AccuLift Reverse Transcriptase First-Strand cDNA Synthesis Kit

Contents

About This Document

This protocol describes how to use the AccuLift™ Reverse Transcriptase 1st-Strand cDNA Synthesis Kit to generate full-length first-strand cDNA from total cellular RNA or poly(A) RNA-enriched samples.

IMPORTANT Before using this kit, read and understand the detailed instructions and safety guidelines in this document. For complete safety information, see Appendix A.

Safety Alert Conventions

Fluidigm documentation uses specific conventions for presenting information that may require your attention. Refer to the following safety alert conventions.

Safety Alerts for Chemicals

For hazards associated with chemicals, this document follows the United Nations Globally Harmonized System of Classification and Labelling of Chemicals (GHS) and uses indicators that include a pictogram and a signal word that indicates the severity level:

Indicator	Description
	Pictogram (see example) consisting of a symbol on a white background within a red diamond-shaped frame. Refer to the individual safety data sheet (SDS) for the applicable pictograms and hazards pertaining to the chemicals being used.
DANGER	Signal word that indicates more severe hazards.
WARNING	Signal word that indicates less severe hazards.

Safety Data Sheets

Read and understand the SDSs before handling chemicals. To obtain the SDS for this kit, contact your Fluidigm representative. To obtain SDSs for other chemicals ordered from Fluidigm, either alone or as part of this system, go to fluidigm.com/sds and search for the SDS using either the product name or the part number.

Some chemicals referred to in this user guide may not have been provided with your system. Obtain the SDSs for chemicals provided by other manufacturers from those manufacturers.

Introduction

The AccuLift Reverse Transcriptase 1st-Strand cDNA Synthesis Kit (PN 10008) is optimized for generating full-length first-strand cDNA from total cellular RNA or poly(A) RNA-enriched samples. The kit features Epicentre's high-activity formulation MMLV Reverse Transcriptase and proprietary 10X RT Reaction Buffer, which together are capable of synthesizing full-length cDNA from mRNA templates longer than 15 kb.

Performance Specifications and Quality Control

The AccuLift 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 ~2 kb poly(A) RNA into full-length cDNA in 30 minutes at 37 °C.

Materials

Required Kit from Fluidigm

Product Name	Part Number	Storage
AccuLift Reverse Transcriptase 1st-Strand cDNA Synthesis Kit (50 Samples) • Reverse Transcriptase, 50 µL (PN 10089) • RNase Inhibitor, 30 µL (PN 10090) • dNTP Premix (5 mM each dNTP), 100 µL (PN 10091) • Oligo(dT)$_{21}$ Primer (10 µM), 100 µL (PN 10092) • Random Nonamers (9-mers; 50 µM), 50 µL (PN 10093) • Nuclease-Free Water, Sterile, 1 mL (PN 10094) • DTT (100 mM), 125 µL (PN 10095) • 10X RT Reaction Buffer, 100 µL (PN 10096)	10008	–20 °C in a freezer without a defrost cycle

Required Consumable

Product	Source	Part Number
0.2 or 0.5 mL sterile microtubes	Major laboratory supplier (MLS)	—

Required Equipment

Product	Source	Part Number
Microcentrifuge	MLS	—
Pipettes and appropriate low-retention, filtered tips*	MLS	—
Vortexer	MLS	—
Water bath or thermocycler	MLS	—

* Recommended: Rainin® pipettes

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® Bioanalyzer® 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 AccuLift 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 AccuLift reverse transcription process include but are not limited to:

- **TRIzol®/TRI Reagent®**, 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 2 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:** The AccuLift Rapid RNA Extraction kit , AccuLift RNA Cleanup and Concentrator kit or the AccuLift RNA Spin Column Micro Prep kit 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 (for example, 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 bioanalyzer 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®. 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, for performing successful cDNA synthesis reactions, it is critical to create an RNase-free work environment and maintain RNase-free solutions. Therefore, we strongly recommend that the user:

- Autoclave all tubes and pipette tips that will be used in the cDNA synthesis reactions.
- Always wear gloves when handling samples containing RNA. Change gloves frequently, especially after touching potential sources of RNase contamination such as doorknobs, pens, pencils, and human skin.
- Always wear gloves when handling kit components. Do not pick up any kit component with an ungloved hand.
- 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 Epicentre® 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 either by using the Oligo(dT)$_{21}$ Primer or Random Nonamers (9-mers) that are provided in the kit, or by 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 in the subsequent PCR reaction. Random primers, however, can be helpful when:

- Synthesizing cDNA from eukaryotic mRNAs that lack a poly(A) tail or have a very short poly(A) tail.
- Priming cDNA synthesis of a poly(A)-enriched RNA sample.
- Synthesizing cDNA from partially degraded RNA samples such as those obtained from laser-captured cells or formalin-fixed paraffin-embedded tissue (FFPE) samples.
- It is necessary to eliminate or reduce 3′ sequence bias that can result when using an oligo(dT) primer.

Gene-specific primers designed and synthesized 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 annealing and extension (reverse transcription) conditions for each primer used.

RT-PCR: Endpoint or Real-Time PCR Amplification and Detection

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

Endpoint PCR products are detected by agarose gel electrophoresis and staining with ethidium bromide or SYBR® Gold. We recommend using at least 10 ng of total RNA in the AccuLift Reverse Transcriptase 1st-Strand cDNA Synthesis reaction when endpoint PCR will be performed.

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

Procedure

The following protocol has been optimized 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 optimization of the reaction.

AccuLift 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 first-strand cDNA synthesis reaction, combine the following components on ice, in a sterile (RNase-free) 0.2 mL or 0.5 mL tube:

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

2 Incubate at 65 °C for 2 min in a water bath or thermocycler with heated lid.

3 Chill on ice for 1 min. Centrifuge briefly in a microcentrifuge.

4 To each first-strand cDNA synthesis reaction, add on ice:

Component	Volume (µL)
10X RT Reaction Buffer	2
100 mM DTT	2
dNTP Premix	2
RiboGuard RNase Inhibitor	0.5
MMLV Reverse Transcriptase	1

5 Mix the reaction gently. Incubate the reaction at 37 °C for 60 min.

6 Terminate the reaction by heating at 85 °C for 5 min.

7 Chill on ice for at least 1 min. Centrifuge briefly in a microcentrifuge.

8 The cDNA can be used immediately, without purification, for endpoint 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 AccuLift Reverse Transcriptase 1st-Strand cDNA Synthesis Kit reaction is sufficient for most 50 µL PCR reactions. 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 reaction 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.

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

Appendix A: Safety

General Safety

In addition to your site-specific safety requirements, Fluidigm recommends the following general safety guidelines in all laboratory and manufacturing areas:

- Use personal protective equipment (PPE): safety glasses, fully enclosed shoes, lab coats, and gloves.
- Know the locations of all safety equipment (fire extinguishers, spill kits, eyewashes/showers, first-aid kits, safety data sheets, etc.), emergency exit locations, and emergency/injury reporting procedures.
- Do not eat, drink, or smoke in lab areas.
- Maintain clean work areas.
- Wash hands before leaving the lab.

Chemical Safety

The responsible individuals must take the necessary precautions to ensure that the surrounding workplace is safe and that instrument operators are not exposed to hazardous levels of toxic substances. When working with any chemicals, refer to the applicable safety data sheets (SDSs) provided by the manufacturer or supplier.

Disposal of Products

Used reagents should be handled and disposed of in accordance with federal, state, regional, and local laws for hazardous waste management and disposal.