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Green Fluorescent Protein

Applications and Protocols

Edited by
Barry W. Hicks



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METHODS IN MOLECULAR BIOLOGY™

Green Fluorescent Protein

Applications and Protocols

Edited by

Barry W. Hicks

*Department of Chemistry,
United States Air Force Academy,
Colorado Springs, CO*

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Cover illustration: The cover shows the crystal structure of the green fluorescent protein displayed using Rasmol software; the PDB ID number is 1EMG. The structure is shown in cartoon display with beta sheets in yellow, alpha helices in magenta, and turns in blue. The chromophore is colored green and depicted in space filling display.

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Amplification of Representative cDNA Samples from Microscopic Amounts of Invertebrate Tissue to Search for New Genes

Mikhail V. Matz

1. Introduction

Recently, we cloned six new green fluorescent protein (GFP)-like fluorescent proteins from five species of Antozoa (*I*), including one red-emitting variant (DsRed), which is now commercially available. This project did not require expeditions and collection of animals on reefs: In all cases, the starting material was just several milligrams of tissue (e.g., a tentacle tip of a sea anemone), collected from a specimen in a private aquarium. This truly noninvasive kind of study was possible because the approach of total cDNA amplification, which is extensively applied to various tasks and biological models in our lab. This chapter outlines several year's of experience in this helpful technique.

The possibility of amplifying total cDNA obtained from small amounts of biological material is not yet routinely considered, despite the fact that obtaining amounts of material suitable for direct processing by standard methods is often time-consuming, expensive, and may be even impossible. Perhaps the most significant obstacle to the full appreciation of the technique is the widespread belief that polymerase chain reaction (PCR) amplification severely distorts the original cDNA profile, so that some cDNA species dramatically rise in abundance, but others diminish, and may even become completely lost. However, we found that there are just a few simple rules that should be followed to ensure that the amplified sample is minimally distorted and fully representative, i.e., contains all types of messages originally present in RNA, even the least abundant ones. This was demonstrated in experiments on differ-

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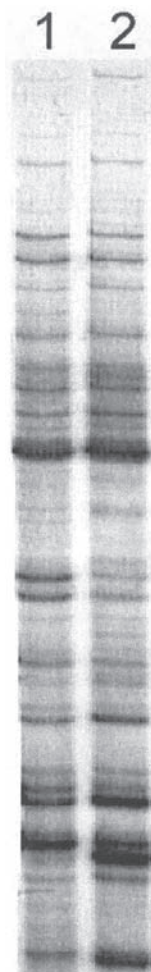


Fig. 1. Differential display patterns obtained according to **ref. 9** for the same total RNA sample, either from the product of total cDNA amplification using a KlenTaq/*Pfu* enzyme mixture (lane 1) or directly from nonamplified double-stranded cDNA (lane 2).

ential display (**Fig. 1**), and elsewhere in application of amplified cDNA as a probe for gene profiling by array technology (2–5). According to our experience in gene hunting in various biological models, amplified cDNA can substitute for normal, nonamplified cDNA in virtually all tasks. Moreover, in PCR-based gene hunting techniques, such as rapid amplification of cDNA ends (RACE) (6,7), subtraction (8), or differential display (9), the amplified cDNA usually outperforms the normal one, because all backgrounds are predictable, and can easily be kept under control.

1.1. Total RNA Isolation

We usually use the following procedure, rather than commercial kits, because this technique is suitable for virtually all animals. It is based on the well-known protocol of Chomczynski and Sacchi (*10*), with one difference: All the procedures are performed at neutral pH, instead of acidic, as was originally suggested. Also, the step of RNA precipitation with lithium chloride (LiCl) is added, because it results in stable RNA preparations and considerably improves the consecutive procedures of cDNA synthesis. We have successfully applied the protocol to RNA isolation from representatives of 13 phyla of multicellular animals. As an alternative, a popular Trizol method (Gibco/Life Technologies) may be used in many cases, although it may not perform well on some nonstandard species, such as jellyfish. Kits for RNA isolation which utilize columns (such as Qiagen's RNeasy kit) are generally not recommended for nonstandard samples. The protocol is designed for rather large tissue samples (tissue vol 10–100 μ L), which normally yield about 10–100 μ g total RNA. The protocol for microscopic amounts of starting material (expected to yield $\sim$ 1 μ g RNA, or less) is the same but does not include second phenol–chloroform extraction (*see Subheading 3.1., step 4*) and LiCl precipitation (*see Subheading 3.1., step 6*). Additionally, the final pellet should be dissolved in 5 μ L, instead of 40 μ L of water and transferred directly to cDNA synthesis, omitting the agarose gel analysis..

