3050 Spruce Street, St. Louis, MO 63103 USA
Tel: (800) 521-8956 (314) 771-5765 Fax: (800) 325-5052 (314) 771-5757
email: techservice@sial.com sigma-aldrich.com

Product Information

Enhanced Avian HS RT-PCR Kit

Catalog Numbers **HSRT20, HSRT100**

TECHNICAL BULLETIN

Product Description
The Enhanced Avian HS RT-PCR Kit combines two powerful and versatile techniques used to convert mRNA transcripts into cDNA and to subsequently amplify the resulting cDNA. The methods are most often used to detect the presence or absence of mRNA transcripts or to amplify specific RNA sequences for cloning, sequencing, and many other applications.

Sigma's Enhanced Avian HS RT-PCR Kit is designed to be flexible and versatile, allowing the user variable applications depending on the individual experiment. The system utilizes an enhanced avian myeloblastosis virus reverse transcriptase (eAMV-RT) enzyme that offers superior performance to standard AMV-RT and Moloney murine leukemia virus reverse transcriptase (MMLV-RT). JumpStart™ AccuTaq™ LA DNA polymerase mix is provided to produce a specific high fidelity second strand cDNA and subsequent PCR amplification. The combination of these two enzymes makes it possible to detect very small amounts of mRNA from just a few cells. Procedures are provided for a one-step RT-PCR reaction and a two-step RT-PCR reaction. These two procedures allow for maximum flexibility in the experimental design. The one-step RT-PCR reaction combines the eAMV-RT with the JumpStart AccuTaq LA. In a single tube, transcription occurs to produce cDNA followed immediately by amplification using PCR. This provides quick, sensitive analysis of RNA. In the two-step reaction, each reaction is optimized for maximum results, giving greater yields with high fidelity.[1] Sigma's eAMV-RT has the ability to transcribe through complex secondary structure at elevated temperatures (up to 65 °C)[2] making it the ideal enzyme for producing high quality full-length cDNA from total RNA or poly(A)$^+$ RNA with complex secondary structure. eAMV-RT is also very efficient at transcribing targets up to 9 kb in length.[3]

JumpStart AccuTaq LA DNA Polymerase Mix is a specially blended enzyme mix for robust, high fidelity long and accurate (LA) PCR. It is an optimal blend of Sigma's Taq DNA polymerase and a small amount of a proofreading DNA polymerase, along with JumpStart Taq antibody that provides a hot start mechanism. This blend increases the specificity, accuracy, length, and yield of the amplified product. JumpStart AccuTaq LA is the ideal enzyme for amplifying first strand cDNA from 0.5 to 15 kb in length.[1] The increased fidelity (up to 6.5 times greater than that of standard Taq DNA polymerase) ensures the highest quality product.

When sequence information is incomplete or absent, or other instances where specific primers are not useful, random nonamers (9-mers) and anchored oligo $(dT)_{23}$ included in the kit may be used, either alone or together. An anchored oligo has 23 thymidine residues and one G, C or A residue (the anchor). This anchor insures that the oligo (dT) primer binds at the very beginning of the message and there is not a long region of unusable sequence. This provides the user with the most flexibility in choice of primer and RT-PCR experimental design.

Note: A control RNA template and primers were previously provided with the Enhanced Avian HS RT-PCR 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.

Reagents Provided	Catalog Number	HSRT20 20 Rxn	HSRT100 100 Rxn
Deoxynucleotide Mix (10 mM dATP, 10 mM dCTP, 10 mM dGTP, 10 mM dTTP)	D7295	50 µL	200 µL
Random Nonamers, 50 µM in water	R7647	100 µL	100 µL
Anchored Oligo $(dT)_{23}$, 0.5 µg/µL in water	O4387	100 µL	100 µL
RNase Inhibitor, 20 U/µL	R1274	25 µL	100 µL
Enhanced AMV Reverse Transcriptase, 20 U/µL in 200 mM KH_2PO_4, pH 7.2, 2 mM DTT, 0.2% Triton, 50% glycerol	A4714	400 U	2 x 1,000 U
10x Buffer for AMV RT, 500 mM Tris-HCl, pH 8.3, 400 mM KCl, 80 mM $MgCl_2$, 10 mM DTT	B1175	1.5 ml	1.5 ml
JumpStart AccuTaq LA DNA Polymerase, 2.5 U/µL in 20 mM Tris-HCl, pH 8.0, 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5% TWEEN® 20, 0.5% IGEPAL® CA-630, 50% glycerol	D5559	50 U	2 x 125 U
10x AccuTaq Buffer, 50 mM Tris-HCl, 150 mM ammonium sulfate, pH 9.3, 25 mM $MgCl_2$, 1% TWEEN 20	B0174	0.5 ml	3 x 0.5 ml
10x PCR Buffer, 100 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.01% gelatin	P2192	1.5 ml	1.5 ml
25 mM $MgCl_2$	M8787	1.5 ml	1.5 ml
PCR Reagent Water	W1754	1.5 ml	4 x 1.5 ml

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.

