and **multiqc**.
2. Initiate a tmux session. Tmux is a terminal window manager that enables the execution of commands in a detached terminal window, allowing processes to continue running even after the terminal is closed.
3. Run fastqc tool via R through the package fastqcr to perform QC on raw reads (.fastq), generating output in html format. To optimize processing time, utilize multiple threads; in our case 20 threads were used.

4. Summarize the fastqc html output files into a single file (.html) using multiqc. Assess the quality based on metrics (a) total number of reads: unique and duplicated, (b) Phred scores across the length, (c) per sequence quality score, (d) per base sequence content, (e) per sequence GC content, (f) per base N content, (g) sequence length distribution, (g) sequence duplication levels, (h) overrepresented sequences, and (i) adapter content.
5. Review the QC results to determine if trimming is necessary. A Phred score of 33 is used as a standard. Adapters are removed in this step with the help of a custom adapter sequence file. Reads shorter than 15 nt are discarded.
6. Set up a new conda environment named "condatrim" and start another tmux session to perform trimming. Run **trimmomatic** on the raw reads (.fastq) with parameters "-threads 6 -phred33 ILLUMINACLIP:adapter_seqs.txt:2:30:5:1:true SLIDINGWINDOW:5:15 LEADING:20 TRAILING:20 MINLEN:15" to generate trimmed reads (.fastq).
7. Reactivate the "condafastqc" environment and run fastqcR in a tmux session to generate the QC for trimmed reads (.fastq) in html format. Summarize these results into a multiqc report (.html).
8. Create a new conda environment named "condabowtie" and initiate a tmux session containing **bowtie**, **samtools**, **picard**, and **bedtools**. Construct indices (.ebwt) named "genome_index" (reference genome, nuclear and mitochondrial genomes combined) and "mito_index" (only the reference mitochondrial genome) using **bowtie-build**. This process requires separate fasta files for the reference genome and the mitochondrial genome.
9. Align the trimmed reads with the mitochondrial reference genome to produce the mapped reads (.fastq) and alignments (.sam). Alignment is done with **bowtie** using the indexed reference genome in the previous step and with parameters "-p 20 -k 10 –best –strata -e 99999 -v 0 -l 15 –chunkmbs 2048 -x mito_index -q trimmed.fastq –al mapped.fastq –sam –no-unal ". Repeat this step and the following steps for analyzing the complete genome (genome_index) with the same trimmed reads.
10. Convert the output.sam files into output.bam file format using **samtools** with the " view -b" function. Then, sort the output.bam files with **samtools** "sort" command. The sorted.bam files are indexed using **samtools** "index" producing a corresponding .bai file.

11. Check alignment quality (.bam) using the **picard CollectAlignment SummaryMetrics** command with parameters "-Xmx5g" using the reference mitochondrial genome. This step generates a log file (.txt).
12. Run the **samtools** "flagstat" command to gain alignment summary metrics (.txt).
13. Switch to the "condafastqc" environment to summarize the alignment QC from **bowtie**, **picard**, and **samtools** into a single file (.html).
14. Using the "condabowtie" environment, retrieve the relevant information from alignment files (.bam) by applying **samtools** "view" in conjunction with the awk command to extract columns 1 (QNAME), 10 (SEQ), 3 (RNAME), 4 (POS), 15 (number of multimapped alignments).
15. Using the **bedtools** intersect command with parameters "-wo -f1", take the alignments (.bam) and the mitochondrial genome features (.gff), and generate an annotated feature file (.bed) for each alignment.
16. Retrieve the number of counts of alignments for each sequence using unix awk command "**samtools** view.bam | awk '{print $10}'| sort | uniq -c". This will operate on the 10th column of the .bam file which contains the SEQ field.
17. Create a new environment "condasports" installing the **sports** tool and its dependencies. Download the relevant curated databases for the species of interest. Run the tool on the mapped reads (.fastq) obtained in step 9 with parameters "-M 2 -g mito_index". This will annotate the reads with up to 2 mismatches.
18. Finally, run the **R script** (available on github) to aggregate the results obtained from the previous steps.
19. To examine in detail the sequences of interest, visually inspect the alignment distribution for any patterns using genome browser tools such as **JBrowse** or **Geneious**. This requires the small RNA alignment .bam files (from step 10), and a genome references (assembly and annotation files). See example provided in Fig. 2.

