cDNA Synthesis Kit Manual

Cat.# 6130
v.0708

TaKaRa

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1. Description:

cDNA Synthesis Kit is designed for the synthesis of double-stranded cDNA using RNA derived mainly from animals and plants. This kit is based on Gubler-Hoffman's method. Using this kit, cDNA library which contains high concentration of full-length cDNA is simply and efficiently obtained without lacking 5'-end sequences, even from small amount of RNA. No need to utilize vector-primer system or to remove hairpin loops by S1 nuclease.

2. Kit Components:

(for 20 μg RNA)

1. Reverse Transcriptase (M-MLV) .....2000 units
2. RNase Inhibitor (20 units/ μl).....200 units
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 (ea. 10 mM)) .....40 μl
7. *E.coli* RNase H / *E.coli* DNA Ligase Mixture .....20 μl
8. *E.coli* DNA Polymerase I (20 units/ μl).....400 units
9. 5x 2nd Strand Synthesis Buffer.....300 μl
10. T4 DNA Polymerase(1 units/ μl).....40 units
11. Diethylpyrocarbonated H_2O (DEPC-H_2O) .....600 μl x 2
12. Control RNA* .....5 μg

*Control RNA
Supplied control RNA is *in vitro* transcribed RNA using SP6 RNA polymerase from DNA fragments (approximately 1.4 kb) containing tetracycline resistant gene in the downstream region of SP6 promoter (pSPTet3). This control RNA is a poly(A)$^+$ RNA (approximately 1.4 kb) containing 30 bases poly(A)$^+$ tail. When full-length double- stranded cDNA is synthesized from this control RNA, tetracycline resistant plasmid is obtained by inserting this cDNA into an appropriate vector.

Reagents not supplied in the kit:

- 10% (w/v) SDS
- 0.25 M EDTA
- Phenol / chloroform (1:1, v/v)
- Ethyl ether
- 4M ammonium acetate
- Isopropanol
- Ethanol
- TE Buffer

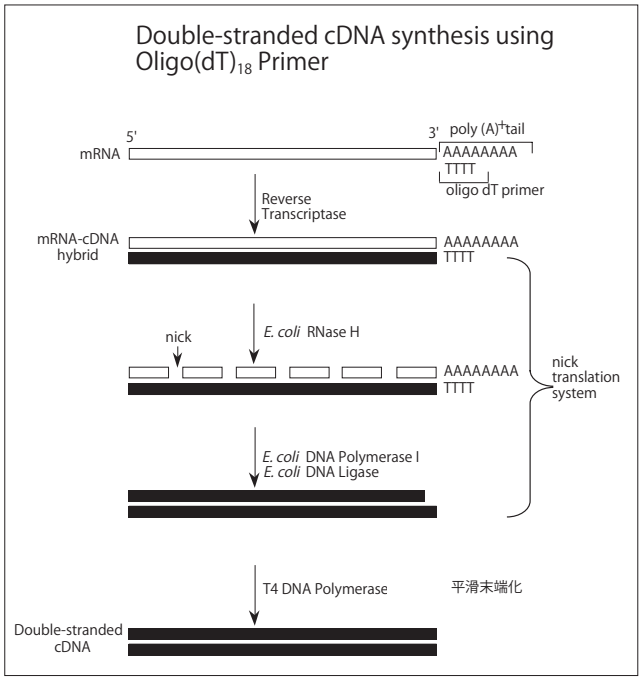
Equipment required:

