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Direct-TRI: High-throughput RNA-extracting Method for All Stages of Zebrafish Development

Kota Ujibe, Kanako Nishimura, Makoto Kashima and Hiromi Hirata*

Department of Chemistry and Biological Science, College of Science and Engineering, Aoyama Gakuin University, Sagamihara, Japan
*For correspondence: hihirata@chem.aoyama.ac.jp

[Abstract] Recent popularization of next-generation sequencing enables conducting easy transcriptome analysis. Nevertheless, substantial RNA isolation work prior to RNA sequencing, as well as the high cost involved, still makes the routine use of large-scale transcriptome analysis difficult. For example, conventional phenol-chloroform RNA extraction cannot be easily applied to hundreds of samples. Therefore, we developed Direct-TRI, a new cost-effective and high throughput RNA-extraction method that uses a commercial guanidine-phenol-based RNA extraction reagent and a 96-well silica column plate. We applied Direct-TRI to zebrafish whole larvae and juvenile samples and obtained comparable RNA qualities by several different homogenization methods such as vortexing, manual homogenizing, and freezing/crushing. Direct-TRI enabled the extraction of 192 RNA samples in an hour with a cost of less than a dollar per sample. Direct-TRI is useful for large-scale transcriptome studies, manipulating hundreds of zebrafish individuals, and may be used with other animal samples.
Keywords: Zebrafish, RNA isolation, RNA-Seq, Gene expression, High throughput

[Background] RNA-sequencing (RNA-Seq) is one of the standard methods in various fields of biology (Cheng *et al.*, 2021; Sun *et al.*, 2021) because it provides comprehensive information about gene expression. Recently, several researchers have developed cost-effective and high-throughput RNA-Seq library preparation protocols such as Lasy-Seq (Kamitani *et al.*, 2019), BRB-Seq (Alpern *et al.*, 2019), and Decode-Seq (Li *et al.*, 2020). Although these techniques and the lower cost of massive parallel sequencing have enabled large-scale RNA-Seq analyses (Hoang *et al.*, 2020; Miller *et al.*, 2013), the RNA extraction process prior to RNA-Seq remains laborious and time-consuming. For example, a conventional RNA extraction method using phenol-chloroform (Peterson *et al.*, 2009) requires careful liquid handling and a number of steps. Another method using silica columns, the RNeasy of Qiagen, provides easy and time-saving operation but is costly. In addition, the lysis solutions included in the kit do not contain phenol and, thus, are less effective in disrupting cells and tissues than phenol-containing lysis solutions. Recently, a new easy and time-saving method, Direct-zol (Sosanya *et al.*, 2013), has been developed by combining these two methods. In the Direct-zol protocol, samples are lysed in phenol-containing solution and subsequently subjected to silica column-based RNA purification. However, the kit is costly (approximately 7 dollars per sample) for a large number of samples. From the viewpoint of high throughput, low cost, and robustness, the aforementioned methods are not feasible for large-scale sampling. Here, we introduce Direct-TRI, a high-throughput, cost-effective, and reliable RNA-extraction method using TRI Reagent-LS and 96-well silica column plate. This protocol consists of

two steps: homogenization of samples in phenol-containing TRI Reagent-LS followed by the direct isolation of RNA from the phenol lysate with handmade washing solutions. Direct-TRI makes it possible to simultaneously process hundreds of RNA samples individually with a simple procedure and at an affordable cost. We show an example of a Direct-TRI application using whole zebrafish samples. Zebrafish is a model organism to study development, behavior, and disease in vertebrates (Goldsmith *et al.*, 2012; Gore *et al.*, 2018; Maeta *et al.*, 2020). The large-scale gene expression analysis of zebrafish is a powerful tool for investigating the molecular basis underlying developmental events and diseases (Lee *et al.*, 2020; Scholz, 2013). The combination of Direct-TRI and the cost-effective and high throughput RNA-Seq library preparation methods will make large-scale transcriptome analysis more useful not only for zebrafish samples but also for other organisms.

