

Chapter 2

Preparation of a High-Quality cDNA Library from a Single-Cell Quantity of mRNA Using Chum-RNA

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Abstract

Unlike exponential amplification using polymerase chain reaction (PCR), linear RNA amplification using T7 RNA polymerase is advantageous for genome-wide analysis of gene expression and for cDNA library preparation from single-cell quantities of RNA. However, the use of RNA polymerase requires a large amount of RNA, as the optimum concentration of the substrate (mRNA), or the Michaelis constant (K_m), is one millionfold higher than the single-cell amount of mRNA. To circumvent this K_m problem, we designed a small mRNA-like dummy molecule, termed chum-RNA, which can be easily removed after the completion of the reaction. Chum-RNA allowed the preparation of a high-quality cDNA library from single-cell quantities of RNA after four rounds of T7-based linear amplification, without using PCR amplification. The use of chum-RNA may also facilitate quantitative reverse-transcription (qRT)-PCR from small quantities of substrate.

Key words: Small RNA, Single-cell cDNA library, T7 RNA polymerase, Sense mRNA amplification, RT-PCR

1. Introduction

Amplification of the RNA isolated from a limited amount of specimen is obligatory to compare gene expression patterns among cells and/or tissues for microarray analysis or cDNA library preparation. Although the polymerase chain reaction (PCR), which is based on exponential amplification, is a powerful method for amplifying a single target DNA, it often produces a biased product because of distinct efficiencies of amplification for transcripts (or cDNAs) of differing lengths, abundance, and/or diversity (1).

The linear amplification method, which was first developed by Van Gelder, Eberwine, and coworkers (2, 3), is an alternative

to PCR that is particularly useful for the amplification of RNA, as it is considered to generate non-biased RNA pools. This technique and subsequent improved protocols, with or without combination with PCR (4–8), have allowed the genome-wide microarray analysis of gene expression using a single-cell amount of RNA as the starting material (9–12). However, most cDNA library preparations from single-cell amounts of mRNA are performed with at least partial assistance of PCR amplification (6, 13, 14), as the use of RNA polymerase alone requires 1 μ g of total RNA after two-round amplification of complementary RNA (cRNA) (15).

We noticed that this is mainly because the optimum concentration, or the Michaelis constant (K_m), of most of the enzymes used in cDNA library preparation is more than 1 μ M; this value exceeds the single-cell amount of mRNA by one millionfold (15). To circumvent this K_m problem, we recently designed a small mRNA-like molecule, termed chum-RNA, and synthesized sense RNA (sRNA) successfully using RNA amplification without the aid of PCR to prepare a high-quality cDNA library from a single-cell amount of mRNA (15). Chum-RNA can be added to the reaction mixture to increase the effective quantity of substrate, thereby increasing the substrate conversion rate of the enzyme, and can be easily removed after the completion of the reaction. Here, we present the detailed protocol for the application of chum-RNA to the preparation of a cDNA library and for reverse transcriptase (RT)-PCR using a small amount of mRNA.

2. Materials

2.1. Phenol/Chloroform Extraction and Ethanol Precipitation

1. Phenol/chloroform: Add 100 g of crystallized DNA-grade phenol and 0.1 g of 8-hydroxyl quinoline to 100 mL of 1 M Tris-HCl (pH 7.5), mix, and dissolve in a water bath at 50°C (see Note 1). Place the solution on a bench for 10 min and then remove the supernatant. Confirm the neutral pH of the supernatant using a pH test strip. If it is still acidic, repeat this step until the pH of the supernatant becomes neutral. Add an equal volume of chloroform, cover the bottle in aluminum foil, and store at 4°C. As 8-hydroxyl quinoline acts as an antioxidant, a partial inhibitor of RNase, and an indicator of the organic phase of the solution (as indicated by its bright yellow color), it is recommended to prepare a fresh stock of phenol/chloroform solution when its color turns to dark yellow.
2. Glycogen carrier: Use glycogen solution as a carrier for the ethanol precipitation of nucleic acids and CHROMA SPIN centrifugation.
3. Dry-ice ethanol bath: Perform ethanol precipitation by cooling the sample (one volume of sample, one-tenth volume of 3 M

Na acetate, and two volumes of ethanol) in a dry-ice ethanol bath for more than 5 min. Alternatively, immerse the sample into ethanol that is kept cool in a vacuum-insulated stainless steel container in a deep freezer set to -85°C .

2.2. Agarose Gel Electrophoresis

1. TAE buffer (50 \times): Prepare a 50 \times stock solution using 726 g Trizma base, 171.3 mL glacial acetic acid, 55.8 g EDTA-2Na, and bring the volume to 3 L with Nanopure water (see Note 2). Store at room temperature. Before use, dilute 20 mL of this solution in 980 mL of Nanopure water.
2. 1% Agarose gel: 1 g Agarose in 100 mL TAE buffer (1 \times).
3. DNA molecular weight marker: *Syl*I-digested lambda phage DNA, which comprises DNA fragments of the following sizes (kb): 19.33, 7.74, 6.22, 4.26, 3.47, 2.69, 1.88, 1.49, 0.93, and 0.42.
4. DNA visualization: Immerse the agarose gel in water containing ethidium bromide (EtBr) at 1 $\mu\text{g}/\text{mL}$ and visualize the fluorescent signal under UV light.

2.3. RT-PCR

1. Enzyme: *ExTaq*TM DNA polymerase (TaKaRa Bio Inc., Ohtsu, Japan) (see Note 3).
2. Primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) detection: Forward (HsGAPDH-F) 5'-CGA GAT CCC TCC AAA ATC AA-3' and reverse (HsGAPDH-R) 5'-AGG GGT CTA CAT GGC AAC TG-3'.
3. It is recommended to perform PCR in a PCR tube (0.2 mL thermo-strip; ABgene Epsom, UK).