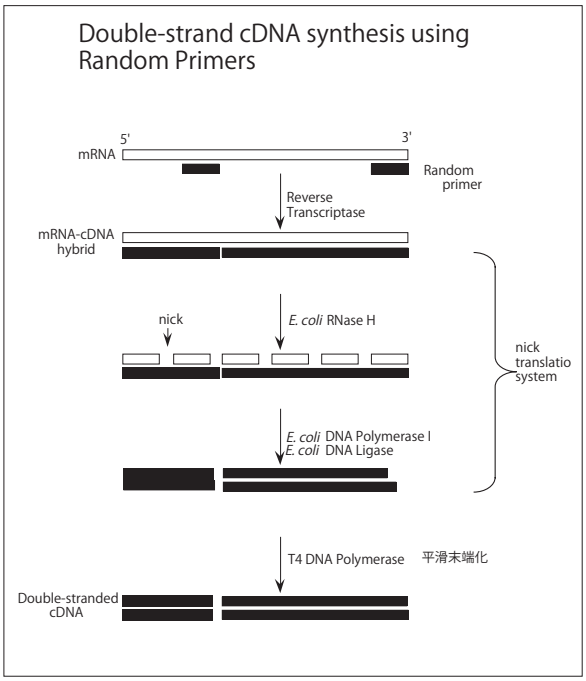
- Microcentrifuge
- 42℃ water bath (or TaKaRa PCR Thermal Cycler)
- 12℃ water bath
- 22℃ water bath
- 70℃ water bath
- 37℃ water bath
- Adjustable pipettes suitable for accurate dispensing 0-5, 10-50, 200-100 μl
- Microcentrifuge tubes and pipette tips

Storage: − 20℃

3. Principle

1) Synthesize first strand cDNA with Reverse Transcriptase (M-MLV).
2) Nick RNA in the mRNA-cDNA hybrid with *E.coli* RNase H. Replace RNA chain by DNA chain and synthesize second strand cDNA with *E.coli* DNA Polymerase I and *E.coli* DNA ligase.
3) Blunt ends with T4 DNA polymerase.





4. Preliminaries for cDNA synthesis reaction: (Preparation of RNase-free equipments and reagents)

A. Equipment

Commercially available sterile, disposable plasticwares are essentially free of RNase and can be used for the RNA preparation. However, microcentrifuge tubes and pipette tips must be autoclaved before use. In the case of glassware or spatulas, they should be treated by heating at 160℃ for more than 2 hours.
Other equipments, not suitable for heat treatment, should be treated with 0.1% diethylpyrocarbonate (DEPC) solution at 37℃ for 12 hours, and then remove DEPC by autoclaving to prevent carboxymethylation of RNA. It is necessary to clearly identify that are to be used only for experiments with RNA and to store separately items of laboratory equipments. A major source of RNase contamination is from the hands of the researcher. Gloves should be worn at all stages during the preparation of materials and solutions used for the isolation and analysis of RNA, and during all manipulations involving RNA.

B. Reagents

The solutions should be treated with 0.1% DEPC as above, with autoclaving before use. Reagents not suitable for autoclaving should be prepared using baked glassware, distilled autoclaved water, then filtrated.

5. Purification and analysis of mRNA

A. Preparation of total RNA

Total RNA shall be prepared with cesium chloride density-gradient centrifugation method, or guanidinium thiocyanate phenol chloroform method, or by using commercially available reagents or kits for RNA isolation.

B. Isolation of Poly $(A)^+$ RNA

Poly$(A)^+$ RNA is isolated from total RNA using affinity chromatography on Oligo(dT) cellulose or Poly(U) Sepharose.

C. Analysis of poly$(A)^+$ RNA

It is important to have a pure and undegraded poly$(A)^+$ RNA preparation to get maximum efficiency of cDNA synthesis. Once isolated, it is advisable to examine the integrity of the RNA population. This can be achieved by the analysis of poly$(A)^+$ RNA sample through electrophoresis of agarose gels containing glyoxal or formaldehyde.

6. Protocol

(1) First strand cDNA synthesis

1. Prepare the following reaction mixture in a microcentrifuge tube to the total volume of 20 μl.

Template RNA solution	2 μg RNA/reaction
5X 1st Strand Synthesis Buffer	4 μl
dNTP Mixture	1 μl
RNase Inhibitor	1 μl
$Oligo(dT)_{18}$ Primer, or Random Primer	2 μl
Reverse Transcriptase (M-MLV)	1 μl
DEPC-H_2O	up to 20 μl

2. Mix gently.
3. Leave at room temperature for 10 min. and move into the 42℃ water bath.
4. Incubate at 42℃ for 1 hour.
5. Place on ice and cool for 2 min.