2.5 Wet-lab validation

Wet-lab experiments can be used to validate and follow-up on findings from small RNA sequencing, as well as to investigate the presence of possible mito-tDRs in the absence of sequencing data. Northern blotting (detailed below) allows for detection of mature mitochondrial tRNAs and their tDRs, and has been the standard for research on nuclear-derived tDRs and tiRNAs. Cellular fractionation (detailed below) can be

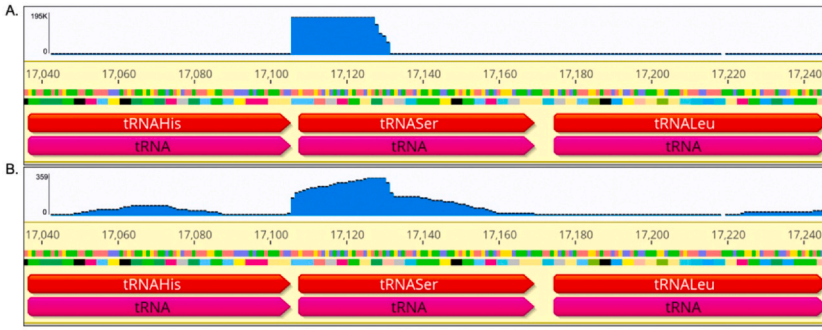

Fig. 2 Visualization of alignment distributions and nucleotide-level coverage for mitosRNAs on the mitochondrial genome of *Austrofundulus limnaeus* using Geneious Prime (version 2024.0.5). Two interesting coverage patterns (blue histograms) can be seen at the region encompassing the histidine, serine, and leucine tRNA gene regions (in red; tRNAHis, tRNASer, and tRNALeu) depending on which alignment files are used as an input. Data are represented from two different developmental stages (Wourms' stage 36 and 40) (Riggs & Podrabsky, 2017). (A) Alignment of all detectable mitosRNA reads (503,708 sequence reads) that represents the overall abundance of sequences (level of expression). (B) Alignment of each unique mitosRNA sequence (45,520 mitosRNAs) that represents the distribution of sequences detected across this portion of the mitochondrial genome. The scale bar at the left of each image represents the mean coverage calculated from the number of characters aligned at each position and spans from (A) 0 to 359 and (B) 0 to 195,000 character alignments.

performed to enrich RNA from the mitochondria in the sample and increase the chance for detection of mito-tDRs by northern blotting or sequencing. Whole mount and cellular in situ hybridization (ISH) can facilitate organ-level and subcellular localization, respectively, of mito-tDRs. Whole mount and cellular in situ localization protocols for mito-tDRs (and miRNAs) in annual killifish embryos have been adapted from protocols developed for miRNA detection in zebrafish embryos and human cell lines (Barrey et al., 2011; Lagendijk et al., 2012; Obernosterer et al., 2007; Riggs, Woll, et al., 2019). Cellular and whole mount in situ hybridizations are only appropriate once the presence of a mito-tDR of interest has been confirmed. Therefore, we are only focusing here on methods of detection and refer the reader to the publications referenced above for localization experiments.

2.5.1 Probe design

Each of these techniques (northern blotting and ISHs) depends on effective probe design. Antisense oligonucleotide probes must robustly bind to the

sequence of interest with limited off-target binding. Choosing the right sequence is not as straightforward for mito-tDRs as it may be for miRNAs, given the range of variants. We observe high sequence variation for mito-tDRs, with many sequences differing by only a couple of nucleotides. While variants could represent functional differences, we recommend initially designing a probe to target the "core" conserved sequence, as shown in red in Fig. 3, for all applications. This probe will bind to the majority of mitosRNAs annotating to that region of the mitochondrial genome. In the case of mito-tDRs, the mature tRNA and other tRNA variants containing the probe target sequence will be detected by northern blot since the target RNA is denatured. This is not expected for in situ analyses, since RNAs are not denatured and therefore a mature tRNA in which the 5' and 3' halves are bound to each other will not permit probe binding. When probing potential mito-tDRs without prior knowledge of sequences from sequencing data, targeting a sequence of 15–17 nts in the middle of either the 5' or 3' half of the tRNA will increase the probability of detection.