2.4. Preparation of a Single-Cell cDNA Library (see Notes 1 and 4)

1. Chum-RNA: 5'-AAU UCG UCU GGA CAC G(A)₂₅-3'. Chum-RNA (1 $\mu\text{g}/\mu\text{L}$) can be purchased from Gene Design Inc., Osaka, Japan (http://www.saito.tv/e/lsp/LSP_GuideList/English/GeneDesign.htm?m3).
2. Enzymes: RNase H, DNA polymerase I, T4 DNA polymerase, T4 DNA ligase, RNase-free DNase I, T7 RNA polymerase, *Xho*I, *Not*I, and RNase inhibitor. Reverse transcriptase (SuperScript III; Invitrogen, San Diego, CA).
3. Nucleotides: NTP and 10 mM rATP (TaKaRa Bio). Prepare a 25 mM NTP mix from ATP, CTP, GTP, and UTP (100 mM each).
4. Linker primer (HPLC grade): 1.6 mg/mL of 5'-(GA)₁₀ A CGC GTC GAC TCG AGC GGC CGC GGA CCG (T)₁₈-3'.
5. Amplification adaptor: Sense T7: 5' CAC TAG TAC GCG TAA TAC GAC TCA CTA TAG GGA ATT CCC CGG G-3'; antisense T7: 5'-pCCC GGG GAA TTC CCT ATA GTG AGT CGT ATT ACG CGT ACT AGT GAG CT-3'.

6. Library adaptor (TaKaRa Bio): *Bam*HI(*Bgl*II)–*Sma*I d(GATCCCCGGG) and p*Sma*I linker: d(pCCCCGGG).
7. 10× First-strand buffer: 500 mM Tris–HCl (pH 8.3), 750 mM KCl, and 30 mM MgCl₂.
8. First-strand mixture: 10 mM dATP, dGTP, and dTTP, and 5 mM 5-methyl-dCTP.
9. 10× Second-strand buffer: 188 mM Tris–HCl (pH 8.3), 906 mM KCl, and 46 mM MgCl₂.
10. Second-strand nucleotide mixture: 10 mM dATP, dGTP, and dTTP, and 25 mM dCTP.
11. 10× T4 DNA polymerase buffer: 500 mM Tris–HCl (pH 8.3), 100 mM MgCl₂, 500 mM NaCl, and 100 mM dithiothreitol (DTT).
12. 10× Ligase buffer: 500 mM Tris–HCl (pH 7.5), 70 mM MgCl₂, and 10 mM DTT.
13. 10× *Not*I buffer supplement: 278 mM NaCl, 8 mM MgCl₂, 1.8 mM DTT, 0.018% BSA, and 0.018% Triton X-100.
14. 10× *Bgl*II buffer: 100 mM Tris–HCl (pH 7.5), 1.0 M NaCl, 70 mM MgCl₂, and 10 mM DTT.
15. 10× T7 Pol buffer: 400 mM Tris–HCl (pH 8.0), 80 mM MgCl₂, 20 mM spermidine, and 50 mM DTT.
16. 10× Bacterial alkaline phosphatase (BAP) buffer: 500 mM Tris–HCl (pH 8.0) and 10 mM MgCl₂.
17. 10× STE: 1 M NaCl, 100 mM Tris–HCl (pH 8.0), and 10 mM ethylenediaminetetraacetic acid (EDTA).
18. Centrifuge column: CHROMA SPIN-400 (Clontech, Palo Alto, CA).
19. Vector DNA: pAP3*neo* (TaKaRa Bio).
20. TE buffer: 10 mM Tris–HCl (pH 7.5) and 1 mM EDTA (see Note 4).
21. 1/10 TE buffer: 1 mM Tris–HCl (pH 7.5) and 0.1 mM EDTA (see Note 4).
22. Centrifuge filter: Ultrafree-C3 (Cat. #UFCP3TK50; Millipore, Bedford, MA) or MINICENT-30 (Cat. #08627; Tosoh SMD, Grove City, OH) (see Note 5).
23. QuickPrep Micro mRNA purification (QMP) kit: Cat. #27-9255-01 (GE Healthcare Bio-Sciences Corp., Piscataway, NJ).
24. Plasmid Maxi kit: Cat. #12162 (QIAGEN, Hilden, Germany).
25. Other reagents: DTT, sodium dodecyl sulfate (SDS), EDTA, dimethyl sulfoxide (DMSO), NaCl, sodium acetate (NaAc), and ethanol.

2.5. Electroporation and Propagation of *Escherichia coli* Cells

1. Electro-MAX DH12S cells: Cat. #18312-017 (Invitrogen).
2. L-Broth (Luria–Bertani medium): Mix 10 g Bacto tryptone (Difco), 5 g Bacto yeast extract (Difco), and 5 g NaCl. Make up the volume of the mixture to ~990 mL using Nanopure water (see Note 2) and stir. Adjust pH to 7.4 with NaOH and then adjust the volume with Nanopure water up to 1 L; stir to mix. Pour into a 1-L bottle and autoclave for 20 min.
3. L-Broth ampicillin plates: Add 15 g Bacto agar (Difco) to 1 L of pH-adjusted L-broth and autoclave for 30 min. Cool to about 65°C, add 0.15 g of ampicillin mix, and pour into plates.
4. L-Broth top agar: Add 0.7 g Bacto agar (Difco) to 100 mL L-broth and autoclave for 20 min.
5. SOB: Mix 20 g Bacto tryptone (Difco), 5 g Bacto yeast extract (Difco), 2 mL 5 M NaCl, and 1.25 mL 2 M KCl. Adjust the volume with Nanopure water up to 1,000 mL and stir. Pour into 1-L bottle and autoclave for 20 min. After autoclaving, add 10 mL of 2 M Mg solution (1 M $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 1 M $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, which were autoclaved separately for 20 min).
6. SOC: Add 1 mL of 2 M glucose to 100 mL of SOB. Filter using a 0.22- μm bottle-top filter. Store at 4°C.