Note: Particular mRNA templates may contain extensive secondary structure which may reduce the efficiency of reverse transcription. The following measure should be taken to reduce the affection of secondary structure.

・Heat the mRNA solution at 65℃ for 5 min, followed by rapid chilling on ice prior to cDNA synthesis.

(2) Second strand cDNA synthesis and conversion to blunt-ended form

1. To the microcentrifuge tube containing 20 μl of the first strand cDNA synthesis mix, add the following components to the total volume of 142 μl.

5x 2nd Strand Synthesis Buffer	30 μl
dNTP Mixture	3 μl
DEPC-H_2O	up to 89 μl

2. Add 2 μl of *E.coli* DNA Polymerase and 2 μl of *E.coli* RNase H / *E.coli* DNA Ligase Mixture. Mix gently.
3. Incubate sequentially at 16 ℃ for 2 hours and 70℃ for 10 min.
4. Add 4 μl of T4 DNA polymerase and mix gently.
5. Incubate at 37℃ for 10 min.
6. Stop the reaction by adding 15 μl of 0.25M EDTA (pH8.0) and 15 μl of 10% SDS solution, followed by gentle mixing.

(3) Purification of 2nd strand cDNA

1. Add 180 μl of phenol/chloroform/isoamylalcohol (25: 24 :1) to 180 μl of 2nd strand cDNA reaction mixture.
2. Mix vigorously by a vortex mixer.
3. Spin for 1 min. in a microcentrifuge to separate the phases.
4. Remove the upper aqueous phase into a new tube, avoiding material at the interface.
5. Repeat the steps 1-4.
6. Add 180 μl of chloroform/isoamylalcohol (24:1) to the aqueous phase and mix well.
7. Spin for 1 min in a microcentrifuge.
8. Remove the upper aqueous phase into a new tube, avoiding material at the interface.
9. Add 60 μl of 10 M ammonium acetate.
10. Add 600 μl of Ethanol.
11. Leave at room temperature for 10 min.
12. Spin for 10 min. in a microcentrifuge.
13. Remove the supernatant, being careful not to disturb the cDNA pellet.
14. Wash the pellet with 70% ethanol by gentle shaking, centrifugation, and dry under vacuum. A few minutes' dry is enough because it is difficult to redissolve pellets excessively dried.
15. Dissolve the pellet in TE buffer.
16. Store at -20℃ .

The above procedure is effective for removal of unincorporated dNTP. Unincorporated nucleotides can also be removed by spin-column chromatography or gel filtration. Size fractionation by an agarose gel electrophoresis is an alternative method for recovery of cDNA of target size, removing unincorporated dNTP.

Depending on the volume of template RNA, total volume of reaction mixture in cDNA synthesis reactions can be prepared in a volume as below:

Template RNA	Reaction mixture in 1st strand cDNA synthesis (inclusive of enzyme)	Reaction mixture in 2nd strand cDNA synthesis (inclusive of enzyme)
2 μg	20 μl	150 μl
3 μg	30 μl	225 μl
4 μg	40 μl	300 μl
5 μg	50 μl	375 μl

7. Calculation of cDNA reaction yields by RI labeling

(1) Labeling Reaction

A. Separate reaction labeling
This enables the labeling of 1st and 2nd strand cDNA independently.

1. Prepare two (20 μl) standard reaction mixture <No.1> and <No.2> by combining the followings. In the 1st strand cDNA synthesis reaction, <No.1> includes [α-^{32}P]dCTP, <No.2> does not.