1.2. cDNA Synthesis

Two alternatives are provided for preparing amplified total cDNA from the isolated RNA: method A and method B (**Fig. 2**). Both methods provide a possibility of amplifying a cDNA fraction corresponding to messenger (poly[A]⁺) RNA, starting from total RNA. The fraction of ribosomal RNA in the amplified sample, as it was determined in an EST sequencing project based on amplified cDNA, is 15–20% represented mostly by small subunit RNA. This is the same figure that is normally obtained with standard methods of cDNA synthesis (*11*).

Method A (“classical”) is to synthesize a double-stranded cDNA by conventional means (employing DNA polymerase I/RNase H/DNA ligase enzyme cocktail for second-strand synthesis), then ligate adaptors and amplify the sample, using adaptor-specific primers. The structure of the adaptors evokes a PCR-suppression effect (*12*), and provides a method for selective amplification of only those cDNA molecules that contain both adaptor sequence and T-primer sequence, corresponding to the poly(A)⁺ fraction of RNA. The principles behind this method are described (*13*). The obvious advantage of this method is its high efficiency. A representative cDNA sample (with representation of 10^7 and higher) can be prepared from as little as 20–30 ng of total RNA. However, the method is rather laborious.

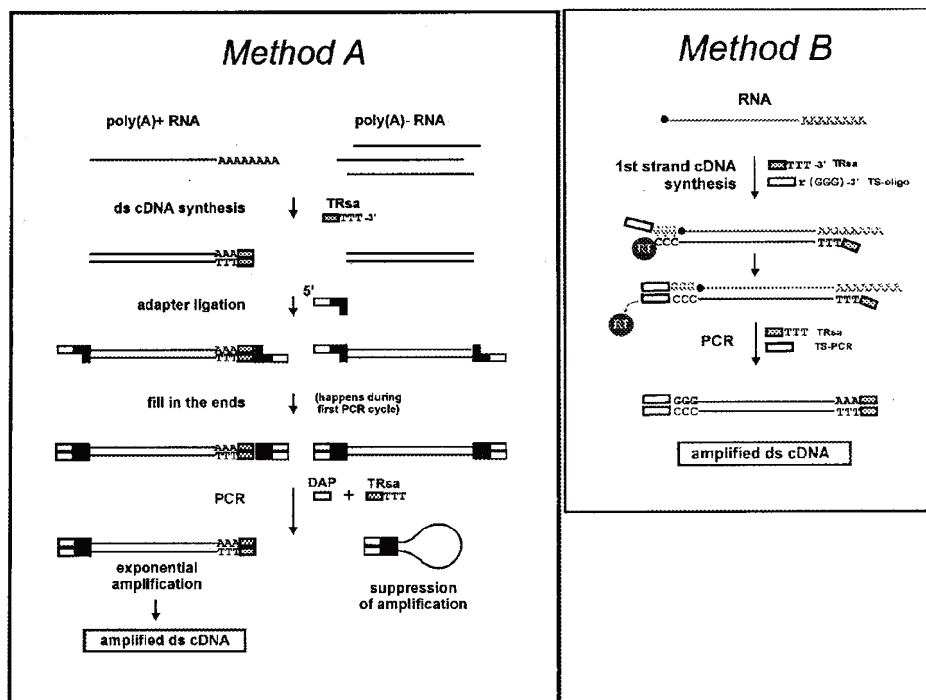


Fig. 2. Schematic outlines of cDNA amplification methods.

Method B is implemented in the SMART cDNA synthesis kit available from Clontech. It utilizes one surprising feature of Moloney murine leukemia virus reverse transcriptase (MMLV RT), its ability to add a few non-template deoxynucleotides (mostly C) to the 3' end of a newly synthesized cDNA strand, upon reaching the 5' end of the RNA template. Oligonucleotide containing oligo(rG) sequence on the 3' end, which is called “template-switch oligo” (TS-oligo), will base-pair with the deoxycytidine stretch produced by MMLV RT when added to the RT reaction. Reverse transcriptase then switches templates and continues replicating using the TS-oligo as a template. Thus, the sequence complementary to the TS-oligo can be attached to the 3' terminus of the first strand of cDNA synthesized, and may serve as a universal 5' terminal site for primer annealing during total cDNA amplification (14). Recently, an improvement to the original procedure was reported (15). Addition of $MnCl_2$ to the reaction mixture after first-strand synthesis, followed by a short incubation, increases the efficiency of nontemplate C addition to the cDNA, and thus results in higher overall yield following cDNA amplification.