2.5.2 Northern blotting for mitosRNA detection

Due to their small size, mito-tDRs can be difficult to detect using standard northern blot protocols. Using DIG-labeled probes detected by an anti-DIG antibody is a safe alternative to radioactive labeling . *This can also help to boost signal due to the high affinity of the anti-DIG antibody and the associated enzymatic amplification of signal generated by the alkaline phosphatase conjugate for detection.*

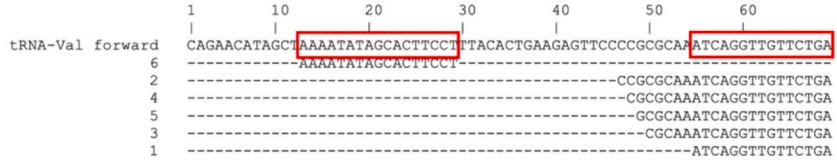

Fig. 3 Example of mito-tDR alignment to mitochondrial tRNA valine. Differentially expressed sequences in response to anoxia in the annual killifish *Austrofundulus limnaeus* are displayed aligned to the mature sequence for mitochondrial tRNA valine. Red boxes indicate possible probe targets. The target on the 5′ side encompasses the full length of the one mito-tDR aligning to the 5′ half. On the 3′ end, several mito-tDRs align, differing in 1–8 nucleotides from the target sequence. In this case, a probe targeting the 15 nt stretch conserved in all of the 3′ mito-tDR-Val variants is proposed. Such a probe will bind to all of the 3′ mito-tDR-Val sequences of interest.

Northern blots can be used to confirm sequencing results and to assess mito-tDR expression in additional conditions once a sequence of interest has been identified. We adapted a miRNA northern blot protocol (Kim et al., 2010) to detect mito-tDRs (as well as other mitosRNAs and miRNAs) in killifish RNA. Key considerations for success are: (1) Design of DNA probes interspersed with LNA (Locked Nucleic Acid) nucleotides; (2) choice of hybridization buffer; (3) cross-linking method; (4) signal amplification and detection methods (Digoxigenin (DIG) labeled probes for detection by anti-DIG antibody is recommended as an alternative to radiolabeling).

Choice of hybridization buffer can also have a large effect on the success of northern blots with mito-tDRs. The effects of different hybridization buffers on miRNA northern blot success is detailed by Kim et al. (2010). There are many reasons why hybridization conditions are important, but the most critical variables are likely the small size of the probes, the highly stable secondary structure of tRNAs, and the effects of chemical modifications on hybridization dynamics. We have had success using the ULTRAhyb™ ultrasensitive hybridization buffer (Thermo Fisher AM8669) and recommend its use for mito-tDR detection.

Cross-linking mito-tDRs to a membrane may be difficult due to their short sequence length and chemical composition. In some cases, using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) to fix small RNAs to the membrane via their 5' phosphate group may be required for reliable detection; however, we have been able to detect mito-tDRs solely with UV crosslinking.

2.5.2.1 Northern blot solutions and supplies

Note: DIG washing, blocking, and detection buffer can be purchased as part of DIG Wash and Block Buffer Set (Roche, 11585762001) or prepared as follows below.

1. **ULTRAhyb™ ultrasensitive hybridization buffer** (Thermo Fisher AM8670) Note: contains formamide which is a reproductive toxin and suspected carcinogen. Handle and dispose of as advised.
2. **1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC)** for crosslinking (prepare fresh)
   24 mL:
   9 mL DEPC-treated (RNase-free) water
   251 µl of 1-methylimidazole to water (yields final concentration of ~

0.340 M), maintaining a pH of 8.0 using 1 M HCl.
0.753 g EDC (= ~163.5 mM; FW = 155.25 g/mol)
Bring up to 24 mL with RNase-free H_2O

