3050 Spruce Street
Saint Louis, Missouri 63103 USA
Telephone 800-325-5832 • (314) 771-5765
Fax (314) 286-7828
email: techserv@sial.com
sigma-aldrich.com

ProductInformation

Enhanced Avian RT First Strand Synthesis Kit

Product Number **STR-1**
Technical Bulletin No. MB-555
Storage Temperature –20 °C

TECHNICAL BULLETIN

Product Description
Sigma's Enhanced Avian First Strand Synthesis Kit utilizes a highly purified avian myeloblastosis virus reverse transcriptase (eAMV-RT) that offers superior performance to standard AMV-RT or standard Moloney murine leukemia virus reverse transcriptase (MMLV-RT). This exceptionally robust eAMV-RT has an enhanced ability to transcribe through difficult secondary structure at elevated temperatures (up to 65 °C)[1,2] making it the ideal enzyme for producing high quality full-length cDNA from total RNA or poly$(A)^+$ RNA with difficult secondary structure. eAMV-RT is also very efficient at transcribing targets up to 9 kb in length.[3]

Sigma's Enhanced Avian RT First Strand Synthesis Kit also provides random nonamers (9-mers) and anchored oligo $(dT)_{23}$ primers, since gene specific primers may not always be useful or possible. The anchored oligo has 23 thymidine residues and one G, C, or A residue (the anchor). The anchor insures that the oligo (dT) primer binds at the very beginning of the message and that there is not a long region of unusable sequence.

Note: A control RNA template and primers were previously provided with the Enhanced Avian RT First Strand Synthesis Kit. The RNA template is no longer commercially available and has been discontinued. Customers that wish to run a control reaction along with their tests may run a human β-actin positive control using the following primer sequences:

Forward Primer
5'- TGC GTG ACA TTA AGG AGA AG-3'
Reverse Primer
5'- CTG CAT CCT GTC GGC AAT G-3'

The expected product size is 316 bp.

Enhanced Avian RT Unit Definition: One unit incorporates one nanomole of TMP into TCA precipitable material in 10 minutes using polyadenylic acid as template and oligo $(dT)_{12-18}$ as a primer.

Reagents Provided
The kit is sufficient for 50 reactions.

- Deoxynucleotide Mix, Product Code D 7295, 50 µl
  10 mM dATP, 10 mM dCTP, 10 mM dTTP, 10 mM dGTP
- Random Nonamers, Product Code R 7647, 100 µl
  50 µM in water
- Anchored Oligo $(dT)_{23}$, Product Code O 4387 100 µl
  0.5 µg/µl (70 µM) in water
- RNase Inhibitor, Product Code R 1274, 50 µl
  20 units/µl
- Enhanced Avian Reverse Transcriptase 1,000 units
  Product Code A 4714
  Solution in 200 mM KH_2PO_4, pH 7.2, 2 mM DTT, 0.2% Triton, 50% glycerol, 20 units/µl, 50 µl
- 10X Buffer for eAMV Reverse Transcriptase, 1.5 ml
  Product Code B1175, 500 mM Tris-HCl, pH 8.3, 400 mM KCl, 80 mM $MgCl_2$, 10 mM DTT
- Water, PCR Reagent, Product Code W 1754 1.5 ml

Items Required but not Provided
- RNA to be transcribed and amplified
- Specific primers for RT (optional, see Primer Design under Preliminary Considerations)
- Dedicated pipettes
- Aerosol resistant pipette tips
- 0.5 or 0.2 ml thin-walled PCR tubes, Product Codes P 3114 and P 3364
- Thermal cycler
- JumpStart AccuTaq LA DNA Polymerase Mix, Product Code D 5809

Precautions and Disclaimer
Sigma's First Strand Synthesis Kit is for R&D use only. Not for drug, household or other uses. Warning statements are included on the label or in the Reagents Provided section of this bulletin where applicable. In addition, when using radioactively labeled nucleic acids, standard procedures for safely handling radioactive materials should be followed.

Storage
All components should be stored at –20 °C.

