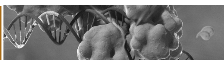
DNA AMPLIFICATION & PCR

ProtoScript® M-MuLV *Taq* RT-PCR Kit

Instruction Manual

NEB #E6400S
Store at –20°C



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Kit Components:

Store RNA at –70°C and all other kit components at –20°C. The kit provides all the reagents required for performing 30 cDNA synthesis reactions (20 µl volume) and 50 PCR reactions (50 µl volume).

Component	Amount
M-MuLV Reverse Transcriptase Reaction Buffer (10X RT Buffer)	100 µl
Deoxynucleotide Solution Mix (dNTP Mix) (2.5 mM each)	200 µl
M-MuLV Reverse Transcriptase (10 units/µl)	35 µl
Oligo $d(T)_{23}VN^*$ Primer (50 µM)**	70 µl
Random Primer Mix (60 µM)**	70 µl
Murine RNase Inhibitor (40 units/µl)	20 µl
Control Total RNA (rat liver) (0.5 µg/µl)	15 µl
Control Primer Set (GAPDH) (10 µM)	15 µl
Nuclease-free H_2O	1 ml
Taq 2X Master Mix	1.25 ml

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

Introduction:

The ProtoScript M-MuLV *Taq* RT-PCR Kit is designed for the sensitive detection of mRNAs in a two-step process. Each reaction is optimized for maximum results, leading to greater sensitivity and higher yield. Multiple transcripts can be detected from a single first-strand cDNA synthesis. Semi-quantitative analysis of the mRNA level can be achieved by agarose gel electrophoresis. In the first step, M-MuLV Reverse Transcriptase (RT) is used to extend a random primer, anchored oligo-dT primer, or gene-specific primer annealed to an RNA sample. In the second step, PCR amplification is performed in a separate tube using gene-specific primers. This Kit includes murine RNase inhibitor, which provides better protection of RNA against RNAses than human RNase Inhibitor. A Random Primer Mix is introduced to provide even and consistent coverage of the RNA template population across a wide range of RNA template concentration. A ready-to-use *Taq* 2X Master Mix is provided for its convenient and consistent amplification performance.

For other applications, such as high-fidelity cloning or long-range amplification, LongAmp® *Taq* DNA Polymerase, Vent™ DNA Polymerase, and Phusion® DNA Polymerase can be obtained from NEB (see Kit Components Sold Separately/Companion Products on page 12).

Quality Controls:

The performance of ProtoScript M-MuLV *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.

Important Factors for Successful RT-PCR Reactions:

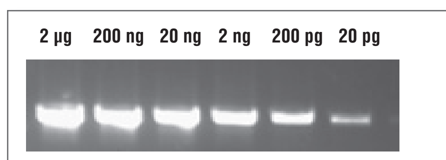
Template RNA

Intact RNA of high purity is essential for sensitive RT-PCR detection.

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).

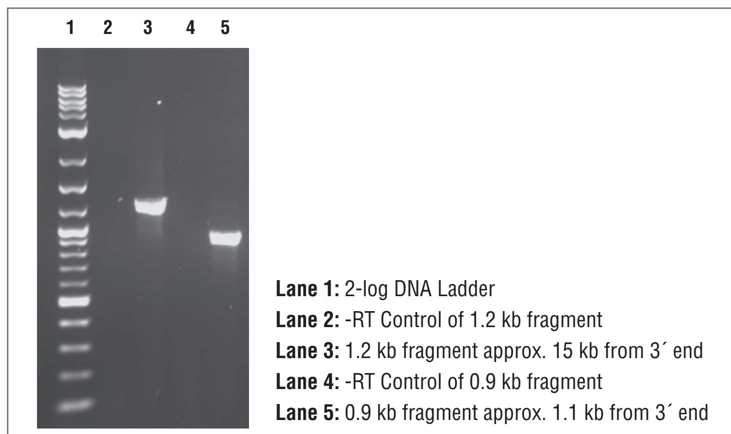
The amount of RNA required for detection depends on the abundance of the transcript of interest. In general 10 ng to 1 µg of total RNA or 1 ng to 100 ng of mRNA are recommended.

