

Simple Approaches for Optimization of RT-PCR

Reverse transcription-polymerase chain reaction (RT-PCR) converts and amplifies a single-stranded RNA template to yield abundant double-stranded DNA product^(1,2,3). RT-PCR is a powerful tool for analysis of gene expression and for characterization of RNA splice variants. RT-PCR is generally carried out as either one-step RT-PCR, in which all reagents are combined in one tube and RT and PCR are conducted sequentially in this tube^(1,3) or two-step RT-PCR, in which RT and PCR are conducted sequentially in separate tubes⁽²⁾. The USB One-Step RT-PCR Kit (PN 78350), Two-Step RT-PCR Kit (PN 78355), and RT Script Kit (PN 78360) are based on M-MLV Reverse Transcriptase⁽⁴⁾ and Taq DNA Polymerase⁽⁵⁾. M-MLV Reverse Transcriptase is useful in RT-PCR due to its naturally low RNase H activity⁽²⁾. The kits are designed for typical routine analyses of gene expression, based on amplification of relatively short (≤ 1.5 kb) RT-PCR products (Fig. 1).

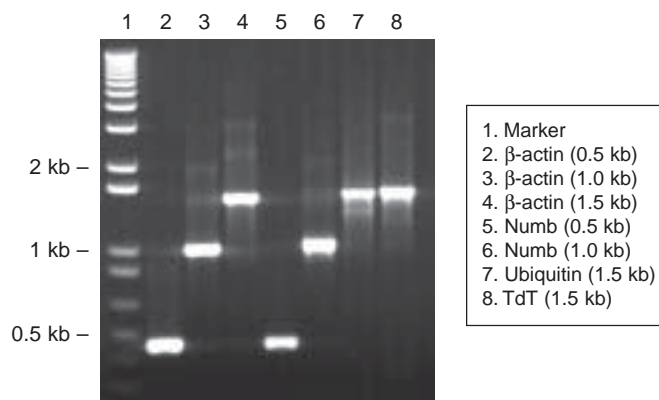


Fig. 1. Amplification of diverse RNA targets by one-step RT-PCR. Target (source): β -actin (100 ng total RNA, human liver), Numb (100 ng total RNA, human liver), Ubiquitin (1 μ g total RNA, Arabidopsis leaf), and Terminal deoxynucleotidyl transferase (TdT) (100 ng polyA RNA, calf thymus). Gene specific primers were designed to generate products of defined sizes.

Optimization of reaction parameters is a crucial aspect of RT-PCR. With new targets, optimization can determine the difference between failure and success in generating product. With familiar targets, optimization can minimize use of sample and reagents and maximize reproducibility and reliability of results. A small investment of effort in optimizing reactions can yield a large payoff in results, particularly if careful attention is also paid to fundamental issues such as primer design^(6,7), RNA isolation^(8,9), and use of RNase free reagents⁽¹⁰⁾. This Tech Tip describes several simple approaches for optimizing sensitivity, specificity, and yield in one-step RT-PCR. The same approaches also apply in two-step RT-PCR.

Method

Reactions (50 μ l) were carried out with the USB One-Step RT-PCR Kit (PN 78350) according to the kit protocol, except as noted. Reactions and primers are described in Table 1. A brief version of the protocol is described here. Note that the reaction master mix can be scaled-up for analysis of multiple samples. Reaction master mixes and assembled reactions should be mixed well by vortexing.

Table 1. Reaction conditions for amplification of various targets by one-step RT-PCR. Targets correspond to human β -actin (Accession: NM_001101), human Numb (Accession: NM_003744), and human Clathrin (Accession: NM_004859). RT-PCR is carried out according to the standard protocol with 100 ng total RNA of human liver, except where indicated.

Gene	Product size	G+C content	Notes on reactions
β -actin	0.5 kb	54.4%	Standard protocol
β -actin	1.0 kb	49.7%	0.1 μ M primers
β -actin	1.5 kb	53.3%	0.1 μ M primers, PCR annealing, 60°C; PCR extension, 2 min
Numb	0.5 kb	46.8%	Standard protocol
Numb	1.0 kb	46.9%	Standard protocol
Numb	1.4 kb	48.9%	1 μ g total RNA; PCR extension, 2 min
Numb	2.1 kb	51.6%	1 μ g total RNA; PCR extension, 2 min
Clathrin	0.5 kb	41.7%	PCR annealing, 50°C
Clathrin	0.9 kb	38.8%	PCR annealing, 50°C; PCR extension, 1.5 min
Clathrin	1.7 kb	42.2%	PCR extension, 2 min

1. **RNA/Primer Mix**—In first tube, combine RNA and oligonucleotide primers to 10 μ l vol on ice.

RNA	x μ l	(total RNA: 1 pg to 1 μ g)
Forward Primer (10 μ M)	2 μ l	(0.4 μ M in 50 μ l reaction)
Reverse Primer (10 μ M)	2 μ l	(0.4 μ M in 50 μ l reaction)
Water (RNase-free)	6-x μ l	(to adjust volume to 10 μ l)

Mix well and collect contents at bottom of tube.