JumpStart AccuTaq LA Unit Definition: One unit incorporates 10 nmol of total dNTPs into acid precipitable DNA in 30 minutes at 74 °C.

Items Reagents Required but Not Provided

- RNA to be transcribed and amplified
- Specific primers for RT and PCR
- Dedicated pipettes
- Aerosol resistant pipette tips
- 0.5 ml or 0.2 ml thin-walled PCR tubes, Catalog Numbers P3114 and P3364
- Thermal cycler
- Dimethyl sulfoxide, Catalog Number D8418 (optional)

Precautions and Disclaimer

Sigma's Enhanced Avian HS RT-PCR Kit is for R&D use only. Not for drug, household, or other uses. Please consult the Material Safety Data Sheet (MSDS) for information regarding hazards and safe handling practices.

Storage/Stability

All components should be stored at –20 °C.

Preparation Instructions

RNA Preparation

The single most important step in assuring success with RT-PCR is high quality RNA preparation. Integrity and purity of RNA template is essential. Either total or poly(A)$^+$ RNA can be used as the 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 the number of copies present.

Primer Design

Sigma's Enhanced Avian HS RT-PCR 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 $d(T)_{23}$ primers, or random nonamers.

The reverse PCR primer is routinely used as the specific primer for RT. This primer transcribes only the specific sequence to which it is homologous. Random nonamers and anchored oligo $(dT)_{23}$ are provided as alternatives for first strand synthesis, cDNA library construction, and other applications. 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.

One-Step or Two-Step Reaction

The **one step method** is recommended for systems where specific primers are used. Addition of the nonamers and/or oligo (dT) may enhance the yield of the cDNA. Random nonamers will not affect the PCR due to their decreased ability to prime at increased temperatures (up to 65 °C). Quick results, with less potential for contamination can be achieved.
Because the RT and PCR reactions can be optimized separately, the **two step method** may be desirable for amplification of less characterized sequences, rare sequences, or problematic templates. A specific primer, random nonamers and oligo (dT) can be used separately or together in the RT reaction of the **two-step method.** For a diverse and nonspecific priming of the RNA sample in the RT reaction of the **two-step method**, random nonamers and oligo (dT) may be combined.

Buffer preparation

The 10x buffer for AccuTaq LA DNA polymerase mix is at a relatively high pH, and magnesium may precipitate as $Mg(OH)_2$. Before use, thaw the buffer at room temperature, then vortex to redissolve any precipitated magnesium hydroxide. Alternatively, warm the buffer at 37 °C for 3-5 minutes, then vortex.

Procedure

The optimal conditions for the concentration of enhanced AMV Reverse Transcriptase, JumpStart AccuTaq LA DNA polymerase, $MgCl_2$, template RNA, primers, and amplification parameters will depend on the system being utilized and should be determined empirically.

One-Step RT-PCR Reaction

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

Volume	Reagent	Final Concentration
q.s.	Water, PCR reagent	
5 µL	10x PCR buffer (Note: Do not use 10x AccuTaq buffer)	1x
3 µL	25 mM $MgCl_2$	3.0 mM (including $MgCl_2$ in PCR buffer*)
1 µL	Deoxynucleotide mix	200 µM each dNTP**
1 µL	RNase inhibitor	0.4 units/µL
1 µL	RNA template	2 pg/µl to 20 ng/µL total RNA or desired amount of poly $(A)^+$ RNA
1 µL	Specific primers	0.4-1 µM each primer
1 µL	eAMV-RT	0.4 units/µL***
1 µL	JumpStart AccuTaq LA DNA polymerase	0.05 units/µL
50 µL	Total Volume	

* **Note**: The recommended magnesium chloride concentration in the one-step RT-PCR control reaction is 3 mM. Magnesium chloride concentration may need to be experimentally optimized for the one-step RT-PCR reaction.
** **Note**: Yields for one-step RT-PCR products under 1 kb may also be enhanced by increasing the concentration of deoxynucleotides from 200 µM to 400 µM for each dNTP.
*** **Note**: If using decreased amounts of RNA template, we recommend diluting the eAMV-RT 10-fold in 1x PCR buffer and using 0.04 units/µl per reaction.

2. Mix gently by vortex and briefly centrifuge to collect all components at 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:

First Strand Synthesis	42-50°C	50 min.
Denaturation/ RT Inactivation	94 °C	2 min.
For Cycles 1-35:		
Denaturation	94 °C	15 sec.
Annealing	55 °C	30 sec.
Extension	68 °C	1 min.
Final Extension	68 °C	5 min.
Hold	4 °C	

   Note: 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. Extension times will vary depending upon the length of products being amplified. Times can be estimated using 1 minute per kilobase. If yields are lower than expected, increase the extension time by 1 additional minute per kb to be amplified. Increasing the extension temperature to 72 °C may also increase the yield for one-step RT-PCR products under 1 kb.

