Cat.# 6111A

For Research Use

TaKaRa

PrimeScript™ Double Strand cDNA Synthesis Kit

Product Manual

v201811Da

Table of Contents

I. Description

cDNA cloning is used for structural analysis of genes and RNAs, and expression of target proteins. Generating a cDNA library involves synthesizing double-stranded cDNA that is complementary to the target mRNA, inserting the cDNA into a vector of bacterial or viral origin, and introducing the vector into bacterial or eukaryotic cells. The cloned cDNA can be then used for analysis, and *in vitro* transcription and translation.

The PrimeScript Double Strand cDNA Synthesis Kit synthesizes double-stranded cDNA from polyA$^+$ RNA of animal or plant origin using the Gubler-Hoffman method [1]. The principles of the system are shown below (Figures 1 and 2).

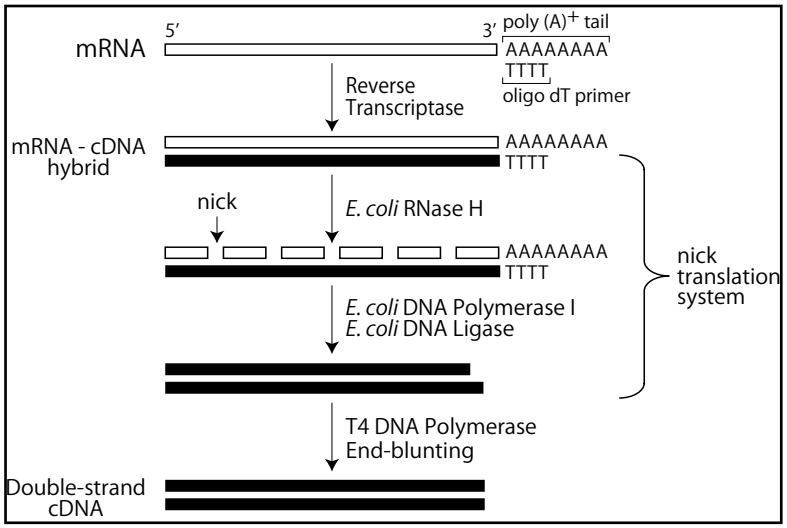


Figure 1. Double Strand cDNA Synthesis using Oligo $(dT)_{18}$ Primer

- 1st strand cDNA is synthesized using PrimeScript RTase and Oligo $(dT)_{18}$ Primer[1-4] or Random Primer[5,6].
- *E. coli* RNase H[7] nicks the RNA of the mRNA-cDNA hybrid. *E. coli* DNA Polymerase I is used for second strand synthesis by nick translation and *E. coli* DNA Ligase repairs any breaks in the second strand[1,8].
- T4 DNA Polymerase is used to blunt the ends.

