Reverse Transcription

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19.1 Introduction and Purpose

Formalin fixation and paraffin embedding has been the method of choice for the processing and long-term storage of diagnostic tissue samples for a century. A number of studies has shown that such archival material is in principle amenable to the analysis of mRNA and several protocols have been established for the retrieval of mRNA from routinely processed paraffin-embedded tissues [1–3]. Several methods are available to analyze mRNA expression in tissues, including Northern hybridization, subtractive hybridization, RNase protection assay, and cDNA microarrays. However, these methods are significantly limited by the requirement of fresh, unfixed tissues to permit isolation of abundant and high-quality mRNA. Conversely, reverse transcription-polymerase chain reaction (RT-PCR) offers a number of advantages because it allows the use of relatively small amounts of molecules that may be fragmented or degraded, such as those obtained from embedded tissues. RT can be primed with oligo-dT, but this approach is not suitable in formalin-fixed and paraffin-embedded (FFPE)-derived material because of the degradation and loss of polyA tails during fixation. For this reason, random oligonucleotides or specific antisense primers are recommended when analyzing these tissues. Complementary DNA synthesis from RNA is a critical step in gene expression analysis and it is a major source of variability for all RT-PCR assays when these latter need to be performed in a quantitative manner (RT-qPCR). In fact, reverse transcription yield can vary up to 100-fold depending on reverse transcriptase [4], priming strategy [5], and quantity of RNA used [6], and the variation can also be gene dependent [4, 5]. The technical variability is further exacerbated when using RNA from FFPE samples due to intrinsic tissue heterogeneity, to the presence of inhibitory copurified components [7], and to

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variable degradation of extracted RNA. Standardization of the assays is necessary to allow the analysis of gene expression at the RNA level in standard diagnostic specimens in a clinical setting. The RT-qPCR assay can be performed combining both RT and PCR enzymes in the same tube (single-step RT-PCR system), using an enzyme endowed with both RT and polymerase activity (as the Tth polymerase: one enzyme/one tube system) or separating RT and amplification steps (two-step RT-PCR). Furthermore, commercial RT kits or home-made reagents can be used. We think that higher sensitivity and potential for optimization make the use of the two-enzyme protocol the best choice especially when using home-made reagents [8].

Herein we propose two protocols which differ for the type of reverse transcriptase and for the priming strategy used. The choice of the protocol depends mainly on RNA availability and on the expected expression levels of the target genes.

19.2 Reverse Transcription with MMLV-RT and Random Hexamers

This protocol combines the use of MMLV-RT (Moloney Murine Leukemia Virus Reverse Transcriptase) with "random priming" strategy. Random hexamers prime RT at multiple points along the transcript; this method is nonspecific but yields the most cDNA and is the most useful for transcripts with significant secondary structures. Furthermore, this strategy generates the least bias in the resulting cDNA, because the same reverse transcription reaction can be used for the detection of several genes in the same sample [9]. It is not the method of choice if the mRNA target is present at low levels, as less abundant targets appear to be reverse transcribed less efficiently than more abundant ones: this is probably due to the lower specific efficiency or to aspecific priming of ribosomal RNA [9]. However, when the starting RNA is low, a specific reverse transcriptase with reduced RNase H activity[1] (e.g., MMLV-RT RNase H minus, Superscript™) can be used. Moreover, recent results obtained by one of the IMPACTS groups showed a clear improvement of RT yield when using high concentration of random primers (3.35 nmol/reaction) in the reaction with MMLV enzyme [10].

The RT reaction step involving MMLV and random hexamers has been optimized specifically for quantitative studies in FFPE samples. This method improves the detection of scarcely expressed genes in paraffin-embedded tissues and should allow comparable results between fresh and fixed samples [11]. Such protocol is described as follows.

19.2.1 Reagents

Reagents from specific companies are reported here, but reagents of equal quality purchased from other companies may be used.

All reagents should be RNase free or DEPC treated. Refer to Chapter "RNA extraction from FFPE Tissues" for precautions against RNase contamination.