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

Two-Step RT-PCR Control Reaction

In a two-step reaction, transcription is optimized for yield and full-length. This is accomplished by utilizing the optimized RT buffer included in the kit. An aliquot can then be amplified with the JumpStart AccuTaq LA DNA polymerase mix. If re-amplification of the first strand cDNA is necessary, another aliquot from the initial RT reaction can be taken. The choice of primer is up to the individual user based upon preference and past performance.

I. Enhanced Avian Reverse Transcription Reaction for Two-Step RT-PCR

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

Volume	Reagent	Final Concentration
q.s.	Water, PCR reagent	
x µL	RNA template	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)
	Anchored oligo $(dT)_{23}$	3.5 µM (In general, use between 1-3.5 µM)
10 µL	Total volume	

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

3. Place the 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 the tube, place on ice, centrifuge and add the following components to the reaction:

Volume	Reagent	Final Concentration
6 µL	Water, PCR reagent	-------
2 µL	10x buffer for AMV-RT	1x
1 µL	RNase inhibitor	1 U/µL
1 µL	Enhanced avian RT	1 U/µL
20 µL Total Volume		

Note: Increased yield for longer templates may be obtained by increasing the concentration of deoxy-nucleotides 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 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. Raising the reverse transcription reaction temperature incrementally (up to 65 °C) is recommended for reverse transcribing templates with complex secondary structure. If the reverse transcription reaction is performed at elevated temperatures, a drop in yield may occur.

7. The first strand cDNA is now ready for subsequent PCR amplification, cloning, library synthesis, etc.

II. <u>PCR Amplification of Target cDNA using JumpStart AccuTaq LA DNA Polymerase for Two-Step RT-PCR</u>

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 on ice:

Volume	Reagent	Final Concentration
q.s.	Water, PCR reagent	----
5 µL	10x AccuTaq buffer	1x
1 µL	DMSO (optional)	2% (v/v)
1 µL	Deoxynucleotide mix	200 µM each dNTP
1 µL	PCR primers	Approximately 0.4 µM each
2-5 µL	Template DNA (cDNA) from RT reaction	-------
1 µL	JumpStart AccuTaq LA DNA polymerase mix	0.05 units/µL
50 µL	Total Volume	

Note: DMSO maintains the single stranded state longer during the elongation process. This is necessary for amplification of DNA that has a GC content >65%. DMSO is not required for use with DNA with a lower GC content.

2. Mix gently by vortex and briefly centrifuge to collect all components at 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:

Denaturation/ RT Inactivation	94 °C	2 min
For Cycles 1-35:		
Denaturation	94 °C	15 sec.
Annealing	55 °C	30 sec.
Extension	68 °C	1 min.
Final Extension	68 °C	5 min.
Hold	4 °C	

Note: Extension times will vary depending upon the length of products being amplified. Times can be estimated using 1 minute per 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 Mueller, E., Hot start RT-PCR results in improved performance of the enhanced avian RT-PCR system, Sigma-Aldrich Corporation's Life Science Quarterly, **2,** 2-5 (2001).
2. Eastlund, E., and Song, K., Sigma's new enhanced avian RT-PCR Kit, Sigma-Aldrich Corporation's Life Science Quarterly, **1,** 15-17 (2000).
3. 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).
4. Don, R.H., et al., 'Touchdown' PCR to circumvent spurious priming during gene amplification, *Nucleic Acids Res.*, **19,** 4008 (1991).

Troubleshooting Guide

Problem	Possible Cause	Solution
There is no first strand synthesis	The RNA is degraded.	Check the RNA by denaturing agarose gel electrophoresis. Poly $(A)^+$ RNA should appear as a smear between 0.5 kb and 2 kb. The total RNA should have two sharp ribosomal RNA bands without notable degradation. For purifying RNA, use RNA isolation kits (See Related Products section) or TRI Reagent, Catalog Numbers T9424, T3809, or T3934).
	eAMV-RT was thermally inactivated.	Add the eAMV-RT to the reaction mix after the initial primer-template denaturation/annealing step.
	There is incomplete removal of guanidinium during RNA isolation.	For any procedure using guanidinium-based lysis solution, remove as much of the residual liquid as possible after the first precipitation and then wash once with 70% alcohol.
	There is incomplete removal of the protease (such as Proteinase K) during RNA isolation.	Proteases used during RNA isolation may be removed by phenol/chloroform extraction and alcohol precipitation.
	The first-strand primer is not designed optimally.	Verify the proper primer design. Consider that the greatest yield of first-strand product is often achieved with random oligonucleotide primers.
	The wrong first strand primer was used.	If using the anchored oligo (dT) primer, make sure that the RNA template has a polyadenylated tail. If using a specific primer, make sure that the 3′ antisense primer is used for first strand synthesis.
Low yield or no PCR product is observed.	A PCR component is missing or degraded.	A positive control should always be run to insure components are functioning. A checklist is also recommended when assembling reactions.
	There are too few cycles performed.	Increase the number of cycles (3-5 additional cycles at a time).
	The annealing temperature is too high.	Decrease the annealing temperature in 2-4 °C increments.