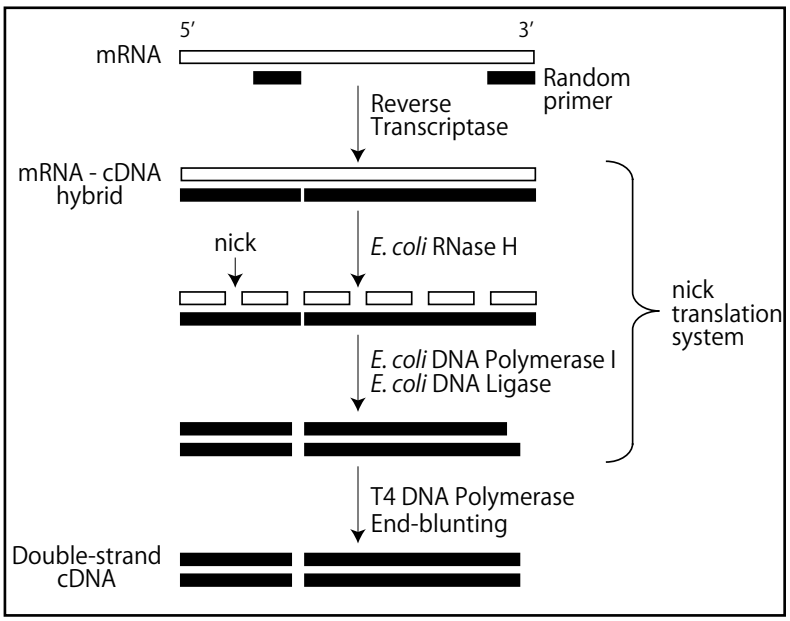


Figure 2. Double Strand cDNA Synthesis using Random Primer

II. Components (10 reactions)

1.	PrimeScript RTase (200 U/μl)	10 μl
2.	RNase Inhibitor (40 U/μl)	10 μl
3.	Oligo $(dT)_{18}$ Primer (1 μg/μl)	20 μl
4.	Random Primer (9 mer) (0.3 μg/μl)	20 μl
5.	5X 1st Strand Synthesis Buffer	40 μl
6.	dNTP Mixture (10 mM each)	40 μl
7.	*E. coli* RNase H/*E. coli* DNA Ligase Mixture	20 μl
8.	*E. coli* DNA Polymerase I (20 U/μl)	20 μl
9.	5X 2nd Strand Synthesis Buffer	300 μl
10.	T4 DNA Polymerase (1 U/μl)	40 μl
11.	RNase free dH_2O	600 μl × 2
12.	Control RNA (1 μg/μl)*	5 μl

* The Control RNA included in this kit is synthesized by *in vitro* transcription with SP6 RNA polymerase from the pSP Tet3 plasmid. The pSP Tet3 plasmid includes a tetracycline resistance gene from pBR322 downstream of the SP6 promoter.
The Control RNA is a 1.4-kb $polyA^+$ RNA from the tetracycline resistance gene that can be used as a positive control. When full-length double strand cDNA is synthesized from this RNA template and cloned, the resulting plasmid confers resistance to tetracycline.

III. Materials Required but not Provided

<Reagents>

- 10% (w/v) SDS
- 0.25 M EDTA (pH 8.0)
- Phenol/Chloroform/Isoamyl Alcohol (25:24:1, v/v/v)
- Chloroform/Isoamyl Alcohol (24 : 1, v/v)
- 10 M Ammonium Acetate
- Isopropanol
- Ethanol
- TE Buffer

<Equipment>

- Microcentrifuge
- 42℃ water bath (or PCR thermal cycler, etc.)
- 16℃ water bath
- 70℃ water bath
- 37℃ water bath
- Micropipette
- Microcentrifuge tubes
- Pipette tips

IV. Storage

-20℃

V. Precautions Prior to Use

1. Sterilization of Equipment

Commercially available sterile disposable plastic equipment can be considered RNase-free and can be used in experiments as is. Equipment such as microtubes, and tips for micropipettes should be autoclaved before use. Glassware and spatula are done dry heat sterilization at 160℃ for at least 2 hours. Items that cannot be sterilized by dry-heating should be treated with 0.1% diethylpyrocarbonate (DEPC) solution for 12 hours at 37℃ and then autoclaved (to prevent carboxymethylation of RNA by the DEPC) before use.
Designate equipment for RNA experiments and separate from other equipment. Additionally, because the most common source of RNase contamination is bare hands, wear plastic gloves and a face mask when performing RNA experiments.

2. Preparation of Reagents

Whenever possible, treat reagents with 0.1% DEPC and autoclave before use. For reagents that cannot be autoclaved, prepare using equipment and water that have been sterilized and then filter sterilize after preparation. Solutions and sterile purified water that are used to prepare reagents should be designated for use in RNA experiments only.

3. Preparation of RNA Samples

It is necessary to prepare highly-pure RNA. Impurities such as polysaccharides and protein may inhibit the cDNA synthesis reaction. In addition, prevent genomic DNA contamination.
Preparation of RNA from tissues and cells should be performed as quickly as possible after sample collection. If this is not possible, store samples at -80℃ or in liquid nitrogen.