3. Methods

3.1. Preparation of mRNA from a Single Mammalian Cell

Chum-RNA may be useful for protecting mRNA from digestion by RNase or from nonspecific binding to the wall of the tubes. Thus, it is recommended to add chum-RNA into the solution used for mRNA purification. This section is a modified version of the QMP kit manufacturer's protocol for the use of chum-RNA in mRNA purification from a single mammalian cell.

1. Transfer a small amount of specimen to a sterile 1.5-mL microfuge (or microcentrifuge) tube containing 0.4-mL of QMP kit extraction buffer and mix by vortexing for 30 s.
2. Add 0.8-mL QMP kit elution buffer and vortex for 5 s.
3. Add 5 μg of chum-RNA if the amount of specimen is very small, to protect the sample RNA from degradation or from nonspecific binding to the wall of the tubes.
4. From the bottle of oligo(dT)-cellulose solution, which was gently shaken to resuspend the cellulose, transfer 1 mL to a sterile 1.5-mL microfuge tube.
5. Centrifuge the samples (see steps 2 and 4 in Subheading 3.1) at maximum speed ($20,000 \times g$; $16,000 \times g$) for 1 min.

6. Remove the supernatant from the cellulose-containing tube (see step 4 in Subheading 3.1) and add the supernatant (~1.2 mL) from the specimen-containing tube (see step 2 in Subheading 3.1).
7. Mix the solution by gently inverting the tube for 3 min to trap the mRNA within the oligo(dT)-cellulose.
8. Centrifuge at $20,000 \times g$ for 10 s.
9. Remove the supernatant, add 1-mL QMP kit high-salt buffer and then repeat steps 7 and 8 in Subheading 3.1.
10. Repeat steps 7–9 in Subheading 3.1 five times.
11. Remove the supernatant, add 1-mL low-salt buffer, mix the solution by inverting the tube (to wash the mRNA), and then centrifuge at $20,000 \times g$ for 10 s.
12. Repeat step 11 in Subheading 3.1.
13. Remove the supernatant, add 0.3-mL low-salt buffer, and transfer the solution to a microfuge cup inserted into the 2-mL receptacle tube.
14. Centrifuge the tube in a microfuge at $20,000 \times g$ for 5 s to remove the wash buffer from the column.
15. Discard the wash buffer (~0.3 mL) in the receptacle tube.
16. Add 0.3-mL low-salt buffer to the microfuge cup and repeat steps 14 and 15 in Subheading 3.1.
17. Repeat step 16 in Subheading 3.1.
18. Transfer the microfuge cup to a fresh receptacle tube.
19. Add 0.2-mL of warmed (65°C) elution buffer and centrifuge at $20,000 \times g$ for 5 s to elute the mRNA from the column.
20. Repeat see step 19 in Subheading 3.1.
21. Add 5 μg chum-RNA, 40 μL 5 M NaCl, and 0.8 mL ethanol to the 0.4 mL elution buffer collected in the receptacle tube. Mix by vortexing and chill in a dry ice/ethanol bath for 15 min.
22. Centrifuge the pellet in a microfuge for 10 min at $20,000 \times g$.
23. Add 500 μL ice-cold 70% ethanol and centrifuge again for 1 min at $20,000 \times g$.
24. Remove the 70% ethanol and centrifuge again for 1 min at $20,000 \times g$.
25. Remove the residual 70% ethanol from around the pellet.
26. The precipitated mRNA can be stored as a pellet in a deep freezer.

3.2. Synthesis of cDNA from a Small Amount of mRNA Using Chum-RNA

Chum-RNA is useful to promote cDNA synthesis using a very small amount of mRNA (15). As chum-RNA may also serve to protect mRNA from degradation and as a carrier during ethanol precipitation, it is practical to add chum-RNA to the reaction mixture from the beginning of the procedure if the amount of

specimen is very small. The procedure described in the next section is illustrated schematically in Figs. 1*a–i–iii* and 2*a*.

1. Mix the following reagents in a sterile 0.5-mL microfuge tube:

5 mM Tris-HCl (pH 7.5)	3.5 μ L
RNA or mRNA sample	1.0 μ L (or pellet from step 26 in Subheading 3.1)
Chum-RNA (1.0 μ g/ μ L)	5.0 μ L (5.0 μ g)

2. Warm the tube at 65°C for 5 min and then quickly immerse in ice-cold water.

3. Add the following reagents and mix by vortexing:

10 \times First-strand buffer	2.5 μ L
0.1 M DTT	2.5 μ L
First-strand mixture	1.5 μ L
Linker primer (1.6 μ g/ μ L)	1.0 μ L (1.6 μ g) (see Note 6)
RNase inhibitor	0.5 μ L
H ₂ O (see Note 1)	6.5 μ L (to 25 μ L with RTase)

