Amplification of representative cDNA samples from microscopic amounts of invertebrate tissue to search for new genes

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Introduction

Recently, we cloned six new GFP-like fluorescent proteins from five species of Antozoa ***(1)***, 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 (for example, a tentacle tip of a sea anemone), collected from a specimen in a private aquarium. This truly non-invasive kind of study was possible due to the approach of total cDNA amplification, which we extensively apply to various tasks and biological models in our lab. In this chapter I will outline our several-year experience in this helpful technique.

The possibility to amplify 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 PCR amplification severely distorts the original cDNA profile, so that some cDNA species dramatically rise in abundance while others diminish and may even become completely lost. However, we found that there are just a few simple rules which 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 our own experiments on differential 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, non-amplified cDNA in virtually all tasks. Moreover, in PCR-based gene hunting techniques such as RACE ***(6, 7)***, subtraction ***(8)*** or differential display ***(9)*** the amplified cDNA usually outperforms the normal one, because all backgrounds are predictable, and can be easily 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 it was originally

suggested. Also, the step of RNA precipitation with lithium chloride (LiCl) is added, because it results in very 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 multi-cellular 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 non-standard objects, such as jellyfish. Kits for RNA isolation that utilize columns (such as Qiagen's RNeasy kit) are generally not recommended for non-standard samples.

The protocol is designed for rather large tissue samples (tissue volume 10-100 μL), which normally yield about 10-100 micrograms of total RNA. The protocol for really microscopic amounts of starting material (expected to yield about 1 μg RNA or less) is the same but does not include second phenol-chloroform extraction (step 4) and LiCl precipitation (step 6). Additionally, the final "pellet" should be dissolved in 5 μL instead of 40 μL of water and transferred directly to cDNA synthesis, omitting the agarose gel analysis.

1. 2. cDNA synthesis

We provide two alternatives for preparing amplified total cDNA from the isolated RNA, method A and method B (**Fig. 2**). Both methods provide a possibility to amplify a cDNA fraction corresponding to messenger (polyA[+]), RNA, starting from total RNA. The fraction of ribosomal RNA in the amplified sample, as it was determined in EST sequencing project based on amplified cDNA, is 15-20 %, represented mostly by small subunit ribosomal 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 a conventional means (employing DNA polymerase I / RNAseH / 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, that is, corresponding to the polyA(+) 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 nanograms of total RNA. However, the method is rather laborious.

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 non-template 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 somewhat reduced efficiency means that a cDNA sample of suitable representation (more than 10^6) requires a minimum of one microgram of total RNA. It should be noted that both techniques (as they are described here) provide material not only for total cDNA amplification, but also for RACE (Rapid Amplification of cDNA Ends), 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. Materials for Total RNA Isolation (*see* **Note 1**)

1. Dispersion buffer ("buffer D"): 4*M* Guanidine thiocyanate, 30 m*M* disodium citrate, 30 m*M* 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 lithium chloride.
7. Co-precipitant: SeeDNA reagent (Amersham) or glycogen.
8. Fresh milliQ water.
9. Agarose gel (1%) containing ethidium bromide.

2.2. Materials for 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 20× PowerScript reverse transcriptase (Clontech) with provided buffer..
2. 0.1*M* DTT.
3. dNTP mix, 10 m*M* each.
4. 5× Second strand buffer: 500 m*M* KCl, 50 m*M* Ammonium sulfate, 25 m*M* $MgCl_2$, 0.75 m*M* β-NAD, 100 m*M* Tris-HCl (pH 7.5), 0.25 mg/ml BSA.
5. 20× Second-strand enzyme cocktail: 6 U/μL DNA polymerase I, 0.2 U/μL RNase H, 1.2 U/μL *E.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 m*M* Tris-HCl pH 8.0, 1 m*M* $MgCl_2$.
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 (Advantage2 polymerase mix by Clontech, LA-PCR by Takara, Expand Taq by Boehringer or equivalent, *see* **Note 4**).
12. 10× PCR buffer: provided with the enzyme mix or, if KlenTaq-based homemade mix is used: 300 m*M* tricine-KOH (pH 9.1), 160 m*M* ammonium sulfate, 30 m*M* $MgCl_2$, 0.2 mg/ml BSA.
13. Yeast tRNA, 10 μg/μL.
14. 3*M* sodium acetate (pH 5).
15. Fresh milliQ water.
16. Agarose gel (1%) containing ethidium bromide.