- *DEPC-treated water (DEPC H_2O)*: Add 1 ml of diethylpyrocarbonate (DEPC) (0.1% final concentration) to 1 l of sterile water at 37°C overnight and then autoclave[2]
- *200 U/µl MMLV-RT*,[3] supplied with 5× MMLV-RT buffer and 0.1 M DTT (Invitrogen)
- *Mix of 10 mM dNTPs*: Add 80 µl of each 100 mM dNTP (dATP, dCTP, dGTP, and dTTP[4]) (Promega) to 480 µl DEPC H_2O and aliquot

[1] RNase H is an endonuclease that specifically degrades the RNA in RNA: DNA hybrids. It is commonly used to destroy the RNA template after cDNA synthesis, as well as in procedures such as nuclease protection assays. Since an RNase H activity is also present in the reverse transcriptases, they have been genetically altered to remove this activity, resulting in an increase of full-length cDNA products.

[2] DEPC is a carcinogen and should be handled with care under a fume hood.

[3] When testing mRNA sequences rich in secondary structures, the use of a thermostable reverse transcriptase such as Superscript III or AMVTM is recommended. In such case, the RT step should be carried out at 60°C if using amplicon-specific primer (see Sect. 19.3) or at lower temperatures if using oligo-dT or random primers.

[4] dUTP instead of dTTP may be used both in RT and in PCR reaction to avoid reamplification of carryover PCR products. In such case, an additional incubation of mixes at 50°C for 2′ with the enzyme uracil N-glycosilase is necessary before the transcription procedure. 10′ incubation at 95°C is performed to heat-inactivate the enzyme. Because UNG is not completely deactivated at 95°C, the PCR reaction temperatures should be kept higher than 55°C.

- *100 ng/µl Pd(N)$_6$* random hexamers (Amersham): Resuspend the lyophilized powder with DEPC H_2O to 1 µg/µl stock solution and then make aliquots of 100 ng/µl each
- *40 U/µl RNase inhibitor* [5](Promega)
- *25 mM* $MgCl_2$ [6]

19.2.2 Equipment

- *Disinfected adjustable pipettes*,[7] range: 2–20 µl, 20–200 µl, 100–1,000 µl
- *Nuclease-free aerosol-resistant pipette tips*
- *1.5 and 0.2 ml tubes* (autoclaved)
- *Centrifuge* suitable for centrifugation/spinning of 0.2 ml tubes at 13,200 or 14,000 rpm
- *Thermal Cycler*

19.2.3 Method

1. All RT assays should include a positive control (e.g., good-quality RNA from cell lines) and a negative one (DEPC H_2O or parallel extraction from a paraffin block without tissues). The amplifiability of genomic DNA (gDNA) in subsequent PCR should be ruled out performing a "no-RT" control, lacking the enzyme, or a control containing only gDNA. Add the following components to a 0.2 ml microcentrifuge tube, to a final volume of 7 µl:

100 ng/µl Random hexamers	2.5–3 µl (250–300 ng final)
Total RNA	1 ng–5 µg[8,9,10]
DEPC H_2O	to 7.05 µl

2. Heat the mixture of RNA, random primers, and water in a thermal cycler at 65°C for 10′ and quick chill on ice.
3. Add the following components to the mastermix, to a final volume of 20 µl:

5× MMLV-RT buffer	4 µl (1× final)
10 mM dNTPs	2 µl (1 mM final)
0.1 M DTT	2 µl (10 mM final)
40 U/µl RNase inhibitor	0.1 µl (4 units final)
200 U/µl MMLV-RT	1.25 µl (250 units final)
25 mM $MgCl_2$	3.6 µl (7.5 mM final, considering the amount contained in the buffer)

4. Use the following Thermal Cycler programme:
   - 1×: 25°C for 10′
   - 1×: 37°C for 60′
   - 1×: 70°C for 15′
   - 1×: hold at 4°C
5. The cDNA can now be used as a template for PCR amplification. Store at –20°C until use. Avoid repeated freeze-thawing. The volume of first-strand reaction mixture to be used in the PCR should not exceed 10% of the amplification mixture volume, since larger amounts may result in decreased PCR product.