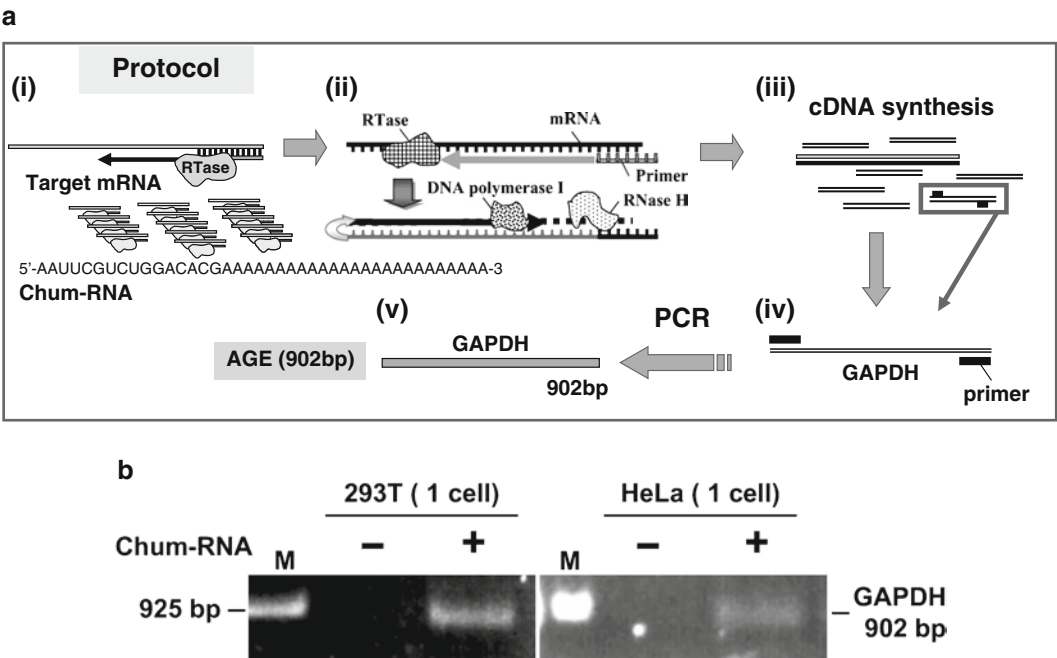


Fig. 1. Chum-RNA facilitates sense-strand mRNA amplification from a single-cell amount of mRNA. The amplified mRNA may be useful for subsequent PCR amplification. (a) Schematic illustration of the protocols described in Subheadings 3.2 and 3.3. (b) Synthesis of cDNA using a single-cell-derived amount of mRNA from 293T (left, 10.5 pg) and HeLa (right, 10.1 pg) cells in the presence (+) or absence (–) of chum-RNA (3 μ M). Successful cDNA synthesis was confirmed by the detection of the GAPDH cDNA, which was detected by PCR (50 cycles at 50°C) using the reaction product of step 26, Subheading 3.2 and was observed as a band of 902 bp on AGE. M denotes the molecular size marker (925 bp).

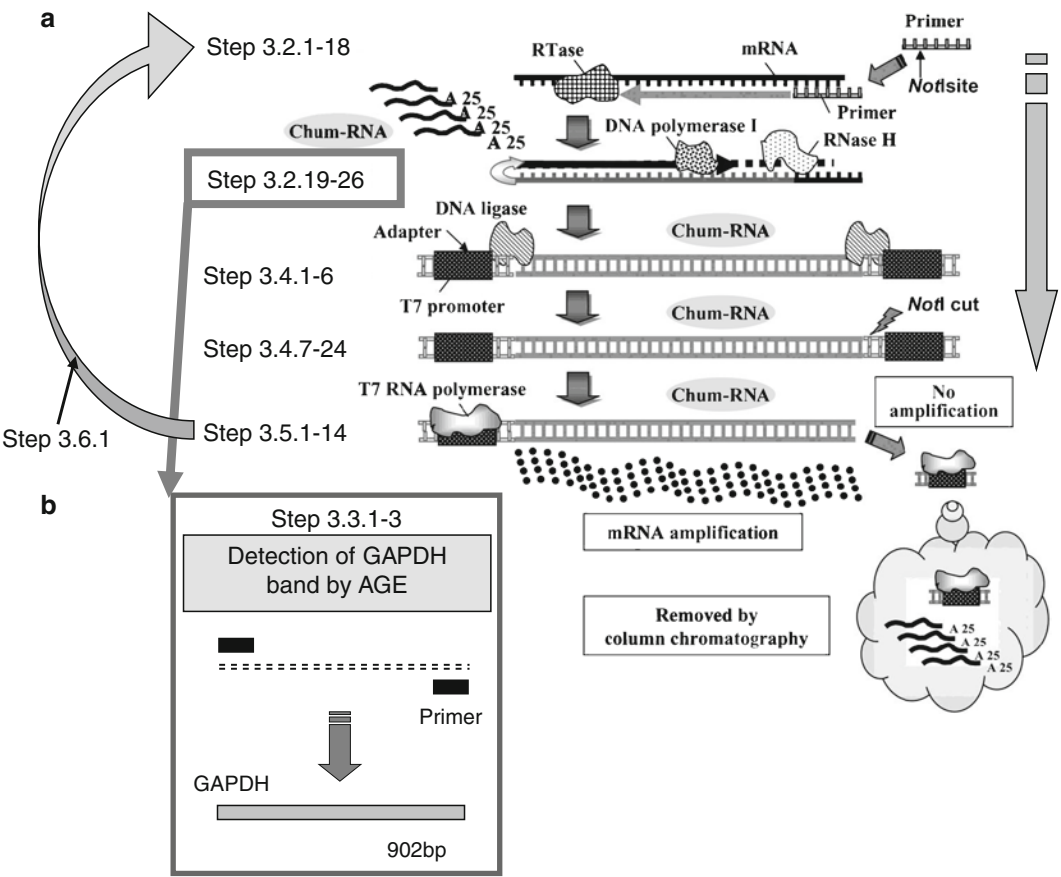


Fig. 2. Schematic depiction of the procedure used for a single round of sense-strand mRNA amplification (from step 1, Subheading 3.2, to step 18, Subheading 3.5). (a) Chum-RNA is present in the reaction mixture throughout. A fraction of the chum-RNA may be converted into chum-cDNA during the procedure. Subsequently, the chum-RNA, the chum-cDNA, and the adapter that cuts by *NotI* digestion are removed using column chromatography (described at step 5 to 17, Subheading 3.5). DNA is indicated by a gray line and mRNA is indicated by a black line. See text for details. (b) Successful cDNA synthesis may be confirmed by PCR (steps 1–3, Subheading 3.3).

