Manual

MMLV Reverse Transcriptase 1st-Strand cDNA Synthesis Kit

For Research Use Only. Not for use in diagnostic procedures.

MMLV Reverse Transcriptase 1st-Strand cDNA Synthesis Kit is part of the Epicentre™ product line, known for its unique genomics kits, enzymes, and reagents which offer high quality and reliable performance.

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GENOMIC ANALYSIS BY LGC

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1. Introduction

The MMLV Reverse Transcriptase 1st-Strand cDNA Synthesis Kit is optimised for generating full-length first-strand cDNA from total cellular RNA or Poly(A) RNA-enriched samples. The kit features the LGC Biosearch Technologies™ high activity formulation MMLV Reverse Transcriptase and proprietary 10X RT Reaction Buffer which together are capable of synthesising full-lengthcDNA from mRNA templates longer than 15 kb.

2. Product designations and kit components

Product	Kit size	Catalogue number	Reagent description	Part number	Volume
MMLV Reverse Transcriptase 1st-Strand cDNA Synthesis Kit	50 rxns	MM070150	MMLV Reverse Transcriptase (50 U/µL)	E0045-50D	50 µL
			10X RT Reaction Buffer	SS000737-D1	100 µL
			RiboGuard RNase Inhibitor (40 U/µL)	E0126-40D5	30 µL
			DTT (100 mM)	SS000065-D5	125 µL
			dNTP Premix (5 mM each dNTP)	SS000753-D	100 µL
			Oligo(dT)$_{21}$ Primer (10 µM)	SS000754-D	100 µL
			Random Nonamers (9-mers; 50 µM)	SS000541-D	50 µL
			Nuclease-Free Water, Sterile	SS000772-D3	1 mL

3. Product specifications

Storage: Store only at -20 °C in a freezer without a defrost cycle.

Performance specifications and quality control: The MMLV 1st-Strand cDNA Synthesis Kit is function-tested in a control reaction using the Oligo(dT)$_{21}$ Primer. In this reaction, the kit converts 200 ng of an ~2 kb poly(A) RNA into full length cDNA in 30 minutes at 37 °C.

Additionally required reagents and equipment: Water bath or thermal cycler.

4. Preparation

Assessing the quality of the total RNA

The success of the cDNA synthesis reaction, and subsequent PCR reaction, is strongly influenced by the quality of the RNA. RNA quality has two components: purity of the RNA (or absence of contaminants) and integrity (intactness) of the RNA. RNA quality should be assessed prior to every cDNA synthesis reaction. RNA integrity can be assessed using an Agilent Bioanyzer using a Nano or Pico assay chip to determine the RNA Integrity Number or with a Tapestation or Alliance Analytical Fragment Analyzer.

RNA purification methods and RNA purity

Total cellular RNA, isolated by a number of methods, can be reverse transcribed successfully using the MMLV Reverse Transcriptase 1st-Strand cDNA Synthesis Kit. However, it is very important that

the purified RNA be free of salts, metal ions, ethanol and phenol, which can inhibit the enzymatic reactions performed in the reverse transcription process. Commonly used RNA extraction and purification methods that are compatible with the MMLV reverse transcription process include but are not limited to:

TRIzol®/TRI Reagent® (Molecular Research Center Inc.), a homogeneous solution of the powerful denaturants guanidinium isothiocyanate and phenol, is very effective at extracting the RNA from the cells. However all traces of guanidinium salts and phenol must be removed from the RNA sample prior to the RNA amplification process. If you precipitate the RNA from TRIzol-extracted cells, be sure to wash the RNA pellet at least two times with cold 70-75% ethanol to remove all traces of phenol and guanidinium salts. Air dry the RNA pellet (do not use a vacuum centrifuge) to remove residual ethanol. Then, resuspend the RNA in RNase-Free water. If you purify the RNA from TRIzol-extracted cells by column purification methods, please read the section "Spin Columns" below.

Spin columns (e.g., the Qiagen RNeasy® MinElute Cleanup Kit and RNeasy Mini Kit or Zymo Research RNAClean and Concentrator columns) are effective in purifying RNA samples that are free of the contaminants that may inhibit the reverse transcription reaction. Spin columns can be used with most RNA extraction procedures (e.g., TRIzol reagent). If using spin columns, follow the manufacturer's instructions closely, especially if an ethanol wash of the RNA is performed prior to the RNA elution step. Then, elute the RNA from the column membrane using RNase-Free water.