Materials and Reagents

1. 1.5 ml microcentrifuge tube (Rikaken, catalog number: RSV-MTT1.5)
2. Transfer pipet, 3 ml (Falcon, catalog number: 357575)
3. 8-strip tube, dome type (Rikaken, catalog number: RS-PCR-8D)
4. Cell culture dish, 100 mm (Nippon Genetics, catalog number: FG-2090)
5. Cell culture dish, 35 mm (Nippon Genetics, catalog number: TR4000)
6. Gemma Micro ZF 75 (Skretting)
7. Otohime B2 (Marubeni Nisshin Feed)
8. Kimwipe (Kimberly Clark)
9. AcroPrep Advance 96-well Long Tip Filter Plate for Nucleic Acid Binding (Pall, catalog number: 8133)
10. A reservoir for AcroPrep Advance (Merck, catalog number: BR701340-50EA)
11. Eppendorf twin.tec 96 well LoBind PCR plates (Eppendorf, catalog number: 0030129512)
12. DuraCross zebrafish breeding tank with divider (Laboratory Product Sales, catalog number: T233795)
13. Zebraifsh (*Danio rerio*)
14. TRI Reagent-LS (Molecular Research Center, catalog number: TS120)
    Notes:
    a. *TRIzol-LS Reagent (Thermo Fisher Scientific, catalog number: 10296028) or ISOGEN-LS (Nippon Gene, catalog number: 311-02621) can also be used.*
    b. *Sepasol-RNA II Super (Nacalai Tesque, catalog number: 30487-46), an equivalent product, could not be used for RNA extraction from adult zebrafish because a precipitate was produced during the freezing and crushing processes.*
15. Ethanol, 99.5% (Nacalai Tesque, catalog number: 08948-25)
16. Nuclease-free water (Merck, catalog number: H20MB1006)
17. BioMasher II (Nippi, catalog number: 320102; Figure 1)

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Figure 1. Image of BioMasher II

18. Liquid nitrogen
19. Pronase (Merck, catalog number: 53702)
20. QuantiFluor RNA System (Promega, catalog number: E3310)
21. Agilent RNA 6000 Pico kit (Agilent Technologies, catalog number: 5067-1513)
22. 10× Loading Buffer (Takara Bio, catalog number: 9157)
23. ExcelBand 1 KB (0.25-10 kb) DNA Ladder (Smobio, catalog number: DM3100)
24. Tricaine (Wako, catalog number: 051-06571)
25. Tris(hydroxymethyl)aminomethane (Rikaken, catalog number: RSP-THA500G)
26. Boric acid (Wako, catalog number: 021-02195)
27. EDTA-2Na (Wako, catalog number: 343-01861)
28. Agarose Powder (Rikaken, catalog number: RSV-AGRP-500G)
29. 10 mg/ml Ethidium bromide (Nippon Gene, catalog number: 315-90051)
30. Ethanol, 80% (see Recipes)
31. 1% agarose gel (see Recipes)
32. Tricaine (see Recipes)
33. E3 medium (see Recipes)
34. 20 mg/ml Pronase (see Recipes)
35. 5× TBE (see Recipes)

Equipment

1. Mortar and pestle, 120 mm (AsOne, catalog number: 6-549-03)
2. Vortex-Genie 2 (Scientific Industries)
3. PlateSpin II (Kubota)

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4. Quantus Fluorometer (Promega, catalog number: E6150)
5. Agilent 2100 Bioanalyzer System (Agilent, catalog number: G2939B)

Procedure

A. Zebrafish maintenance and larvae collection
   1. Maintain adult zebrafish in a recirculation system at 28.5°C under 14:10 h light:dark cycle according to the standard protocol (Westerfield, 2000).
   2. Place female and male adult zebrafish in a breeding tank with a divider that separates fish to avoid immediate mating.
   3. Obtain fertilized eggs [embryos, 0 days post fertilization (dpf)] by removing the divider the next morning.
   4. Raise zebrafish embryos in E3 medium in a 100 mm cell culture dish at 28.5°C.
   5. At 1 dpf, transfer embryos with 2.5 ml of E3 medium into a 35 mm cell culture dish and add 150 µl of 20 mg/ml pronase.
   6. After 15 min incubation at room temperature (RT), dechorionate embryos by stirring the dish.
   7. Transfer embryos to a 100 mm cell culture dish with 20 ml of E3 medium and raise them up to 5 dpf at 28.5°C.
   8. Place zebrafish larvae (5 dpf larvae) in the recirculation system (30 larvae/tank).
   9. Feed zebrafish larvae in the recirculation system both paramecia and Gemma Micro ZF 75 twice a day from 5 to 30 dpf.
   10. Feed juveniles (30-90 dpf) and adults (~90 dpf) both brine shrimp and Otohime B2 twice a day after 30 dpf (Wakamatsu *et al.*, 2019).

B. RNA extraction from zebrafish larvae and juveniles

   Note: Use nuclease-free tips in all RNA extraction steps.