3. **Low stringent buffer** (2x SSC, 0.1 % SDS):
   50 mL:
   5 mL 20x SSC
   0.5 mL 10 % SDS
   Bring up to 50 mL total volume with nuclease-free water
4. **High stringent buffer** (0.1x SSC, 0.1 % SDS):
   50 mL:
   0.25 mL 20x SSC
   0.5 mL 10 % SDS
   Bring up to 50 mL total volume with nuclease-free water
5. **DIG washing buffer** (0.1 M maleic acid, 0.15 M NaCl, 0.3 % Tween 20, pH = 7.5):
   50 mL:
   0.58 g maleic acid (FW = 116.07 g/mol)
   0.43 g NaCl (FW = 58.44 g/mol)
   1.5 mL 10 % Tween-20
   Bring up to 50 mL total volume with nuclease-free water
6. **DIG blocking buffer** (5 % normal sheep serum, 2 mg/mL BSA in maleic acid buffer):
   50 mL:
   5 mL 10x maleic acid buffer (in kit) or 0.1 M maleic acid, 0.15 M NaCl, 0.3 % Tween 20, pH = 7.5
   2.5 mL normal sheep serum
   0.1 g bovine serum albumin (note: does *not* need to be fatty acid free, unlike BSA for mitochondrial isolation)
   Bring up to 50 mL total volume with nuclease-free water
7. **DIG detection buffer** (0.1 M Tris-HCl, 0.1 M NaCl, pH = 9.5):
   For 50 mL:
   0.788 g Tris-HCl (FW = 157.60 g/mol)
   0.2922 g NaCl (FW = 58.44 g/mol)
   Bring up to 50 mL total volume with nuclease-free water
8. **SYBR Gold** (Thermofisher # S11494)
9. **Special Equipment**
   UV Crosslinker
   Hybridization Oven

2.5.2.2 Northern blot protocol for mitosRNA detection

Day 1: Run RNA gel, transfer to membrane, begin hybridization

Separate RNA on a gel

1. Prepare samples: Mix 5 μg of total RNA with 2 μL 6x RNA loading dye II (Thermo Fisher) and water to a total volume of 12 μL.
2. Heat RNA-dye samples to 95 °C for 10 min and then put on ice immediately. Centrifuge briefly.
3. Prepare 15 % urea-TBE gel. Use 0.5x TBE for running buffer to clear wells. Optional: pre-run gel for 60 min at 200 V.
4. Load samples and 5 μL DIG labeled Blue Color Marker for Small RNA (Diagnocine, #FNK-NM270).
5. Run gel at 160 V for ~80 min or until lower blue band is about 1.5 cm above bottom of gel. BPB and cyanol in the loading dye run around 15 bases and 60 bases, respectively. Careful to not run gel too long - this will result in loss of small RNAs.
6. Optional gel visualization. After running gel, rinse a couple of times with 0.5x TBE and stain in 20 mL of 0.5x TBE mixture with 2 μL SYBR gold for 15-30 min rocking at RT in the dark. Visualize the gel with a UV imager.

Transfer RNA to a membrane

7. Transfer the RNA to a positively charged nylon membrane using the BioRad Trans-Blot Turbo Transfer system. Pre-soak filter stacks and membrane in 0.5x TBE. Assemble from bottom up: transfer stack, membrane, gel, transfer stack. Transfer at 0.3 A, max volt 25, for 30 min. Note: transfer can also be performed overnight using a Whatman™ TurboBlotter. Note: During the transfer, set the hybridization oven to 37 °C. Warm **ULTRAhyb™ ultrasensitive hybridization buffer** (Thermo Fisher AM8670) in the oven.
8. Crosslink RNA to membrane: remove top transfer stack and gel. Keep membrane and bottom transfer stack together. Cross link with UV and/or EDC.
   a. Place in UV Crosslinker. Use the "optimal crosslink" setting to crosslink the RNA to the membrane.
   b. EDC crosslinking
      i. Prepare **EDC** crosslinking solution up to 2 h before use.
      ii. Saturate 3 MM Whatman chromatography paper with EDC solution in clean pipet box lid.

iii. Disassemble transfer apparatus. Set membrane on top of EDC-saturated Whatman paper. RNA-side up.
iv. Carefully wrap Whatman paper and membrane in Saran wrap and incubate at 60 °C for 1–2 h to facilitate RNA to membrane cross-linking.
v. Unwrap membrane and discard Whatman paper. Remove residual EDC by rinsing membrane with distilled water 2 × 5 min at RT.