Preliminary Considerations
RNA Preparation
The single most important step in assuring successful reverse transcription is high quality RNA preparation. Integrity and purity of RNA template is essential. Either total or poly$(A)^+$ RNA can be used as template for the reverse transcription reaction. All RNA preparations should be DNA-free to assure that product is derived from RNA. Amplification Grade DNase I (Product Code AMP-D1) is recommended for the digestion of contaminating DNA in the RNA preparation before the first strand synthesis reaction. The minimum amount of RNA that can be amplified is both primer and template dependent. For total RNA or poly$(A)^+$ RNA, amplified product is obtained using as little as 10-100 pg of starting material, depending on number of RNA copies present.

Primer Design
Sigma's First Strand Synthesis kit allows the user to choose the desired primer based upon experimental design or personal preference. For the RT reaction, the choices are specific primer (user defined), anchored oligo $(dT)_{23}$ primers or random nonamers.

The reverse PCR primer is routinely used as the specific primer for RT. This primer transcribes only specific sequence to which it is homologous. Random nonamers and anchored oligo $(dT)_{23}$ are provided with the kit as alternatives for first strand synthesis, cDNA library construction and other applications. They can be used separately or together. Specific primers for RT and PCR should be designed with the aid of primer design software to eliminate the complications introduced with primer-dimers and secondary structures. In addition, selection of primers that span an intron will greatly reduce the possibility of amplifying from the genomic DNA. This will also allow genomic amplification products to be identified by their larger size.

Procedure
I. Reverse Transcription
The optimal conditions for the concentration of Enhanced AMV Reverse Transcriptase, template RNA, and primers will depend on the system being utilized and should be determined empirically.

1. Add the following reagents to a thin-walled 200 µl or 500 µl PCR microcentrifuge tube on ice:

Volume	Reagent	Final Concentration
x µl	RNA template	In general 0.005-0.25 µg/µl total RNA or desired amount of poly $(A)^+$ RNA
1 µl	Deoxynucleotide mix	500 µM each dNTP
1 µl	Random nonamers -or-	2.5 µM (In general, use between 1-4 µM)
	3' Antisense specific primer -or-	1 µM (In general, use between 0.5-1 µM)
	Oligo $(dT)_{23}$	3.5 µM (In general, use between 1-3.5 µM)
--- µl	Water, PCR reagent	q.s.
10 µl	Total Volume	

2. Mix gently and briefly centrifuge to collect all components to the bottom of the tube.

3. Place tube in the thermal cycler at 70 °C for 10 minutes.

   Note: This 70 °C incubation step before the reverse transcription reaction is optional. This step may denature RNA secondary structure, which will allow for more efficient reverse transcription. All of the reverse transcription components may be added together in one tube and placed immediately at the optimal reverse transcription temperature unless random primers are being used. If using random nonamers, a 15 minute incubation at 25 °C is required (see step 5).

4. Remove tube, place on ice, centrifuge and add the following components to the reaction:

Volume	Reagent	Final Concentration
2 µl	10X buffer for eAMV-RT	1X
1 µl	Enhanced avian RT	1 unit/µl
1 µl	RNase inhibitor	1 unit/µl
6 µl	Water	-------
20 µl	Total Volume	

   Note: Increased yield for longer templates may be obtained by increasing the concentration of deoxynucleotides from 500 µM to 1 mM for each dNTP.

5. Incubate the reaction tubes at 25 °C for 15 minutes if using random nonamers. If using oligo$(dT)_{23}$ or a specific primer, this step is not needed. This preincubation step allows these primers to be extended by the enhanced avian RT before incubating at a temperature between 42-50 °C.

6. Place tubes at a temperature between 42-50 °C for 50 minutes.

   Note: The optimal reaction temperature should be determined empirically. It is suggested that the reaction be run at a temperature between 42-50 °C initially. Raising the transcription reaction temperature incrementally (up to 65 °C) is recommended for transcribing templates with difficult secondary structure. If the transcription reaction is run at elevated temperatures, a drop in yield may occur.

7. The first strand cDNA is now ready for subsequent PCR amplification.

II. PCR amplification of the cDNA using JumpStart AccuTaq LA DNA polymerase Mix

The amplification parameters will vary depending on the primers and the thermal cycler used. It may be necessary to optimize the system for individual primers, template, and thermal cycler.