4. Incubate at room temperature (~18°C) for 10 min to allow the linker primer to anneal to the mRNA.
5. Add 1.0 µL RTase (SuperScript III) and incubate at 42°C for 45 min.
6. Add 1.0 µL RTase and incubate at 55°C for 30 min.
7. Immerse the sample tube in ice-cold water and cool for 5 min.
8. Add the following reagents:

10× Second-strand buffer	20 µL
0.1 M DTT	7.5 µL
Second-strand nucleotide mixture	3.0 µL
H ₂ O (ice cold) (see Note 1)	133 µL (to 200 µL with RNase H)

9. Incubate in ice-cold water for 5 min.
10. Add 1.5 μL (2 U) RNase H and 10 μL (50 U) *E. coli* DNA Polymerase I.
11. Incubate at 16°C for 150 min.
12. Add an equal volume (200 μL) of phenol/chloroform and vortex for 5 s.
13. Centrifuge the tube in a microfuge for 1 min at 20,000 $\times g$.
14. Transfer the supernatant to a fresh 1.5-mL microfuge tube. Add 0.1 volumes (20 μL) of 3 M sodium acetate (NaAc) and 2.5 volumes (500 μL) of ice-cold ethanol. Mix and chill in a dry ice/ethanol bath for 15 min.
15. Centrifuge the pellet in a microfuge for 10 min at 20,000 $\times g$.
16. Add 500 μL ice-cold 70% ethanol and centrifuge again for 1 min at 20,000 $\times g$.
17. Remove the 70% ethanol and centrifuge again for 1 min at 20,000 $\times g$.
18. Remove the residual 70% ethanol from around the pellet.
19. Add the following reagents to the pellet:

10 \times T4 DNA polymerase buffer	10 μL
2.5 mM dNTP mixture	5 μL
H ₂ O (see Note 1)	81.5 μL (to 100 μL with enzyme)

20. Incubate in ice-cold water for 5 min.
21. Add 3.5 μL (5 U) of T4 DNA polymerase and incubate at 37°C for 30 min.
22. Add an equal volume (100 μL) of phenol/chloroform and vortex for 5 s.
23. Centrifuge the tube in a microfuge for 1 min at 20,000 $\times g$.
24. Transfer the supernatant to a fresh 1.5-mL microfuge tube and add 0.1 volumes (10 μL) of 3 M sodium acetate (NaAc) and 2.5 volumes (250 μL) of ice-cold ethanol. Mix and chill in a dry ice/ethanol bath for 15 min.
25. Centrifuge the pellet in a microfuge for 10 min at 20,000 $\times g$.
26. The reaction can be stopped here: refer to either Subheading 3.3 or 3.4. The precipitated double-stranded DNA (dsDNA) can be stored at -80°C.

3.3. Application of Chum-RNA to RT-PCR Using a Small Amount of mRNA

Chum-RNA may be useful for RT-PCR using a small amount of mRNA (Figs. 1a-iv and -v and 2b). Thus, the sample obtained at the end of step 26 in Subheading 3.2 may be used for PCR amplification, as described below. Two examples of the result of this procedure are shown in Fig. 1b.

1. Dissolve the pellet (see step 26 in Subheading 3.2) in 50 μL TE buffer; this template will be useful for 50 \times PCR.
2. Add the following reagents to a PCR tube (see Subheading 2.3):

10 \times <i>ExTaq</i> TM buffer	1.0 μL (see Note 3)
dNTP	0.8 μL
Primer Fw (10 pmol/ μL)	1.0 μL (see Subheading 2.3)
Primer Rv (10 pmol/ μL)	1.0 μL (see Subheading 2.3)
<i>ExTaq</i> TM DNA polymerase	0.1 μL (see Note 3)
Template cDNA	1.0 μL (see step 1 in Subheading 3.3)
H ₂ O (see Note 1)	5.1 μL (to 10 μL)

3. Perform PCR at an annealing temperature of 50 or 55°C and 30, 40, or 50 amplification cycles.
4. Analyze the reaction product corresponding to the GAPDH cDNA (at 902 bp) using 1% agarose gel electrophoresis, as shown in Fig. 1b.

3.4. Adapter Ligation, NotI Digestion, and Spin Centrifugation

1. Mix the following reagents with the precipitate (see step 26 in Subheading 3.2):

dsDNA (step 26 in Subheading 3.2)	Precipitate
10 \times Ligase buffer	2.0 μL
10 mM rATP	2.0 μL
Amplification adapter (0.35 $\mu\text{g}/\mu\text{L}$)	1.0 μL (0.35 μg) (see Note 7)
H ₂ O (see Note 1)	13.5 μL (to 18.5 μL with DNA ligase)

2. Add 1.5 μL (4 U) T4 DNA ligase.
3. Incubate at 8°C overnight.
4. Heat the tube at 70°C for 30 min to denature the T4 DNA ligase.
5. Centrifuge in a microfuge for 1 min at 20,000 $\times g$.
6. Transfer the supernatant to a fresh 0.5-mL microfuge tube.
7. Add the following reagents:

<i>NotI</i> buffer supplement	27 μL (see Note 8)
<i>NotI</i>	3.0 μL (~50 U)

8. Incubate at 37°C for 90 min.

9. Add 5 μL 10 \times STE and 1 μL glycogen carrier. Mix by vortexing. Incubate in ice-cold water until use (see step 15 of this Subheading 3.4 below).
10. Mix a CHROMA SPIN-400 column by inverting it a couple of times, cut off the top and bottom lids, and then allow it to stand on a receptacle tube (a 1.5-mL microfuge tube with lid removed) at room temperature for 10 min to drain the extra TE solution from the column.
11. Add 1 mL of 1 \times STE to the top of the column, set the column and receptacle tube into a 15-mL plastic tube, and centrifuge for 3 min at 700 $\times g$ using, for example, Beckman's J6-HC centrifuge with a swing-basket rotor 20 cm diameter rotating at 1,800 rpm (700 $\times g$).
12. Discard the solution that was spun into the receptacle tube.
13. Centrifuge again for 3 min at 700 $\times g$ to remove the residual solution from the column completely.
14. Discard the small amount of solution that was spun into the receptacle tube.
15. Transfer the column to a fresh receptacle tube and load the sample (step 9 of this section) onto the top (at the center) of the column, drop by drop (~ 10 μL per drop). Never let the solution touch the inner wall of the column, to avoid the solution from passing free of the resin packed into the column.
16. Set the column and receptacle tube into a 14-mL round-bottom plastic tube (used as a protector to hold the column tubes in a swing basket) and centrifuge for 3 min at 700 $\times g$.
17. Transfer the fractionated sample solution collected at the bottom of the receptacle tube (~ 50 μL) to a fresh 0.5-mL microfuge tube, add 50 μL of phenol/chloroform, and vortex for 5 s.
18. Centrifuge the tube in a microfuge for 1 min at 20,000 $\times g$.
19. Transfer the supernatant to a fresh 0.5-mL microfuge tube. Add 1 μL glycogen carrier, 4 μL 5 M NaCl, and 100 μL ice-cold ethanol. Mix and chill in a dry ice/ethanol bath for 15 min.
20. Centrifuge the pellet in a microfuge for 10 min at 20,000 $\times g$.
21. Add 500 μL ice-cold 70% ethanol and centrifuge for 1 min at 20,000 $\times g$.
22. Remove the 70% ethanol and centrifuge for 1 min at 20,000 $\times g$.
23. Remove the residual 70% ethanol from around the pellet.
24. The wet precipitated DNA can be stored at -20 or -85°C until it is used in the procedure described in Subheading 3.5.