Although method B is simpler and faster than method A, its reduced efficiency means that a cDNA sample of suitable representation (more than 10^6)

requires a minimum of 1 µg of total RNA. Both techniques (as they are described here) provide material not only for total cDNA amplification, but also for RACE, a procedure for obtaining unknown flanks of a fragment. This procedure is indispensable for cloning complete coding regions of proteins. Different RACE techniques are available for each of the methods of cDNA amplification described here (**refs. 6** and **7** for methods A and B, respectively), both based on a PCR suppression effect (**12**).

2. Materials

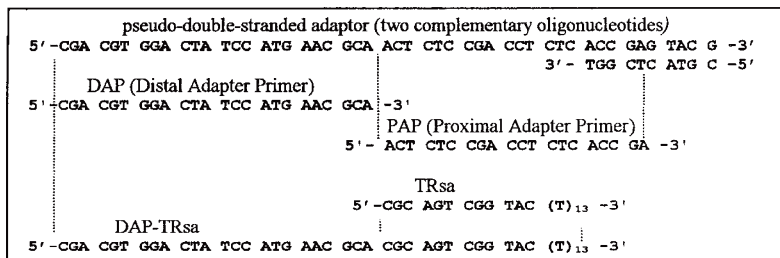
2.1. Total RNA Isolation (see Note 1)

1. Dispersion buffer (buffer D): 4 M guanidine thiocyanate, 30 mM disodium citrate, 30 mM β-Mercaptoethanol, pH 7.0–7.5 (see **Note 2**).
2. Buffer-saturated phenol, pH 7.0–8.0 (Gibco-Life Technologies).
3. Chloroform–isoamyl alcohol mix (24:1).
4. 96% Ethanol.
5. 80% Ethanol.
6. 12 M LiCl.
7. Co-precipitant: SeeDNA reagent (Amersham) or glycogen.
8. Fresh MilliQ water.
9. Agarose gel (1%) containing ethidium bromide (EtBr).

2.2. cDNA Synthesis

2.2.1. Method A Using Conventional Second-Strand Synthesis (see **Note 3**)

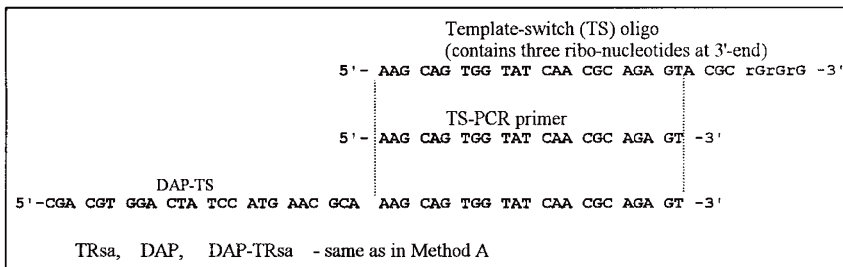
1. SuperScript II reverse transcriptase, 200 U/µL (Life Technologies) or 20X PowerScript reverse transcriptase (Clontech) with provided buffer.
2. 0.1 M Dithiothreitol (DTT).
3. dNTP mix, 10 mM each.
4. 5X Second strand buffer: 500 mM KCl, 50 mM ammonium sulfate, 25 mM MgCl₂, 0.75 mM β-NAD, 100 mM Tris-HCl (pH 7.5), 0.25 mg/mL bovine serum albumin (BSA).
5. 20X Second-strand enzyme cocktail: 6 U/µL DNA polymerase I, 0.2 U/µL RNase H, 1.2 U/µL *Escherichia coli* DNA ligase.
6. T4 DNA polymerase (1–3 U/µL).
7. T4 DNA ligase 2–4 U/µL with provided buffer (New England Biolabs or equivalent).
8. T/M buffer: 10 mM Tris-HCl, pH 8.0, 1 mM MgCl₂.
9. Buffer-saturated phenol, pH 7.0–8.0 (Gibco-Life Technologies).
10. Chloroform–isoamyl alcohol mix (24:1).
11. Long-and-Accurate PCR enzyme mix (Advantage 2 polymerase mix by Clontech, LA-PCR by Takara, Expand *Taq* by Boehringer, or equivalent, see **Note 4**).
12. 10X PCR buffer: provided with the enzyme mix or, if KlenTaq-based homemade mix is used: 300 mM tricine-KOH, pH 9.1, 160 mM ammonium sulfate, 30 mM MgCl₂, 0.2 mg/mL BSA.