17. Oligonucleotides: *see* **Box 1** and **Note 5**.

2.2.2 Method B using template-switching effect

1. SuperScript II reverse transcriptase, 200 U/μL (Life Technologies) or 20× PowerScript reverse transcriptase (Clontech) with provided buffer.
2. 20 m*M* $MnCl_2$.
3. 0.1*M* DTT.
4. dNTP mix, 10 m*M* each.
5. Long-and-Accurate PCR enzyme mix with buffer (*see* **Note 4**).
6. 10× PCR buffer: provided with the enzyme mix or, if KlenTaq-based homemade mix is used: 300 m*M* tricine-KOH (pH 9.1), 160 m*M* ammonium sulfate, 30 m*M* $MgCl_2$, 0.2 mg/ml BSA.
7. Agarose gel (1%) containing ethidium bromide.
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 maxumum speed on table microcentrifuge for 5 minutes 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 1/5 volume of chloroform-isoamyl alcohol (24:1) and vortex the sample. Two distinct phases will separate. Vortex three to four more times with about one-minute intervals between steps. Incubate the tube on ice between steps. Spin at maxumum speed on table microcentrifuge for 30 minutes at +4 ºC. 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 of co-precipitant, and then add an equal volume of 96% ethanol and mix. Spin immediately at maxumum speed on table microcentrifuge at room temperature for 10 minutes. 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 of fresh milliQ water. If the pellet cannot be dissolved completely, remove the debris by spinning the sample at maxumum speed on table microcentrifuge for 3 minutes at room temperature. Transfer the supernatant to a new tube, then add equal volume of 12 M LiCl and chill the solution at –20 ºC for 30 minutes. Spin at maxumum speed on table microcentrifuge for 15 minutes 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 µL of fresh milliQ water.
8. Load 2 µl of the solution onto a standard (non-denaturing) 1% agarose gel to check the amount and integrity of the RNA. Add ethidium bromide (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. Methods for cDNA Synthesis

3.2.1. Method A ("classical")

3.2.1.1. Method A first strand cDNA synthesis

1. To 5 µL RNA solution in water (0.03-3 µg of total RNA), add 1 µL of 10 µM primer TRsa and cover with mineral oil. Incubate at 65 ºC for 3 minutes, and then put the tube on ice.
2. Add 2 µL 5× first-strand buffer (provided with reverse transcriptase), 1 µL of 0.1*M* DTT, 1 µL of reverse transcriptese, 0.5 µL of dNTP mix (10 mM each) and incubate at 42 ºC for 1 hour, then put the tube on ice.

3.2.1.2. Method A second strand cDNA synthesis

3. To the first-strand cDNA solution, add 49 µL of milliQ H_2O, 1.6 µL of dNTP mix (10 mM each), 16 µL of 5× second strand reaction buffer, and 4 µL of 20× second-strand enzyme cocktail. (The total volume of the reaction mix is about 80 µL). Incubate at 16 ºC for 1.5 hours, and then put the tube on ice.
4. Add 1 µL T4 DNA polymerase, incubate 0.5 hours at 16 ºC to polish ends.
5. Stop the reaction by heating at 65 ºC for 5 minutes.
6. Take the reaction mix from under the oil, put in new tube and add 0.5 volume phenol then 0.5 volume chloroform-isoamyl alcohol (24:1). Vortex the solution and spin at maxumum speed on table microcentrifuge for 10 minutes. Transfer the upper, aqueous phase into new tube.
7. Add carrier (SeeDNA, Amersham, or glycogen) and precipitate DNA by adding 0.1 volume (8 µL) 3*M* sodium acetate (pH 5) and 2.5 volume (200 µL) 95% ethanol at room temperature. Spin immediately for 15 minutes at maxumum speed on table microcentrifuge at room temperature.
8. Wash the pellet with 80% ethanol; air-dry the pellet for about 5 minutes at room temp. Dissolve pellet in 6 µL H_2O.

3.2.1.3. Method A adaptor ligation

9. To the 6 µL of ds-cDNA, add 2 µL of adaptor (10 µ*M*), 1 µL of 10× ligation buffer, 1 µL T4 DNA ligase and incubate overnight at 16 ºC.
10. 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 of T/M buffer. Alternatively, dilute the ligation mixture 5-fold by adding 40 µL of milliQ water to it (*see* **Note 11**).

