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Not for use in diagnostic procedures.



cDNA Synthesis System

 **Version: 24**

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For the synthesis of double-stranded cDNA from total RNA or mRNA

Cat. No. 11 117 831 001 1 kit
up to 10 reactions

Store the kit at -15 to -25°C

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1. General Information


1.1. Contents

Vial	Label	Function/Description	Content
1	AMV RT Buffer	5x conc.	1 vial, 500 µl
2	AMV Reverse Transcriptase	25 U/µl	1 vial, 20 µl
3	DTT	0.1 M	1 vial, 1 ml
4	Protector RNase Inhibitor	25 U/µl	1 vial, 10 µl
5	Oligo(dT) ₁₅ Primer	200 µM = 1 µg/µl	1 vial, 20 µl
6	Oligo[(dT) ₂₄ T7Prom] ₆₅ Primer	100 µM = 2 µg/µl Sequence of the oligo[(dT) _{24VN} T7prom] ₆₅ 5' GGC CAG TGA ATT GTA ATA CGA CTC ACT ATA GGG AGG CGG (T) ₂₄ VN 3'	1 vial, 20 µl
7	dNTP Mixture	10 mM of each nucleotide	1 vial, 110 µl
8	Neo mRNA Poly (A) ⁺	0.2 µg/µl	1 vial, 25 µl
9	2 nd Strand Synthesis Buffer	5x conc.	1 vial, 500 µl
10	2 nd Strand Enzyme Blend	Mixture of DNA polymerase I, <i>E. coli</i> ligase, RNase H	1 vial, 65 µl
11	T4 DNA Polymerase	1 U/µl	1 vial, 200 µl
12	Water, PCR Grade	To adjust the final reaction volume.	1 vial, 1 ml
13	RNase I	10 U/µl	1 vial, 15 µl
14	Proteinase K, recombinant, PCR Grade	50 U/ml	1 vial, 50 µl

1.2. Storage and Stability

Storage Conditions (Product)

When stored at –15 to –25°C, the kit is stable through the expiration date printed on the label.

Vial / Bottle	Label	Storage
14	Proteinase K, recombinant, PCR grade	 Store Proteinase K (Vial 14) at +2 to +8°C.

 The kit is shipped on dry ice.

1.3. Additional Equipment and Reagents Required

Cleaning the ds cDNA

- Phenol
- TE buffer (10 mM Tris/HCl, pH 8; 1 mM EDTA pH 8.0)
- Phenol/chloroform/isoamylalcohol (25:24:1)
- Chloroform/isoamylalcohol (24:1)
- 5 M NH₄OAc
- 100% ethanol (–15 to –25°C)
- 70% ethanol
- Water, PCR Grade

i Roche recommends using the Roche High Pure PCR Product Purification Kit* instead of purifying the cDNA with phenol-chloroform extraction (Footitt, S. et al., 2003).

Second Strand Synthesis

- EDTA 0.2 M, pH 8.0

1.4. Application

Optimized one-tube procedure for the synthesis of ds cDNA from total RNA or mRNA. The ds cDNA can be used for:

- The construction of non-directional cDNA libraries (Current Protocols in Molecular Biology, 5.6, 1987).
- As a starting point for subtractive hybridization experiments to enrich differentially expressed genes (Hubank, M., and Schatz, D.G., 1994, and Lavery, D.J. et al., 1997).
- For the *in vitro* transcription of whole cDNA populations to generate labeled cRNA for hybridization on DNA microarrays (Mahadevappa, M. and Warrington, J.A., 1999).

1.5. Preparation Time

Assay Time

1st strand synthesis: 60 minutes

2nd strand synthesis: 2 hours

2. How to Use this Product

2.1. Before you Begin

Sample Materials

Construction of an optimal double-stranded cDNA begins with the preparation of high-quality RNA. For best results, optimize the isolation of total RNA or mRNA from eukaryotic tissues or from cell cultures, and to prevent adventitious introduction of RNases into a preparation.

- For the construction of cDNA libraries and subtractive hybridization protocols, use mRNA as starting sample material.
 - For expression profiling on DNA microarrays, use total RNA as starting material.
- Roche recommends using the High Pure RNA Isolation Kit* for the isolation of total RNA and the mRNA Isolation Kit* for mRNA isolation.

Control Reactions

A control RNA (Neo mRNA) is included in the cDNA Synthesis System to verify that the system is functioning correctly. For best results, use the control RNA by simply substituting your RNA with 5 µl (1 µg) control RNA in the first strand reaction.