Box 1**Invertebrate-optimized sets of oligos for total cDNA amplification and RACE****For Method A:*****Oligos used in cDNA amplification:***

TRsa - for 1st strand cDNA synthesis
 adapter - to ligate to ds cDNA
 DAP, TRsa - to amplify adapter-ligated cDNA

Oligos used in RACE from amplified cDNA (according to [6]):

DAP - universal primer for 5' RACE;
 PAP - nested universal primer for 5' RACE
 DAP-TRsa - universal primer for 3'-RACE
 TRsa - nested universal primer for 3'-RACE

For Method B:***Oligos used in cDNA amplification:***

TRsa, TS-oligo - for 1st strand cDNA synthesis
 TRsa, TS-PCR - to amplify 1st strand cDNA

Oligos used in RACE from amplified cDNA (according to [7]):

DAP + DAP-TS ("step-out mixture") - universal for 5'-RACE
 TS-PCR - nested for 5'-RACE
 DAP-TRsa - universal primer for 3'-RACE
 TRsa - nested universal primer for 3'-RACE

Box 1

13. Yeast tRNA, 10 µg/µL.
14. 3 M sodium acetate, pH 5.0.
15. Fresh MilliQ water.
16. Agarose gel (1%) containing EtBr.
17. Oligonucleotides: *see* **Box 1** and **Note 5**.

2.2.2. Method B Using the Template-Switching Effect

1. SuperScript II reverse transcriptase, 200 U/µL (Life Technologies) or 20X PowerScript reverse transcriptase (Clontech) with provided buffer.
2. 20 mM MnCl₂.
3. 0.1 M DTT.
4. dNTP mix, 10 mM each.
5. Long-and-Accurate PCR enzyme mix with buffer (*see* **Note 4**).
6. 10X PCR buffer: provided with the enzyme mix or, if KlenTaq-based homemade mix is used: 300 mM tricine-KOH, pH 9.1, 160 mM ammonium sulfate, 30 mM MgCl₂, 0.2 mg/mL BSA.
7. Agarose gel (1%) containing EtBr.
8. Fresh MilliQ water.
9. Oligonucleotides: *see* **Box 1** and **Note 5**.

3. Methods

3.1. Total RNA Isolation

1. Dissolve the tissue sample in buffer D (*see* **Note 6**).
2. Spin the sample at maximum speed on table microcentrifuge for 5 min at room temperature, to remove debris. Transfer the supernatant to a new tube.
3. Put the tube on ice, add equal volume of buffer-saturated phenol, and mix. There will be no phase-separation at this moment. Add one-fifth vol chloroform–isoamyl alcohol (24:1), and vortex the sample. Two distinct phases will separate. Vortex 3–4× more with ~1-min intervals between steps. Incubate the tube on ice between steps. Spin at maximum speed on table microcentrifuge for 30 min at 4°C. Remove and save the upper, aqueous phase. Take care to avoid warming the tube with your fingers, or the interphase may become invisible.
4. Repeat **step 3**.
5. Add 1 µL co-precipitant, then add an equal vol of 96% ethanol and mix. Spin immediately at maximum speed on table microcentrifuge at room temperature for 10 min. The precipitate may not form a pellet, being instead spread over the back wall of the tube and thus being almost invisible even with co-precipitant added. Wash the pellet once with 0.5 mL 80% ethanol. Dry the pellet briefly, until no liquid is seen in the tube (do not over-dry).
6. Dissolve the pellet in 100 µL fresh MilliQ water. If the pellet cannot be dissolved completely, remove the debris by spinning the sample at maximum speed on table microcentrifuge for 3 min at room temperature. Transfer the supernatant to a new tube, then add equal volume of 12 M LiCl, and chill the solution at –20°C