   1. Larvae (approximately 4-12 dpf, size smaller than 5 mm)
      a. Anesthetize a zebrafish larva in 20 ml of 0.2 mg/ml Tricaine at RT for 3 min.
      b. Prepare 100 µl of TRI Reagent-LS in 8-strip tube.
      c. Place a single zebrafish larva inside of a cap of the 8-strip tube and remove water using a micropipette (Figure 2).

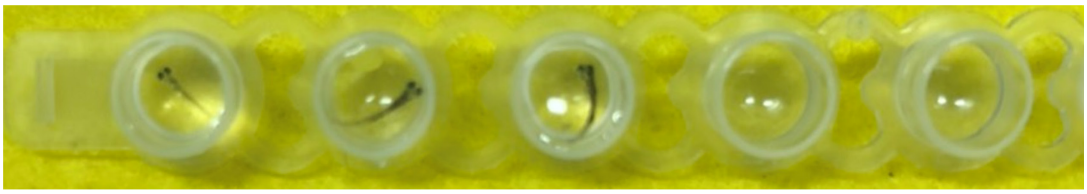

Figure 2. Zebrafish larvae placed in the tip of the 8-strip tube dome cap

      d. Assemble the cap to the 8-strip tube with each well containing the TRI Reagent, and lyse

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larvae completely by vortexing for 1 min at RT.

e. Move to the RNA purification steps immediately or store the 100 µl lysates at -80°C for 1 month.

2. Larva (approximately 14-38 dpf, size smaller than 10 mm)
   a. Anesthetize a zebrafish larva in 20 ml of 0.2 mg/ml Tricaine at RT for 3 min.
   b. Transfer a zebrafish larva in a 1.5 ml microcentrifuge tube with a Biomasher II and remove excess water using a micropipette.
   c. Add 50 µl of TRI Reagent-LS to the larva and immediately mash it at RT using a pestle until it is completely crushed.
   d. Add 50 µl of TRI Reagent-LS and vortex the sample for 1 min at RT.
   e. Move to the RNA purification steps immediately or store the 100 µl lysates at -80°C for 1 month.
3. Juvenile (approximately 38 dpf, size larger than 10 mm)
   a. Anesthetize a zebrafish juvenile in 20 ml of 0.2 mg/ml Tricaine at RT for 3 min.
   b. Place a juvenile zebrafish in a 120 mm mortar and remove water by wiping the juvenile using Kimwipes.
   c. Pour liquid nitrogen to quickly freeze the juvenile.
   d. Grind the frozen juvenile into powder using a pestle (Figure 3).

Figure 3. Image of a juvenile zebrafish on a mortar for grinding

   e. Add 1 ml of TRI Reagent-LS to the frozen powder and mix using a pestle until the mixture becomes liquid.

   Note: TRI Reagent-LS is frozen before the completion of tissue lysis. To lyse tissues completely, defrosting the regents is essential. Placing the mortar on a water bath (40-50°C)

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is sufficient to defrost the regents in a short time. The integrity of the RNA is not affected by this warming.

f. Transfer 100 µl of the lysate to a new 1.5 ml microcentrifuge tube.

g. Move to the RNA purification steps immediately or store the 100 µl lysates at -80°C for 1 month.

Note: For reuse, the mortar should be washed with distilled water until the lysate is completely removed.

4. RNA Purification

a. Add 100 µl of 99.5% ethanol to the 100 µl lysate and mix by vortexing at RT.

Note: When the lysate is stored in -80°C, thaw it at RT prior to this step.

b. Prepare the AcroPrep Advance 96-Well Filter Plate on a reservoir (Figure 4).

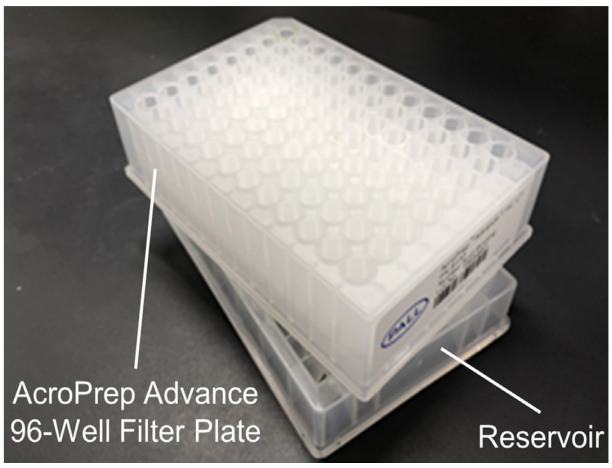

Figure 4. AcroPrep Advance 96-Well Filter Plate on a reservoir

c. Transfer the 200 µl mixture into each well of the AcroPrep Advance plate.