Primers

Do not proceed with the protocol until a primer for reverse transcription has been selected. If cRNA synthesis from double-stranded cDNA is the goal, choose the oligo [(dT)₂₄ T7promotor]₆₅ primer (Vial 6) for the first strand synthesis since this primer also has the promoter for T7 RNA Polymerase.

General Considerations

Precautions

Successful cDNA synthesis demands an RNase-free environment. Use only RNase-free autoclaved tubes, aerosol-free pipette tips, and use only DEPC-treated water and autoclaved or sterile-filtered solutions. Wear gloves to avoid RNase contamination during handling with RNA.

Analysis of RNA Preparation

The quality of RNA used as template significantly influences the size distribution and yield of the first strand product. After preparing total RNA or mRNA, evaluate its integrity on a formaldehyde denaturing agarose/EtBr gel. mRNA shows a smear from approx. 7 kb down to 200 bp, often with faint banding. The gel should reveal a smear of fluorescent material centered in the 1 to 3 kb range for mRNA, with some discrete fragments corresponding to abundant mRNAs or residual 18S or 28S rRNA. For total RNA, the major bands will be 18S rRNA (approx. 2 kb) and 28S rRNA (approx. 5 kb), as well as a background smear centered in the 1 to 3 kb range. If neither rRNA is visible and the distribution of mRNA is not centered at the 1 to 3 kb range, then you will need to consider troubleshooting for RNA isolation or cell storage.

First Strand Reaction

AMV Reverse Transcriptase is used for first strand cDNA synthesis. The initiation of the first strand synthesis depends upon hybridization of a primer to the mRNA, usually at the poly(A)⁺ tail. The kit includes two different oligo dT primers. Oligo(dT)₁₅ Primer (Vial 5) for standard cDNA synthesis followed by cloning or restriction enzyme digestion, and Oligo[(dT)₂₄ T7promotor]₆₅ Primer (Vial 6) for dsDNA synthesis followed by *in vitro* transcription of the cDNA population by T7 RNA polymerase. The reaction conditions for first strand synthesis have been optimized for yield and size of the cDNAs. The optimal temperature for reverse transcription is +42°C.

2. How to Use this Product

Amount of RNA

The amount of mRNA in an assay can be as high as 2 µg; the amount of total RNA up to 20 µg. We recommend using at least 1 µg of total RNA in a 40 µl reaction volume.

Enzyme concentration

The ratio of Reverse Transcriptase AMV (50 U/assay) to RNA is not critical up to 20 µg total RNA. If less than 1 µg total RNA is available, use 25 U Reverse Transcriptase AMV per assay and halve the reaction volume to 20 µl, also adjusting the components to this volume.

Second Strand Reaction

The first and second strand syntheses are performed in the same tube, which speeds up the synthesis procedure and maximizes recovery of cDNA. Synthesis for the second strand takes place using the DNA/RNA hybrid as substrate. Mild treatment with RNase H inserts nicks into the RNA, providing 3' OH-primers for DNA polymerase I present in the 2nd strand enzyme cocktail.

The 5'→3' exonuclease activity of DNA polymerase I removes the primer stretches in the direction of synthesis, which are then replaced with new nucleotides by the polymerase activity. *E. coli* Ligase links the gaps to the ds cDNA strand. The last step in the cDNA synthesis is to ensure that the termini of the cDNA are blunt. This is done by adding T4 DNA Polymerase, which removes any remaining overhanging 3' ends on the ds cDNAs.

2.2. Protocols

cDNA Synthesis from Total RNA or mRNA

i Since the conditions described for the first strand reaction are optimized, do not change volumes and concentrations.

1 Thaw all necessary components and place them on ice.

2 Pipet into a sterile 1.5 ml reaction tube when using 40 µl total RT reaction volume. For 20 µl total RT reaction volume, we recommend using a thin-walled PCR tube and a thermal cycler for the first strand synthesis.