19.3 Reverse Transcription with AMV-RT and Specific Reverse Primer

It is possible to prime the RT using an antisense oligonucleotide, designed to target a specific mRNA transcript. Since specific primers' melting temperature is higher than that of random oligos, the use of a thermostable enzyme, such as AMV (Avian Myeloblastosis Virus Reverse Transcriptase) or Superscript is advisable.

[5]The use of RNase inhibitor is critical for the successful processing of RNA as naked RNA from tissue samples is extremely susceptible to degradation by endogenous ribonucleases (RNases).

[6]Additional $MgCl_2$ may be useful if the RNA has been previously digested by DNase according to the protocol described in the chapter about DNase digestion followed by heat inactivation, because the chelating properties of EDTA, used to inactivate DNase, can reduce free Mg^{2+} concentration

[7]Clean the pipettes with a disinfectant (e.g., Meliseptol®rapid) and leave them under the UV lamp for at least 10 min. Alternatively it is possible to autoclave the pipette depending on the provider instructions.

[8]Do not add more than 5 µg of total RNA per reaction because efficiency of cDNA synthesis can be reduced by higher RNA quantities. Use the same amount of RNA in all reactions.

[9]Total RNA is preferred to polyA RNA when reverse transcription is performed on RNA extracted from FFPE as polyA enrichment requires additional purification steps that cause RNA loss.

[10]When low amounts of RNA are obtained from microdissected or from very small FFPE samples, a fixed volume instead of a fixed quantity can be used for RT reaction.

This protocol uses the AMV reverse transcriptase associated with a specific reverse primer. Target-specific primers synthesize the most specific cDNA and provide the most sensitive method of quantification [12], so it is the method of choice for the detection of scarcely expressed genes. The main disadvantage is that this approach requires separate priming reactions for each target gene, which is wasteful if only limited amounts of RNA are available. The use of more than one specific reverse primer in a single reaction tube (multiplex) is possible, but it requires careful experimental design and optimization of reaction conditions.

19.3.1 Reagents

Reagents from specific companies are reported here, but reagents of equal quality purchased from other companies may be used.

All reagents should be RNase free[11] or DEPC treated

- *DEPC-treated water (DEPC H_2O)*: add 1 ml of diethylpyrocarbonate (DEPC) (0.1% final concentration) to 1 l of sterile water at 37°C overnight and then autoclave[12]
- *10 U/µl AMV-RT*, supplied with *5× AMV-RT buffer* (Promega)
- *Mix of 10 mM dNTPs* (Promega): add 80 µl of each 100 mM dNTP (dATP, dCTP, dGTP, and dTTP)[13] to 480 µl DEPC H_2O and aliquot
- *30 pmol/ul specific reverse primer*[14]: resuspend the lyophilized powder with DEPC H_2O to 300 pmol/µl stock solution and then make diluted aliquots of 30 pmol/µl each
- *40 U/µl RNase inhibitor*[15] (Promega)

19.3.2 Equipment

- *Disinfected adjustable pipettes*,[16] range: 2–20 µl, 20–200 µl, 100–1,000 µl
- *Nuclease-free aerosol-resistant pipette tips*
- *1.5 and 0.2 ml tubes* (autoclaved)
- *Centrifuge* suitable for centrifugation/spinning of 0.2 ml tubes at 13,200 or 14,000 rpm
- *Thermal Cycler*

19.3.3 Method

1. All RT assays should include a positive control (e.g., good-quality RNA) and a negative one (DEPC H_2O). The amplifiability of gDNA in subsequent PCR should be ruled out performing a "no-RT" control, lacking the enzyme, or a control containing only gDNA.
   Add the following components to a 0.2 ml microcentrifuge tube, to a final volume of 10 microliters:

5× AMV-RT buffer	2 µl (1× final)
10 mM dNTPs	1 µl (1 mM final)
30 pmol/µl Specific reverse primer	0.5 µl (15 pmol final)
10 U/µl AMV-RT	0.2 µl (2 units final)
40 U/µl RNase inhibitor	0.1 µl (4 units final)
DEPC H_2O	to 10 µl
Total RNA	1 ng–2 µg[17,18,19]

[11] Refer to chapter dedicated to RNA extraction from FFPE for precautions against RNases contamination.