	<No.1>	<No.2>
Template RNA solution	1~2 μg	1~2 μg
5x 1st Strand Synthesis Buffer	4 μl	4 μl
dNTP Mixture	1 μl	1 μl
RNase Inhibitor	1 μl	1 μl
Oligo(dT)$_{18}$ primer or Random Primer	2 μl	2 μl
Reverse Transcriptase (M-MLV)	1 μl	1 μl
[α-^{32}P] dCTP*	1 μl	-
DEPC-H_2O	up to 20 μl	up to 20 μl

*[α-^{32}P] dCTP : GE Healthcare (110 TBq/mmol)

2. Proceed with 1st strand synthesis as described in '6. Protocol (1) steps 2-5'. (page 4)
3. Take 1 μl of sample from <No.1> and process as described in '(3) Calculation of percentage incorporation'(page 7).
4. Prepare 2nd strand synthesis reaction mixture by adding the followings into <No.2>.

5x 2nd Strand Synthesis Buffer	30 μl
[α-^{32}P] dCTP	1 μl
dNTP Mixture	3 μl
DEPC-H_2O	88 μl

5. Add 2 μl of *E.coli* DNA Polymerase I and 2 μl of *E.coli* RNase H / *E.coli* DNA Ligase Mixture. Mix gently.
6. Incubate sequentially at 16℃ for 2 hours, 22℃ for 1 hour, and 70℃ for 10 min.
7. Add 4 μl of T4 DNA Polymerase and mix gently.
8. Incubate at 37℃ for 10 min.
9. Stop the reaction by adding 15 μl of 0.25 M EDTA (pH8.0) and 15 μl of 10% SDS.
10. After reaction has been stopped, take 1 μl of <No.2> and process as described in `(3) Calculation of percentage incorporation'(page 7).

B. Sequential reaction labeling
This method is used to monitor the success of the whole synthesis reactions.

1. Prepare the reaction mixture by combining the followings.

Template RNA solution	1~2 μg
5x 1st Strand Synthesis Buffer	4 μl
dNTP Mixture	1 μl
RNase Inhibitor	1 μl
Oligo(dT)$_{18}$ primer or Random Primer	2 μl
Reverse Transcriptase (M-MLV)	1 μl
[α-^{32}P] dCTP	0.5 μl
DEPC-H_2O	up to 20 μl

2. Proceed with 1st strand synthesis labeling as described in '6. Protocol (1) steps 2-5'. (page 4)
3 Take 1 μl of sample and process as described in '(3) Calculation of percentage incorporation' below.
4. Set up the 2nd strand synthesis reaction mixture by adding the followings.

5x 2nd Strand Synthesis Buffer	30 μl
[α -^{32}P] dCTP	5 μl
dNTP Mixture	3 μl
DEPC-H_2O	84 μl

5. Proceed separate reaction labeling as described in 'A. Separate labeling reaction', steps 5-9.
6. Take 1 μl of sample and process as described in '(3) Calculation of percentage incorporation' below.

(2) Measurement of percentage of radioactivity incorporated in cDNA synthesis

1. Take 1 μl of 1st strand cDNA reaction mixture or 2nd strand mixture and dilute 10-50 times with H_2O or 0.2 M EDTA.
2. Spot a known volume, for example 5 μl, onto the centre of each of two discs of Whatmann DE81 (φ 2.4 cm). Designate them filter disc A and disc B.
3. Dry filter disc A thoroughly with a lamp. Filter A is used to measure the total radioactivity (Total cpm) in the sample.
4. Wash filter disc B in the steps below.

5% Na_2HPO_4	5 min. X 6 times
Deionized water	1 min. X 2 times
70% ethanol	1 min. X 2 times

5. Dry the filter disc B thoroughly, for example using a lamp. Filter disc B is used to measure the radioactivity incorporated into nucleic acids (Sample cpm).
6. Add the dried fliter disc A or B in aqueous scintillation cocktail and count cpm by liquid scintillation counter.