Figure 1:



RT-PCR amplification of the GAPDH gene from human spleen total RNA. First strand cDNA was carried out in the presence of oligo dT23VN, and about 1/10th of the cDNA reaction was amplified in a 35-cycle PCR reaction using the Taq 2X Master Mix (-RT control not shown).

Figure 2:



Amplification of different regions of human guanine nucleotide exchange factor p532 (15,164 bp, GenBank accession number U50078). Approximately 2 µg of human spleen total RNA was reverse transcribed using dT23VN. After 30 cycles of amplification using 1/20th of the cDNA product, 5 µl was analyzed on a 1% agarose gel. (Note: In the -RT control reactions, no reverse transcriptase was added.)

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 (dT23VN) 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.

1. Random primers provide random priming sites throughout the entire RNA templates including both mRNAs and non-polyadenylated RNAs such as ribosomal RNAs. Traditional random priming by hexamer is sensitive to the ratio of primer to RNA amount. In contrast, Random Primer Mix is an optimized mixture of hexamers and anchored-dT primer ($dT_{23}VN$). A mixture of hexamers and anchored-dT primer provides even and consistent coverage of the RNA template population across a wide range of RNA template concentration. We recommend using Random Primer Mix for reverse transcription of the following RNA templates:

- RNA without poly(A) tail
- RNA with strong secondary structures
- Partially degraded RNA samples
- Targets regions at 5´ end of a long messenger RNA transcript.

OR

2. 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:

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 may be omitted in many cases (Xu, Y., unpublished observations).

PRIMER	Oligo $d(T)_{23}VN$ PRIMER (50 µM)	RANDOM PRIMER MIX (60 µM)	SPECIFIC PRIMER
Amount	100 pmol (2 µl)	120 pmol (2 µl)	10–20 pmol

We recommend incubation at 42°C for one hour for maximum cDNA yield and length. However, many targets can be detected after a much shorter incubation time. For example, a 10 minute incubation time is enough for a 2 kb cDNA synthesis.

PCR Primers

For best results, specific primers for PCR should be designed with the aid of a primer design computer program, such as PrimerSelect™ (DNAStar Inc, Madison, MI) or Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). To minimize complications introduced by contaminating genomic DNA, use primers that span an exon-exon boundary of the mRNA. In general, primers with 40–60% GC and temp. of 55–68°C are preferred.

PCR Amplification

- Most targets can be efficiently amplified using 1/10th (2 µl of 20 µl) of the cDNA synthesis reaction, or 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 and 1 µM.
- The recommended extension step using the *Taq* 2X Master Mix is 68°C with an extension time of one minute per kb.
- The final Mg^{2+} concentration of the *Taq* 2X Master Mix is 1.5 mM, which is optimal for most RT-PCR applications. However, the Mg^{2+} concentration can be further optimized in 0.2 mM increments.
- A manual hot-start my increase PCR sensitivity and yield. This is done by assembling reactions in thin wall 0.2 ml PCR tubes placed on ice. Tubes are then transferred to a PCR machine with a block preheated at 95°C, and cycler is immediately started.

RT-PCR Protocols

First Strand cDNA Synthesis

Proper precautions should be used to avoid ribonuclease contamination. This includes the use of autoclaved tubes, baked glassware, ultra-pure solutions, sterile pipette tips and latex gloves during manipulations (3).

Thaw system components and place on ice. The 10X RT Buffer can be warmed briefly at 42°C and vortexed to dissolve any precipitate. (Note: It is important to set up a -RT control reaction (no reverse transcriptase) to insure there is no DNA contamination).

