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I. Description：

RNA LA PCR Kit Ver.1.1 is designed to achieve longer and more accurate PCR with *TaKaRa LA Taq* ™ following reverse transcription. *TaKaRa LA Taq* ™ is the newly developed enzyme by TaKaRa as the key component of LA (long and accurate) PCR ＊. Based on the advantage of LA Technology, this kit performs reverse transcription of RNA to cDNA using AMV (Avian Myeloblastosis Virus) Reverse Transcriptase and subsequent amplification of long cDNA using *TaKaRa LA Taq* ™ all in a single tube. Also by eliminating the dilution step of Reverse Transcriptase, this kit allows simple and efficient analysis of RNA.

The supplied Oligo dT - Adaptor Primer is designed to allow more efficient cDNA synthesis from 3' - termini of poly (A) $^+$ RNA. This enables amplification of unknown 3' - termini utilizing 3' - RACE System.

＊：U.S.Patent 5,436,149 for LA Technology is owned by TAKARA BIO INC..

II. Kit components (100 reactions ＊) ：

1.	AMV Reverse Transcriptase XL ＊＊ (originated from Avian Myeloblastosis Virus)	(5 units / μ l)	50 μ l
2.	RNase Inhibitor	(40 units / μ l)	25 μ l
3.	Random 9 mers ＊＊＊	(50 pmol / μ l)	50 μ l
4.	Oligo dT - Adaptor primer ＊＊＊	(2.5 pmol / μ l)	50 μ l
5.	RNase Free distilled H_2O		1 ml
6.	*TaKaRa LA Taq* ™	(5 units / μ l)	25 μ l
7.	M13 primer M4 ＊＊＊	(20 pmol / μ l)	50 μ l
8.	10x RNA PCR Buffer 100 mM Tris - HCl (pH 8.3) 500 mM KCl		120 μ l
9.	10X LA PCR Buffer II (Mg^{2+} free)		500 μ l
10.	dNTP Mixture	(ea. 10 mM)	150 μ l
11.	$MgCl_2$	(25 mM)	1 ml
12.	Control R - 1 primer ＊＊＊ (downstream primer for positive control RNA)	(20 pmol / μ l)	25 μ l
13.	Control F - 1 primer ＊＊＊ (upstream primer for positive control RNA)	(20 pmol / μ l)	25 μ l
14.	Positive control RNA ＊＊＊＊ (transcribed poly (A) $^+$ RNA of pSPTet3 plasmid)	(2 x 10^5 copies / μ l)	25 μ l

＊：This kit is desighed for 100 reactions. (the total reaction volume; RT reaction 10 μ l, PCR 50 μ l) In the previous protocol of this kit, it was desighed for 50 reactions. (the total reaction volume; RT reaction 20 μ l, PCR 100 μ l) The number of times of reaction is changed as mentioned above this time, but the volume of all component in this kit is not changed.

＊＊：Manufactured by Life Science Co.

＊＊＊：Primers Sequence

- Random 9 mers： 5' - NNNNNNNNN - 3'
- Oligo dT - Adaptor primer：
  The original primer includes dT and complementary region to M13 primer M4.
- Control F - 1 primer： 5' - CTGCTCGCTTCGCTACTTGGA - 3'
- Control R - 1 primer： 5' - CGGCACCTGTCCTACGAGTTG - 3'
- M13 primer M4： 5' - GTTTTCCCAGTCACGAC - 3'

＊＊＊＊：Positive control RNA

Supplied control RNA is in vitro transcribed RNA using SP6 RNA polymerase from plasmid pSPTet3 inserted with DNA fragment (approximately 1.4 kb) having tetracycline resistant gene, originated frompBR322, in the downstream of SP6 promoter.
This control RNA is a poly (A) $^{+}$ RNA containing 30 bases of poly (A) at the tail.
When full - length double - stranded cDNA is synthesized from this control RNA, tetracycline resistant plasmid is obtained by inserting this cDNA.