d. Centrifuge at 1,300 × *g* for 4 min at RT. Discard the flowthrough.

e. Add 400 µl of 99.5% ethanol and centrifuge the plate at 1,300 × *g* for 4 min at RT. Discard the flowthrough.

f. Repeat adding 400 µl of 99.5% ethanol and centrifuge the plate at 1,300 × *g* for 4 min at RT. Discard the flowthrough.

g. Add 700 µl of 80% ethanol to the plate and centrifuge at 1,300 × *g* for 10 min at RT. Discard the flowthrough.

h. Elute the RNA by adding 30 µl of nuclease-free water to the center of each well followed by incubation for 1 min at RT. Then, centrifuge at 1,300 × *g* for 4 min at RT. RNA solution can be stored at -80°C.

C. Determination of the RNA concentration

Determine RNA concentration with the QuantiFluor RNA System kit and Quantus Fluorometer according to the manufacturer's manual. Typical results are shown in Note 1.

Note: The expected RNA concentration from an individual is too low to be measured with a spectrophotometer (e.g., NanoDrop).

D. RNA quality check

We recommend checking the quality of RNA through agarose gel electrophoresis or with the Bioanalyzer for some but not all samples because the amounts of RNA are low in some cases.

1. Agarose gel electrophoresis
   a. Add 15 µl (300 ng/lane) of the RNA sample into a well of 1% agarose gel.
   b. Run electrophoresis at 135 V for 25 min in 0.5× TBE. Typical results are shown in Note 2.
2. Bioanalyzer

   This procedure requires the use of the Bioanalyzer.

   Check the RNA quality with the Agilent 2100 Bioanalyzer and the Agilent RNA 6000 Pico kit according to the manufacturer's manual. Typical results are shown in Note 3.

Notes

1. Typical yields for RNA isolation with the Direct-TRI and phenol-chloroform are shown in Table 1. Owing to the limitation in the capacity of RNA to bind to the AcroPrep Advance 96 Filter Plate (approximately 3 µg), the yield of Direct-TRI tends to be smaller than that of the phenol-chloroform method (Peterson *et al.*, 2009) when adult zebrafish are used.

   Table 1. Total RNA amounts obtained with the Direct-TRI and phenol-chloroform methods

	6 dpf (Vortex)	17 dpf (Biomasher)	84 dpf (Freezing homogenize)
Direct-TRI	330 ng	3480 ng	2.8 µg
Phenol-chloroform	170 ng	1500 ng	132 µg

   Note: RNA concentration was determined with the Quantus™ Fluorometer.

2. A typical result of the RNA quality check through electrophoresis is shown in Figure 2. Zebrafish (6 dpf) were used to extract RNA using the TRI Reagent-LS to compare two different RNA-extracting methods, and then the RNA quality was checked. Two major bands (28S and 18S rRNA) indicate that RNA was not degraded (Figure 5). The small-size RNA was successfully obtained with the Direct-TRI method but not with the conventional phenol-chloroform method.

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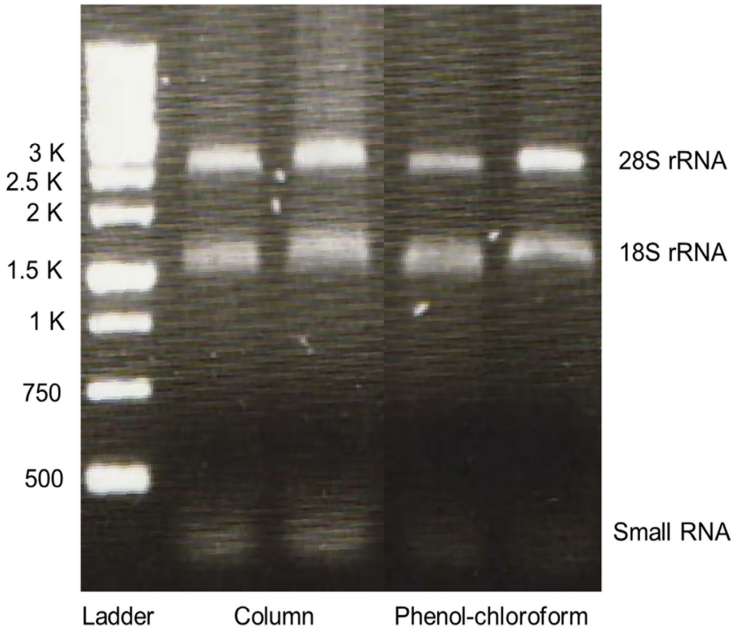

