One*Taq*® RT-PCR Kit

NEB #E5310S

30 reactions

Version 5.0_10/20

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Kit Components

All kit components should be stored for one year at –20°C except where noted.

M-MuLV Reaction Mix (2X)	400 µl
M-MuLV Enzyme Mix (10X)	60 µl
Oligo d(T)$_{23}$VN* (50 µM)**	70 µl
Random Primer Mix (60 µM)**	70 µl
One*Taq* Hot Start 2X Master Mix with Standard Buffer	1.25 ml
Nuclease-free H_2O	1 ml

*V = A,G or C; N = A, G, C or T
**Contains 1 mM dNTP

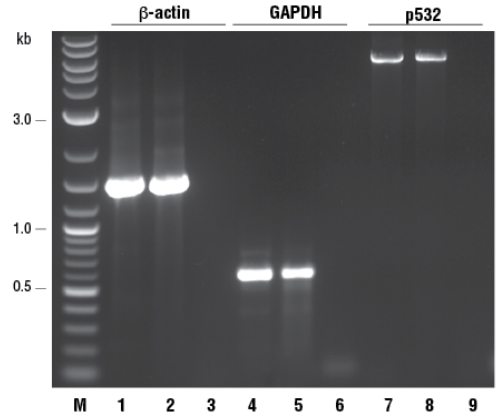
Introduction

One*Taq* RT-PCR Kit combines two powerful mixes, M-MuLV Enzyme Mix and One*Taq* Hot Start 2X Master Mix with Standard Buffer for 2-step RT-PCR applications. The two mixes require minimal handling during reaction setup and yet offer consistent and robust RT-PCR reactions.

The first strand cDNA synthesis is achieved by using two optimized mixes, M-MuLV Enzyme Mix and M-MuLV Reaction Mix. M-MuLV Enzyme Mix combines M-MuLV Reverse Transcriptase and Murine RNase Inhibitor while M-MuLV Reaction Mix contains dNTPs and an optimized buffer. The kit also contains two optimized primers for reverse transcription and nuclease-free water. An anchored oligo-dT primer [d(T)$_{23}$VN] forces the primer to anneal to the beginning of the polyA tail. The optimized Random Primer Mix provides random and consistent priming sites covering the entire RNA templates including both mRNAs and non-polyadenylated RNAs.

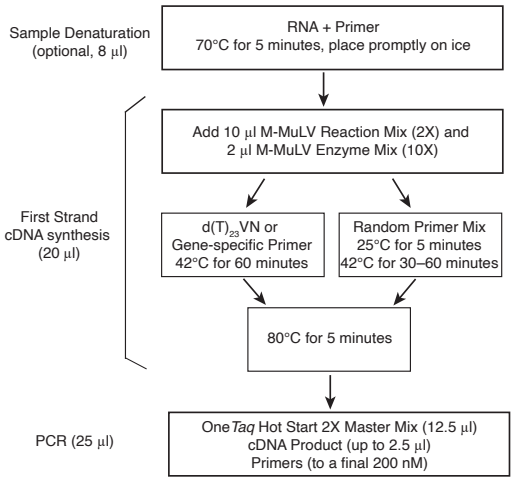
The amplification step features a One*Taq* Hot Start DNA Polymerase in a master mix format. One*Taq* Hot Start DNA Polymerase offers higher fidelity than *Taq* and better amplification. RT-PCR product up to 6 kb can be generated (Figure 1).

Figure 1: First Strand Synthesis.



First strand cDNA synthesis was carried out in the presence of 1X M-MuLV Enzyme Mix at 42°C using 0.5 µg of human spleen total RNA in the presence of $dT_{23}VN$ (lanes 1, 4 and 7) or Random Hexamer Mix (lanes 2, 5 and 8). No-RT controls were lanes 3, 6 and 9. OneTaq Hot Start 1X Master Mix was used to amplify a 1.5 kb fragment of beta-actin gene, a 0.6 kb fragment of GAPDH gene, and a 5.5 kb fragment from p532 gene in 35 cycles. The marker lane (M) contains 1 kb Plus DNA Ladder (NEB #N3200).