**3.5. Amplification
of mRNA by T7 RNA
Polymerase Using
Chum-RNA**

1. Mix the following reagents with the precipitate (see step 24 in Subheading 3.4):

dsDNA (from step 24 in Subheading 3.4)	Precipitate
10× T7 Pol buffer	10 µL
Chum-RNA (1.0 µg/µL)	5.0 µL (5.0 µg)
25 mM NTP mix	8.0 µL
H ₂ O (see Note 1)	72 µL (to 95 µL)

2. Add 5 µL (50 U) of T7 RNA polymerase.
3. Incubate at 37°C for 90 min.
4. Add 1 µL (10 U) T7 RNA polymerase and incubate at 37°C for 30 min (go to step 10 of this section below).
5. During the reaction, mix a CHROMA SPIN-400 column (Clontech) by inverting it a couple of times, cut off the top and bottom lids, and then allow it to stand on a receptacle tube (a 1.5-mL microfuge tube with lid removed) at room temperature for 10 min, to drain out the extra TE solution from the column.
6. Add 1 mL of 1× STE to the top of the column, set the column and receptacle tube into a 15-mL plastic tube, and centrifuge for 3 min at 700×g (at 1,800 rpm using Beckman J6-HC centrifuge, in a swing-basket rotor 20 cm in diameter).
7. Discard the solution that was spun into the receptacle tube.
8. Centrifuge for 3 min at 700×g to remove the residual solution from the column completely.
9. Discard the small amount of solution that was spun into the receptacle tube.
10. Transfer the column to a fresh receptacle tube and load the sample (step 4 of this section) onto the top (at the center) of the column, drop by drop (~10 µL per drop). Never let the solution touch the inner wall of the column to avoid the solution from passing free of the resin packed into the column.
11. Set the column and receptacle tube into a 14-mL round-bottom plastic tube (used as a protector to hold the column tubes in a swing basket) and centrifuge for 3 min at 700×g.
12. Transfer the fractionated sample solution collected at the bottom of the receptacle tube (~50 µL) to a fresh 0.5-mL microfuge tube, add 50 µL of phenol/chloroform, and vortex for 5 s.
13. Centrifuge the tube in a microfuge for 1 min at 20,000×g to obtain a pellet.
14. Add 0.5-mL ice-cold 70% ethanol and centrifuge for 1 min at 20,000×g.

15. Remove the 70% ethanol and centrifuge for 1 min at 20,000×g.

16. Remove the residual 70% ethanol around the pellet.

17. The wet pellet (i.e., precipitated amplified mRNA) can be stored at −20°C until use.

3.6. Synthesis of cDNA Using Amplified mRNA for Construction of Single-Cell cDNA Library

1. Mix the following reagents in a sterile 0.5-mL microfuge tube:

5 mM Tris-HCl (pH 7.5)	3.5 μL
RNA or mRNA sample	1.0 μL (or pellet from step 14 in Subheading 3.5)
Chum-RNA (1.0 μg/μL)	5.0 μL (5.0 μg)

2. Incubate at 65°C for 5 min and then quickly immerse tube in ice-cold water.
3. Mix the following reagents:

10× First-strand buffer	2.5 μL
0.1 M DTT	2.5 μL
First-strand mixture	1.5 μL
Linker primer (1.6 μg/μL)	1.0 μL (1.6 μg)
RNase inhibitor	0.5 μL
H ₂ O (see Note 1)	6.5 μL (to 24 μL)

4. Repeat steps 4–6 in Subheading 3.2 to synthesize double-stranded cDNA using amplified mRNA.
5. The amplification procedure (steps 1–4 in Subheading 3.5) may be repeated four times to obtain a quantity of amplified mRNA that would be sufficient for the preparation of a cDNA library (Fig. 3).
6. The wet precipitated amplified mRNA can be stored at −20°C until use (see step 1 in Subheading 3.8).

3.7. Preparation of an Insertion-Ready Vector

1. In a sterile 0.5-mL microfuge tube (on ice), mix the following reagents:

Vector DNA (pAP3neo)	100 μg
10× NotI buffer	20 μL
H ₂ O (see Note 1)	To 200 μL

2. Add 50 U NotI, incubate the reaction for 2 h at 37°C, add 20 U of NotI, and incubate for 1 h at 37°C.
3. Add an equal volume (~210 μL) of phenol/chloroform and vortex for 5 s.



Fig. 3. Schematic representation of the procedure used for the preparation of a cDNA library from a single-cell-derived amount of mRNA using chum-RNA (Subheadings 3.6–3.9). **(a)** Illustration of the procedure used for a single round of chum-RNA amplification. **(b)** Illustration of the procedure used for the preparation of a single-cell cDNA library after four rounds of chum-RNA amplification.