1. Make the RNA/primer/dNTP mix by combining the following components in a sterile RNase-free microfuge tube:

Component	Volume	
Total RNA	1–10 µl	(1 ng–1 µg)
Primer $dT_{23}VN$	2 µl	
dNTP Mix	4 µl	
nuclease-free H_20	variable	
Total volume	16 µl	

2. Heat for 5 minutes at 70°C. Spin briefly and promptly chill on ice.

3. Add the following components to the 16 µl RNA/primer/dNTP solution and mix well by pipetting up and down:

		-RT control
10X RT Buffer	2 µl	2 µl
Murine RNase Inhibitor	0.5 µl	0.5 µl
M-MuLV Reverse Transcriptase	1 µl	–
Nuclease-free H_2O	0.5 µl	1.5 µl
Final volume	20 µl	20 µl

4. Incubate the 20 µl cDNA synthesis reaction at 42°C for one hour. If random primers are used, an incubation step at 25°C for 5 minutes is recommended prior to the 42°C incubation.
5. Inactivate the enzyme at 80°C for 5 minutes.
6. Bring the reaction volume to 50 µl with water. The cDNA product should be stored at –20°C.

PCR Amplification

We recommend 2–5 µl of the diluted cDNA product per 50 µl PCR reaction.

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

Taq 2X Master Mix	25 µl	(mix well by inverting before use)
Forward Primer (10 µM)	1 µl	(final concentration 200 nm)
Reverse Primer (10 µM)	1 µl	(final concentration 200 nm)
Diluted cDNA	2–5 µl	
H_2O	variable	
total volume	50 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.

INITIAL DENATURATION	95°C	1 MINUTE
25–35 Cycles	94°C 45–68°C 68°C	30 seconds 10–30 seconds 1 minute per kb
Final Extension	68°C	5–10 minutes

4. Analyze 5 µl of the PCR product by agarose gel electrophoresis.

Control Reactions

1. The following control reactions can be used to examine the quality of kit components and RT-PCR products produced by the kits. The positive control reaction should give a 327 bp fragment, and no product is detectable in the -RT Reaction (Figure 3). If a PCR product is detected in the -RT control reaction, it is due to either the contamination of genomic DNA or a carry-over PCR product (see troubleshooting guide).

	Positive Control	-RT Control
10X RT Buffer	2 µl	2 µl
Murine RNase Inhibitor	0.5 µl	0.5 µl
Rat Liver Total RNA (500 ng/µl)	1 µl	1 µl
dT_{23} VN (50 µM)	2 µl	2 µl
dNTP mix (2.5 mM)	4 µl	4 µl
M-MuLV Reverse Transcriptase	1 µl	–
Nuclease-free H_20	9.5 µl	10.5 µl
Total Volume	20 µl	20 µl

2. Mix well by pipetting and incubate at 42°C for one hour.
3. Inactivate the reverse transcriptase by heating at 80°C for 5 minutes.
4. Dilute the cDNA by adding 30 µl H_2O, and add the diluted DNA to the following PCR reaction:

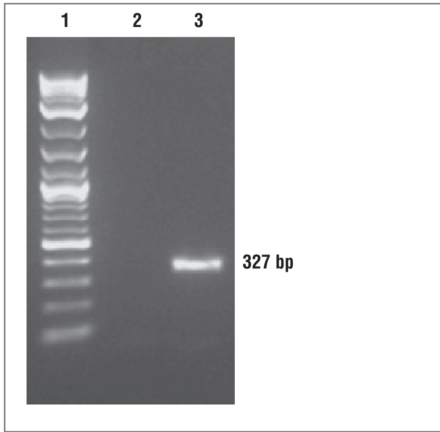
Taq 2X Master Mix	25 µl
GAPDH Primer Set (10 µM)	1 µl
Diluted cDNA	2 µl
H_20	22 µl
total volume	50 µl

The following PCR cycling conditions are recommended:

INITIAL DENATURATION	94°C	2 MINUTES
30 Cycles	94°C 55°C 68°C	30 seconds 15 seconds 30 seconds
Final Extension	68°C	5 minutes

7. Analyze 5 µl of the reaction on a 1% agarose gel, stained with ethidium bromide.

Figure 3:



Analysis of control reactions on a 1% agarose gel. Lane 1: 2-log DNA Ladder, Lane 2: 5 µl of the -RT control reaction, Lane 3: 5 µl of the positive control reaction.