Figure 2: First Strand DNA Synthesis



Quality Controls

The performance of One*Taq* RT-PCR Kit is tested in an RT reaction using human Jurkat total RNA with primer $d(T)_{23}VN$. The sensitivity of the kit is verified by the detection of GAPDH transcript in 20 pg total RNA after 35 cycles. The length of cDNA achieved is verified by the detection of a 5.5 kb amplicon of the p532 gene.

First Strand cDNA Synthesis Reaction

- Denaturation of RNA and primer at 70°C for 5 minutes can remove secondary structures that may impede long cDNA synthesis. However, this step can be omitted in some cases (unpublished results).
- We recommend incubation at 42°C for one hour for maximum yield and length. However, many targets can be detected after a much shorter incubation time. For example, 10 minutes incubation is enough for a 2 kb cDNA synthesis.

Choice of Primers for Reverse Transcription

- Oligo-dT priming is preferred for most applications because it ensures that all cDNA copies terminate at the 3′ end of the mRNA and produces the longest contiguous cDNA. An anchored oligo-dT primer [$d(T)_{23}VN$] forces the primer to anneal to the start of the polyA tail, thereby preventing priming at internal sites in the polyA tail (1).
- The Random Primer Mix is an optimized mix of hexamer and $d(T)_{23}VN$ primers. It provides random priming sites covering the entire RNA templates including both mRNAs and non-polyadenylated RNAs (such as ribosomal RNAs). The Random Primer Mix yields shorter cDNAs on average and can be used for the detection of multiple short RT-PCR products. Random Primer Mix offers good performance in a wide range of RNA templates.
- When a gene-specific primer is used in a cDNA synthesis reaction, the cDNA product can be used only for amplification of that transcript. This priming method gives good results when the amount of RNA is limiting (below 10 ng) and only one particular cDNA is desired.
- Recommended primer concentration:

PRIMER	$D(T)_{23}VN$ PRIMER	RANDOM PRIMER MIX	SPECIFIC PRIMER
Final Concentration	5 µM	6 µM	0.1-1 µM

Important Factors for Successful RT-PCR

Template RNA

- Intact RNA of high purity is essential for sensitive RT-PCR detection. High quality total RNA has a ratio of OD_{260}/OD_{280} greater than 2.
- Both total RNA and mRNA can be used in the reverse transcription reaction. Total RNA is generally sufficient for most RT-PCR analysis. However, if desired, mRNA can be easily obtained using a PolyA Spin™ mRNA Isolation Kit (NEB #S1560).
- The amount of RNA required for detection depends on the abundance of the transcript-of-interest. In general 1 ng to 1 µg total RNA or 50 pg to 100 ng mRNA are recommended.

RNA-Priming Choices

- Oligo-dT priming is recommended for most applications. It ensures that all cDNA copies terminate at the 3′ end of the mRNA and produces the longest contiguous cDNA. An anchored oligo-dT primer [$d(T)_{23}VN$] forces the primer to anneal to the start of the polyA tail, thereby preventing priming at internal sites in the polyA tail (1). However, two other priming choices are possible if desired.
- The Random Primer Mix is an optimized mix of hexamer and $d(T)_{23}VN$ primers. It provides random priming sites covering the entire RNA templates including both mRNAs and non-polyadenylated RNAs (such as ribosomal RNAs). The Random Primer Mix yields shorter cDNAs on average and can be used for the detection of multiple short RT-PCR products. Random Primer Mix offers good performance in a wide range of RNA templates.
- When a gene-specific primer is used in a cDNA synthesis reaction, the cDNA product can be used only for amplification of that transcript. This priming method gives good results when the amount of RNA is limiting (below 10 ng) and only one particular cDNA is desired.
- Recommended primer amount for a 20 µl cDNA synthesis reaction:

PRIMER	$D(T)_{23}VN$ PRIMER	RANDOM PRIMER MIX	SPECIFIC PRIMER
Final Concentration	5 µM	6 µM	0.1-1 µM

cDNA Synthesis Reaction

- Denaturation of RNA and primer at 70°C for 5 minutes can remove secondary structures that may impede long cDNA synthesis. However, this step can be omitted in many cases (unpublished results).
- We recommend incubation at 42°C for one hour for maximum yield and length. However, many targets can be detected after a much shorter incubation time. For example, 10 minutes incubation is enough for a 2 kb cDNA synthesis.