3.2.1.4. Method A cDNA amplification

11. Prepare the PCR mixture (note that final concentration of primers is 0.1 μ*M*) as follows: add 3 μL of 10× PCR Buffer, 1 μL of dNTP mix (10 m*M* of each), 1.5 μL of 2 μ*M* TRsa primer, 1.5 μL of 2 μ*M* DAP primer, 1 μL of 5-fold dilution of ligation mixture or 20 μL of Qia-Quick purified sample of adapter-ligated cDNA, H_2O to 30 μL, and KlenTaq/Pfu homemade polymerase mixture corresponding to 8 U of KlenTaq (*see* **Note 3**). When using commercial polymerase mixtures, follow manufacturer's recommendations.
12. Perform cycling: 94 ºC 30 seconds – 65 ºC 1 minute – 72 ºC 2 minutes 30 seconds (block control); 95 ºC 10 seconds – 65 ºC 30 seconds – 72 ºC 2 minutes 30 seconds (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 5 more cycles. If the product is barely visible do only 3 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 template-switching effect.

3.2.2.1. Method B first-strand cDNA synthesis

1. To 4 μl RNA solution in water (1-3 μg of total RNA) add 1 μL of 10-μ*M* primer TRsa and cover with mineral oil. Incubate at 65 ºC 3 minutes, put on ice.
2. Add 2 μL 5× first-strand buffer provided with reverse transciptase, 1 μL of 0.1*M* DTT, 1 μL of 5 μ*M* TS-oligo, 1 μL reverse transcriptase, 0.5 μL dNTP mix (10 mM each). Incubate at 42 ºC for 1 hour, then add 1 μL of 20 m*M* $MnCl_2$ and incubate for an additional 15 minutes at 42 ºC. Heat to 65 ºC and incubate for 3 minutes to stop the reaction. The product can be stored at -20 ºC for several months.

3.2.2.2. cDNA amplification

3. Prepare the PCR mixture (final concentration of primers is 0.1 μ*M*) as follows: 3 μL of 10× PCR Buffer, 1 μL of dNTP mix (10 m*M* each), 1.5 μL of 2 μ*M* TRsa primer, 1.5 μL of 2 μ*M* TS-PCR primer, 1.5 μL of five-fold dilution of first-strand cDNA (from step 2), milliQ H_2O to 30 μL, and KlenTaq/Pfu homemade polymerase mixture corresponding to 8 U of KlenTaq (see Note 4). When using commercial polymerase mixtures, follow manufacturer's recommendations.
4. Perform cycling: 94 ºC 30 seconds – 65 ºC 1 minute – 72 ºC 2 minutes 30 seconds (block control); 95 ºC 10 seconds – 65 ºC 30 seconds – 72 ºC 2 minutes 30 seconds (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 about 17 cycles if there was 1 μg of 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 ten-fold larger volume (i.e. making it in ten 30 μL tubes instead of one) and then pooling them. In this way ten times more product of first-strand synthesis may be put into PCR, while avoiding the background problems due to non-incorporated cDNA synthesis oligomers. However, this approach leads to slightly more distorted cDNA sample in comparison 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 rather stable and, ironically, too much anti-RNAse treatment can become a source of problems. This especially applies to DEPC-treating of aqueous solutions, which often leads to RNA preparations that are very 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 guanindine or disodium citrate. The buffer may be stored for years at +4 ºC in the dark.
3. For cDNA synthesis, it is recommended to use reagents (except oligonucleotides) provided in the Marathon kit (Clontech).
4. For LA-PCR enzyme mixtures I strongly recommend enzyme the ones based on KlenTaq polymerase (Ab Peptides) or its analogs (such as AdvanTaq polymerase, Clontech) instead of non-truncated 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 of cloned Pfu polymerase (Stratagene) for every 30 units of KlenTaq polymerase. Calculate the required amount of mix assuming that 25 units of 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 1/5 of the buffer D volume. 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 minutes 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 ºC for several days (exceptions, such as *Balanoglossus* - acorn worm, phylum *Hemichordat*a, - that 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 due to 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 (for example, 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 volume of solution containing 20% cysteine chloride and 50 m*M* tricine-KOH, pH 7 (takes a lot of titration!). The cysteine solution should be freshly prepared. After dissolving the tissue, incubate the sample for 2 hours at +4 ºC, and then proceed with the above protocol.