2. **Heat Treatment of RNA/Primer (optional)**—Heat RNA/primer mixture at 75°C, 5 min, then cool immediately on ice for at least 5 minutes and store on ice. Note that this step may be omitted in some cases but may be necessary in others.

3. **Reaction Master Mix**—In second tube, combine RT-PCR reagents to 40 μ l on ice.

Water (RNase-free)	27 μ l
RT-PCR Buffer (5X)	10 μ l
PCR Nucleotide Mix (10mM)	1 μ l
RNase Inhibitor (4 units/ μ l)	1 μ l
RT-PCR Enzyme Mix (50X)	1 μ l

Mix contents of tube by vortexing, collect contents at bottom of tube, and store on ice.

4. **Complete Reaction Mix**—Combine 10 μ l RNA/primer and 40 μ l reaction master mix in one tube on ice for complete 50 μ l reaction. Mix contents of tube by vortexing, collect contents at bottom of tube, and store on ice.

5. **Thermal Cycle**—Design a thermal cycler program appropriate for RT-PCR amplification of specific target.

Step 1: 42°C, 30 min

Step 2: 95°C, 3 min

Step 3: 95°C, 30 sec

Step 4: 55°C, 30 sec (or choose temperature based on primers)

Step 5: 72°C, 1 min (or 1 to 2 min/kb)

Step 6: Go to step 3, 39 more times

Step 7: 72°C, 3 min

6. **Reaction**—Start thermal cycler program. When thermal cycler block reaches 42°C, transfer reaction tube from ice to block. Upon completion of thermal cycler program, transfer tubes to ice.
7. **Analysis**—Analyze sample (typically 1 to 10 μ l aliquots) by agarose gel electrophoresis with staining by ethidium bromide. Visualize PCR product in gel with a UV transilluminator or fluorescence imager.

Results and Discussion

Optimizing sensitivity

Optimal sensitivity may be achieved by designing RT-PCR primers to accomplish detection of a target of any length based on amplification of a short (~0.5 kb) fragment of the target. In general, for each ~0.5 kb increase in the length of the designed RT-PCR product, approximately 10 to 100-fold more RNA should be used in order to detect the target (Fig. 2). Thus, the use of primers designed to amplify short fragments results in lower requirements for input RNA and greater ease in detection of targets.

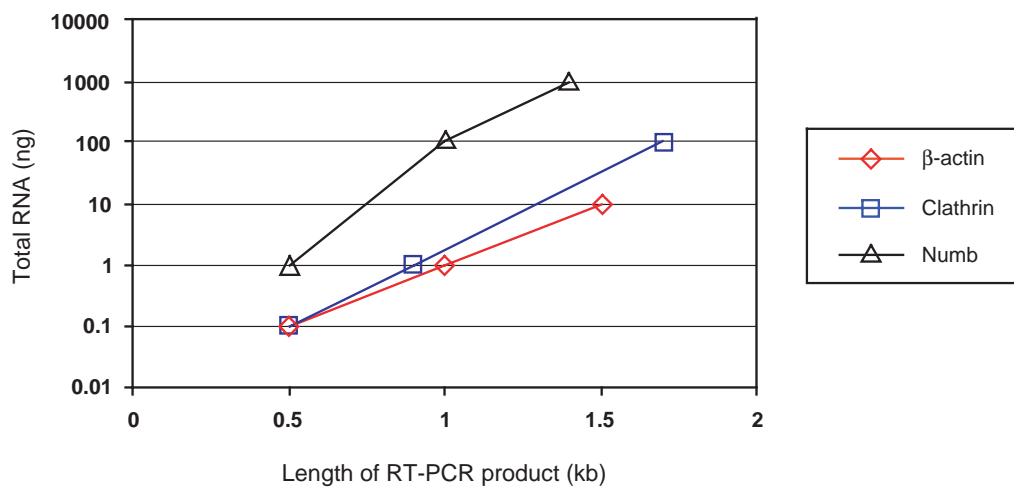


Fig. 2. Relationship between size of RT-PCR product and amount of total RNA sufficient for reaction. Data refer to human β -actin, Clathrin, and Numb targets, as detected in total RNA from human liver. Note that these genes are expressed at different levels, which accounts for the difference in amounts of total RNA required in reactions. Reactions were carried out as described in Table 1.

Sensitivity may also be improved by the use of relatively high amounts of primers in RT-PCR reactions. For relatively short (0.5 kb) RT-PCR products in particular, increasing the concentration of each primer from 0.2 μ M to 1.2 μ M can improve sensitivity approximately 10-fold (Fig. 3). Note that this approach tends not to work as well with relatively high amounts of RNA or relatively long (1.5 kb) RT-PCR products. In such cases relatively high primer concentrations may result in decreased specificity and/or yield (see for example Fig. 3, 1 ng RNA, 1.2 μ M each primer).