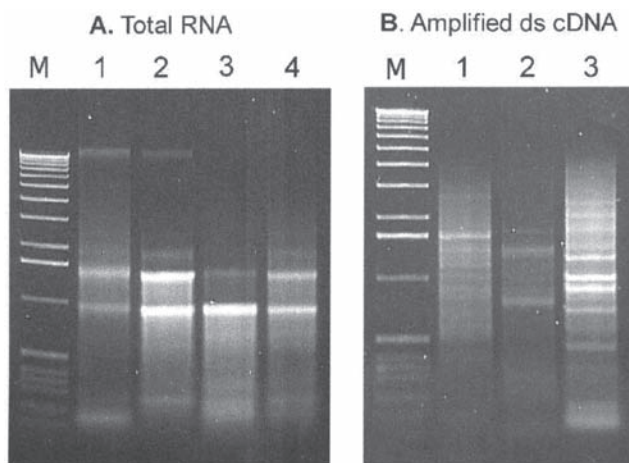


Fig. 3. **(A)** Nondenaturing agarose electrophoresis of total RNA from various invertebrate sources. Lane 1, unidentified sponge; lane 2, comb jelly *Bolinopsis infundibulum* (phylum Ctenophora); lane 3, planarian *Girardia tigrina* (phylum Platyhelminthes); lane 4, stony coral *Montastraea cavernosa* (phylum Cnidaria). M, 50 ng 1-kb DNA ladder (Gibco-Life Technologies). **(B)** Amplified total cDNA from various sources. Lane 1, comb jelly; lane 2, planarian; lane 3, mollusk *Tridacna sp.* M, 50 ng 1-kb DNA ladder (Gibco-Life Technologies). Product on lane 2 needs one more PCR cycle; product on lane 3 is already slightly overcycled (by 1–2 cycles), but is still well-suitable for further manipulations.

for 30 min. Spin at maximum speed on table microcentrifuge for 15 min at room temperature. Wash the pellet once with 0.5 mL 80% ethanol, and dry as previously done. The precipitated RNA is usually invisible, since co-precipitant does not precipitate in LiCl.

7. Dissolve the pellet in 40 μ L fresh MilliQ water.
8. Load 2 μ L solution onto a standard (nondenaturing) 1% agarose gel to check the amount and integrity of the RNA. Add EtBr to the gel to avoid the additional (potentially RNase-prone) step of gel staining. Load a known amount of some DNA on a neighboring lane to use as standard for determining the RNA concentration. Intact RNA should exhibit sharp band(s) of ribosomal RNA (see Fig. 3A and Notes 7–10).

3.2. cDNA Synthesis

3.2.1. Method A (“Classical”)

3.2.1.1. FIRST-STRAND cDNA SYNTHESIS

1. To 5 μ L RNA solution in water (0.03–3 μ g total RNA), add 1 μ L of 10 μ M primer TRsa, and cover with mineral oil. Incubate at 65°C for 3 min, then put the tube on ice.

2. Add 2 μL 5X first-strand buffer (provided with reverse transcriptase), 1 μL 0.1 M DTT, 1 μL reverse transcriptase, 0.5 μL dNTP mix (10 mM each), and incubate at 42°C for 1 h, then put the tube on ice.

3.2.1.2. SECOND-STRAND cDNA SYNTHESIS

1. To the first-strand cDNA solution, add 49 μL of MilliQ water, 1.6 μL dNTP mix (10 mM each), 16 μL 5X second-strand reaction buffer, and 4 μL 20X second-strand enzyme cocktail (the total volume of the reaction mix is about 80 μL). Incubate at 16°C for 1.5 h, and then put the tube on ice.
2. Add 1 μL T4 DNA polymerase, incubate 0.5 h at 16°C to polish ends.
3. Stop the reaction by heating at 65°C for 5 min.
4. Take the reaction mix from under the oil, put in new tube, and add 0.5 vol phenol, then 0.5 vol chloroform–isoamyl alcohol (24:1). Vortex the solution and spin at maximum speed on table microcentrifuge for 10 min. Transfer the upper, aqueous phase into new tube.
5. Add carrier (SeeDNA, Amersham, or glycogen) and precipitate DNA by adding 0.1 vol (8 μL) 3 M sodium acetate (pH 5.0) and 2.5 vol (200 μL) 95% ethanol at room temperature. Spin immediately for 15 min at maximum speed on table microcentrifuge at room temperature.
6. Wash the pellet with 80% ethanol; air-dry the pellet for ~5 min at room temperature. Dissolve pellet in 6 μL water.

3.2.1.3. ADAPTOR LIGATION

1. To the 6 μL double-stranded cDNA, add 2 μL of adaptor (10 μM), 1 μL of 10X ligation buffer, 1 μL T4 DNA ligase, and incubate overnight at 16°C.
2. To the ligation mixture, add 90 μL MilliQ water and 10 μg yeast tRNA. Purify by QiaQuick PCR purification kit (Qiagen, follow manufacturer's instructions), elute with 40 μL T/M buffer. Alternatively, dilute the ligation mixture fivefold, by adding 40 μL MilliQ water to it (*see Note 11*).