1. Add the following reagents to a thin-walled 200 µl or 500 µl PCR microcentrifuge tube in the following order:

Volume	Reagent	Final Concentration
--- µl	Water, PCR reagent	q.s.
5 µl	10X PCR buffer	1X
1 µl	Deoxynucleotide mix	200 µM each dNTP
x µl	PCR Primers	Approximately 0.4 µM each
1 µl	JumpStart AccuTaq LA DNA Polymerase (2.5 units/µl)	0.05 units/µl
1-5 µl	Template DNA (cDNA) from RT reaction	------

50 µl Total Volume

   Note: If amplifying transcripts up to 3 kb, JumpStart Taq DNA polymerase (Product Codes D 9307 or D 4184) may be used instead of JumpStart AccuTaq LA DNA polymerase.

2. Mix gently by vortex and briefly centrifuge to collect all components to the bottom of the tube.

3. The amplification parameters will vary depending on the primers and the thermal cycler used. It may be necessary to optimize the system for individual primers, template and thermal cycler

Cycling parameters that have been found optimal for a 316 bp fragment of β-actin transcribed from Human Total RNA (cell line HeLa S-3) are as follows:

Step	Temperature	Time	Cycles
Denaturation/ RT Inactivation	94 °C	2 min	
Denaturation	94 °C	15 sec	
Annealing	55 °C	30 sec	30 cycles
Extension	72 °C	1 min	
Final Extension	72 °C	5 min	1 cycle
Hold	4 °C		

Note: Extension times will vary depending upon the length of products being amplified. Times can be estimated using 1 minute/kilobase. These cycling parameters were optimized using a PE9700 thermal cycler. A 45 second or longer incubation time is needed at each temperature if using Stratagene's RoboCycler, which has no ramp time.

4. Evaluate the PCR product by sequencing or agarose gel electrophoresis and subsequent ethidium bromide staining.

References

1. Eastlund, E., and Song, K., Sigma's new enhanced avian RT-PCR Kit, Sigma-Aldrich Corporation's Life Science Quarterly*,* **1**, 15-17, (2000).

2. Brooks, E.M., et al. Secondary structure in the 3' UTR of EGF and the choice of reverse transcriptases affect the detection of message diversity by RT-PCR. Biotechniques **19,** 806-812 (1995).

3. Eastlund, E., and Mueller, E., Hot Start RT-PCR results in improved performance of the enhanced Avian RT-PCR, Sigma-Aldrich Corporation's Life Science Quarterly, **2**, 2-5, (2001).

Related Products

RNA Isolation

GenElute™ Mammalian Total RNA Purification Kit, for isolating total RNA from tissue or cells, Product Codes RTN10, RTN70 and RTN350
GenElute Direct mRNA Miniprep Kit, for isolating mRNA from cells or tissue, Product Codes DMN10 and DMN70
GenElute mRNA Miniprep Kit, for isolating mRNA from total RNA, Product Codes MRN10 and MRN70
TRI Reagent®, for isolating total RNA from Tissue Product Code T 9424
TRI Reagent® BD, for isolating total RNA from whole blood, Product Code T 3809
TRI Reagent® LS, for isolating total RNA from fluid samples, Product Code T 3934
RNaseZAP®, a cleaning product for removing RNase from laboratory surfaces, Product Code R 2020
Deoxyribonuclease I, amplification grade, for removing DNA from RNA preps, Product Code AMP-DI
RNA*later*™ for long-term RNA storage, Product Code R 0901

PCR Products

Product Code D5809, JumpStartAccuTaq LA DNA Polymerase Mix with 10X reaction buffer containing $MgCl_2$
Product Code D9307, JumpStart Taq DNA Polymerase with 10X reaction buffer containing $MgCl_2$
Product Code D4184, JumpStart Taq DNA Polymerase with 10X reaction buffer without $MgCl_2$
Product Code. GEN-PCR, GenElute PCR DNA Purification Kit for purification of PCR products
Product Code OPT-2, PCR Optimization Kit II

RT-PCR Products

Enhanced Avian RT-PCR Kits for both one-step and two-step RT-PCR reactions, Product Codes HS-RT100 (100 reactions) and HS-RT20 (20 reactions)

†The PCR process is covered by patents owned by Hoffman-LaRoche, Inc.

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