Figure 5. Typical results of the RNA quality check through agarose electrophoresis

3. A typical result of the RNA quality check using the Bioanalyzer is shown in Figure 6. RNA was isolated from a 15 dpf larva with Direct-TRI, and the RNA quality was checked with the Bioanalyzer. Two RNA peaks (18S and 28S rRNA) were clearly observed, indicating that the RNA was not degraded (RIN 9.10). In the Direct-TRI method, the RIN value was approximately 7-9.5.

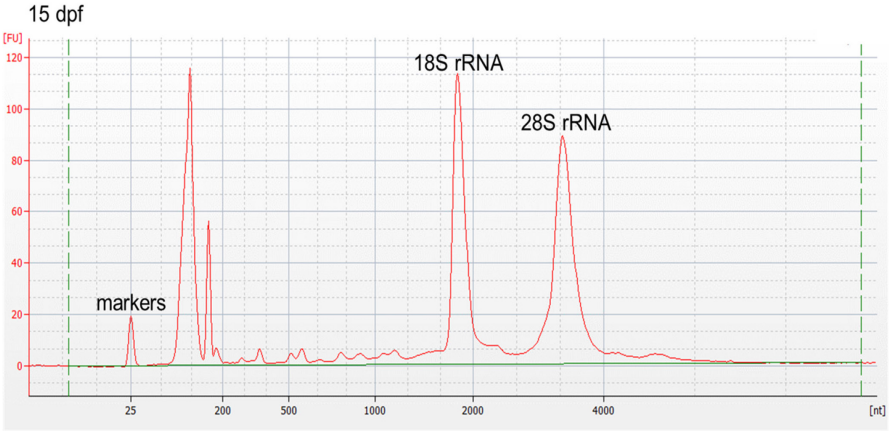

Figure 6. Typical results of the RNA quality check with the Bioanalyzer

Recipes

1. E3 medium

   50× E3 stock solution

NaCl	14.65 g (500 mM)

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KCl	0.63 g (17 mM)
$CaCl_2 \cdot 2H_2O$	2.43 g (33 mM)
$MgSO_4 \cdot 7H_2O$	4.07 g (33 mM)
Distilled water	mess up to 1 L
Adjust pH to 7.2 with ~100 µl of 0.1 M NaOH	
Total	1 L
Autoclave at 121°C for 20 min	
Store at RT	

E3 medium (1× E3 working solution)

50× E3 stock solution	20 ml
Distilled water	mess up to 1 L
Total	1 L
Store at RT	

2. 20 mg/ml Pronase

Pronase	1 g
Distilled water	mess up to 50 ml
Total	50 ml
Divide into 1 ml aliquots	
Store at -20°C	

3. Tricaine (Ethyl 3-aminobenzoate methanesulfonate)

10 mg/ml Tricaine (Ethyl 3-aminobenzoate methanesulfonate)

Tricaine	1 g
Distilled water	mess up to 100 ml
Total	100 ml
Divide into 1 ml aliquots	
Store at -20°C	

0.2 mg/ml Tricaine (Ethyl 3-aminobenzoate methanesulfonate)

10 mg/ml Tricaine	1 ml
Distilled water	mess up to 50 ml
Total	50 ml

4. 80% Ethanol

Ethanol, 99.5%	40 ml
Nuclease-free water	10 ml
Total	50 ml
Store at RT	

5. 5× TBE

Tris(hydroxymethyl)aminomethane	54.0 g

Boric acid	27.5 g
EDTA-2Na	1.85 g
Distilled water	mess up to 1 L
Total	1 L

Store at RT

6. 1% agarose gel

5× TBE	35 ml
Agarose Powder	3.5 g
Distilled water	315 ml
Total	350 ml

Autoclave at 121°C for 1 min and cool to 50°C

Add 17.5 µl of 10 mg/ml Ethidium bromide and pour into a gel plate

Acknowledgments

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Competing interests

No competing interests declared.

Ethics

This study was approved by the Animal Care and Use Committee of the Aoyama Gakuin University (A9/2020) and conducted according to the Aoyama Gakuin University Animal Care and Use Guidelines and the Animal Research of *In VIVO* Experiments (ARRIVE) guidelines.

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