3.2.1.4. cDNA AMPLIFICATION

1. Prepare the PCR mixture (note that final concentration of primers is 0.1 μM) as follows: Add 3 μL 10X PCR buffer, 1 μL dNTP mix (10 mM of each), 1.5 μL 2 mM TRsa primer, 1.5 μL 2 μM DAP primer, 1 μL fivefold dilution of ligation mixture or 20 μL of QiaQuick purified sample of adapter-ligated cDNA, water to 30 μL , and KlenTaq–Pfu homemade polymerase mixture, corresponding to 8 U KlenTaq (*see Note 3*). When using commercial polymerase mixtures, follow manufacturer's recommendations.
2. Perform cycling: 94°C 30 s, 65°C 1 min, 72°C 2 min 30 s (block control), 95°C 10 s, 65°C 30 s, 72°C 2 min 30 s (tube control or simulated tube control). Check 2 μL of the product on a 1% agarose gel after 12 cycles, keeping the PCR tube at room temperature while the electrophoresis runs. If nothing is seen, put the tube back into thermal cycler and do five more cycles. If the product is barely visible,

do only three more cycles. It is very important to determine the minimal number of cycles required to amplify the product until it is readily detectable on an agarose gel with EtBr staining (*see* **Notes 12–15**).

3.2.2. Method B for cDNA Synthesis Using the Template-Switching Effect

3.2.2.1. FIRST STRAND cDNA SYNTHESIS

1. To 4 μL RNA solution in water (1–3 μg total RNA), add 1 μL 10 mM primer TRsa, and cover with mineral oil. Incubate at 65°C for 3 min; put on ice.
2. Add 2 μL 5X first-strand buffer provided with reverse transcriptase, 1 μL 0.1 M DTT, 1 μL 5 μM TS-oligo, 1 μL reverse transcriptase, 0.5 μL dNTP mix (10 mM each). Incubate at 42°C for 1 h, then add 1 μL 20 mM MnCl_2 , and incubate for an additional 15 min at 42°C. Heat to 65°C, and incubate for 3 min to stop the reaction. The product can be stored at –20°C for several months.

3.2.2.2. cDNA AMPLIFICATION

1. Prepare the PCR mixture (final concentration of primers is 0.1 mM) as follows: 3 mL of 10X PCR Buffer, 1 mL of dNTP mix (10 mM each), 1.5 mL of 2 mM TRsa primer, 1.5 mL of 2 mM TS-PCR primer, 1.5 mL of five-fold dilution of first-strand cDNA (from **step 2**), milliQ water to 30 mL, and KlenTaq/Pfu homemade polymerase mixture corresponding to 8 U of KlenTaq. When using commercial polymerase mixtures (*see* **Note 4**), follow manufacturer's recommendations.
2. Perform cycling: 94°C 30 s, 65°C 1 min, 72°C 2 min 30 s (block control); 95°C 10 s, 65°C 30 s, 72°C 2 min 30 s (tube control or simulated tube control). To determine the exact number of PCR cycles required to amplify cDNA, use the same strategy as described above for method A, but do 17 cycles before the first check on agarose gel. Typically, it takes ~17 cycles, if there was 1 μg total RNA at the start. If the number of cycles is 22–24, a 10^6 -representation sample still can be accumulated by making amplification in 10-fold-larger volume (i.e., making it in 10 30- μL tubes instead of one), then pooling them. In this way, 10X more product of first-strand synthesis may be put into PCR, while avoiding the background problems caused by nonincorporated cDNA synthesis oligomers. However, this approach leads to slightly more distorted cDNA sample compared to direct less-than-20 amplification (*see* **Notes 12–15**).

4. Notes

1. There is widespread belief that RNA is very unstable and therefore all the reagents and materials for its handling should be specially treated to remove possible RNase activity. We have found that purified RNA is stable and, ironically, that too much anti-RNase treatment can become a source of problems. This especially applies to diethyl pyrocarbonate-treatment of aqueous solutions, which often leads to RNA preparations that are stable but completely unsuitable for cDNA

synthesis. Simple precautions, such as wearing gloves, avoiding speech over open tubes, using aerosol-barrier tips, and using fresh MilliQ water for all solutions, are sufficient to obtain stable RNA preparations. All organic liquids (phenol, chloroform, and ethanol) can be considered essentially RNase-free, by definition, as well as the dispersion buffer containing 4 M guanidine thiocyanate.

