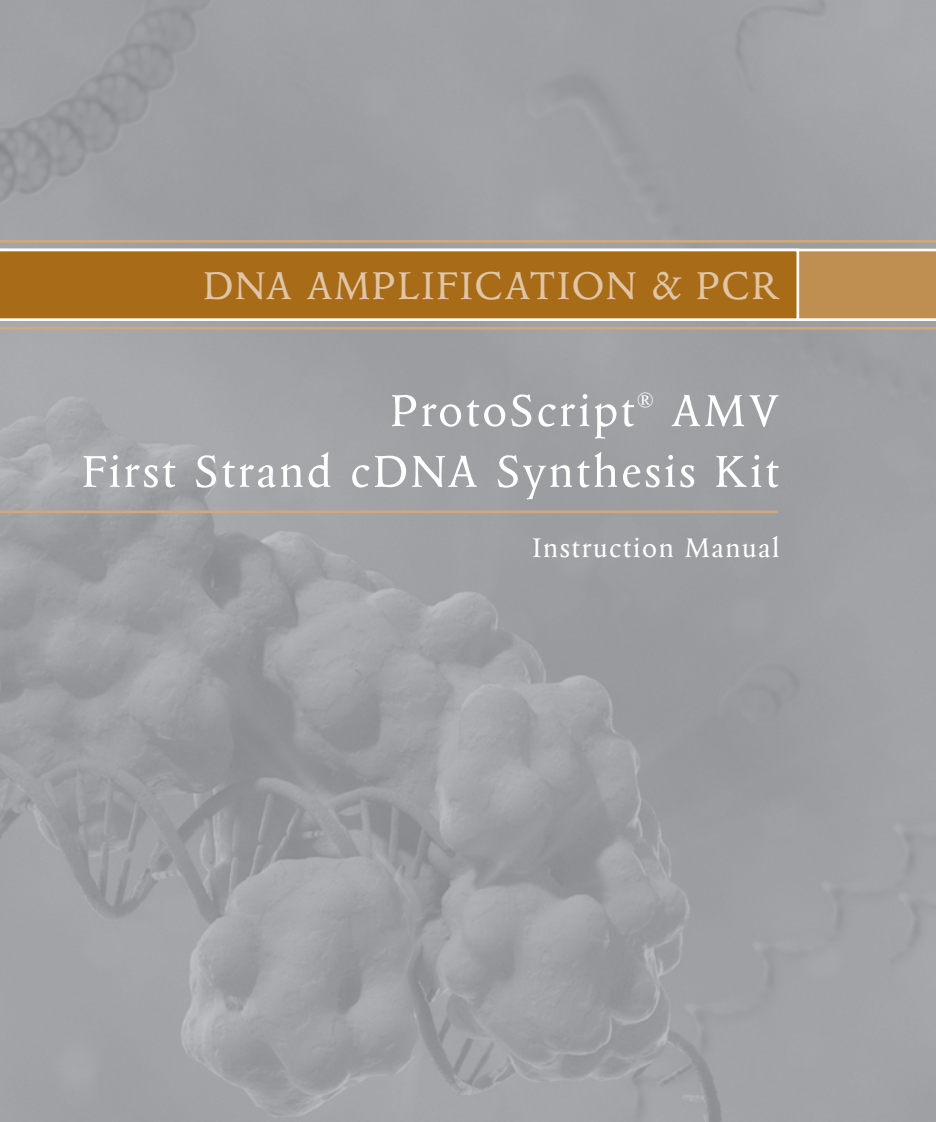
DNA AMPLIFICATION & PCR

ProtoScript® AMV First Strand cDNA Synthesis Kit

Instruction Manual



NEB #E6550S
Store at –20°C

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Kit Components:

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

AMV Enzyme Mix (10X)	60 µl
AMV Reaction Mix (2X)	300 µl
Oligo $d(T)_{23}VN$* (50 µM)**	70 µl
Random Primer Mix (60 µM)**	70 µl
Nuclease-free H_2O	1 ml

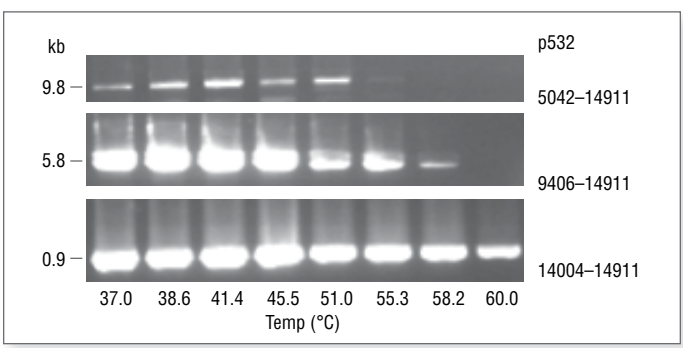
*V = A,G or C; N = A, G, C or T
**Contains 1 mM dNTP

Introduction:

The ProtoScript® AMV First Strand cDNA Synthesis Kit combines AMV Enzyme Mix and AMV Reaction Mix. The AMV Enzyme Mix is an optimized blend of AMV Reverse Transcriptase and a Murine RNase Inhibitor. AMV Reverse Transcriptase provides first strand cDNA synthesis reactions with a broader optimal reaction temperature from 37°C to 50°C. The murine RNase inhibitor is less sensitive to oxidation than the human RNase inhibitor, leading to better protection of RNA templates. The AMV Reaction Mix is an optimized buffer including dNTP. In the presence of the 1X AMV Reaction Mix, the AMV Enzyme Mix produces long cDNA products with high yield (up to 10 kb).