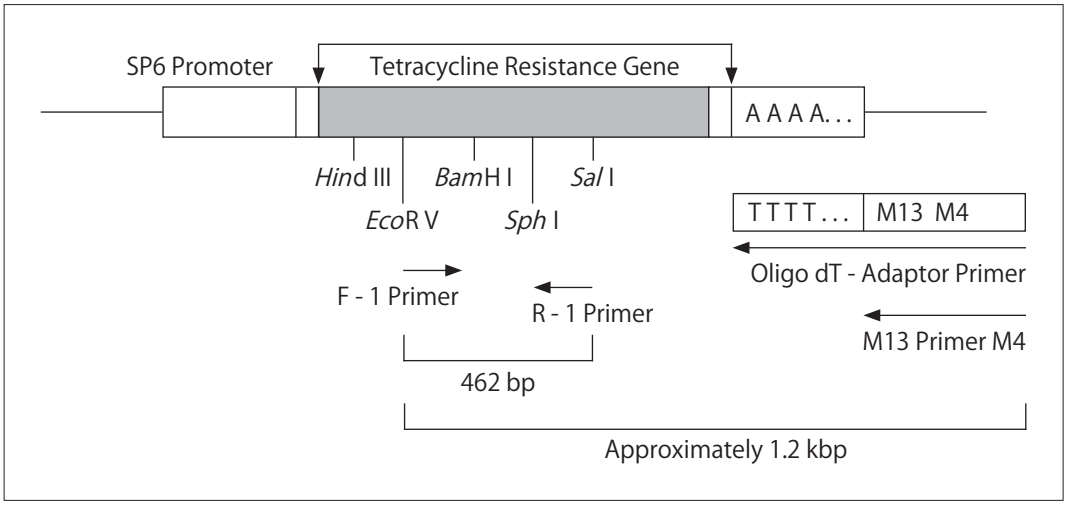


Fig. 1 Amplified DNA fragments using control RNA and several primers

III. Reagents not supplied in the kit：

1. Mineral Oil (If necessary)
2. Agarose gel
   ex. NuSieve® 3：1 Agarose (Cambrex Biosciences Corp.)

IV. Equipment required：

1. Authorized thermal cycler
   ex. TaKaRa PCR Thermal Cycler Dice ™ Gradient / Standard (Cat.#TP600 / TP650)
2. Microcentrifuge tubes (made of polypropylene)
3. Agarose gel electrophoresis apparatus
   ex. Mupid® - 2plus (Cat.#AD110)
4. Microcentrifuge
5. Micropipets and pipette tips (autoclaved)

V. Storage： − 20 ℃

VI. References：

1) Kawasaki, E. S. and Wang, A. M. (1989) PCR Technology (Erlich, H. A. ed.) , Stockton Press, 89 - 97.
2) Lynas, C., Cook, S. D., Laycock, K. A., Bradfield, J. W. B., and Maitland, N. J. (1989) *J. Pathology*, **157**, 285 - 289.
3) Frohman, M. A., Dush, M. K., Martin, G. R. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 8998 - 9002.