4. Centrifuge the tube in a microfuge for 1 min at $20,000\times g$.
5. Transfer the supernatant to a fresh 0.5-mL microfuge tube, add an equal volume (210 μL) of phenol/chloroform, and vortex for 5 s.
6. Centrifuge the tube in a microfuge for 1 min at $20,000\times g$.
7. Transfer the supernatant to a fresh 1.5-mL microfuge tube, add 17 μL of 3 M sodium acetate (NaAc) and 420 μL of ice-cold ethanol, and then mix and chill in a dry ice/ethanol bath for 15 min.
8. Centrifuge the pellet in a microfuge for 10 min at $20,000\times g$.
9. Add 500 μL of ice-cold 70% ethanol to wash the pellet, and centrifuge for 1 min at $20,000\times g$.
10. Remove the 70% ethanol and centrifuge for 1 min at $20,000\times g$.
11. Remove the residual 70% ethanol from around the pellet.
12. Add the following reagents to the wet pellet and mix by vortexing:

10 \times <i>Not</i> I buffer	20 μL
H ₂ O (see Note 1)	To 200 μL

13. Add 20 U of *Not*I and incubate for 1 h at 37°C.
14. Confirm the complete digestion of DNA by *Not*I using 1% agarose gel electrophoresis. A single band at 4.3 kb is expected.
15. Repeat steps 3–11 in Subheading 3.7.
16. Add the following reagents to the wet pellet and mix by vortexing:

10 \times BAP buffer	20 μL
H ₂ O (see Note 1)	To 200 μL

17. Add 1 U of BAP and incubate for 30 min at 65°C.
18. Repeat steps 3–11 in Subheading 3.7.
19. Add the following reagents to the wet pellet and mix by vortexing:

10 \times <i>Bgl</i> II Buffer	20 μL
H ₂ O (see Note 1)	To 200 μL

20. Add 100 U of *Bgl*II and incubate for 1 h at 37°C.
21. Add 10 μL 10% SDS and 20 μL 0.25 EDTA and mix by vortexing.

- 22. Add an equal volume (~240 μL) of phenol/chloroform and vortex for 5 s.
- 23. Centrifuge the tube in a microfuge for 1 min at $20,000\times g$.
- 24. Transfer the supernatant to a fresh 0.5-mL microfuge tube, add an equal volume (~240 μL) of chloroform, and vortex for 5 s to remove the phenol from the sample.
- 25. Centrifuge the tube in a microfuge for 1 min at $20,000\times g$.
- 26. Transfer the supernatant to a centrifuge filter (Ultrafree-C3 or Minicent-30) and centrifuge in a microfuge for 20 min (or until all solutions are spun to the receptacle tube) at $13,000\times g$, which is the maximum speed that allows avoidance of filter fracture.
- 27. Add TE (100 μL) to the upper chamber of the filter cup and centrifuge for 20 min at $13,000\times g$.
- 28. Repeat step 27 in Subheading 3.7.
- 29. Add 1/10 TE (90 μL) to the upper chamber of the filter cup and mix by pipetting three to five times using a 200- μL pipette tip.
- 30. Transfer the solution to a fresh 0.5-mL microfuge tube, add 10 μL of 10 \times *Bgl*II buffer and 10 U of *Bgl*II, and then incubate for 1 h at 37°C.
- 31. Add 10 μL of 10 \times STE to the reaction mixture and mix by vortexing.
- 32. Remove the small DNA fragments produced by the *Not*I–*Bgl*II digestion using a CHROMA SPIN-400 column as described in steps 10–24 in Subheading 3.4.
- 33. Add TE to yield 0.1 $\mu\text{g}/\mu\text{L}$ of *Not*I–*Bgl*II-digested vector solution by measuring the optical density (OD) at 260 nm. Note that $\text{OD}_{260}=1.0$ equals 50 μg of DNA.

3.8. Adapter Ligation and Insertion to a Cloning Vector

- 1. Mix the following reagents with the precipitate from step 6 in Subheading 3.6:

dsDNA (step 6 in Subheading 3.7)	Pellet
10 \times Ligase buffer	2.0 μL
10 mM rATP	2.0 μL
Library adapter (0.35 $\mu\text{g}/\mu\text{L}$) (see Note 9)	1.0 μL (0.35 μg)
H ₂ O (see Note 1)	13.5 μL (to 18.5 μL)

- 2. Add 1.5 μL (4 U) of T4 DNA ligase and incubate at 8°C overnight.
- 3. Repeat steps 4–24 in Subheading 3.4.

4. Add the following reagents to the wet precipitate:

10× Ligase buffer	3 μ L
10 mM rATP	3 μ L
<i>NotI</i> / <i>BglII</i> digested vector (step 33 in Subheading 3.7)	1 μ L (100–300 ng)
H ₂ O (see Note 1)	22 μ L (to 30 μ L)

5. Add 1 μ L (4 U) T4 DNA ligase and incubate at 12°C overnight.
6. The reaction product can be directly used in the next step (see step 1 in Subheading 3.9) or stored at –20°C until use.

3.9. Electroporation to Generate a Single-Cell cDNA Library

Dispense 2 mL of SOC into each of the five 14-mL polypropylene round-bottom tubes (Falcon #2059; Becton, Dickinson and Company, Franklin Lakes, NJ) and incubate at 37°C.

1. Warm the sample from step 6 in Subheading 3.8 at 70°C for 10 min.
2. Add TE (70 μ L) and phenol/chloroform (100 μ L), and mix by vortexing for 5 s.
3. Centrifuge in a microfuge at 20,000 $\times g$ for 1 min.
4. Transfer the supernatant to a fresh 0.5-mL microfuge tube, add 100 μ L chloroform, and mix by vortexing for 5 s to remove the phenol.
5. Centrifuge in a microfuge at 20,000 $\times g$ for 1 min.
6. Transfer the supernatant (~100 μ L) to a centrifuge filter (Ultrafree-C3 or Minicent-30) and centrifuge in a microfuge for 20 min at 13,000 $\times g$, which is the maximum speed that allows avoidance of filter fracture (see Note 5).
7. Add TE (100 μ L) to the upper chamber of the filter cup and centrifuge for 20 min at 13,000 $\times g$.
8. Repeat step 7 in Subheading 3.9.
9. Add TE (20 μ L) to dissolve the unfiltered DNA that remains in the upper chamber of the filter cup and mix by pipetting three to five times using a 200- μ L pipette tip.
10. Invert the 1.5-mL tube over the filter cup and centrifuge at 13,000 $\times g$ for 10 s to collect the DNA-containing solution (20–25 μ L) (see Note 10).
11. Transfer two or three tubes (see Note 10) of ElectroMAX DH12S cells (100 μ L; GIBCO-BRL) from the deep freezer into ice-cold water, add 10 μ L of sample (step 10 in Subheading 3.9) to each tube, gently mix by vortexing for 1 s, and keep the tubes in ice-cold water.