(1) Preparation of Total RNA
The guanidium thiocyanate phenol chloroform method (AGPC method), or a commercial RNA reagent or kit for the isolation and purification of RNA may be used.
Examples: RNAiso Plus (Cat. #9108/9109)
NucleoSpin RNA (Cat. #740955.10/.50/.250)

(2) Purification of polyA$^+$ RNA (for Eukaryotes)
Typically, polyA$^+$ RNA is isolated from total RNA using oligo (dT) cellulose or poly (U) sepharose. *Oligotex-dT30 <Super>* (Cat. #W9021)* and *Oligotex-dT30 <Super>* mRNA Purification Kit (From Total RNA) (Cat. #9086)* allow easy isolation of high-purity polyA$^+$ mRNA.

* Not available in all geographic locations. Check for availability in your area.

(3) Testing RNA Purity

For maximum cDNA synthesis, it is important to use intact, high-purity RNA templates. RNA purity testing before cDNA synthesis is recommended.

1) Agarose Electrophoresis (total RNA)

Heat denature 1-2 μg of total RNA (65℃ , 10 minutes) and perform agarose gel electrophoresis. Total RNA that has not been degraded will have two clear ribosomal RNA bands (28S and 18S in eukaryotes, and 23S and 16S for prokaryotes) in a roughly 2 : 1 ratio. When the ribosomal RNA bands are diffuse, there is a possibility of RNase contamination and the total RNA should not be used. If there are high molecular weight bands, genomic DNA contamination is likely. In this case, treat the RNA sample with Recombinant DNase I (RNase free) (Cat. #2270A/B) prior to cDNA synthesis.

Testing can also be performed using an Agilent 2100 Bioanalyzer.

2) Absorbance (total RNA and polyA$^+$ RNA)

Use RNA samples with a A_{260}/A_{280} ratio of 1.8 - 2.1; samples with a ratio of less than 1.7 should not be used. Use 10 mM Tris-HCl, 0.1 mM EDTA (pH 7.5) to measure absorbance.

VI. Protocol

VI-1. 1st Strand cDNA Synthesis

1. Prepare a total of 10 μl of the following reaction solution in a microtube.

Template polyA$^+$ RNA	2 μg
dNTP Mixture	1 μl
Oligo $(dT)_{18}$ Primer or Random Primer	2 μl
RNase free dH_2O	to 10 μl

2. Incubate at 65℃ for 5 minutes, then cool on ice.
3. Add the following solution to a total volume of 20 μl.

5X 1st Strand Synthesis Buffer	4 μl
RNase Inhibitor	1 μl
PrimeScript RTase	1 μl
RNase free dH_2O	to 20 μl

4. Mix gently.
5. Incubate at 42℃ for 1 hour.
6. Transfer to ice and cool for 2 minutes.

VI-2. 2nd Strand cDNA Synthesis and Blunting of Terminal Ends

1. After 1st strand cDNA synthesis, add the following solution to obtain a total volume of 142 μl.

5X 2nd Strand Synthesis Buffer	30 μl
dNTP Mixture	3 μl
RNase free dH_2O	89 μl

2. Add 2 μl of *E. coli* DNA Polymerase I and 2 μl of *E. coli* RNase H/*E. coli* DNA ligase mixture and mix gently.
3. Incubate at 16℃ for 2 hours.
4. Incubate at 70℃ for 10 minutes.
5. Add 4 μl of T4 DNA Polymerase and mix gently.
6. Incubate at 37℃ for 10 minutes.
7. Add 15 μl of 0.25 M EDTA (pH 8.0) and 15 μl of 10% SDS solution and mix to stop the reaction.