VII. Principles：

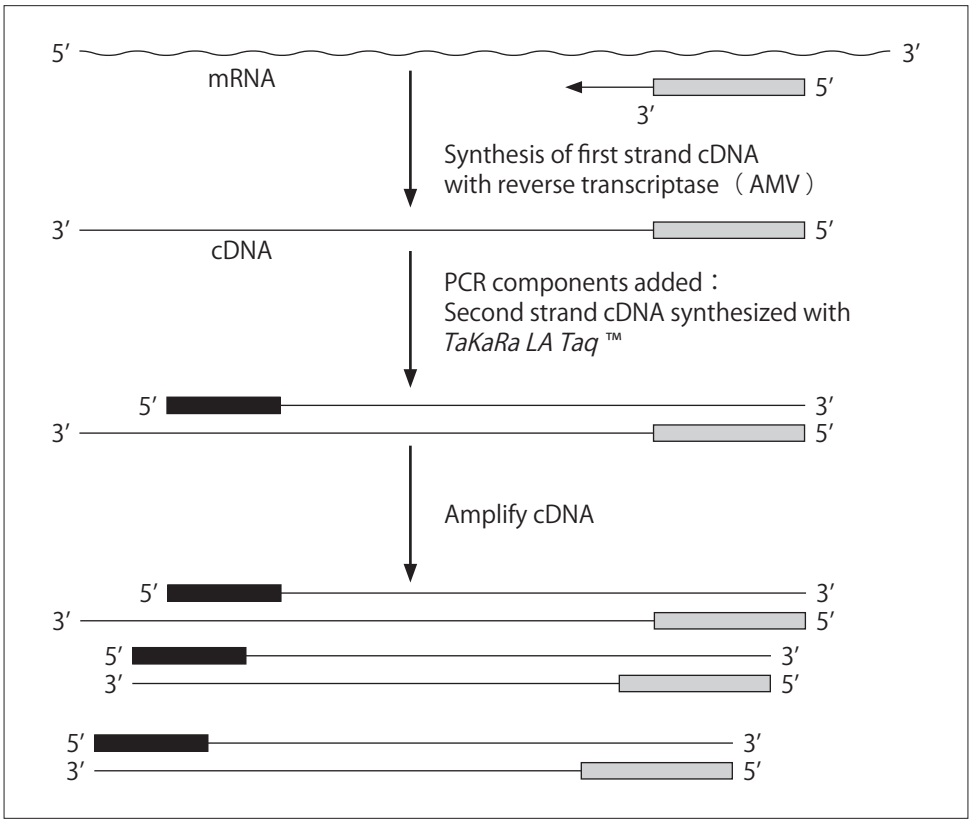
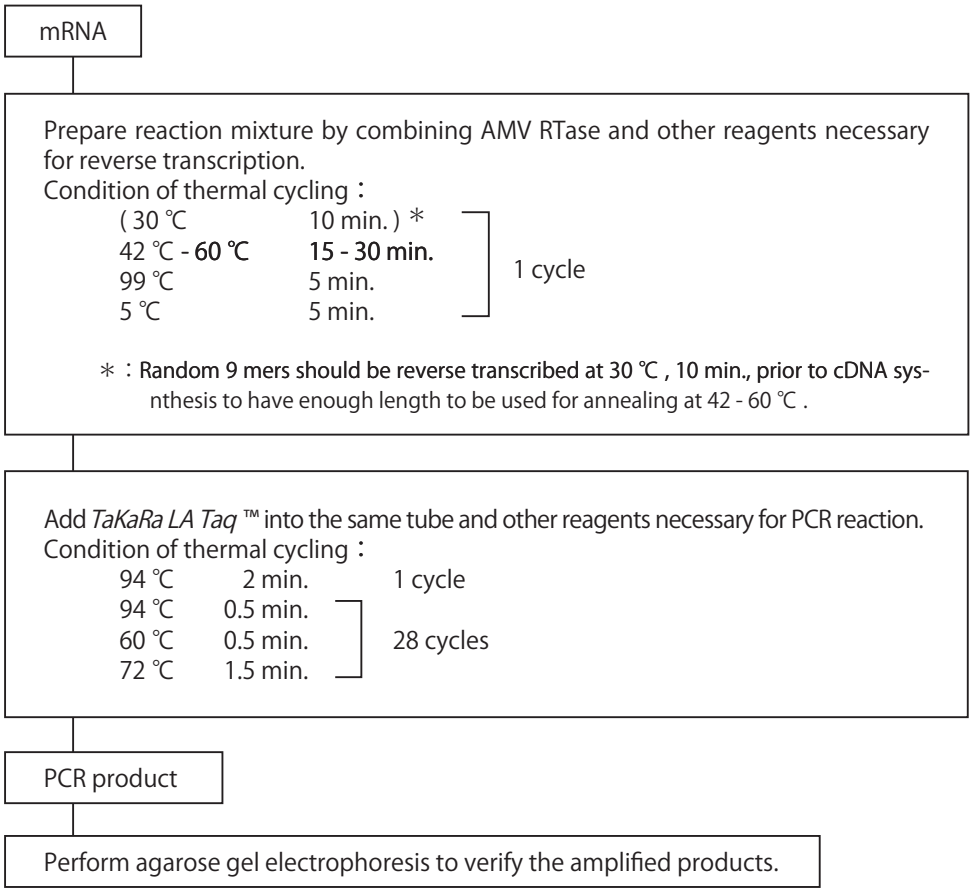