2. Normally, the dispersion buffer does not require titration. If pH comes out significantly lower than 7.0, try another batch of guanidine or disodium citrate. The buffer may be stored for years at 4°C in the dark.
3. For cDNA synthesis, we recommend the use of reagents (except oligonucleotides) provided in the Marathon kit (Clontech).
4. For LA-PCR enzyme mixtures, I strongly recommend enzymes based on KlenTaq polymerase (Ab peptides) or its analogs (such as Advantaq polymerase, Clontech), instead of nontruncated *Taq* variants. In our experience, this enzyme produces the least distortion to the cDNA sample during amplification. The LA mixture can be prepared by adding 1 *Pfu* unit cloned *Pfu* polymerase (Stratagene) for every 30 U KlenTaq polymerase. Calculate the required amount of mix, assuming that 25 U KlenTaq are required for 100 µL PCR.
5. The set of oligonucleotides presented in Box 1 has been extensively tested on a number of various invertebrates and consistently produced good results. It is primarily designed for cDNA amplification and RACE, but can be also successfully applied to preparation of samples for suppression subtractive hybridization (7), since the potentially interfering oligo-derived flanking sequences are removed by *RsaI* digestion.
6. The volume of tissue should be not more than one-fifth vol buffer D. To avoid RNA degradation, tissue dispersion should be done as quickly and completely as possible, ensuring that cells do not die slowly on their own. To adequately disperse a piece of tissue usually takes 2–3 min of triturating, using a pipet, taking all or nearly all volume of buffer into the tip each time. The piece being dissolved must go up and down the tip, so it is sometimes helpful to cut the tip to increase the diameter of the opening for larger tissue pieces. Tissue dispersion can be done at room temperature. The dispersed samples can be stored at 4°C for several days (exceptions, such as *Balanoglossus* [acorn worm, phylum *Hemichordata*] which contain high concentrations of highly reactive iodine in its tissues, are rare). The tissue dispersed in buffer D produces a highly viscous solution. The viscosity is usually caused by genomic DNA. This normally has no effect on the RNA isolation (except for dictating longer periods of spinning at the phenol–chloroform extraction steps), unless the amount of dissolved tissue was indeed too great. However, in some cases (e.g., freshwater planarians or mushroom anemones), mucus produced by the animal contributes to viscosity. This substance tends to co-purify with RNA, making it very difficult to collect the aqueous phase at the phenol–chloroform extraction step. It likewise lowers the efficiency of cDNA synthesis. The RNA sample contaminated with such mucus, although completely dissolved in water, does not enter agarose gel during electrophoresis. The EtBr-stained material stays in the well, probably because the mucus adsorbs RNA.

Including cysteine in buffer D can diminish the mucus problem. To buffer D add 0.1 vol solution containing 20% cysteine chloride and 50 mM tricine-KOH, pH 7.0 (requires a great deal of titration). The cysteine solution should be freshly prepared. After dissolving the tissue, incubate the sample for 2 h at 4°C, then proceed with the above protocol.

7. RNA degradation can be assessed using nondenaturing electrophoresis. The first sign of RNA degradation on the nondenaturing gel is a slight smear starting from the rRNA bands and extending to the area of shorter fragments, such as seen on **Fig. 3**, lanes 3 and 4. RNA showing this extent of degradation is still good for further procedures. However, if the downward smearing is so pronounced that the rRNA bands do not have a discernible lower edge, the RNA preparation should be discarded. The amount of RNA can be roughly estimated from the intensity of rRNA staining by EtBr in the gel, assuming that the dye incorporation efficiency is the same as for DNA (the rRNA may be considered a double-stranded molecule, because of its extensive secondary structure).
8. The rule for vertebrate rRNA, that in intact total RNA the upper (28s) rRNA band should be twice as intense as the lower (18s) band, does not apply to invertebrates. The overwhelming majority have 28s rRNA with a so-called “hidden break” (**16**). It is actually a true break in the middle of the 28s rRNA molecule, which is called “hidden,” because under nondenaturing conditions the rRNA molecule is being held in one piece by the hydrogen bonding between its secondary structure elements. The two halves, should they separate, are each equivalent in electrophoretic mobility to 18s rRNA. In some organisms, the interaction between the halves is weak, so the total RNA preparation exhibits a single 18s-like rRNA band even on nondenaturing gel (**Fig. 3A**, lane 3). In others, the 28s rRNA is more robust, so it is still visible as a second band, but it rarely has twice the intensity of the lower one (**Fig. 3A**, lanes 1, 2, and 4).
9. Curiously, genomic DNA contamination is reproducible for a particular species, but varies between species. However, it never exceeds the amount seen at **Fig. 2A**, lanes 1 and 2, a weak band of high molecular weight. Such extent of contamination does not affect further procedures. In fact, the methods of cDNA amplification described here tolerate genomic DNA up to 50% of the total sample mass, without losing specificity or efficiency.
10. To store the isolated RNA, add 0.1 vol of 3 M sodium acetate and 2.5 vol of 96% ethanol to the RNA in water, and mix thoroughly. The sample may be stored for several years at –20°C.
11. Using the Qia-Quick purified ligation mixture removes excess nonincorporated adapter oligomers, and prevents them from interfering in the subsequent PCR. The step is necessary when the starting amount of RNA was lower than 0.3–0.5 µg, to make it possible to take all the generated cDNA into subsequent PCR. For higher initial amounts, the purification step may be replaced by fivefold dilution of the mixture, followed by PCR, starting with 1 µL of the dilution. Thus the nonincorporated oligos are simply diluted to a noninterfering concentration. In this case, only one-fiftieth part of the available adapter-ligated cDNA goes into