RNA integrity

Synthesis of full-length cDNA is dependent on an RNA sample that contains intact Poly(A) RNA. Presently, the most frequently used methods for assaying RNA integrity are by denaturing agarose gel electrophoresis or using an Agilent 2100 Bioanalyzer.

The advantages of denaturing agarose gel electrophoresis are its low cost and ready availability of the reagents required. Denaturing gel electrophoresis separates the RNAs by size (electrophoretic mobility) under denaturing conditions. Denaturing conditions are necessary to eliminate inter- and intra-molecular secondary structure within the RNA sample which may cause degraded RNA to appear intact. Following electrophoresis, the denaturing gel is stained with, for example, ethidium bromide, when using a eukaryotic RNA sample, the user looks for the highly stained 18S and 28S rRNAs. These bands should be sharp and discrete with an absence of smearing under either. Based on these visual observations, the user infers that the mRNA in the sample is equally intact. In a degraded RNA sample, the rRNA bands appear smeared. Ideally, the ethidium bromide stained 28S rRNA band should appear to be about twice as intense as the 18S rRNA band.

The Agilent 2100 Bioanalyzer is currently the preferred method for evaluating the integrity of an RNA sample. Like a denaturing gel, the bioanalyser separates the RNAs by size (electrophoretic mobility). However, in contrast to a denaturing gel, the 2100 Bioanalyzer consumes as little as 5 ng of total RNA per well when using the manufacturer's RNA 6000 Nano LabChip® (Caliper Technologies Corp.). When analyzing the RNA sample using the Agilent 2100 Bioanalyzer, the 18S and 28S rRNA species should appear as distinct, sharp peaks on the electropherogram. A slightly increased baseline, indicative of the 1-5% Poly(A) RNA contained in the sample, can be seen between the two peaks.

Maintaining an RNase-free environment

Ribonuclease contamination is a significant concern for those working with RNA. The ubiquitous RNase A is a highly stable and active ribonuclease that can contaminate any lab environment and is present on human skin. However, creating an RNase-free work environment and maintaining RNase-free solutions is critical for performing successful cDNA synthesis reactions. Therefore, we strongly recommend that the user:

1) Autoclave all tubes and pipette tips that will be used in the cDNA synthesis reactions.
2) Always wear gloves when handling samples containing RNA. Change gloves frequently especially after touching potential sources of RNase contamination such as door knobs, pens, pencils and human skin.
3) Always wear gloves when handling kit components. Do not pick up any kit component with an ungloved hand.
4) Keep all kit components tightly sealed when not in use. Keep all tubes containing RNA tightly sealed during the incubation steps.
   Additionally, the kit includes Biosearch Technologies' RiboGuard™ RNase Inhibitor which we strongly recommend be added to each reaction.

Choice of primer for first-strand cDNA synthesis

First-strand cDNA synthesis can be primed using either the Oligo(dT)$_{21}$ Primer or Random Nonamers (9-mers) that are provided in the kit or using gene-specific primers (provided by the user).

An oligo(dT) primer is the most commonly used method for priming first-strand cDNA synthesis from a eukaryotic RNA sample. Oligo(dT) primes cDNA synthesis from the poly(A) tail present at the 3′ end of most eukaryotic mRNAs. Priming cDNA synthesis with an oligo(dT) primer precludes the need to enrich the RNA sample for poly(A) RNA. We recommend using the Oligo(dT)$_{21}$ Primer for most applications.

Random Nonamers (9-mers) initiate cDNA synthesis from all RNA species (rRNA and mRNA) contained in a total cellular RNA sample. Since rRNA, which constitutes >95% of the RNA in a total RNA sample, is converted to cDNA using random primers, the complexity of the resulting cDNA will be much greater than when priming the reaction with the Oligo(dT)$_{21}$ Primer. The more complex cDNA sample can result in reduced sensitivity and specificity of the subsequent PCR. Random primers, however, can be helpful when:

1) Synthesising cDNA from eukaryotic mRNAs that lack a Poly(A) tail or have a very short Poly(A) tail.
2) Priming cDNA synthesis of a Poly(A)-enriched RNA sample.
3) Synthesising cDNA from partially degraded RNA samples such as those obtained from laser-captured cells or formalin-fixed paraffin-embedded tissue (FFPE) samples.
4) It is necessary to eliminate or reduce 3′-sequence bias that can result when using an oligo(dT) primer.
   Gene-specific primers, designed and synthesised by the user, provide the greatest specificity when priming cDNA synthesis of a specific mRNA. However, the user frequently must determine empirically the optimal primer annealling and extension (reverse transcription) conditions for each primer used.