PCR Primers

- Specific primers for PCR should be designed with the aid of a primer design computer program to achieve best results, such as PrimerSelect™ (DNAStar Inc, Madison, MI) and Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). To minimize the complication introduced by contaminating genomic DNA, use primers that span an exon-exon boundary of the mRNA.

PCR Amplification

- Most targets can be efficiently amplified using 1/10 (2 µl out of 20 µl cDNA synthesis reaction) or much less of the cDNA product (2).
- A final concentration of 0.2 µM for each primer is recommended for PCR; however, it can vary between 0.05 µM to 1 µM.
- The extension step using One*Taq* Hot Start 2X Master Mix is recommended at 65–68°C with an extension rate of 1 minute per kb. We recommend a 30 seconds incubation time for the annealing step.
- For GC-rich targets, we recommend One*Taq* Hot Start 2X Master Mix with GC Buffer (NEB #M0489).
- Use of thin-wall 0.2 ml PCR tubes and a manual hot-start may increase the PCR sensitivity and yield. A manual hot-start is done by assembling reactions into tubes placed on ice. The reaction tubes are transferred to a PCR machine with a block preheated at 95°C. Upon placement of the tubes, the cycler is immediately started.

2-Step RT-PCR Protocols

First strand cDNA synthesis

Thaw system components and put on ice. A control reaction without reverse transcriptase is recommended to examine the DNA contamination in the samples.

1. Make the RNA/primer/dNTP mix by combining the following components in two sterile RNase-free microfuge tubes.

REAGENT	VOLUME
Total RNA	1-6 µl
$d(T)_{23}VN$ (50 µM)	2 µl
Nuclease-free Water	To a total volume of 8 µl

2. Denature RNA for 5 minutes at 70°C. Spin briefly and put promptly on ice. This step is optional. However, it improves the cDNA yield for long messenger RNAs and GC-rich RNA regions.

3. Add the following components to one tube containing 8 µl RNA/primer/dNTP solution and mix well by pipetting up and down.

REAGENT	VOLUME
M-MuLV Reaction Mix (2X)	10 µl
M-MuLV Enzyme Mix	2 µl

Add the following components to the second tube containing the no RT negative control reaction.

REAGENT	VOLUME
M-MuLV Reaction Mix (2X)	10 µl
Nuclease-free Water	2 µl

4. Incubate the 20 µl cDNA synthesis reaction at 42°C for one hour. **If Random Primer Mix is used, an incubation step at 25°C for 5 minutes is recommended before the 42°C incubation.**

5. Inactivate the enzyme at 80°C for 5 minutes. Dilute reaction to 50 µl with 30 µl H_2O and ready for PCR. The cDNA product should be stored at –20°C. For downsteam PCR amplification, the volume of cDNA product should not exceed 1/10 of the PCR reaction volume.

PCR Amplification

We recommend 2-5 µl of the diluted cDNA product for a 25 µl PCR reaction. Mix the One*Taq* Hot Start 2X Master Mix by inverting before use.

1. Mix the following components in a PCR tube on ice:

REAGENT	VOLUME
One*Taq* Hot Start 2X Master Mix	12.5 µl
10 µM Forward Primer	0.5 µl
10 µM Forward Primer	0.5 µl
Diluted cDNA	2-5 µl
H_2O	to a total volume of 25 µl

2. Mix gently. Overlay with mineral oil if the thermal cycler lacks a heated lid.
3. The following PCR cycling conditions are recommended for 0.2 ml thin-wall PCR tubes on Bio-Rad iCycler or similar thermocyclers. For other PCR tubes or cyclers, it may be necessary to modify the cycle times.

