First-Strand cDNA Synthesis Kit

For the generation of full-length first-strand cDNA from an RNA template using a variety of primers

Product Booklet

Code: 27-9261-01

Page finder

1. Legal

http://www.gelifesciences.com

Amersham Place, Little Chalfont,
Buckinghamshire. HP7 9NA UK.

2. Handling

2.1. Safety warnings and precautions

Warning: ***For research use only***.
Not recommended or intended for diagnosis of disease in humans or animals. Do not use internally or externally in humans or animals.

All chemicals should be considered as potentially hazardous. We therefore recommend that this product is handled only by those persons who have been trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice. Wear suitable protective clothing, such as laboratory overalls, safety glasses and gloves. Care should be taken to avoid contact with skin or eyes. In the case of contact with skin or eyes, wash immediately with water. See material safety data sheet(s) and/or safety statement(s) for specific advice.

Note that the assay protocol requires the use of Diethyl Pyrocarbonate (toxic, irritant), Phenol (toxic, corrosive), Chloroform (harmful, irritant) and Isoamyl Alcohol (flammable, harmful, irritant). Please follow the manufacturers' safety data sheets relating to the safe handling and use of these materials.

2.2. Storage

The kit should be stored at -15°C to -30°C.

2.3. Expiry

Stable for at least 3 months when stored under recommended conditions.

3. Components

Bulk first-strand cDNA reaction mixes (5)
Cloned, FPLC*pure* murine reverse transcriptase, RNAguard (porcine), RNase/DNase-free BSA, dATP, dCTP, dGTP, and dTTP in aqueous buffer.

DTT solution
200 mM aqueous solution.

pd(N)$_6$ primer
Random hexadeoxynucleotides at 0.2 µg/µl in aqueous solution.

Not *I-d(T)$_{18}$ bifunctional primer*
5′-d[AAC TGG AAG AAT TCG CGG CCG CAG GAA T_{18}]-3′ in aqueous solution at 5 µg/µl.

mRNA standard
Synthetic mRNAs of defined sizes (9, 6, 5, 4, 3, 2.5, 2, 1.5, 1 and 0.5 kilobases); 1 µg in aqueous solution at 50 µg/ml.

RNase-free water
Treated with Diethyl Pyrocarbonate (DEPC).

4. Other materials required

- **DEPC-treated water:** A 0.1% solution of Diethyl Pyrocarbonate (DEPC) in distilled water, stirred for a minimum of 2 hours at room temperature then autoclaved.

For monitoring the cDNA synthesis reaction (optional):

- **[α-^{32}P]dCTP:** 10 mCi/ml, 3000 Ci/mmol.
- **Phenol:** A saturated solution of redistilled phenol in TE buffer containing 8-hydroxy quinoline (6).
- **Chloroform/isoamyl alcohol:** Reagent-grade chloroform and isoamyl alcohol, mixed 24:1.
- **Phenol/chloroform:** Equal parts of phenol and chloroform/isoamyl alcohol (24:1); see above.
- **STE buffer:** 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 150 mM NaCl.

5. Description

First-strand cDNA synthesis is catalyzed by Moloney Murine Leukaemia Virus (M-MuLV) reverse transcriptase. The conditions of this reaction have been optimized to permit full-length transcription of RNAs 7 kilobases or more in length. The preassembled bulk first-strand cDNA reaction mixes require only the addition of DTT, RNA, and a primer of choice. The first-strand reaction may be primed with either of the primers provided with the kit: the *Not* I-d$(T)_{18}$ bifunctional primer or pd$(N)_6$ primer. Custom primers complementary to a specific mRNA sequence may also be used to prime first-strand synthesis.

Following synthesis of the first-strand cDNA, the resulting double-stranded RNA:cDNA heteroduplex can be used directly for second-strand cDNA synthesis. Second-strand synthesis may be accomplished by the standard Gubler-Hoffman technique (1) in which RNase H nicks the RNA strand of the RNA:cDNA heteroduplex and DNA polymerase I uses these nicks to replace the RNA with DNA by nick translation.

Alternatively, the completed first-strand reaction may be amplified directly by PCR (2–5). In preparation for this technique, the double-stranded RNA:cDNA heteroduplex is heat-denatured to allow the cDNA strand to be used as a template for polymerization. The specificity of the PCR amplification process is based on two amplification primers which flank the cDNA segment to be amplified and hybridize to complementary strands. Repeated cycles of denaturation, primer annealing and primer extension by DNA polymerase can result in exponential amplification of a target cDNA. Even cDNA made from relatively rare transcripts may be successfully amplified using this technique.