(3) Calculation of percentage incorporation

A. Separate reaction labeling
Percentage incorporation (%) = (Sample cpm / Total cpm) x 100%

B. Sequential reaction labeling
Assume Total cpm, Sample cpm in the 1st strand synthesis are
Total cpm [1], Sample cpm [1],

and those in the 2nd strand synthesis are
Total cpm [2], Sample cpm [2]

Since the 1st strand synthesis reaction mixture is 1/9 of the volume of the 'stopped' 2nd strand mixture, percentage incorporation is calculated by the following formula:

Percentage incorporation during 1st strand cDNA syntheis = (Sample cpm [1] / Total cpm [1])x 100%

Percentage incorporation during 2nd strand cDNA synthesis = (Sample cpm [2] - Sample cpm [1] /9) / (Total cpm [2] - Total cpm [1] / 9) x 100%

(4) Calculation of yield of cDNA

[1 st strand cDNA synthesized]

・Percentage of labeled [α -^{32}P]dCTP incorporated → A(%)

・ Amount of cold dCTP in 1st strand and cDNA synthesis reaction mixture → 10 nmol

・Amount of cold dCTP incorporated, assuming that [α -^{32}P]dCTP and cold dCTP are incorporated at the same ratio → 10 X A/100 = A/10 nmoles

・ Amount of total dNTP incorporated, assuming that other dNTPs (dATP, dGTP, dTTP) are incorporated at the same ratio as dCTP → 4 X A/10 nmoles

・ Weight of cDNA synthesized, assuming that residue molecular weight of dNMP (1 mole) is 330 g → 330 g X 4/10 X A X 10^{-9} = 132 X A (%) ng

Therefore,

・ Amount of 1 st strand cDNA synthesized =Percentage incorporation (%) x 132 ng

[2 nd strand cDNA synthesized]

・Percentage of labeled [α -^{32}P]dCTP incorporated → A(%)

・ Amount of cold dCTP in 1st strand and cDNA synthesis reaction mixture → 40 nmol

・Amount of cold dCTP incorporated, assuming that [α -^{32}P]dCTP and cold dCTP are incorporated at the same ratio → 40 X A/100 = 2/5 X A nmoles

・ Amount of total dNTP incorporated, assuming that other dNTPs (dATP, dGTP, dTTP) are incorporated at the same ratio as dCTP → 4 X 2/5 X A nmoles

・ Weight of cDNA synthesized, assuming that residue molecular weight of dNMP (1 mole) is 330 g → 330 g X 4 X 2/5 X A X 10^{-9} = 528 X A (%) ng

Therefore,

・ Amount of 2nd strand cDNA synthesized =Percentage incorporation (%) x 528 ng

・% yield of 1st strand cDNA synthesized = $\frac{\text{Amount of 1st strand cDNA synthesized x 100\%}}{\text{Amount of template RNA}}$

・ % yield of 2nd strand cDNA synthesized = $\frac{\text{Amount of 2nd strand cDNA synthesized X 100\%}}{\text{Amount of 1st strand cDNA}}$

8. Gel electrophoresis analysis

Alkaline agarose gel electrophoresis is recommended for the analysis of the products of the cDNA synthesis reactions.

(1) Preparation of sample

1. Into the cDNA synthesis reaction mixture, add 20 μg of carrier DNA (tRNA from yeast), 2/3 volume of 5M ammonium acetate, and 2.5 volume of ethanol, and then mix thoroughly.
2. Leave at -20℃ for 30 min and centrifuge for 20 min.
3. Remove the supernatant and dry the pellet.
4. Dissolve the pellet in 30 μl of TE buffer.
5. Add 20 μl of 5M ammonium acetate and 125 μl of ethanol, and mix thoroughly.
6. Leave at -20℃ for 30 min and centrifuge for 20 min.
7. Remove the supernatant, and dry the pellet.
8. Suspend the pellet in 10-20 μl of alkaline agarose gel loading buffer.*
9. Add 1/5 volume into sample (containing cDNA pellet) 6X Alkaline gel loading buffer.**

* Alkaline gel electrophoresis buffer
50 mM NaOH
1 mM EDTA (pH8.0)