7. RNA degradation can be assessed using non-denaturing electrophoresis. The first sign of RNA degradation on the non-denaturing gel is a slight smear starting from the rRNA bands and extending to the area of shorter fragments, such as seen on Fig. 2, lanes 3 and 4. The 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 the rRNA staining by ethidium bromide in the gel, assuming that the dye incorporation efficiency is the same as for DNA (the ribosomal RNA may be considered a double-stranded molecule due to 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 right in the middle of the 28s rRNA molecule, which is called hidden because under non-denaturing 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 rather weak, so the total RNA preparation exhibits a single 18s-like rRNA band even on non-denaturing gel (Fig. 2A, 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. 2A, 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 volume of 3*M* sodium acetate and 2.5 volumes of 96% ethanol to the RNA in water, and mix thoroughly. The sample may be stored for several years at -20 ºC.

11. Using the Qia-Quick purified ligation mixture removes the excess of non-incorporated 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 five-fold dilution of the mixture followed by PCR starting with 1 μL of the dilution. Thus the non-incorporated oligos are simply diluted to a non-interfering concentration. In this case only 1/50 part of the available adapter-ligated cDNA goes into PCR, but due to 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 you find that cDNA amplification requires too many cycles (more than 20, see Note 11), purify the remaining ligation mixture by QiaQuick and take it all into PCR. It is important to use T/M buffer, which contains 10 m*M* tris-HCl and 1 m*M* $MgCl_2$, for elution. Elution with plain water leads to denaturation of DNA due to electrostatic repulsion of strands in low-salt conditions, which decreases the specificity of amplification and promotes background stemming from genomic DNA.

12. The number of PCR cycles required to amplify a visible amount of cDNA (i.e. about 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 10^6 molecules (a representation sufficient for most cDNA tasks) or more would require 20 or less PCR cycles to be amplified. In other words, if it took less than 20 PCR cycles to amplify your 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 about 1 kb (see Fig. 2B). 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 due to poor quality of reverse transcriptase. Try another batch of it. Alternatively, something may be wrong with the PCR system. Most probably the polymerase mixture is bad, but it is better to replace all the reaction components.

14. It is recommended to store the product of amplification as a master sample. The unpurified PCR product produced by a KlenTaq-based enzyme mixture can be stored at –20 ºC for several years. If large amount of cDNA is required for further procedures (for example, 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 of the dilution per each 20 μL of the PCR mixture prepared as at step 10. Do exactly 10 PCR cycles, this will generate a product in a concentration equal to the master sample. Do not apply more cycles attempting to generate more material as over-cycling produces

the most pronounced distortions of cDNA profile. Instead, prepare a large volume of PCR mixture, distribute it into several tubes (30 μL per tube), and pool them after amplification is over. If pure DNA is required for further procedures, the amplified cDNA may be cleaned by Qia-Quick PCR purification kit (Qiagen) according to the provided protocol, but using T/M buffer for elution instead of the provided buffer.

15. If you intend to clone the product of cDNA amplification, it is necessary to perform a "chase" step after the product is amplified. The conditions for amplification recommended here include using low working concentration of primers (0.1 μM), which greatly enhance the specificity of polyA+-fraction amplification. However, there is a high chance that a substantial fraction of the sample will be denatured at the end of PCR, since there will be already no primers available to initiate the synthesis of complementary strand (especially if slight over-cycling 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 72^0 C, inject additional amount of primers there (up to 0.2 μM more of each), and perform two non-denaturing "chase" cycles: 77^0 C 1 minute - 65^0 C 1 minute, 72^0 C 3 minutes. Purify the product by Qia-Quick PCR purification kit (Qiagen) before cloning (use T/M buffer for elution).

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Figure legends

Fig. 1. Differential display patterns obtained according to ***(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 non-amplified double-stranded cDNA (lane 2).

Fig 2. Schematic outlines of cDNA amplification methods.

Fig 3. **A**. Non-denaturating 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 of 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 of 1 kb DNA ladder (GIBCO/Life Technologies). Product on lane 2 needs one more PCR cycle, while product on lane 3 is already slightly over-cycled (by 1-2 cycles), but is still well suitable for further manipulations.

Figure 1.

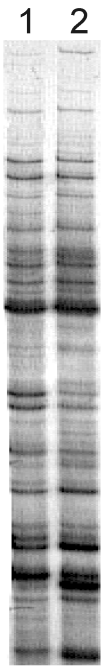


Figure 2.