6. Critical parameters

- Avoid ribonuclease contamination by wearing clean gloves at all times and by autoclaving or DEPC-treating all glass and plastic ware
- Avoid repeated thawing of the kit components: place individual reagents on ice as they are needed, leaving the rest in the freezer.
- When performing PCR, exercise extreme care to prevent DNA contamination. Always use sterile pipette tips and microcentrifuge tubes, and avoid cross-over contamination of stock solutions.

7. Protocol

7.1. Introduction

The use of intact, undegraded mRNA is of primary importance to the success of first-strand cDNA synthesis. We strongly recommend the use of our mRNA purification kits for preparing high-quality mRNA.

For rapid purification of mRNA directly from cells or tissues, we recommend Sera-Mag Oligo dT superparamagnetic particles (38152103010150) in conjunction with the MagRack 6 (28-9489-64) for samples up to 1.5 ml and MagRack Maxi (28-9864-41) for samples up to 50 ml.

If the cDNA portion of the heteroduplex is to be used directly as template in Gulber-Hoffman second-strand cDNA synthesis, we recommend using cloned *E.coli* ribonuclease H and DNA polymerase I. For both second-strand synthesis and PCR, we recommend the DNA polymerization mix as the source of ultrapure deoxyribonucleotides.

Following second-strand cDNA synthesis of PCR, the synthesized DNA fragments can be cloned using a variety of reagents offered by GE. To engineer fragments with *Not* I and *Eco*R I overhangs at opposite ends, you can use the *Not* I-d$(T)_{18}$ bifunctional primer (included in the first-strand cDNA synthesis kit) along with *Eco*R I cohesive-end adaptors and *Not* I endonuclease.

To purify PCR products from primers, nucleotides and traces of mineral oil, use illustra™ MicroSpin columns directly following PCR. To purify PCR products after agarose gel electrophoresis, use the illustra GFX™ PCR DNA and Gel Band Purification Kit. Clone the inserts following PCR using Lambda ExCell *Not* I/*Eco*R I/CIP.

*Covered by U.S. Patent No. 5,459,253 and foreign equivalents

Notes:
Upon storage at -15°C to -30°C, BSA in the bulk first-strand reaction mix may fall out of solution, causing a small white precipitate to form. The formation of the precipitate in no way affects the performance of the kit, and most can easily be dissolved back into solution by gently mixing the bulk first-strand reaction mix prior to use.

7.2. First-strand cDNA synthesis

Depending on the intended use of the first-strand cDNA, synthesis may be performed using different volumes of **bulk first-strand cDNA mix,** and different types and amounts of primer and RNA. If the cDNA is to be used for second-strand synthesis by the Gulber-Hoffman reaction, we recommend you use 11 µl of the **bulk reaction mix**, 1–5 µg of mRNA in 20 µl (see Table 1), and the appropriate amount of primer (see Table 2).

If on the other hand the cDNA is to be amplified, either 5 µl or 11 µl of the **bulk reaction mix** may be used, and 1–5 µg of *total* RNA, or 20–150 ng of *mRNA*, should be sufficient. Choose the appropriate amount of primer from Table 2.

If you wish to monitor first-strand synthesis, refer to page 15.

- Place the RNA sample in a microcentrifuge tube and add **RNAse-free water,** if necessary, to bring the RNA to the appropriate volume (either 8 µl or 20 µl, see Table 1).
- Heat the RNA solution to 65ºC for 10 minutes, then chill on ice.
- Gently pipette the **bulk first-strand cDNA reaction mix** to obtain a uniform suspension. (Upon storage, the BSA may precipitate in the **mix**; this precipitate will dissolve during incubation). Add the appropriate volume of the **bulk first-strand cDNA reaction mix** (either 5 µl or 11 µl, see Table 1) to a sterile 1.5 or 0.5 ml microcentrifuge tube. To this tube add 1 µl of **DTT solution**,

1 µl of your chosen primer at the appropriate concentration (see Table 2), and the heat-denatured RNA. Pipette up and down several times to mix. Incubate at 37°C for 1 hour.

- The completed first-strand cDNA reaction product is now ready for immediate second-strand cDNA synthesis or PCR amplification.