Hybridize probe to RNA

9. Roll membrane long-ways with RNA side in and insert into a 50 mL conical tube. Ensure that the membrane is all the way into the tube and will not interfere with cap closing.
10. Add 7.5 mL pre-warmed (37 °C) **ULTRAhyb™ ultrasensitive hybridization buffer** and pre-hybridize for at least 30 min, rotating in hybridization oven at 37 °C. Check that the tube is not leaking.
11. Prepare probes for hybridization
    a. Thaw LNA/DNA probe(s) for RNAs of interest at RT.
    b. Denature probe (10 μM stock) by heating at 95 °C for 5 min and plunging into ice immediately afterwards. Add denatured probe directly to tube with membrane and hybridization buffer to yield desired final concentration of 2 nM.
12. Return tube to hybridization oven, rotating at 37 °C. Check that the tube is not leaking and temperature is stable. Hybridize overnight.

Day 2: Stringency washes, anti-DIG antibody incubation, and imaging

Antibody detection and imaging

1. Prepare and warm low stringency, high stringency, and DIG washing buffers to 37 °C in the hybridization oven.
2. Discard hybridization buffer from pervious overnight incubation into formamide waste.
3. Wash membrane twice with **Low stringent buffer (2x SSC, 0.1 % SDS)** at hybridization temp (37 °C) rotating for 15 min.
4. Wash the membrane twice with **High stringent buffer (0.1x SSC, 0.1 % SDS)** at hybridization temp (37 °C) rotating for 5 min.
5. Wash the membrane with **DIG washing buffer** at hybridization temp (37 °C) rotating for 10 min.
6. During washes, prepare 10 mL **DIG blocking buffer**.
7. Carefully transfer membrane to square petri dish with 10 mL blocking buffer. Rock at RT for at least 1 hr to block.

8. Add 1 μL of anti-digoxigenin-AP, Fab fragments antibody (Roche, 11093274910) to achieve 1:10,000 dilution. Rock at RT for 45–60 min.
9. Wash membrane 4x in **DIG washing buffer**, 15 min each rocking at RT.
10. Incubate membrane in **DIG detection buffer** for 5 min, rocking at RT.
11. Place the membrane on a clear sheet protector and add 1 mL CDP-Star chemiluminescent substrate (Roche, 12041677001) directly to the blot. Cover with top sheet protector. Incubate in the dark for 15 min.
12. Image on chemidoc or on film.
13. Keep blot in Detection Buffer if planning to re-image or strip and re-probe. Store in the dark.

2.5.3 Mitochondrial isolation to enrich for mitosRNAs

A strategy to increase the likelihood of detecting mitosRNAs is to perform northern blotting on a mitochondrial fraction. Though mitochondrial fractionation is imperfect, it will enrich the sample for RNA in the mitochondria and thus aid in detection of mitosRNAs, including mito-tDRs.

For mitochondrial isolation the osmotic strength of the isolation media and method of homogenization are of key importance. The following protocol is a mitochondrial isolation protocol established for *A. limnaeus* embryos (Duerr & Podrabsky, 2010) adapted for mitochondrial isolation from adherent cells in culture.

2.5.3.1 Mitochondrial isolation solutions

1. **Cell scraping buffer** (1X PBS with 2.7 mM EDTA)
2. **Mitochondria isolation buffer** (20 mM TES, 117 mM KCl, 5 mM EGTA, 2 % BSA, pH = 7.4):
   125 mL:
   1.09 g KCl (FW = 74.55 g/mol)
   0.58 g TES buffer (FW = 229.25 g/mol)
   0.21 g EGTA (FW = 380.35 g/mol)
   2.5 g BSA (fatty acid free) (Sigma-Aldrich, 126575)
   Adjust the pH to 7.4

   Add the BSA last and stir the mixture until the BSA goes into solution. The osmotic pressure should be physiologically relevant for the system, for killifish embryos this is ~270 mOsm.

3. Special Supplies:

Dounce tissue grinders, Wheaton (DWK Life Sciences, 357542). Ideal size depends on cell suspension volume.

2.5.3.2 Mitochondria isolation protocol

See Fig. 4 for a step-by-step visual of mitochondrial isolation.