12. Transfer ~55 μ L of sample into an electroporation cuvette (2-mL Gene Pulser cuvette, Bio-Rad, Hercules, CA) and immediately set it in the electroporation apparatus (Gene Pulser, Bio-Rad, or Glectro cell manipulator, BTX, Holliston, MA). Begin pulsing at 2.5 kV and 129 Ω .
13. Transfer the solution into 2 mL of pre-warmed SOC (see Subheading 2.5) using a sterile Pasteur pipette or a 10- μ L pipette tip. Rinse the cuvette bottom with SOC to increase the recovery rate.
14. Shake vigorously in a rotary shaker (200–250 rpm) at 37°C for ~60 min.
15. Repeat steps 13–14 in Subheading 3.9 for the remainder of the sample solutions, one at a time (step 12 of this section).
16. Transfer the SOC of all tubes (step 15 of this section) into 100 mL L-broth placed in a 500-mL flask supplemented with 50 mg/L ampicillin, and mix. Take aliquots (4, 20, 100, and 500 μ L) and plate onto LB-ampicillin plates after mixing with pre-warmed (50°C) LB top agar, incubate at 37°C overnight, and count the number of *E. coli* colonies. Eight colonies on a 4- μ L plate indicate a cDNA library complexity (independent colony) of one million.
17. Shake the 500-mL flask in a rotary shaker (~200 rpm) at 37°C for several hours (or overnight), until OD₆₀₀ reaches a value of 1.0–2.0.
18. Transfer 30 mL the *E. coli* culture into SOC, add 2.1 mL DMSO, mix, and dispense into a 1.5-mL stock tube for storage in liquid nitrogen (or in a deep freezer).
19. *E. coli* cells in DMSO can be stored for years in liquid nitrogen (–196°C), but 90% die within months in a deep freezer (–80°C).
20. Prepare plasmid DNA from the remainder of the 70 mL of the *E. coli* culture using the Qiagen plasmid DNA purification kit.
21. Digest the plasmid DNA with appropriate restriction enzymes (*Bam*HI for pAP3*neo* vector) to assess the size distribution of the cDNA inserts.
22. Add an equal volume of ethanol to the remaining plasmid DNA and store at –20°C. This can be stored for years in a deep freezer.

4. Notes

1. Unless otherwise stated, all solutions used in the biochemical reactions were prepared in water that was purified using the

following method. First, pre-filtered tap water was purified using the reverse osmosis (RO) method, which was further purified using an electric heating Distilled Water Apparatus (A4D, Sigma-Aldrich, Milwaukee, WI). This was further purified using a Barnstead's Nanopure system (Thermo Fisher Scientific Inc., Waltham, MA; this water has a resistivity of 18.2 M Ω cm and its total organic content is very low). This standard is referred to as "H₂O" in this text. For small-scale reactions, it is recommended to store H₂O in aliquots in 1.5-mL microfuge tubes at -20°C, which may be thawed immediately prior to use.

2. For preparation of agarose gel buffer (TAE) and *E. coli* propagation solutions, we skipped the process of "double-distilled water" preparation (see Note 1). This standard is referred to as "Nanopure water" in this text.
3. *ExTaq*TM DNA polymerase and *ExTaq*TM buffer can be replaced by other PCR kits available commercially.
4. The solutions used for biochemical reactions, including TE, should be stored frozen in aliquots (in 1.5-mL microfuge tubes) at -20°C, which may be thawed immediately prior to use.
5. Minicent-30 (molecular weight cut-off: 30,000 Da) can be purchased from Tosoh SMD (Cat. #08627), Grove City, OH.
6. Oligo(dT)₁₈₋₂₂ primers or random primers may not be useful for chum-RNA-mediated amplification, as the chum-RNA/primer hybrid lacks the protruding portion of oligonucleotide that is used as a scaffold for the enzyme.
7. Mix the following oligonucleotides in equal molar ratios to reach 0.35 μ g/ μ L in the annealing buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA, and 10 mM MgCl₂); 5' CAC TAG TAC GCG TAA TAC GAC TCA CTA TAG GGA ATT CCC CGG G-3' (sense T7) and 5'-pCCC GGG GAA TTC CCT ATA GTG AGT CGT ATT ACG CGT ACT AGT GAG CT-3' (antisense T7). Incubate at 65°C for 2 min, at 37°C for 10 min, and at room temperature (~18°C) for 5 min. This solution can be stored at -20°C.
8. Never use the bovine serum albumin (BSA) that accompanies the commercially available restriction enzymes, as it could be contaminated with traces of DNA fragments of *E. coli* or of the bovine genome.
9. Mix the following oligonucleotides in equal molar ratios to yield 0.35 μ g/ μ L in the annealing buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA, and 10 mM MgCl₂): *Bam*HI(*Bgl*II)-*Sma*I d(GAT CCC CGG G) and p*Sma*I linker d(pCCC GGG). Incubate at 65°C for 2 min, at 37°C for 10 min, and

at room temperature ($\sim 18^{\circ}\text{C}$) for 5 min. This solution can be stored at -20°C .

10. The collected sample volume may be more than 20 μL due to the residual TE solution on the wet filter. Thus, it is recommended to thaw three tubes of ElectroMAX DH12S cells at this step.

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