VI-3. Purification of 2nd Strand cDNA

1. After stopping the reaction at Step VI-2-7, add 180 μl of phenol/chloroform/isoamyl alcohol (25 : 24 : 1) to the 180 μl reaction and vortex for 5 - 10 seconds.
2. Centrifuge at 15,000 rpm for 1 minute at room temperature and transfer the aqueous layer to a new tube. Avoid aspirating the middle layer.
3. Add 180 μl of chloroform/isoamyl alcohol (24 : 1) and vortex 5 - 10 seconds.
4. Centrifuge at 15,000 rpm for 1 minute at room temperature and transfer the aqueous layer to a new tube.
5. Add 60 μl of 10 M ammonium acetate.
6. Add 2.5 volumes of ethanol (600 μl) and mix well.
7. Incubate for 10 minutes at room temperature.
8. Centrifuge at 15,000 rpm for 15 minutes at 4℃ and remove the supernatant, being careful not to aspirate the pellet.
9. Add 70% ethanol and centrifuge at 15,000 rpm for 5 minutes at 4℃.
10. Remove the supernatant, and dry the pellet.
11. Dissolve the pellet in a sufficient amount of TE buffer.
12. Store at -20℃

When the amount of cDNA synthesized is small, add a coprecipitating agent such as Dr. GenTLE Precipitate Carrier (Cat.# 9094) during ethanol precipitation.

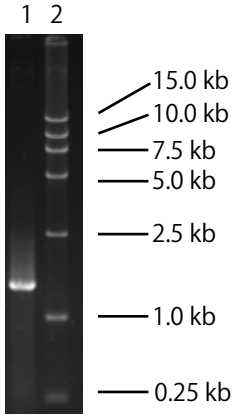
Column purification can also be used to purify the 2nd strand cDNA. It is also possible to use agarose gel electrophoresis and gel purification to isolate the target cDNA.

Additionally, this protocol uses 2 μg of template polyA$^+$ RNA, but it is possible to increase the cDNA synthesis reaction proportionately with the amount of template for both the 1st strand and 2nd strand reactions (refer to the table below).

Amount of RNA Template Used (polyA$^+$ RNA)	1st Strand cDNA Synthesis Reaction Volume	2nd Strand cDNA Reaction Volume (Including Enzyme Solution)
2 μg	20 μl	150 μl
3 μg	30 μl	225 μl
4 μg	40 μl	300 μl
5 μg	50 μl	375 μl

VII. Control Reaction

Perform the 1st strand cDNA synthesis reaction using Oligo $(dT)_{18}$ primer with 2 μg of the Control RNA (approximately 1.4 kb) as the template according to the protocol (refer to section VI). Using half of the 1st strand reaction, perform 2nd strand cDNA synthesis reaction and end blunting.
Purify the reaction solution using phenol/chloroform extraction and ethanol precipitation. Treat the 1st strand cDNA product with RNase H to degrade RNA. Analyze products by agarose gel electrophoresis.



Lane 1 : Product of 2nd Strand cDNA synthesis
2 : Marker

(Results) Bands for double-stranded cDNA from the 2nd strand cDNA synthesis should be observed.

Figure 3. cDNA Synthesis with the Control RNA

VIII. Experimental Example

VIII-1. cDNA cloning

cDNA Cloning Using an Adaptor

Following adaptor ligation, it is possible to directly insert the cDNA into the vector without methylation or restriction enzyme digestion. Below is an example using *EcoR* I-*Not* I-*Bam*H I adaptors.

A. Adaptor Ligation

1. Prepare the following reaction solution in a microtube.[*1]

0.01 - 0.1 pmol	double-stranded cDNA
>100 times the amount of double-stranded cDNA (molar ratio)	*Eco*R I-*Not* I-*Bam*H I adaptor
66 mM	Tris-HCl (pH 7.6)
6.6 mM	$MgCl_2$
10 mM	DTT
0.1 mM	ATP
350 U	T4 DNA Ligase
Total	10 μl

2. Incubate at 16℃ for 2 hours to overnight.[*1]

*1 For adaptor ligation, DNA Ligation Kit, Ver. 2.1 (Cat.# 6022) or DNA Ligation Kit < Mighty Mix > (Cat.# 6023) can be used.