Reagent	Volume	Final conc.
Total RNA	x µl	1 to 20 µg
mRNA	x µl	0.3 to 2 µg
Oligo[(dT) ₂₄ T7 promotor] ₆₅ primer 100 pmol/µl (Vial 6) or Oligo(dT) ₁₅ primer 200 pmol/µl (Vial 5)	2 µl	200 pmol
Water, PCR Grade (Vial 12)	x µl	
Total volume	21 µl	

3 Incubate at 70°C for 10 min in a water bath, then place the tube immediately on ice.

4 Add the following components and mix gently:

Reagent	Volume	Final conc.
AMV RT Buffer, 5x conc. (Vial 1)	8 µl	1x
DTT, 0.1 M (Vial 3)	4 µl	10 mM
AMV, 25 U/µl (Vial 2)	2 µl	50 U
Protector RNase Inhibitor, 25 U/µl (Vial 4)	1 µl	25 U
dNTP Mix, 10 mM each (Vial 7)	4 µl	1 mM each

5 Incubate 60 min at 42°C.

6 Place the tube on ice to terminate the reaction.

7 Continue immediately with the Second Strand Synthesis reaction protocol below.

i The amount and size distribution of cDNA obtained should be analyzed by alkaline agarose gels, revealing a distribution from 0.5 to >7 kb. If inferior results are obtained in the first strand reaction, the control RNA can be used to verify that the system components are working properly (see Fig. 1).

2. How to Use this Product

Second Strand Synthesis

1 Thaw all required components, mix, and place on ice.

2 For one, 150 µl reaction, pipet the following components into the first strand reaction tube on ice and mix gently:

Reagent	Volume
cDNA from RT reaction	40 µl
2 nd Strand Synthesis Buffer, 5x conc. (Vial 9)	30 µl
dNTP mix, 10 mM each (Vial 7)	1.5 µl
2 nd Strand Enzyme Blend (Vial 10)	6.5 µl
Water, PCR Grade (Vial 12)	72 µl
Total Volume	150 µl

3 Incubate 2 h at 16°C.

4 – Add 20 µl (20 U) T4 DNA Polymerase (Vial 11).
– Incubate 5 min at 16°C.

5 Stop reaction by adding 17 µl EDTA, 0.2 M, pH 8.0.

i The second strand reaction is generally efficient, and yields of 80% to 100% are common (relative to the amount of first strand cDNA synthesized). The distribution of second strand cDNA products should resemble the distribution of the first strand cDNA products when analyzed by alkaline agarose gel electrophoresis (see Fig. 1). The performance of the system components can be checked by the supplied control reaction (Control RNA, Vial 8).

Digestion of RNA

For total RNA as starting material, digestion of residual RNA after ds cDNA synthesis is recommended:

1 Add 1.5 µl (15 U) RNase I (Vial 13) to the reaction tube.

2 Digest 30 minutes at 37°C.

3 – Add 5 µl (0.25 U) Proteinase K, recombinant (Vial 14) to the reaction.
– Incubate another 30 minutes at 37°C.

4 Follow the **Cleaning the ds cDNA Protocol** to clean the double-stranded cDNA.

i It is important to use phenol for the cleaning procedure to ensure safe elimination of the RNase I and Proteinase K, recombinant.

Cleaning the ds cDNA

i Instead of purifying the ds cDNA with Phenol-Chloroform Extraction you can use alternatively the High Pure PCR Product Purification Kit*.

The proper cleaning of ds cDNA is important in all steps below:

- 1 Add 200 µl phenol, vortex 10 s, centrifuge 15 s, and transfer supernatant to a new tube.

- 2 Wash phenol phase by adding 50 µl TE (to minimize loss of DNA), vortex 10 s, centrifuge 15 s, and save supernatant.

- 3 Combine supernatant from Steps 1 and 2.

- 4 Add 200 µl phenol/chloroform/isoamylalcohol (25:24:1), vortex 10 s, centrifuge 15s, and transfer supernatant to a new tube.

- 5 Wash the supernatant with 200 µl chloroform/isoamylalcohol (24:1), vortex 10 s, centrifuge 15 s, save supernatant.

- 6 Repeat Step 5.

- 7 Precipitate DNA by adding 0.6 vol of 5 M NH₄OAc and 2.5 vol of 100% ethanol (–15 to –25°C).

- 8 Store approx. 1 hour at –60°C or below.

- 9 Pellet the DNA by centrifugation at high speed for 10 min, discard the supernatant carefully.

- 10 Wash pellet by overlaying with 300 µl 70% ethanol (–15°C to –25°C).

- 11 Centrifuge at high speed for 10 min, discard the supernatant carefully.

- 12 Air dry pellet by evaporating residual ethanol.

- 13 Dissolve pellet in an appropriate volume with Water, PCR Grade, depending on the following procedure.

Analysis of cDNA Products

Monitoring

The synthesis of cDNA or double-stranded cDNA can be monitored if control mRNA or mRNA was used by running a 1% agarose gel. Use an aliquot calculated from a starting amount of 100 ng mRNA.

i *If total RNA has not been digested after ds cDNA synthesis, the analysis of cDNA on a gel is not possible due to the high background smear of the starting RNA.*

Radioactive analysis

For the analysis of synthesis rates by radioactive nucleotides, see (Current Protocols in Molecular Biology, 5.5, 1987).