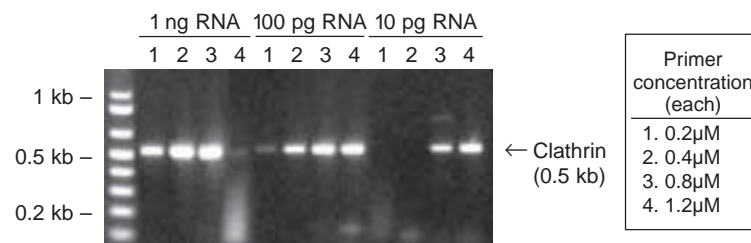


Fig. 3. Enhancement of sensitivity in one-step RT-PCR. For relatively short products, such as 0.5 kb Clathrin, sensitivity may be improved by increasing the primer concentration. Compare results for 0.2 μ M versus 0.8 μ M for each RNA concentration (1 ng, 100 pg, 10 pg). Note however that the combination of relatively high amounts of RNA and primers may yield poor results (1 ng RNA, 1.2 μ M primers). Target was detected from human liver total RNA.

Optimizing specificity

Optimal specificity may be achieved by using the optimal annealing temperature for a given pair of primers in the PCR step of RT-PCR. The optimal annealing temperature for a pair of primers may be determined as follows. First, estimate the primer melting temperature (T_m) for each primer based on the number of G, C, A, and T residues in each primer, according to the following equation: $T_m = [(G+C) \times 4^\circ\text{C}] + [(A+T) \times 2^\circ\text{C}]$. Second, conduct RT-PCR with the pair of primers using an annealing temperature that is well below the lower T_m for the two primers (5 to 10°C lower, for example). Third, increase the primer annealing temperature in increments of 2 to 3°C in subsequent or parallel RT-PCR experiments, until optimal specificity is achieved. For best results, use pairs of primers that are well matched in terms of their T_m values.

Specificity may also be improved by use of relatively low amounts of primers in RT-PCR reactions. For relatively long (≥ 1.5 kb) RT-PCR products in particular, decreasing the concentration of each primer from $0.4\mu\text{M}$ to $0.075\mu\text{M}$ can have a positive effect on specificity (Fig. 4). Note that this approach tends not to work as well with relatively short (0.5 kb) RT-PCR products. In such cases low primer concentrations may result in decreased sensitivity and yield (Fig. 3).

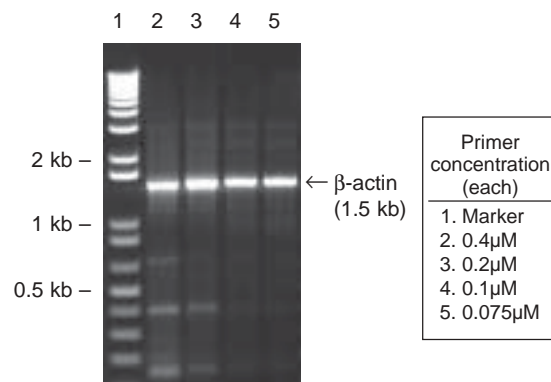


Fig. 4. Enhancement of specificity in one-step RT-PCR by decreasing the concentration of primers. Compare results for $0.4\mu\text{M}$ to $0.075\mu\text{M}$ for each primer. Target corresponds to 1.5 kb β -actin, amplified from 100 ng total RNA.

Optimizing yield

Excellent yields may be obtained by optimizing factors such as product length (shorter products may result in higher yields), RNA concentration (more RNA may result in higher yields), and MgCl_2 concentration (1.5mM to 2.5mM typically works well in one-step RT-PCR). One additional approach, which works well for some targets, is to increase the amount of RT-PCR enzyme mix in the reaction. For some relatively long (≥ 1.5 kb) RT-PCR targets, doubling the amount of RT-PCR enzyme mix in the reaction can also dramatically increase the yield of product (Fig. 5).

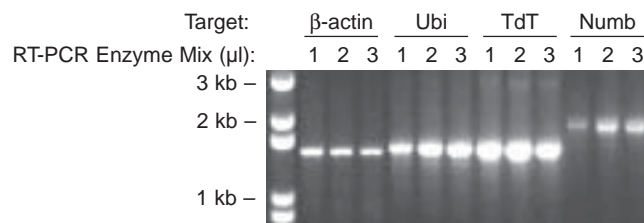


Fig. 5. Enhancement of yields in one-step RT-PCR. For some relatively long products, yields may be enhanced by increasing the amount of RT-PCR enzyme mix in the reaction. Compare results for enzyme mix volumes of 1 μl , 2 μl , and 3 μl in reaction volume of 50 μl . Additional RT-PCR enzyme mix improves results for Ubi and Numb in particular. Targets were detected from the following: β -actin, human liver total RNA (10 ng), Ubi: Arabidopsis leaf total RNA (100 ng); TdT, calf thymus polyA RNA (50 ng); Numb, human liver total RNA (1 μg).

Conclusion

Simple factors in RT-PCR, such as primer concentration, primer annealing temperature, and enzyme amount, have profound effects on sensitivity, specificity, and yield. Optimization of these factors is an excellent starting point for achieving desirable RT-PCR results.

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