Fig. 2 Schematic diagram of RNA LA PCR



TaKaRa RNA LA PCR Kit Ver.1.1 allows reverse transcription from RNA to cDNA using AMV RTase and subsequent amplification in the same tube utilizing *TaKaRa LA Taq ™*.

Radom 9 mers, Oligo dT - Adaptor Primer, or a specific downstream primer which act as an anti - sense primer in PCR process can be used for cDNA synthesis. Oligo dT - Adaptor Primer is used for 3' - RACE System.

VIII. Features：

Template RNA	General
RNA segment to be transcribed and later amplified	at least ≦ 12 kbp
Reverse Transcriptase	AMV Reverse Transpriptase (in the range of 42 ～ 60 ℃)
DNA Polymerase	*TaKaRa LA Taq* ™
RNase Inhibitor	Supplied in the kit
Primer for 1st strand cDNA synthesis	Random 9 mers, or Oligo dT - Adaptor Primer or Specific downstream PCR primer
3' - RACE System	This kit is available for 3' - RACE System by using Oligo dT - Adaptor Primer in RT, and by using M13 Primer M4 in PCR.
Protocol	Single tube reaction (RTase is heat inactivated prior to PCR)

IX. Preparation of RNA sample：

TaKaRa RNA LA PCR Kit Ver.1.1 is designed to perform the reverse transcription of RNA to cDNA and subsequent amplification.
The purity of RNA sample will affect the yield of cDNA synthesis. So it is essential to inhibit the activity of RNase in the cells and also to prevent the contamination of RNase derived from equipments and solutions used.
Extra precautions should be taken during the sample preparation; put on clean disposable gloves, dedicate a table to exclusive use for RNA preparation, and avoid unnecessary talks during the operations to prevent the contamination of RNase from operators' sweat or saliva.

A. Equipment

Disposable plastic equipments shall be used. In case using glass tools, treat the glass tools with DEPC (diethylpyrocarbonate) prior to use.

(1) Treat glass tools with 0.1 % DEPC solution at 37 ℃ , 12 hours.

(2) Autoclave at 120℃ , 30 min., to remove DEPC.

It is recommended to prepare all the equipments as the exclusive use for RNA preparation.

B. Reagent

Reagents for RNA preparation, including distilled water, shall be prepared with heat sterilized glass tools (180 ℃ , 60 min.) , or if possible those treated with 0.1 % DEPC solution and autoclaved. Reagents and distilled water should be exclusively used for RNA preparation.

C. Preparation method

Simple purification methods can yield enough amount of RNA for reverse

transcription and subsequent PCR. However, it is recommended to use highly purified RNA obtained by GTC (Guanidine thiocyanate) method, etc.

D. RNA Sample Amount

Approximately 500 ng of total RNA is appropriate per one reaction.

X. Note：

1) For both reverse transcription and PCR amplification, master mix of reagents (containing RNase - free sterilized distilled water, buffers, dNTP mixtures, $MgCl_2$ solution, etc) for all samples can be prepared first, then aliquoted to individual tubes. Using such mixtures will allow accurate reagents dispense： minimize reagents pipetting losses, and avoid repeat dispensing and mixing of the each reagent. This helps to minimize variation of the data among the experiments.