Downstream Applications

In vitro transcription of T7 RNA polymerase

ds cDNA from up to 20 µg total RNA can be used for *in vitro* transcription and labeling of cRNA by incorporation of modified UTP (*e.g.*, DIG-11-UTP*, Biotin-16-UTP*, Fluorescein-12-UTP*).

For *in vitro* transcription and labeling, we recommend using total RNA for the cDNA synthesis.

Generation of cDNA libraries

The next step in cDNA library construction is the ligation of specific adaptors to the blunt end cDNA with T4 DNA Ligase. The selection of the adaptors depends on the vector used. Very common adaptors are Eco RI (*Not* I) adaptors (Current Protocols in Molecular Biology, 5.6, 1987).

Restriction digest of ds cDNA

In a typical restriction digest, 1 µg of the ds cDNA is digested with approximately 20 U of restriction enzyme under conditions recommended by the supplier.

3. Results

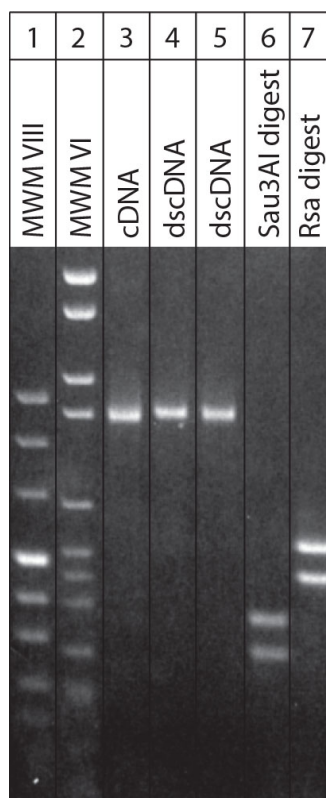


Fig. 1: Performance of the control reaction with Neo mRNA. Analysis of Neo mRNA performed using the cDNA Synthesis System. One microgram of neo mRNA (Vial 8) has been transcribed into ds cDNA according to the Instructions for Use. Aliquots of 500 ng starting material were analyzed on a 1 – 2% agarose gel.

4. Additional Information on this Product

4.1. Test Principle

The double-stranded cDNA Synthesis System is a specially designed one-tube system (Gubler, K. and Hoffman, B.J., 1983). The system is optimized to reduce manipulation steps, allowing the rapid and reliable synthesis of full-length cDNAs, especially from total RNA. The reaction conditions of the cDNA Synthesis System have been optimized to maximize length and yield of double-stranded cDNAs by monitoring the cDNA efficiency on certain RNAs by PCR (see Fig. 2).

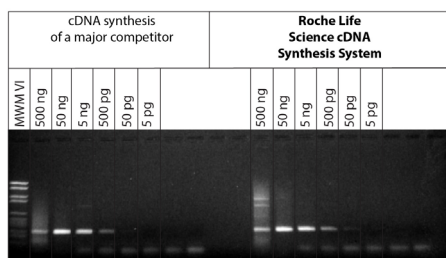


Fig. 2: Comparison of cDNA efficiency of the Roche Life Science cDNA Synthesis System and a cDNA Synthesis Kit of a major competitor. Ten microgram of total human placenta RNA was transcribed into cDNA. To measure yield and efficiency of cDNA synthesis, we amplified the far 5' end of the Insulin Receptor cDNA (10,006 bp). Various amounts of cDNA (500 ng – 5 pg) were amplified for 35 cycles with the Expand High Fidelity PCR System* and primers located on the far 5' end of Insulin Receptor mRNA. The figure shows 1% agarose gel electrophoresis of PCR products from Insulin Receptor cDNA synthesized by two different cDNA synthesis systems.

Results: This clearly shows that the Roche Life Science cDNA Synthesis Systems yields approximately 10 fold more cDNA than the competitor product.