** 6X Alkaline gel loading buffer
300 mM NaOH
6 mM EDTA
18% Ficoll
0.15% Bromocresol green
0.25% Xylene cyanol FF

(2) Preparation of end-labeled molecular weight markers

End-labeled molecular weight markers are used to estimate the product size of synthesized cDNA. λ-*Hin*d III digest (Takara Cat.#3403), or φX174-*Hae* III digest (Takara Cat.#3405) are recommended depending on the molecular weight of the cDNA. Restriction fragments are labeled quickly and conveniently by using MEGALABEL (Takara Cat.#6070) or by using T4 Polynucleotide Kinase (Takara Cat.#2021) in combination with [γ-^{32}P] ATP (GE Healthcare).

(3) Electrophoresis

A. Preparation of the gel

The product size distribution can be analyzed by electrophoresis on a 1.4% agarose gel under denaturing conditions.

Since addition of NaOH to hot agarose solutions may cause hydrolysis of agarose, gels are prepared in a neutral unbuffered solutions (50 mM NaCl / 1 mM EDTA) and are equilibrated in alkaline gel electrophoresis buffer before running.

1. Add powdered agarose to the solutions of 50 mM NaCl / 1 mM EDTA, to give a 1.4% gel. Thin gels are easy to be equilibrated with alkaline buffer.
2. Dissolve the agarose thoroughly by heating in a boiling water or micro wave oven.
3. Pour the gel and solidify it in a gel electrophoresis apparatus.
4. Set the gel to a electrophoresis apparatus, add sufficient alkaline electrophoresis buffer.
5. Allow the buffer to soak into the gel for at least 30 min to equilibrate the gel with the buffer.
6. Before loading samples, remove the excess alkaline electrophoresis buffer almost to the same level as the gel.

B. Loading and running the gel

1. Load the DNA sample using a micropipette.
2. Electrophoresis is carried out at voltages of <7.5 V/cm until the dye (bromocresol green) has migrated approximately 1/3-1/2 of the length of the gel. As bromocresol green diffuses out of alkaline gels into the electrophoresis buffer, place the glass plate directly on top of the gel before continuing electrophoresis just after the dye has migrated out of the loading slot.
3. Remove the gel from the tank and soak it in 7% trichloroacetic acid for 30 min. at room temperature.
4. Wash the gel gently with deionized water before dry.
5. Place the gel on Whatmann 3MM paper to remove water on the surface of the gel. The dried gel is covered with wrap.
6. Dry the gel using a slab gel drying apparatus.
7. The DNA can be detected by autoradiography at room temperature, or at -80℃ with an intensifying screen.
   When cDNA fragments to be cloned have cleavage sites of restriction enzyme, protection of cDNA with corresponding methylase is useful to insert target cDNA into vetor without cutting the fragments. Following is the protocol of the cDNA cloning using *Eco*R I methylation system.

9. Application Examples

cDNA cloning

(1) cDNA cloning with Methylation system

A. Methylation of cDNA

1. Prepare the reaction mixture in a microcentrifuge tube by combining the followings to adjust their final concentration.

100 mM	Tris-HCl (pH8.0)
10 mM	EDTA
2 mM	DTT
80 µM	S-adenosylmethionine
0.5~1.0 µg	2nd strand cDNA
20 units	Methylase *Eco*R I
Total	20 µl

2. Incubate at 37℃ for 1 hour.
3. Extract with the same volume (20 µl) of phenol / chloroform. Repeat the extraction.
4. Add 1/10 volume (2 µ l) of 3M sodium acetate.
5. Add 2 volume (40 µl) of ethanol.
6. Chill at -20℃ for 30 min.
7. Spin for 20 min in a microcetrifuge.
8. Wash the pellet with 80% ethanol and dry under vacuum.
9. Dissolve cDNA in TE buffer.
10. Store at -20℃ .