2) Enzymes such as RTase, *TaKaRa LA Taq* ™, and RNase Inhibitor shall be mixed gently by pipetting. Avoid generating bubbles. Gently spin down the solution prior to mixing. Pipette enzymes carefully and slowly as the viscosity of the 50% glycerol in the buffer can lead to pipetting errors.

3) Keep enzymes at － 20 ℃ until just before use and return into the freezer promptly after use.

4) Use new disposable pipette tips to avoid contamination between samples.

5) PCR condition

Optimum PCR condition varies depending on an used thermal cycler. It is recommended to perform a control experiment to determine the condition prior to using a sample.

6) Primer Selection

Depend on many factors, the primer for reverse transcription should be selected from either of Random 9 mers, or Oligo dT - Adaptor primer, or specific downstream PCR primer. For short mRNAs with no hairpin structure, any one of the above three primers can be used.

[General guideline of the primer selection]

・Random 9 mers：

Use for long transcription of long RNAs or of RNA with hairpin structure. Also can be used to reverse transcribe all RNA (rRNA, mRNA, and tRNA) . Any pairs of PCR primers work equally well in PCR of cDNA synthesized with Random 9 mers.

・Specific downstream primer (anti - sense primer in PCR)

Use for the target RNA which sequence is already determined.

・Oligo dT - Adaptor primer

Use only for mRNAs with poly (A) tails (Note： Prokaryotic RNA, eukaryotic rRNA and tRNA, and some eukaryotic mRNA do not have poly (A) tails) . This primer was designed originally by Takara for efficient cDNA synthesis. This primer will allow 3' - RACE method utilizing M13 primer M4 which is complementary to Adaptor region after reverse transcription.

XI. Protocol：

1. Typical RT - PCR example

A. Reverse Transcription

1. Prepare the reaction mixture in a tube by combining the reagents in the proportions shown. The primer for a cDNA synthesis should be chosen from either of Random 9 mers, Oligo dT - Adaptor primer, or specific downstream primer. For the control experiment, use R - 1 primer. (See "Note (5) Primer Selection" for selection of primer to use.)

Reagents	Volume	Final concentration
$MgCl_2$	2 μl	5 mM
10x RNA PCR Buffer	1 μl	1 X
RNase Free distilled H_2O	4.25 μl	
dNTP Mixture	1 μl	1 mM
RNase Inhibitor	0.25 μl	1 unit / μl
Reverse Transcriptase ＊1	0.5 μl	0.25 units / μl
Random 9 mers		2.5 μM
or		
Oligo dT - Adaptor Primer	0.5 μl	0.125 μM
or		
Specific downstream PCR primer (R - 1 primer)		1.0 μM
Positive control RNA		[1 x 10^5 copies]
or	0.5 μl	or
Experimenvtal Sample		[≦ 500 ng total RNA]
Total volume	10 μl per sample	

2. Ovelay mineral oil to avoid the evaporation of the reaction mixture. (Some thermal cyclers do not require mineral oil.)

3. Place all tubes in a Thermal Cycler and set the parameters by the following condition.

(30 ℃ ,	10 min.) ＊2	1 cycle
42 ～ 60℃ ＊3	15 ～ 30 min.	
99 ℃	5 min.	
5 ℃	5 min.	

＊1：AMV RTase binds to cDNA and inhibits PCR amplification. Heat treatment of 99 ℃ , 5 minutes inactivates the reverse transcriptase and removes the inhibitory effect on PCR. If the concentration of AMV RTase increases, inactivation of AMV RTase becomes difficult.
Therefore, for long RNA, it is advisable to increase the incubation

time during AMV RTase rather than increase the amount of AMV RTase added.

＊2：When using Random 9 mers, perform reverse transcription in advance at 30 ℃ for 10 minutes to obtain enough length to anneal with primer at 42 ～ 60 ℃ .