Troubleshooting Guide (continued)

Problem	Possible Cause	Solution
Low yield or no PCR product is observed (continued).	The primers are not designed optimally.	Confirm the accuracy of the sequence information and the specificity of the primer sequence to non-target sequences.
	There is not enough template.	After increasing the number of cycles has shown no success, repeat the reaction with a higher concentration of template.
	The template is of poor quality.	Evaluate the template integrity by agarose gel electrophoresis.
	The denaturation temperature is too high or too low.	Optimize the denaturation temperature by increasing or decreasing the temperature in 1 °C increments.
	The denaturation time is too long or too short.	Optimize the denaturation time by increasing or decreasing it in 10 second increments.
	The extension time is too short.	Increase the extension time in 2 minute increments, especially for long templates.
	The reaction does not have enough enzyme.	2.5 units are sufficient for most applications. It is recommended that the cycling parameters be optimized before the enzyme concentration is increased. In rare cases, the yields can be improved by increasing the enzyme concentration. However, if the enzyme concentration is above 5 units, higher background may be seen.
	Mg^{++} concentration is suboptimal.	Experimentally optimize the magnesium concentration.
	Deoxynucleotides are degraded.	Try to avoid multiple freeze/thaw cycles. Once thawed keep nucleotides on ice.
	The target template is complex.	In most cases, inherently complex targets are due to unusually high GC content and /or secondary structure. In some cases, the addition of 1-4% DMSO may help.
	(One-Step method) Inhibition of PCR by excess eAMV-RT.	Excess eAMV-RT in the one-step method can prevent PCR amplification. Dilute the eAMV-RT by a minimum of 10-fold using 1x PCR buffer, and use one-tenth the number of units per reaction.
Multiple Products	There are too many cycles performed.	By reducing the cycle number, the nonspecific bands may be eliminated.
	The annealing temperature is too low.	Increase the annealing/extension temperature in increments of 2-3 °C.
	The primers are not designed optimally.	Confirm the accuracy of the sequence information and the specificity of primer sequence to non-target sequences.
	Touchdown PCR may be required.[4]	"Touchdown" PCR significantly improves the specificity of many PCR reactions in various applications. Touchdown PCR uses an annealing/extension temperature that is higher than the T_m of the primers during the initial PCR cycles. The annealing/ extension temperature is then reduced to the primer T_m for the remaining PCR cycles. The change can be performed in a single step or in increments over several cycles.
The PCR product is the wrong size	Genomic DNA is contaminating the RNA template in the reverse transcriptase reaction.	Digest the RNA with RNase-free DNase (Product Code AMP-D1).
	The primers are not designed optimally.	Confirm the accuracy of the sequence information and the specificity of primer sequence to non-target sequences.
	Extension times are too short.	Increase the extension times in 2 minute increments or use touchdown PCR.

Related Products

RNA Isolation

GenElute™ Mammalian Total RNA Purification Kit, for isolating total RNA from tissue or cells, Catalog Nos. RTN10, RTN70 and RTN350

GenElute Direct mRNA Miniprep Kit, for isolating mRNA from cells or tissue, Catalog Nos. DMN10 and DMN70

GenElute mRNA Miniprep Kit, for isolating mRNA from total RNA, Catalog Nos. MRN10 and MRN70

TRI Reagent®, for isolating total RNA from Tissue Catalog Number T9424

TRI Reagent® BD, for isolating total RNA from whole blood, Catalog Number T3809

TRI Reagent® LS, for isolating total RNA from fluid samples, Catalog Number T3934

RNaseZAP®, a cleaning product for removing RNase from laboratory surfaces, Catalog Number R2020

Deoxyribonuclease I, amplification grade, for removing DNA from RNA preps, Catalog Number AMPDI

RNA*later*™ for long-term RNA storage, Catalog Number R0901

PCR Products

JumpStart AccuTaq LA DNA Polymerase Mix with 10x reaction buffer containing $MgCl_2$, Catalog Number D5809

GenElute PCR Clean-Up Kit, for purification of PCR products Catalog Number NA 1020

PCR Optimization Kit II, Catalog Number OPT2

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