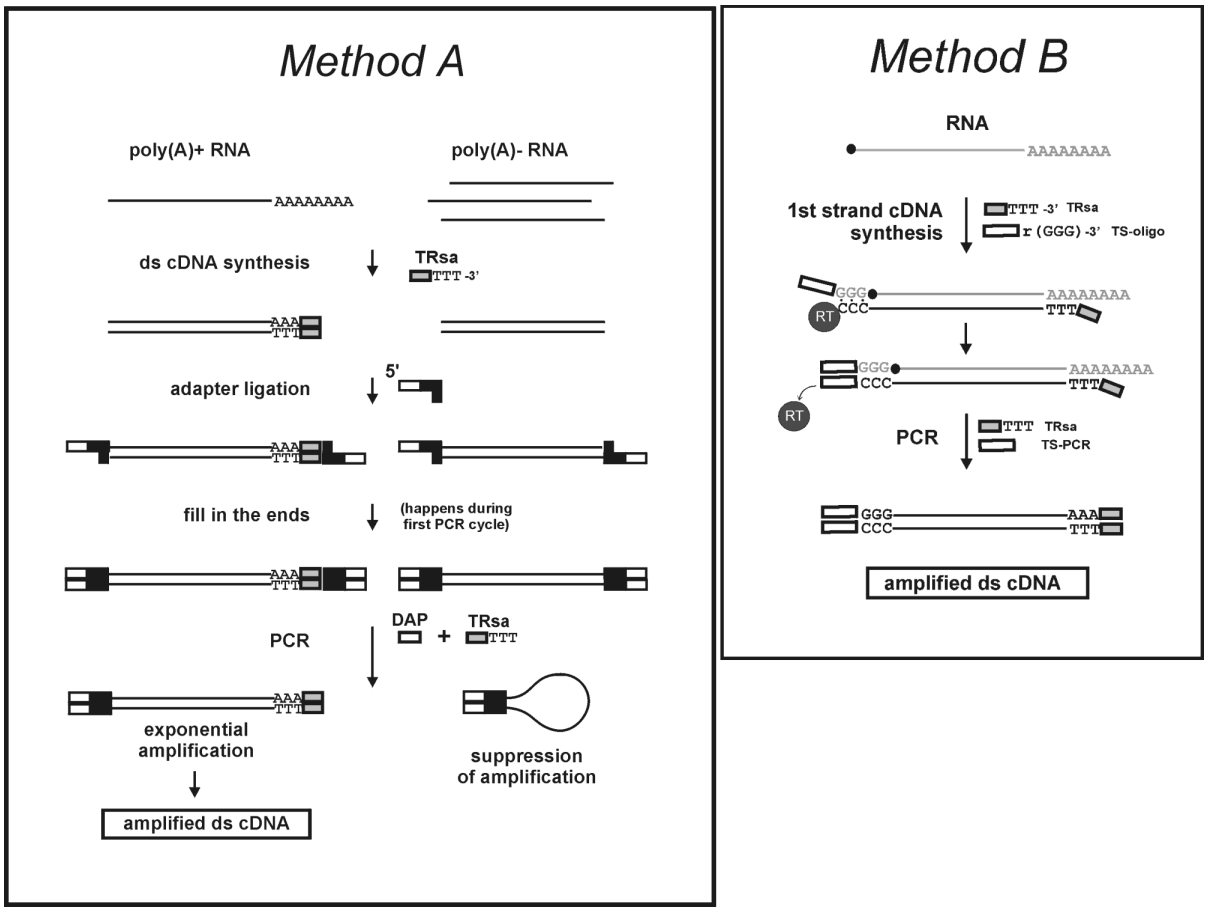
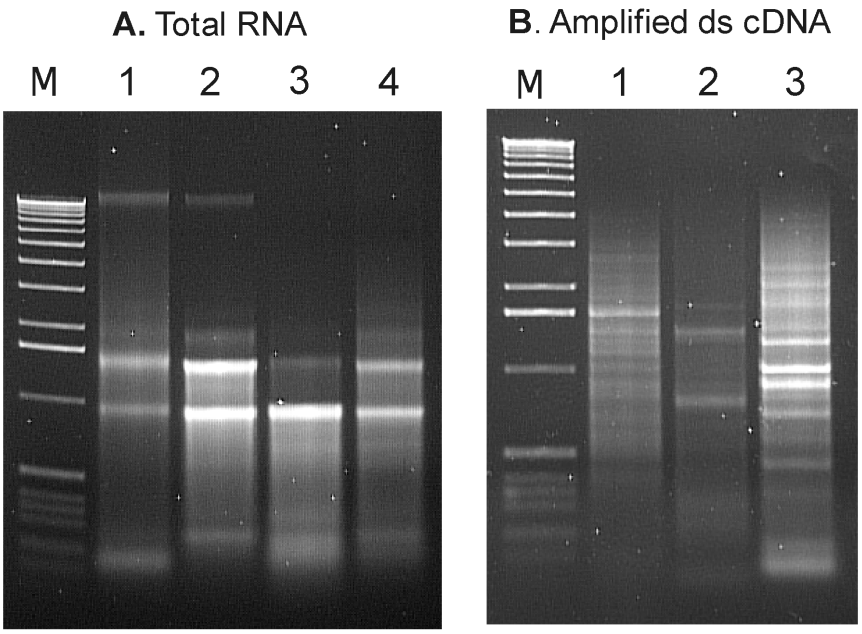