＊3：AMV Reverse Transcriptase can work at 60 ℃ . However, when using long RNA segments (≧ 2kb) , it is advisable to perform reverse transcription at around 42 ℃ . When positive control RNA is used as template, reverse transcription at 55 ℃ is recommended.

B. PCR

1. Prepare reaction mixture by combining the following reagents.
   When the reaction mixture is added into the RT reactant for thesubsequent PCR, the mixture should not contact with mineral oil. It is recommended to pour the mixture with a pipette into the underlayer of mineral oil.

Reagents	Volume	Final concentration (per 50 μl mixture)
$MgCl_2$	3 μl	2.5 mM
10x LA PCR Buffer II （Mg^{2+} free ）	4 μl	1x
Sterilized distilled water	31.75 μl	
TaKaRa LA Taq ™	0.25 μl	1.25 U / 50 μl
Upstream PCR Primer (F - 1 Primer for Control RNA)	0.5 μl	0.2 μM
Downstream PCR Primer ＊	0.5 μl	0.2 μM
(For Control RNA, R - 1 Primer or M13 primer M4 when Oligo dT - Adaptor primer is used in reverse transcription)		
Total volume	40 μl per sample	

＊：When downstream PCR primer is used in reverse transcription, add 0.5 μl of sterilized distilled water instead of downstream primer.

2. Add 40 μl of the mixture into a tube containing the cDNA obtained at A.

3. Spin for approximately 10 seconds with a microcentrifuge.

4. Place the tubes in a Thermal Cycler and perform ampification under the optimal condition. ＊＊

94 ℃ , 2 min.	1 cycle
94 ℃ , 30 sec. 60 ℃ , 30 sec. 72 ℃ , 1.5 min.	28 cycles

＊＊：Conditions for PCR

1) Annealing Temperature：60 ℃ is optimal for amplification of control RNA. It may be necessary to lower or raise (within the range of 37 ℃ ～ 65 ℃) the annealing temperature for RNA samples. (The optimal temperature need to be determined empirically by testing temperatures within the range of 37 ℃ to 65 ℃) .

2) Extension time：The length of the target sequence will affect the required extension time. Usually, *TaKaRa LA Taq* ™ extends 1 kb per 1 - 2 min at 72 ℃ .

3) Number of cycles： When small volume of cDNA is used, the repetition of 40 ～ 50 cycles are required for PCR amplification.

5. After the amplification is completed, apply 5 ～ 10 μ l of the reactant for agarose gel electrophoresis to verify the amplified DNA fragments. ＊＊＊

＊＊＊：The PCR amplified product samples can be stored frozen until subsequent analysis.

2. Application example

RT - PCR of long mRNA fragment (approx. 12 kb)

Template RNA： total human heart mRNA
Target DNA： human dystrophin
Amplified DNA fragment size： 12 kbp

A. Reverse Transcription

1. Prepare the reaction mixture in a tube by combining the reagents in the proportions shown as below.

Reagents	Volume	Final concentration
$MgCl_2$	2 μ l	5 mM
10x RNA PCR Buffer	1 μ l	1 X
RNase Free distilled H_2O	4.5 μ l	
dNTP Mixture	0.25 μ l	1 mM
RNase Inhibitor	0.5 μ l	1 unit / μ l
Reverse Transcriptase	0.5 μ l	0.25 units / μ l
Random 9 mers	0.25 μ l	2.5 μ M
total human heart mRNA	0.25 μ l	0.25 μ g / 10 μ l RT
Total volume	10 μ l per sample	

2. Place the tubes in a Thermal Cycler and perform the reaction by the following condition.

30 ℃ ,	10 min.	┐
42 ℃ ,	50 min.	┘ 1 cycle
70 ℃ ,	15 min.	

