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cDNA generation (RT) using RevertAid reverse transcriptase

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We use this protocol and it's working

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Disclaimer

> This protocol is shared “as is” without warranty of any kind, either expressed or implied. The entire risk as to the quality and performance of the protocol is with you. Intended for basic research, only.

Abstract

This protocol is used for generating cDNA from purified RNA. It is based on RevertAid's manufacturer's protocol, with minor modifications. It can be used with other common RT enzymes such as M-MVL, just make sure to incubate the enzyme at the right temperature.

Using this protocol, we have successfully generated cDNA from eukaryotic RNA from several sources. The cDNA can be further used for qPCR, clonning, end-point PCR, etc.

Materials

- 50 mM Random hexamers **Integrated DNA Technologies, Inc. (IDT)**
- RevertAid RT **Thermo Fisher Scientific** **Catalog #**EP0441
- 10 mM dNTPs **New England Biolabs** **Catalog #**N0447
- 50 uM Oligo dT **Integrated DNA Technologies, Inc. (IDT)**

Safety warnings

> Follow standard molecular biology safety guidelines. While handling RNA, always use clean gloves, as well as RNAse-free tips and tubes. Work on a clean area (cleaning the area with 10% bleach should get rid of most RNAses).

Before start

Make sure you have good quality RNA. You can check RNA integrity via bleach gel (see: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3699176/) or using a bioanalyzer or any similar method. Ideally, you need to have 500 ng of RNA, but we have successfully generated cDNA from as little as 100 ng.

cDNA can be generated using oligo dT (for RT of poly-A RNA), random hexamers or both. Using both can increase the amount of obtained cDNA and dramatically enhance the efficiency of retro transcription if RNA is somewhat degraded. However, random hexamers should only be used if the primers used for (q)PCR are designed exon-exon and if genomic DNA contamination is not a concern. Otherwise, only use oligo dT

Before starting, thaw all components on ice, except for RevertAid, which should be taken out of the fridge only when it's needed. Briefly vortex and spin all components (except for RevertAid!) once thawed.

It is advisable to avoid repeated thaw-freeze cycles of all components. Preferably, make small aliquots of buffer, primers and dNTPs. A mix of dNTPs, oligo dT and random hexamers can also be prepared and frozen beforehand.

We usually use PCR tubes and perform all incubations in a thermocycler (PCR machine). However, using eppendorf tubes and a regular thermomixer should work as well.

1 Thaw RNA, RT buffer, oligo dT, and random hexamers (optional) on ice 2m

STEP CASE

Random hexamers and oligo dT 9 steps

Random hexamers plus oligo dT can increase the amount of obtained cDNA and dramatically enhance the efficiency of retro transcription if the RNA is somewhat degraded. However, they should only be used if the primers used for (q)PCR are designed exon-exon and if genomic DNA contamination is not a concern. Otherwise, only use oligo dT

2 Dilute 500 ng of RNA to a final volume of 12.8 µL in nuclease-free water. 1m

> **Note**
>
> We use PCR tubes, but any nuclease-free sterile tube should work.

3 Add 1 µL of [M] 10 millimolar (mM) dTNPs 1m

Add 1 µL of [M] 50 micromolar (µM) oligo dT (18t)

Add 1 µL of [M] 50 micromolar (µM) random hexamers

> **Note**
>
> You can also pre-mix dNTPs, oligo dT and random hexamers and add 3 µL of the mix to each RNA sample.

RNA heating 7m

4 This step is optional but highly advisable. Heating your RNA will avoid the formation of secondary structures that could hinder RevertAid's ability to efficiently generate cDNA from genes/regions with high GC or stem-loop structures. 5m

Heat the prepared RNA at 50 °C for 00:05:00

Note

While the RNA is heating, you can proceed to step 5

4.1 Immediately transfer all the tubes to ice and chill them for about 2 min. 2m

Note

Make sure the tubes are cold before adding the enzyme mix!

Enzyme addition 3m

5 Per each sample, prepare the following enzyme mix: 1m

4 µL of 5X RT buffer

0.2 µL of RevertAid

If you have more than one sample multiply by number of samples + 0.2 and mix in a single master mix. So, if you have 4 samples, multiply by 4.2. This will give you a little extra that will ensure you will not run out of enzyme master mix.

6 Add 4.2 µL of the enzyme mix to each sample for a final volume of 20 µL 2m

Incubation 1h 25m

7 Incubate your samples: 1h 25m

	A	B	C
	25 °C	10 min	Random hexamer annealing
	42 °C	60 min	Retrotranscription (if using a different enzyme to RevertAid, modify the temperature if needed)
	70 °C	15 min	Enzyme inactivation

	A	B	C
	10 °C	Hold	

cDNA dilution

2m

8 Add 180 µL of nuclease-free water for a 1:10 dilution. 2m

Optionally transfer to an eppendorf tube.

Note

Some components of the RT buffer may inhibit the efficiency of many (q)PCR polymerases. Most protocols advice not to include more than 10% of the final (q)PCR volume directly from the RT reaction. Because we always perform our qPCRs in 10ul, and we always add 1ul of cDNA, we are dangerously close to the tolerated limit. Thus, we always dilute our cDNAs with nuclease-free water and get excellent CTs and plenty of cDNA! We always recommend diluting the cDNA at least 1:2. Remember that in molecular biology, sometimes less is more.

9 Store at -20 °C or proceed to (q)PCR.

Protocol references

RevertAid RT, thermo fisher: https://www.thermofisher.com/order/catalog/product/EP0441