Table 1. Volumes of components in first-strand reaction

Bulk first-strand reaction mix	Primer[a]	DTT solution	RNA	Final volume first-strand reaction
11 µl	1 µl	1 µl	20 µl	33 µl
5 µl	1 µl	1 µl	8 µl	15 µl

Table 2. Recommended quantities of primer for first-strand synthesis (in 1 µl)

	Intended application	
First-strand cDNA primer	Second-strand synthesis	PCR-amplified synthesis
pd(N)$_6$	0.2–0.02 µg[b]	0.2 µg
Not I-d(T)$_{18}$	5 µg	0.2 µg[c]
Specific primer	40-400 pmol	20-40 pmol

Combined notes for Tables 1 and 2:

[a] The primer must be added to the reaction in a volume of 1 µl; in some cases, dilution may be required (see Table 2). If dilution is required, perform the dilution using **RNase-free water**, and use 1 µl of the diluted primer for first-strand cDNA synthesis.

[b] undiluted →1:10 dilution. When using random primers to prime first-strand synthesis prior to Gubler-Hoffman second-strand synthesis, the size of the cDNAs obtained will depend on the amount of $pd(N)_6$ primer added. In general the more $pd(N)_6$ added, the shorter the cDNAs produced.

[c] 1:25 dilution

8. Additional information

8.1. Suggested amplification conditions

PCR amplification of cDNA involves the enzymatic synthesis of specific cDNA target sequences using two oligonucleotide primers which flank the cDNA region of interest and hybridize to opposite strands. The 'upstream' PCR primer should be complementary to the 3' flanking region of the first-strand cDNA (or identical to the 5' flanking region of the mRNA). This primer will prime synthesis of the second-strand cDNA. The 'downstream' primer, which is complementary to the 3' flanking region of the second-strand cDNA (or to the 3' end of the mRNA) is used to prime synthesis of the first-strand cDNA.

The ***Not* I-d(T)$_{18}$ bifunctional primer** is a hybrid primer composed of a string of d(T) residues as well as an 'anchor' domain which contains a *Not* I restriction site (see sequences on page 2). If this primer is used for first-strand cDNA synthesis, it can then be used as the 'downstream' primer in subsequent PCR. Note, however, that it will not function efficiently as a PCR primer for cDNA synthesized using a non-d(T) primer, such as the **pd(N)^6 primer** supplied with the kit. Nor will it always function efficiently for PCR even if it was the primer used for cDNA synthesis: long stretches of d(T) do not always anneal efficiently at elevated temperatures. If this appears to be a problem for you, you may wish to construct your own PCR primer consisting of the 'anchor' portion without the d(T) residues - refer to the sequence of the hybrid primer on page 5.

The volume of the first-strand cDNA reaction used for amplification will depend on the relative abundance of the cDNA of interest in the final reaction mixture. For cDNAs from very rare messages, you may choose to use the entire first-strand reaction for amplification. For more abundant messages, you may be able to use less. However,

if you use less than 10 µl of the first-strand reaction, it will be necessary to adjust the amplification reaction conditions according to the tables on page 15.

The buffer and dNTP conditions in the completed first-strand cDNA reaction are 45 mM Tris (pH 8.3), 68 mM KCl, 15 mM DTT, 9 mM $MgCl_2$, 0.08 mg/ml BSA and 1.8 mM each dNTP.

When amplifying the entire first-strand reaction (either 33 µl or 15 µl), you will need to add only the PCR primers and *Taq* DNA polymerase. When amplifying less than 10 µl of the first-strand reaction, you will also need to add PCR buffer [10 x PCR buffer is 500 mM KCl, 100 mM Tris (pH 9.0), 15 mM $MgCl_2$] and dNTP mix (e.g. DNA polymerization mix from GE, code number 27-2094-01).

Overleaf are two tables which can be used as guidelines when using various amounts of the first-strand reaction for PCR. Refer to Table 3 if the final volume of your first-strand cDNA reaction is 15 µl. Refer to Table 4 if the final volume of your first-strand cDNA reaction is 33 µl.

- Heat the completed first-strand reaction to 90°C for 5 minutes to denature the RNA-cDNA duplex and inactivate the reverse transcriptase. Chill on ice.
- Mix the appropriate volumes of reagents in a sterile 0.5 ml microcentrifuge tube and layer mineral oil over the sample to prevent evaporation.
- Place the samples into a thermal cycler and cycle 20–50 times depending on the abundance of the target. A common PCR 'cycle' consists of denaturing at 90°C for 1 minute, primer annealing at 55°C for 30 seconds, and polymerization at 72°C for 30 seconds, for a total of 30 cycles.