The first strand cDNA products generated by AMV Enzyme Mix are suitable for downstream gene cloning, quantitative analysis by standard PCR or real-time PCR.

Figure 1: Temperature profile of AMV



2 Units of AMV were used in 20 µl first-strand cDNA synthesis reactions using 1 µg human spleen total RNA. Reaction time was 1 hour at temperatures shown. About 1/10 of the cDNA product was amplified using LongAmp™ Taq 2X Master Mix. The p532, guanidine exchange factor (15.14 kb) fragments amplified are indicated.

Quality Controls:

The performance of ProtoScript AMV First Strand cDNA Synthesis 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 Protocols

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

1. Mix RNA sample and primer $d(T)_{23}VN$ in two sterile RNase-free microfuge tubes.

Total RNA	1–6 µl (10 pg–1 µg)
$d(T)_{23}VN$ (50 µM)	2 µl
nuclease-free H_2O	variable
Total Volume	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.

AMV Reaction Mix	10 µl
AMV Enzyme Mix	2 µl

To the negative control tube, add the following:

AMV Reaction Mix	10 µl
H_2O	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 min 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 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.

General Information for Successful cDNA Synthesis:

Template RNA

Intact RNA of high purity is essential for sensitive RT-PCR detection. RNA should have a minimum A_{260}/A_{280} ratio of 1.7 or higher.

Either total RNA or mRNA can be used in the reverse transcription reaction. Total RNA is generally sufficient for most RT-PCR analyses. However, if desired mRNA can be easily obtained using a PolyA Spin mRNA Isolation Kit (NEB #S1560) or Magnetic mRNA Isolation Kit (NEB #S1550).

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 0.1–100 ng mRNA are recommended.

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, 5 minutes incubation is enough for a 2 kb cDNA synthesis.

Higher reaction temperature up to 60°C can be used for difficult targets of high secondary structures.

Choice of Primers for Reverse Transcription:

Oligo d(T) 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-d(T) 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 concentration:

PRIMER	OLIGO $d(T)_{23}VN$	RANDOM PRIMER MIX	SPECIFIC PRIMER
Final conc.	5 µM	6 µM	0.1–1 µM

Downstream Applications Following cDNA Synthesis:

The first strand cDNA products are suitable for following applications:

- Standard PCR
- Cloning
- Real-time PCR

Troubleshooting Guide:

Problem	Suggestion
Low Yield of cDNA	Check the integrity of the RNA by denaturing agarose gel electrophoresis (2).
	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 (2).
	Phenol/chloroform extraction and ethanol extraction can remove contaminant proteins such as proteases (2).
	Some target RNA may contain strong pauses for RT; Use random priming instead of $d(T)_{23}VN$.
	Use sufficient amount of RNA.

References:

1. Liao, J. and Gong, Z. (1997) *Biotechniques* 23, 368–370.
2. Sambrook, J. and Russel, D.W. (2001). *Molecular Cloning: A Laboratory Manual,* (3rd ed.), (pp. 8.46–8.53 and 11.37–11.42). Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Appendix:

Supplied Components:

1X AMV Enzyme Mix

0.1 unit/µl AMV Reverse Transcriptase
1 unit/µl Murine RNase Inhibitor

1X AMV Reaction Mix

50 mM Tris-Acetate (pH 8.4)
75 mM KOAc
10 mM $Mg(OAc)_2$
2.5 mM DTT
1 mM dNTPs each

Ordering Information

PRODUCT	NEB #	SIZE
ProtoScript® AMV First Strand cDNA Synthesis Kit	E6550S	30 reactions
COMPANION PRODUCTS		
polyA Spin mRNA Isolation Kit	S1560S	8 isolations
AMV Reverse Transcriptase	M0277S/L	200/1,000 units
Magnetic mRNA Isolation Kit	S1550S	25 isolations
Murine RNase Inhibitor	M0314S/L	3,000/15,000 units
Oligo $d(T)_{23}$Magnetic Beads	S1419S	25 mg
Random Primer Mix	S1330S	1.0 A_{260} units
LongAmp® *Taq* 2X Master Mix	M0287S/L	100/500 reactions
ProtoScript® M-MuLV *Taq* RT-PCR Kit	E6400S	30 reactions
Oligo $d(T)_{23}$VN	S1327S	1.0 A_{260} units

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