[12] DEPC is a carcinogen and should be handled with care under a fume hood.

[13] dUTP instead of dTTP may be used both in RT and in PCR reaction to avoid reamplification of carryover PCR products. In such case, an additional incubation of mixes at 50°C for 2′ with the enzyme uracil N-glycosilase is necessary before the transcription procedure. 10′ incubation at 95°C is performed to heat-inactivate the enzyme. Because UNG is not completely deactivated at 95°C, the PCR reaction temperatures should be kept higher than 55°C.

[14] As a general rule, primers should be between 15 and 25 bases long to maximize specificity, with a G/C content of around 50%. Avoid primers with secondary structures or with sequence complementarities at the 3′ ends that could form dimers. Specific software can be used for the design of primers for both endpoint and real-time PCR (e.g., Primer3, http://frodo.wi.mit.edu/primer3/, or IDTDNA, http://eu.idtdna.com/scitools/scitools.aspx).

[15] The use of RNase inhibitor is critical for the successful processing of RNA, as naked RNA from tissue samples is extremely susceptible to degradation by endogenous ribonucleases (RNases).

[16] Clean the pipettes with a disinfectant (e.g., Meliseptol®rapid) and leave them under the UV lamp for at least 10 min. Alternatively it is possible to autoclave the pipette depending on the provider instructions.

[17] Do not add more than 2 µg of total RNA per reaction because efficiency of cDNA synthesis can be reduced by higher RNA quantities. Use the same amount of RNA in all reactions.

[18] Total RNA is preferred to polyA RNA when reverse transcription is performed on RNA extracted from FFPE tissues as polyA enrichment requires additional purification steps that cause RNA loss and because polyA is degraded in these tissues.

[19] When low amounts of RNA are obtained from microdissected or from very small FFPE samples, a fixed volume instead of a fixed quantity can be used for the RT reaction.

Table 19.1 Problems and solutions with the RT reaction

Problem	Possible reason	Solution
No amplification	Low RNA or RNA degradation	Increase RNA content; check RNA integrity; use an RT enzyme with reduced RNase H activity
	Secondary structure	Incubate RNA at 65°C for 10′ before RT. Perform RT at a higher temperature and/or use a thermostable enzyme
	Wrong primer design	Change primers
Aspecific amplification	Too high primer concentration	Reduce primer concentration
	Use of oligo-dT or random primers	Use specific primer
Lower amplification than expected	Inhibitory effect of cDNA solution on PCR reaction	Dilute cDNA solution before PCR amplification Perform a phenol-chloroform extraction of RT mixture
	Presence of RT inhibitors in the RNA solution	Improve RNA extraction method
Higher amplification than expected	Use of random hexamers in RT reaction	Use specific primer in the RT reaction
Positive no-RT control	DNA contamination	Perform DNase treatment, use intron-spanning primers, avoid target genes with pseudogenes

2. Use the following Thermal Cycler programme:
   - 1×: 42°C for 60′
   - 1×: hold at 4°C
3. The cDNA can now be used as a template for amplification in PCR. The whole reverse transcription product can be used, provided that amplification reaction is performed in 50 µl in order to avoid inhibition of PCR. In such conditions, a PCR buffer without $MgCl_2$ is required.

19.4 Troubleshooting

Possible troubleshooting in the reverse transcription step is detected after PCR amplification (in this context, an optimized PCR assay is assumed). Refer to Table 19.1 for the most common problems and solutions concerning the cDNA synthesis step.

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