CYCLE STEP	TEMP	TIME	CYCLES
Initial Denaturation	95°C	30 seconds	1
Denaturation	94°C	15-30 seconds	25-40
Annealing	45-68°C	30 seconds	
Extension	68°C	1 minute per kb	
Final Extension	68°C	5 minutes	1

4. Analyze 5 µl of PCR products by agarose gel electrophoresis.

Troubleshooting

PROBLEM	POSSIBLE CAUSE(S)	SOLUTION(S)
	Low Yield of cDNA	• Check the integrity of the RNA by denaturing agarose gel electrophoresis (3). • RNA should have a minimum A_{260}/A_{280} ratio of 1.7 or higher. Ethanol precipitation followed by a 70% ethanol wash can remove most contaminants such as EDTA and guanidinium. Precipitation with lithium chloride can remove polysaccharides (3).
		• Phenol/chloroform extraction and ethanol extraction can remove contaminant proteins such as proteases (3).
		• Some target RNA may contain strong pauses for RT; Use random priming instead of $d(T)_{23}VN$.
		• Use sufficient amount of RNA (maximum 2 µg total RNA).
	Low Yield of PCR Product	• Check the primer design using computer software.
		• Optimize the annealing temperature in a 1–2°C step.
		• A primer concentration of 0.2 µM is satisfactory for most PCR reactions. However, sensitivity and yield of RT-PCR reactions can be improved by increasing the primer concentration to above 0.5 µM. Lower primer concentration between 0.07 µM to 0.2 µM may improve specificity.
		• Increase cycling numbers up to 45 cycles.
		• Do a manual hot-start.
		• Use thin-wall 0.2 ml PCR tubes.
		• Try a touch-down PCR protocol (4).
	Products of wrong size	• Always do a negative control reaction using noRT Master Mix.
		• In cases where the RNA sample is contaminated with genomic DNA, treat with DNase I before cDNA synthesis (5).
		• Design primers spanning an exon-exon boundary.

References

1. Liao, J. and Gong, Z. (1997) *Biotechniques,* 23, 368–370.
2. Van Gilst, M.R. et al. (2005) *PLoS Biology*, 3, 301–312.
3. Sambrook, J. and Russel, D.W. (2001) Molecular Cloning: A Laboratory Manual (3rd Ed.) Cold Spring Harbor: Cold Spring Harbor Laboratory Press.
4. Don, R.H. et al. (1991) *Nucleic Acid Research,* 19, 4008.
5. Aguila et al. (2005) *BMC Molecular Biology,* 6, 9.

Appendix: Supplied Components

M-MuLV Enzyme Mix (1X)

0.5 unit/µl M-MuLV Reverse Transcriptase
1 unit/µl RNase Inhibitor, Murine

M-MuLV Reaction Mix (1X)

50 mM Tris-Acetate (pH 8.3)
75 mM KOAc
3.1 mM $Mg(OAc)_2$
0.5 mM dNTPs each

One*Taq* Hot Start 2X Master Mix with Standard Buffer (1X)

20 mM Tris-HCl
22 mM KCl
0.2 mM dNTPs
1.8 mM $MgCl_2$
5% Glycerol
0.05 % Tween®20
25 units/ml One*Taq* Hot Start DNA Polymerase
22 mM NH_4Cl_2
0.06% IGEPAL® CA-630

Ordering Information

NEB #	PRODUCT	SIZE
E5310S	One*Taq* RT-PCR Kit	30 reactions
KIT COMPONENTS SOLD SEPARATELY		
E6300S/L	ProtoScript® First Strand cDNA Synthesis Kit	30/150 reactions
S1330S	Random Primer Mix	100 µl (60 µM)
M0484S/L	One*Taq* Hot Start 2X Master Mix with Standard Buffer	100/500 reactions

COMPANION PRODUCTS

NEB #	PRODUCT	SIZE
M0253S/L	M-MuLV Reverse Transcriptase	10,000/50,000 units
M0314S/L	RNase Inhibitor, Murine	3,000/15,000 units
N0446S	Deoxynucleotide Solution Mix	25 µmol of each
S1560S	PolyA Spin mRNA Isolation Kit	8 isolations
M0485S/L	One*Taq* Hot Start 2X Master Mix with GC Buffer	100/500 reactions

Revision History

REVISION #	DESCRIPTION	DATE
1.0		N/A
1.1		
1.2		5/13
1.3		3/14
1.4		7/14
1.5		11/14
1.6		10/15
1.7		12/17
2.0		6/18
3.0	Update to new manual format	1/20
4.0	Update legal text	3/20
5.0	Update legal text	10/20

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