Troubleshooting Guide:

Problem	Suggestion
Low yield of cDNA	First, try the control reactions. If the control RNA and GAPDH reactions produce the expected signals, it indicates that RT-PCR reagents and procedure are good.
	RNA of low quality is the most common reason of failed RT-PCR reaction. 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. 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 followed by ethanol precipitation can remove contaminant proteins such as proteases (3).
	Some target RNA may contain strong pauses for RT; Use random priming instead of $dT_{23}VN$.
	Detection of rare transcripts may fail due to insufficient amount of RNA. Increase the amount of RNA template.
Low yield of PCR product	Check the primer design using computer software. Good primers typically have 40-60% GC content, matching Tm and no self-complementary regions.
	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 concentrations between 0.07 µM to 0.2 µM may improve specificity.
	Increase number of cycles (up to 45).
	Optimize the Mg^{2+} concentration.
	Do a manual hot-start.
	Use thin-wall 0.2 ml PCR tubes.

Problem	Solution
Low yield of PCR product (cont.)	Check your reagents with the control reaction for GAPDH.
	Try a touch-down PCR protocol (4).
Products of wrong size	The presence of a PCR product larger than expected is often due to the contamination of genomic DNA. Treat with DNase I prior to cDNA synthesis (5).
	The presence of a PCR product of the correct size in the -RT negative control is either due to contaminating genomic DNA or carryover PCR product. Use separate areas for reaction assembly and product analysis. Use primers spanning an exon-exon boundary.
	Non-specific PCR products can be eliminated by optimizing PCR reactions. This involves the following: (1) check your primers with a computer program, (2) increase annealing temperatures in 1°C increments, and (3) lower primer concentration to 75 nm.

References:

1. Nam, D.K. et al. (2002) *PNAS* 99, 6152–6156.
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, E. et al. (2005) *BMC Molecular Biology* 6, 9.

Appendix:

Supplied Components:

10X RT Buffer
500 mM Tris-HCl (pH 8.3 @ 25°C)
750 mM KCl
30 mM $MgCl_2$
100 mM DTT

dNTP Mix
2.5 mM dATP
2.5 mM dGTP
2.5 mM dTTP
2.5 mM dCTP

M-MuLV Reverse Transcriptase
10 units/µl in:
150 mM NaCl
50 mM Tris-HCl (pH 7.6)
1 mM DTT
0.1 mM EDTA
2% NP-40
50% glycerol

***Taq* 2X Master Mix**
20 mM Tris-HCl (pH 8.6)
100 mM KCl
3 mM $MgCl_2$
0.4 mM dNTPs each
10% glycerol
1 mM DTT
0.16% NP-40
0.1% Tween-20
50 units/ml *Taq* DNA Polymerase

GAPDH Control Primers

Primer	Concentration
Sense: 5´ TGC (A/C)TC CTG CAC CAC CAA CT 3´	10 µM
Anti-sense: 5´ (C/T)GC CTG CTT CAC CAC CTT C 3´	10 µM

Ordering Information

PRODUCT	NEB #	SIZE
ProtoScript M-MuLV *Taq* RT-PCR Kit	E6400S	30 reactions
COMPANION PRODUCTS		
M-MuLV Reverse Transcriptase	M0253S/L	10,000/50,000 units
Taq 2X Master Mix	M0270S/L	100/500 reactions
Murine RNase Inhibitor	M0314S/L	3,000/15,000 units
M-MuLV Reverse Transcriptase Reaction Buffer	B0253S	6 ml
Oligo $d(T)_{23}VN$	S1327S	1.0 A_{260} units
Random Primer Mix	S1330S	1.0 A_{260} units
RNA Ladder	N0362S	25 gel lanes
1 kb DNA Ladder	N3232S/L	200/1,000 gel lanes
100 bp DNA Ladder	N3231S/L	100/500 gel lanes
Deoxynucleotide Solution Mix	N0447S/L	8/40 µmol of each
Taq DNA Polymerase with Standard *Taq* Buffer	M0273S/L	400/2,000 units
LongAmp *Taq* DNA Polymerase	M0323S/L	500/2,500 units
Crimson *Taq* DNA Polymerase	M0324S/L	200/1,000 reactions
Quickload *Taq* 2X Master Mix	M0271S/L	100/500 reactions
Vent DNA Polymerase	M0254S/L	200/1,000 units

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