Table 3. For cDNA synthesis in a final volume of 15 µl

Volume of cDNA rxn	10x PCR buffer	20 mM dNTP mix	Upstream primer (10–40 pmol)	Downstream primer[a] (10–40 pmol)	Water	*Taq* pol
1–5 µl	5 µl	1 µl	X µl	X µl	to 50 µl	2.5 U
15 µl	0	0	X µl	X µl	to 50 µl	2.5 U

Table 4. For cDNA synthesis in a final volume of 33 µl

Volume of cDNA rxn	10x PCR buffer	20 mM dNTP mix	Upstream primer (10–40 pmol)	Downstream primer[a] (10–40 pmol)	Water	*Taq* pol
1–5 µl	10 µl	1 µl	X µl	X µl	to 100 µl	2.5 U
6–10 µl	10 µl	0	X µl	X µl	to 100 µl	2.5 U
33 µl	0	0	X µl	X µl	to 100 µl	2.5 U

Combined notes for Tables 3 and 4:

[a] 0.3 µg of ***Not* I-d(T)$_{18}$ bifunctional primer** ≈ 21 pmol.

8.2. Monitoring first-strand synthesis

The most reliable way to monitor first-strand cDNA synthesis is to incorporate radioactive nucleotides during polymerization, separate the reaction products by gel electrophoresis, and analyze their size by autoradiography. In order to assay first-strand cDNA synthesis,

follow the directions below using the mRNA standard provided in the kit or 1–5 µg of your own RNA.

- Heat 20 µl of an RNA solution (=1 µg for the **mRNA standard**) to 65°C for 10 minutes, and then chill on ice.
- Gently pipette a **bulk first-strand cDNA reaction mix** and spin briefly to remove any solution from the sides of the tube. Place 11 µl of the **bulk first-strand cDNA reaction mix** in a sterile 1.5 ml microcentrifuge tube, add 20 µCi of [α-^{32}P]dCTP (2 µl of 10 mCi/ml, see page 6), 1 µl of **DTT solution,** 1 µl of the appropriate primer, and the heat-denatured RNA. Pipette up and down several times to mix. Incubate at 37°C for 1 hour.
- Bring the final volume to 100 µl by adding 65 µl of STE buffer (see page 6). Add 100 µl of phenol/chloroform, vortex, then centrifuge for 1 minute.
- Collect the upper (aqueous) phase and remove any unincorporated radioactive nucleotides by either spun-column chromatography (e.g. using illustra MicroSpin columns) or standard ethanol precipitation.

Using established procedures (6), analyze 50–100 µl of the sample by electrophoresis and autoradiography of a 1% agarose gel.

8.3. Function testing

Each batch of the first-strand cDNA synthesis kit is tested using the mRNA standard and both the pd(N)$_6$ primer and the *Not* I-d(T)$_{18}$ bifunctional primer to verify its ability to generate full-length cDNA molecules. Further, the kit is tested for its ability to generate rabbit globin cDNA suitable for amplification by PCR.

9. References

1. Gubler, U. and Hoffman, B. J., *Gene* **25**, 263 (1983).

2. Berchtold, Martin W., *Nucl. Acids Res.* **17**, 453 (1989).

3. Noonan, E. and Roninson, I. B., *Nuc. Acids Res.* **16**, 10366 (1988).

4. Chelley *et al., Nature* **333**, 858 (1988).

5. Rappolee *et al., Science* **241**, 708 (1988).

6. Maniatis, T., Fritsch, E. F. and Sambrook, J., *Molecular Cloning: A laboratory manual*, Cold Spring Harbor Laboratory, (1982).

10. Related products

Product	Pack size	Product number
illustra PuReTaq™ Ready-To-Go™ PCR beads (0.5 ml tubes)	100 reactions	27-9558-01
illustra PuReTaq Ready-To-Go PCR beads (0.2 ml tubes/plate)	96 reactions	27-9557-01
	5 x 96 reactions	27-9557-02
illustra PuReTaq Ready-To-Go PCR beads (0.2 ml hinged tube with cap)	96 reactions	27-9559-01
illustra QuickPrep *Micro* mRNA Purification Kit	24 purifications	27-9255-01
illustra mRNA Purification Kit	4 purifications	27-9258-02
DNA polymerization mix	10 µmol of each dNTP	28-4065-57
	4 x 10 µmol of each dNTP	28-4065-58

Product	Pack size	Product number
illustra MicroSpin S-200 HR Columns	50 columns	27-5120-01
illustra MicroSpin S-300 HR Columns	50 columns	27-5130-01
illustra MicroSpin S-400 HR columns	50 columns	27-5140-01
illustra GFX™ PCR DNA and Gel Band Purification Kit	100 purifications	28-9034-70
	250 purifications	28-9034-71
Hyperfilm™ MP	Various sizes	

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