3. Add 1 μl of 0.5 M EDTA to stop the reaction.
4. Add an equal amount (11 μl) of phenol/chloroform.
5. Add 1/10 volume (1.1 μl) of 3 M sodium acetate.
6. Add 2 volumes of ethanol (24.2 μl).
7. Incubate at -20℃ for 30 minute.
8. Centrifuge for 20 minutes in a microcentrifuge.
9. After washing the pellet in 80% ethanol, dry the pellet using a vacuum.
10. Dissolve the cDNA in a suitable amount of TE buffer.

B. Adaptor Phosphorylation

1. Prepare the following reaction solution in a microtube.

50 mM	Tris-HCl (pH 8.0)
10 mM	$MgCl_2$
5 mM	DTT
0.1 mM	ATP
0.01-0.1 pmol	double-strand cDNA with adaptor
5-20 U	T4 Polynucleotide Kinase
Total	50 μl

2. Incubate at 37℃ for 30 minutes.
3. Add 5 μl of 0.5 M EDTA to stop the reaction.
4. Incubate at 70℃ for 5 minutes.
5. Add an equal amount (55 μl) of phenol/chloroform.
6. Add 1/10 the amount (5.5 μl) of 3M sodium acetate.
7. Add 2 volumes of ethanol (121 μl).
8. Incubate at -20℃ for 30 minutes.
9. Centrifuge at 15,000 rpm for 20 minutes at 4℃ in a microcentrifuge.

10. After washing the pellet in 80% ethanol, dry the pellet under a vacuum.
11. Dissolve the cDNA in a suitable amount of TE buffer.
12. Remove the unligated adaptor by gel filtration, spin column chromatography, or agarose gel electrophoresis.
13. Recover the cDNA.
14. Ligate into a suitable vector. A λ vector is generally used. (See "VIII-2. Ligation of cDNA into λ Vectors" .)

VIII-2. Ligation of cDNA into λ Vectors

1. Prepare the reaction solution below in a microtube.

0.01 - 0.1 pmol	Double-stranded cDNA fragments with adaptor
2 times the amount of cDNA (molar ratio)	λ -vector-DNA (*Eco*R I-digested, dephosphorylated fragments)

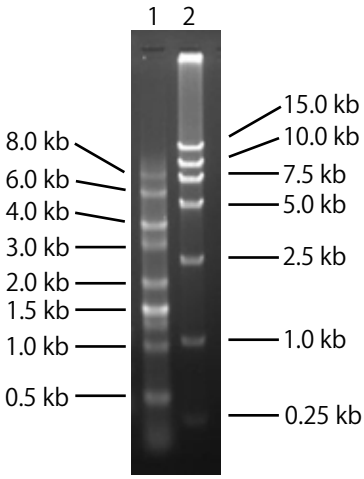
2. Add 1/10 volume of 3 M Sodium acetate
3. Add 2 volumes of Ethanol
4. Incubate at -20℃ for 30 minutes.
5. Centrifuge for 20 minutes in a microcentrifuge.
6. After washing the pellet with 80% ethanol, dry the pellet.
7. Dissolve in a suitable amount of TE buffer.
8. Add the following reagents to the cDNA fragment/ λ -vector DNA for the ligation reaction.[*2]

66 mM	Tris-HCl (pH 7.6)
6.6 mM	$MgCl_2$
10 mM	DTT
0.1 mM	ATP
350 U	T4 DNA Ligase
Total	10 μl

9. Incubate at 16℃ for 2 hours to overnight.[*2]
   *2 DNA Ligation Kit <Mighty Mix> (Cat. # 6023) can be also used for λ -vector ligation.
10. All or part of the reaction mixture can be used for *in vitro* phage packaging using a commercial kit.
11. Transfect a suitable *E. coli* host to form plaques on appropriate medium plate.
12. The cDNA library can be screened by plaque hybridization, etc.