B. Linker Ligation and digestion with restriction enzyme

1. Prepare the reaction mixture in a microcentrifuge tube by combining the followings.

Volume, or final concentraiton	Reagent
0.01~0.1 pmol	Methylated 2nd strand cDNA
Over 100 fold molar excess relative to cDNA	p*Eco*R I Linker
66 mM	Tris-HCl (pH7.6)
6.6 mM	$MgCl_2$
10 mM	DTT
0.1 mM	ATP
350 units	T4 DNA Ligase
Total	10 µl

3. Incubate at 65℃ for 10 min.
4. Add the followings to carry out digestion with *Eco*R I.

Linker-ligated cDNA	10 µl
Reaction buffer supplied with *Eco*R I	5 µl
*Eco*R I	20~100 units (<5 µl)
Distilled water	up to 50 µl

5. Incubate at 37℃ for 2 hours.
6. Extract with the same volume (50 µl) of phenol / chloroform. Repeat extraction.
7. Add 1/10 volume (5 µl) of 3M sodium acetate.
8. Add 2 volumes (100 µl) of ethanol.
9. Chill at -20℃ for 30 min.
10. Spin for 20 min in a microcentrifuge.
11. Wash the pellet with 80% ethanol and dry under vacuum.
12. Dissolve cDNA in TE buffer.
13. Recover cDNA fragments by removing excess linkers through gel filtration, or spin-column chromatography, or agaose gel electrophoresis.
14. Insert the recovered cDNA into a vector. We recommend to use λ -DNA vector. Refer to the 'Insertion of cDNA into λ -DNA' (page 12)

(2) cDNA cloning using adaptors

After ligation of adaptors to cDNA, cDNA does not need to be methylated or digested with restriction enzymes. The adapted, phosphated cDNA is ready for direct ligation to plasmid vectors.

A. Adaptor ligation

1. Prepare the reaction mixture by combining the followings to adjust their final concentration.

Final concentration	Reagent
0.01~0.1 pmol	2nd strand cDNA solution
Over 100-volume molar excess relative to cDNA	*Eco*R I-*Not* I-*Bam*H I adaptor
66 mM	Tris-HCl (pH7.6)
6.6 mM	$MgCl_2$
10 mM	DTT
0.1 mM	ATP
350 units	T4 DNA Ligase
	Total 10 µl

2. Incubate at 16℃ for 2 hours~overnight.
3. Stop reaction by adding 1 µl of 0.5M EDTA.
4. Extract with an equal volume (11 µl) of phenol / chloroform. Repeat the extraction.
5. Add 1/10 volume (1.1 µl) of 3M sodium acetate.
6. Add 2 volumes (22 µl) of ethanol.
7. Chill at -20℃ for 30 min.
8. Spin for 20 min in a microcentrifuge.
9. Wash the pellet with 80% ethanol and dry under vacuum.
10. Dissolve cDNA in TE buffer.

B. Phosphorylation of adaptors

1. Prepare the reaction mixture by combining the followings to adjust the final concentration.

Final concentration	Reagent
50 mM	Tris-HCl (pH8.0)
10 mM	$MgCl_2$
5 mM	DTT
0.1 mM	ATP
0.01~0.1 pmol	Adaptor-ligated 2nd strand cDNA
5~20 units	T4 Polynucleotide Kinase
	Total 50 µl

2. Incubate at 37℃ for 30 min.
3. Stop the reaction by adding 5 µl of 0.5 M EDTA.
4. Incubate at 70℃ for 5 min.
5. Extract with an equal volume (55 µl) of phenol / chloroform.
6. Add 1/10 volume (5.5 µl) of 3M sodium acetate.
7. Add 2 volumes (110 µl) of ethanol.
8. Chill at -20℃ for 30 min.

9. Spin for 20 min in a microcentrifuge.
10. Wash the pellet with 80% ethanol and dry under vacuum.
11. Dissolve the cDNA in TE buffer.
12. Recover cDNA fragments by removing excess adaptors through gel filtration, or spin-column chromatography, or agarose gel electrophoresis.
13. Insert the recovered cDNA into a vector. We recommend to use λ -DNA. Refer to 'Insertion of cDNA into λ -DNA' as below.