4.2. References

- Footitt S, Ingouff M, Clapham D, von Arnold S - Expression of the viviparous 1 (Pavp1) and p34cdc2 protein kinase (cdc2Pa) genes during somatic embryogenesis in Norway spruce (*Picea abies* [L.] Karst) (2003) *Journal of Experimental Botany* **388**, 1711-1719
- Gubler U, Hoffman BJ - A simple and very efficient method for generating cDNA libraries (1983) *Gene* **2**, 263-269
- Hubank M, Schatz DG - Identifying differences in mRNA expression by representational difference analysis of cDNA (1994) *Nucleic Acids Research* **25**, 5640-5648
- John Wiley & Sons Inc - Current Protocols in Molecular Biology (2003) , 593-598
- Lavery DJ, Lopez-Molina L, Fleury-Olela F, Schibler U - Selective amplification via biotin- and restriction-mediated enrichment (SABRE), a novel selective amplification procedure for detection of differentially expressed mRNAs (1997) *Proceedings of the National Academy of Sciences of the United States of America* **13**, 6831-6836
- Mahadevappa M, Warrington JA - A high-density probe array sample preparation method using 10- to 100-fold fewer cells (1999) *Nature biotechnology* **11**, 1134-1136

4.3. Quality Control

The cDNA synthesis and the 2nd strand synthesis are controlled using the supplied *in vitro*-transcribed Control Neo mRNA (Vial 8), the Oligo(dT)₁₅ primer, and the Oligo[(dT)₂₄ T7promotor]₆₅ primer, respectively. After double-stranded cDNA synthesis, the product is digested with *Sau3A*I. All steps are monitored on an agarose gel. In addition, the double-stranded cDNA synthesized using the Oligo(dT)₂₄ T7 promoter primer, is reverse transcribed using T7 RNA Polymerase to verify the function of the T7 promoter.

4.4. Other Parameters

Specific Activity

Proteinase K, recombinant, PCR Grade: Approx. 2.5 U /mg, when assayed with the Chromozym assay (equivalent to 30 U/mg with the hemoglobin assay).

Temperature Optimum

+42°C

Reverse transcription

Volume Activity



Proteinase K, recombinant, PCR Grade: 50 U/ml

One unit is the enzyme activity which cleaves at +25°C in 1 min, 18 mmol Chromozym TRY (equivalent to 600 U/ml with the hemoglobin assay).

5. Supplementary Information

5.1. Conventions

To make information consistent and easier to read, the following text conventions and symbols are used in this document to highlight important information:

Text convention and symbols	
	<i>Information Note: Additional information about the current topic or procedure.</i>
	Important Note: Information critical to the success of the current procedure or use of the product.
① ② ③ etc.	Stages in a process that usually occur in the order listed.
① ② ③ etc.	Steps in a procedure that must be performed in the order listed.
* (Asterisk)	The Asterisk denotes a product available from Roche Diagnostics.

5.2. Changes to previous version

Editorial changes.

5.3. Ordering Information

Product	Pack Size	Cat. No.
Reagents , kits		
Digoxigenin-11-UTP	250 nmol, 25 µl (10 mM)	11 209 256 910
	200 nmol, 57 µl (3.5 mM)	03 359 247 910
Biotin-16-UTP	250 nmol, 25 µl (10 mM)	11 388 908 910
Fluorescein-12-UTP	250 nmol, 25 µl (10 mM)	11 427 857 910
Expand High Fidelity PCR System	100 U, 3.5 U/µl, 40 reactions in a final volume of 50 µl	11 732 641 001
	500 U, 2 x 250 U, 200 reactions in a final volume of 50 µl	11 732 650 001
	2,500 U, 10 x 250 U, 1,000 reactions in a final volume of 50 µl	11 759 078 001
Water, PCR Grade	25 ml, 25 x 1 ml	03 315 932 001
	25 ml, 1 x 25 ml	03 315 959 001
	100 ml, 4 x 25 ml	03 315 843 001
mRNA Isolation Kit	1 kit	11 741 985 001
High Pure PCR Product Purification Kit	1 kit, up to 50 purifications	11 732 668 001
	1 kit, up to 250 purifications	11 732 676 001
High Pure RNA Isolation Kit	1 kit, 50 isolations	11 828 665 001

5.4. Trademarks

EXPAND and HIGH PURE are trademarks of Roche.

All third party product names and trademarks are the property of their respective owners.

5.5. License Disclaimer

For patent license limitations for individual products please refer to: **List of LifeScience products**

5.6. Regulatory Disclaimer

For life science research only. Not for use in diagnostic procedures.

5.7. Safety Data Sheet

Please follow the instructions in the Safety Data Sheet (SDS).

5.8. Contact and Support

To ask questions, solve problems, suggest enhancements or report new applications, please visit our **Online Technical Support** Site.

To call, write, fax, or email us, visit **sigma-aldrich.com** and select your home country. Country-specific contact information will be displayed.