IX. Evaluation of cDNA Synthesis

Using 2 μg of polyA$^+$ RNA ladder as the template, 1st strand cDNA synthesis was performed using Oligo $(dT)_{18}$ Primer. 2nd strand cDNA synthesis was performed using half of the 1st strand cDNA reaction solution. cDNA synthesis was analyzed on a 1% agarose gel electrophoresis.



Lane 1 :Product of 2nd Strand cDNA synthesis
2 : Marker

(Result) Clear bands were present for the target molecular sizes, confirming that double-stranded cDNA were generated.

Figure 4. Electrophoresis of cDNA

X. Troubleshooting

In the unlikely event that the experiment is not successful, consider the following issues.

1. Control RNA

   This kit includes pSP Tet3 polyA$^+$ RNA as the control RNA. Use this RNA as a positive control to ensure 1st strand and 2nd strand cDNA synthesis (see section VII. Control Reaction).

2. PolyA$^+$ RNA Purity

   To maximize the efficiency of cDNA synthesis, it is important that intact polyA$^+$ RNA be used as a template. Before cDNA synthesis, check RNA purity by ensuring an A_{260}/A_{280} value of 1.8 - 2.1 and by gel electrophoresis.

3. RNase Contamination

   Avoid RNase contamination in RNA samples. Use sterile or autoclaved equipment and reagents whenever possible and wear gloves when handling RNA samples.

XI. References

1) Gubler U and Hoffman B J. *Gene* (1983) **25**: 263.
2) Wokdnarfilipowicz A, *et al*. *Proc Natl Acad Sci USA.* (1984) **81**: 2295.
3) Howells R D, *et al*. *Proc Natl Acad Sci USA.* (1984) **81**: 7651.
4) Schneider C, *et al*. *Nature.* (1984) **311**: 675.
5) Haymerle H, *et al*. *Nucleic Acids Res*. (1986) **14**: 8615.
6) Koike S, Sakai M, and Muramatsu M. *Nucleic Acids Res*. (1987) **15**: 2499.
7) Leis P, *et al*. *Proc Natl Acad Sci USA.* (1973) **70**: 466.
8) Okayama H and Berg P. *Mol Cell Biol*. (1982) **2**: 161.
9) Buell C, *et al. J Biochem*. (1978) **235**: 2471.

XII. Related Products

PrimeScript™ Reverse Transcriptase (Cat. #2680A/B)
Recombinant RNase Inhibitor (Cat. #2313A/B)
DNA Polymerase I (*E. coli*) (Cat. #2130A/B)
T4 DNA Polymerase (Cat. #2040A/B)
T4 DNA Ligase (Cat. #2011A/B)
T4 Polynucleotide Kinase (Cat. #2021S/A/B)*
Random Primer (nonadeoxyribonucleotide mixture; pd $(N)_9$) (Cat. #3802)
Adaptor, *Eco*R I-*Not*I-*Bam*H I (Cat. #4510)
dNTP Mixture (Cat. #4030)
pHY Marker (Cat. #3404A/B)
DNA Ligation Kit, Ver.2.1 (Cat. #6022)
DNA Ligation Kit <Mighty Mix> (Cat. #6023)
RNAiso Plus (Cat. #9108/9109)*
NucleoSpin RNA (Cat. #740955.10/. 50/. 250)
Oligotex-dT30 <Super> (Cat. #W9021A/B)*
Oligotex-dT30 <Super> mRNA Purification Kit (From Total RNA) (Cat. #9086)*
Dr. GenTLE™ Precipitation Carrier (Cat. #9094)
Mupid-2plus (Cat. #AD110)*
Mupid-exU (Cat. #AD140)*

* Not available in all geographic locations. Check for availability in your area.