Insertion of cDNA into λ -DNA

1. Prepare the reaction mixture by combining the followings.

0.01~0.1 pmol	Linker (Adaptor)-ligated 2nd strand cDNA solution
2 fold molar excess relative to cDNA	*Eco*R I digested dephosphorylated λ -DNA
1/10 volume of reaction mixture	3M sodium acetate
2 volumes of reaction mixture	Ethanol

2. Chill at -20℃ for 30 min.
3. Spin for 20 min. in a microcentrifuge.
4. Wash the pellet with 80% ethanol and dry under vacuum.
5. Dissolve the pellet in TE buffer.
6. Add the following reagents to cDNA / λ -DNA vector mixture and adjust the concentration as shown below.

66 mM	Tris-HCl(pH7.6)
6.6 mM	$MgCl_2$
10 mM	DTT
0.1 mM	ATP
350 units	T4 DNA Ligase
Total	10 µl

7. Incubate at 16℃ for 2 hours~overnight.
8. Take the reactant and proceed with *in vitro* packaging by using a commercially available kit.
9. Transfect to a host cell and form plaques on the medium.
10. Plaques are used for screening by plaque hybridization.

10. Trouble shooting

Takara's cDNA Synthesis kit is subjected to careful quality control before despatch to ensure efficient performance. Should poor results be obtained, we recommend the following points for consideration.

1. Control poly(A)$^+$ mRNA

pSP Tet3 poly(A)$^+$ RNA is supplied with this cDNA Synthesis kit as control RNA. It is supplied ready to use as a control to check both the first and second strand synthesis reactions by liquid scintillation counter, or by agarose gel electrophoresis.

2. Purity of mRNA

It is important that mRNA should be as pure as possible to obtain the maximum efficiency of cDNA synthesis. Before proceeding with cDNA synthesis, it is advisable to check the integrity of the mRNA by gel electrophoresis, and by measuring absorbance at 260 nm(A260) and at 280 nm(A280)*.
(*The absorbance ratio of A260/A280 = 1.7~1.9 is recommended.)

3. RNase contamination

Extreme care should be taken to avoid RNase contamination.
Wherever possible, all instruments, reagents should be sterilized by heat sterilization or by autoclaving, and plastic gloves should be worn at all stages during the manipulation involving RNA. RNase Inhibitor is included with this kit to ensure full protection of the template mRNA from RNase.

4. Efficiency of reverse transcription
Individual species of mRNA may be reverse transcribed with different efficiencies. Particular mRNA templates may contain extensive secondary structure which may reduce the efficiency of reverse transcription. If this is suspected, treat mRNA by the following procedures prior to cDNA synthesis to obtain high efficiency in reverse transcription.

・Heat the mRNA solution at 65℃ for 5 min, followed by rapid chilling on ice.

5. Optimal enzyme / mRNA ratio
If it is observed that a particular template mRNA continually results in poor efficiency of 1st strand synthesis that can not be impromed by reference to notes 3) and 4) of this section, then it may be necessary to determine the optimal enzyme to mRNA ratio. This may be carried out as follows:

1) Set up a series of 1st strand standard reaction mixtures of 20 µl containing 1 µl of [α -^{32}P]dCTP (110 TBq/mmol) and 200 units of M-MLV reverse transcriptase. Add mRNA in a range of concentrations and incubate at 42℃ for 1 hour. Take samples and calculate the yield of cDNA synthesized in each tube by a liquid scintilation counter as described in '6. Calculation of cDNA reaction yields by RI labeling'. Calculate the enzyme/ mRNA ratio (units / µg) and determine the optimal condition by selecting the concentrations which gives largest value.

2) If sufficient amounts of mRNA is available, set up a series of 1st strand synthesis reaction mixtures containing 1 µg of mRNA with increasing amounts of M-MLV reverse transcriptase. Proceed with 1st strand synthesis and carry out the analysis as in note 1) above.

For both options a no-enzyme incubation should be included as a control.

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