RT-PCR: end-point or real-time PCR amplification and detection

The cDNA product of a MMLV Reverse Transcriptase 1st-Strand cDNA Synthesis Kit reaction can be used in either end-point (standard) or real-time quantitative PCR (qPCR). Detection of real-time PCR products is significantly more sensitive than detection of PCR products from end-point PCR. Therefore, the amount of total RNA to use in a MMLV Reverse Transcriptase 1st-Strand cDNA Synthesis Kit reaction may be dependent on the type of PCR the user intends to perform.

End-point PCR products are detected by agarose gel electrophoresis and staining with ethidium bromide or SYBR® Gold (Molecular Probes Inc.). We recommend using at least 10 ng of total RNA in the MMLV Reverse Transcriptase 1st-Strand cDNA Synthesis reaction when end-point PCR will be performed.

Real-time PCR is much more sensitive than end-point PCR. When performing real-time PCR, as little as 100 pg of total RNA, equivalent to 10 mammalian cells, can be used in the MMLV Reverse Transcriptase 1st-Strand cDNA Synthesis Kit reaction.

5. Procedure

The following protocol has been optimised to convert 100 pg to 1 µg of total cellular RNA to first-strand cDNA using the Oligo(dT)$_{21}$ Primer or Random Nonamers (9-mers) provided in the kit. Gene-specific primers (provided by the user) may require additional optimisation of the reaction.

MMLV 1st-strand cDNA synthesis

Gently mix and briefly centrifuge all kit components prior to dispensing.

1. Anneal the selected primer(s) to the RNA sample. For each 1st-strand cDNA synthesis reaction, combine the following components on ice, in a sterile (RNase-free) 0.2-mL or 0.5-mL tube:

x µL	Nuclease-Free Water, Sterile
x µL	Total RNA sample (up to 1 µg)
2 µL	Oligo(dT)$_{21}$ Primer (10 µM)
	- or -
1 µL	Random Nonamers (9-mers) (50 µM)
	- or -
x µL	Gene-specific primers
12.5 µL	Total reaction volume

2. Incubate at 65 °C for 2 minutes in a water bath or thermal cycler with heated lid.
3. Chill on ice for 1 minute. Centrifuge briefly in a microcentrifuge.

4. To each 1st-strand cDNA synthesis reaction, add on ice:
   - 2 µL 10X RT Reaction Buffer
   - 2 µL 100 mM DTT
   - 2 µL dNTP PreMix
   - 0.5 µL RiboGuard RNase Inhibitor
   - 1 µL MMLV Reverse Transcriptase

5. Mix the reaction gently. Incubate the reaction at 37 °C for 60 minutes.
6. Terminate the reaction by heating at 85 °C for 5 minutes.
7. Chill on ice for at least 1 minute. Centrifuge briefly in a microcentrifuge.
8. The cDNA can be used immediately, without purification, for end-point or real-time PCR or stored at -20 °C for future use.

PCR amplification of the cDNA

Typically, 2 µL (10%) of the 20 µL MMLV Reverse Transcriptase 1st-Strand cDNA Synthesis Kit reaction is sufficient for most 50 µL PCRs. However, if detecting a rare mRNA or reverse transcribing a minute amount of total RNA (<100 pg) it is possible to add up to 10 µL (50%) of the 20 µL cDNA synthesis reaction to a 50-µL PCR in order to increase detection sensitivity. A maximum of 25% of the MMLV cDNA synthesis reaction can be added to a PCR reaction without purification.

End-point PCR products are detected by agarose gel electrophoresis and staining with, for example, ethidium bromide or SYBR Gold.

6. Further support

If you require any further support, please do not hesitate to contact our Technical Support Team: techsupport@lgcgroup.com.

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