Method A
poly(A)+ RNA
poly(A)- RNA
AAAAAAAA
ds cDNA synthesis
TRsa
TTT -3'
AAA
TTT
adapter ligation
5'
fill in the ends
(happens during first PCR cycle)
PCR
DAP
+
TRsa
TTT
exponential amplification
suppression of amplification
amplified ds cDNA
Method B
RNA
AAAAAAAA
1st strand cDNA synthesis
TTT -3' TRsa
r (GGG) -3' TS-oligo
GGG
CCC
RT
AAAAAAAA
TTT
PCR
TTT TRsa
TS-PCR
GGG
CCC
AAA
TTT
amplified ds cDNA

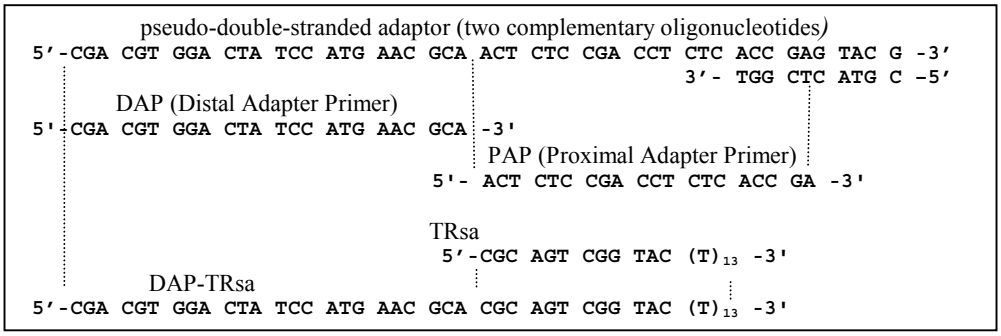
Figure 3.



Box 1

Invertebrate-optimized sets of oligos for total cDNA amplification and RACE

For Method A:



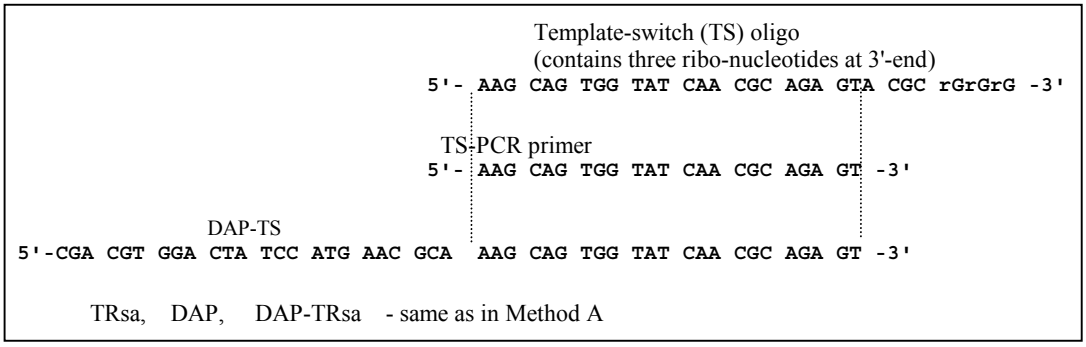
Oligos used in cDNA amlification:

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 amlification:

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 2

Amplification guidelines

$$N = 2^{(40-n)}$$ *(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