1. Grow cells to ~80 % confluence in 100 mm cell culture dishes in normal growth medium. Note: In our experience, pooling killifish cells from 12 plates yields sufficient mitochondrial RNA (~5–10 μg) for northern blot analysis. However, the amount of starting material will likely require optimization for other cell lines or tissues.
2. Harvest cells for fractionation:
   a. Add 1.5 mL ice-cold **cell scraping buffer** to each 100 mm plate and let sit for 5 min.
   b. Scrape cells with a cell scraper and transfer all cells to a 15 mL conical tube. Pool cells from plates of the same treatment.
   c. Spin at 100 × g at 4 °C for 7 min to pellet cells. Discard supernatant.

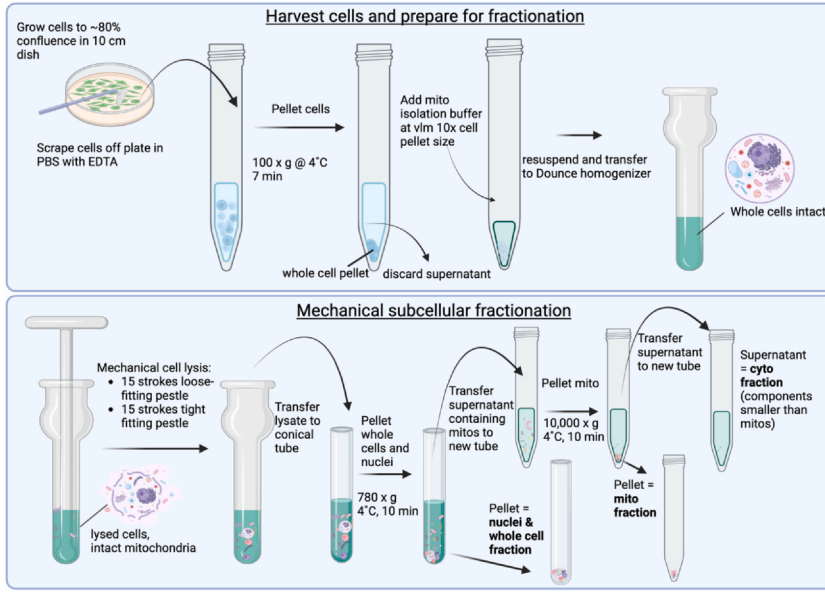

Fig. 4 Overview of protocol for subcellular fractionation and mitochondrial isolation. Source: Figure prepared using BioRender.com.

3. Resuspend cells in **mitochondria isolation buffer** at ~10x volume of pellet (e.g. if pellet is about 250 μL, add 2.5 mL of isolation buffer). Gently pipet up and down to resuspend.
4. Transfer cell suspension into a Wheaton® Dounce Tissue Grinder (DWK Life Sciences, 357542). Note, the ideal homogenizer size will depend on the volume of cell suspension. Cell suspension should *not* enter 'fish bowl' portion of homogenizer when homogenizing.
5. Homogenize with loose-fitting Teflon pestle. About 15 slow strokes (up and down) with the pestle. Avoid forming bubbles. This will break open cells, but not burst the mitochondria.
6. Repeat homogenization with the tight fitting pestle.
7. Transfer solution into a round bottom conical tube without a cap.
8. Centrifuge 10 min at 4 °C at 780 × g to pellet whole cells and nuclei. The supernatant contains mitochondria - do NOT discard.
9. Transfer supernatant into a 15 mL conical tube. Centrifuge for 10 min at 10,000 × g at 4 °C to pellet mitochondria. The supernatant contains organelles and cytoplasmic components smaller than mitochondria. Transfer the supernatant to a new 15 mL conical tube and save.
10. Add 1 mL TRIzol™ Reagent (Invitrogen, 15596026) to the mitochondrial pellet and resuspend.
11. Add 1 mL TRIzol™ to the whole cell and nuclei pellet and resuspend.
12. Add TRIzol™ LS (Invitrogen, 10296010) (0.75 mL per 0.25 mL sample) to cytoplasmic suspension containing components smaller than mitochondria.
13. Isolate RNA as described above for cells.
14. Quantify RNA.
15. Enrichment of mitochondria can be confirmed by assessing the presence of mitochondrial-specific RNA by northern blot analysis or small RNA sequencing Proceed as desired.

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