PCR, but, because of the excess of RNA at the start, this is usually enough to generate a representative cDNA sample. If you are not quite sure which variant to choose, start with dilution. If cDNA amplification requires too many cycles (more than 20, *see* below), purify the remaining ligation mixture by QiaQuick, and take it all into PCR. It is important to use T/M buffer, which contains 10 mM Tris-HCl and 1 mM MgCl₂, for elution. Elution with plain water leads to denaturation of DNA, caused by electrostatic repulsion of strands in low-salt conditions. This will decrease the specificity of amplification and promote background stemming from genomic DNA.

12. The number of PCR cycles required to amplify a visible amount of cDNA (i.e., ~5–10 ng/μL) is a key parameter to assess the representation of an amplified sample. There is a simple link between initial number of target DNA molecules and number of PCR cycles required to amplify the sample (**Box 2**), as it was empirically determined during the work on in vitro cloning (*17,18*). Using these guidelines, it can be calculated that a sample consisting of 10⁶ molecules (a representation sufficient for most cDNA tasks) or more would require 20 or fewer PCR cycles to be amplified. In other words, if it took <20 PCR cycles to amplify the cDNA, this is a well-representative sample. In our practice, we prefer to achieve at least one-order-of-magnitude-higher representation (i.e., get robust cDNA product in 16–17 cycles) to ensure that we have even the most rare messages.
13. The amplified cDNA at agarose gel should look like a smear (which may contain some bands, corresponding to the most abundant cDNA species) with the average length ~1 kb (*see Fig. 3B*). If it comes out much less, this may be a sign of pronounced RNA degradation during cDNA synthesis (if the total RNA was confirmed to be intact), which is usually the result of poor quality of reverse transcriptase. Try another batch of it. Alternatively, something may be wrong with the PCR system. Probably the polymerase mixture is bad, but it is better to replace all the reaction components.
14. We recommend storing the product of amplification as a master sample. The unpurified PCR product produced by a KlenTaq-based enzyme mixture can be stored at –20°C for several years. If a large amount of cDNA is required for further procedures (e.g., cloning), use aliquots of the master sample to amplify more material. Dilute the aliquot of the master sample 50-fold in deionized water, and add 1 μL dilution/each 20 μL of the PCR mixture, prepared as at **step 10**. Do exactly 10 PCR cycles, which will generate a product in a concentration equal to the master sample. Do not apply more cycles, attempting to generate more material, because overcycling produces the most pronounced distortions of cDNA profile. Instead, prepare a large volume of PCR mixture, distribute it into several tubes (30 μL/tube), and pool them after amplification is over. If pure DNA is required for further procedures, the amplified cDNA may be cleaned by QiaQuick PCR purification kit (Qiagen) according to the provided protocol, but using T/M buffer for elution, instead of the provided buffer.
15. If one intends to clone the product of cDNA amplification, it is necessary to perform a “chase” step after the product is amplified. The conditions for ampli-

Box 2 **Amplification guidelines**

$$N = 2^{(40-n)} \quad (\text{Lukyanov-Matz equation})$$

N - number of DNA molecules at the start of amplification, **n** - number of PCR cycles required to amplify the product to the concentration 5-10 ng/μl.

1 DNA molecule 1 kb long weights about 10^{-18} g

1 molecule	→	40 cycles	determined experimentally
1000 molecules	→	30 cycles	calculated
(1 pg) 10^6 molecules	→	20 cycles	calculated & confirmed
(1 ng) 10^9 molecules	→	10 cycles	calculated & confirmed

Box 2

Amplification recommended here include using low working concentration of primers (0.1 μM), which greatly enhances the specificity of poly(A)⁺-fraction amplification. However, there is a great chance that a substantial fraction of the sample will be denatured at the end of PCR, since there will already be no primers available to initiate the synthesis of complementary strand (especially, if slight overcycling occurred). Obviously, for cloning, it is highly desirable to have the entire PCR product double-stranded. To ensure this without sacrificing the specificity of amplification, do the following. Run the PCR with low primer concentration, as recommended, until the product is amplified; then, keeping the completed PCR reaction in the thermocycler at 72°C, inject an additional amount of primers there (up to 0.2 μM more of each), and perform two nondenaturing chase cycles: 77°C 1 min, 65°C 1 min, 72°C 3 min. Purify the product by QiaQuick PCR purification kit (Qiagen) before cloning (use T/M buffer for elution).

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