B. PCR

1. Prepare reaction mixture for PCR by combining the following reagents.

Reagents	Volume	Final concentration (per 50 μl mixture)
$MgCl_2$	3 μl	2.5 mM
10x LA PCR Buffer II （Mg^{2+} free）	4 μl	1x
Sterilized distilled water	31.75 μl	
TaKaRa LA Taq ™	0.25 μl	1.25 U / 50 μl
Primers	1 μl	ea. 0.2 μM
Total volume	40 μl per sample	

2. Add 40 μl of the mixture into a tube containing the cDNA obtained at A.

3. Place the tubes in a Thermal Cycler and perform PCR under the following condition.

94 ℃ ,	30 sec.	┐
65 ℃ ,	15 min.	┘ 30 cycles

4. After the amplification is completed, apply 5 μl of the reactant for agarose gel electrohporesis to verify the amplified DNA fragments. The target cDNA is verified with the amplified fragment of 12 kbp.

3. 3' - RACE System

Template RNA： human HL60 total RNA
Target DNA： Transferrin receptor (TFR)
Amplified DNA fragment size： 1105 bp

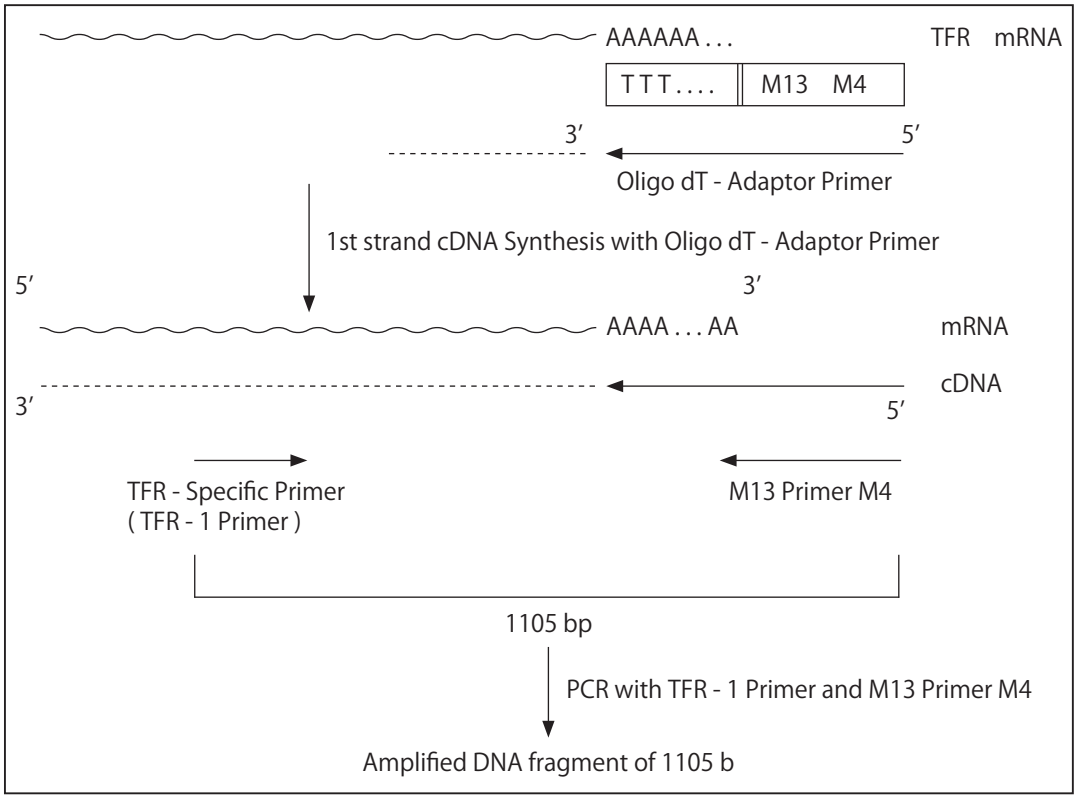


Fig. 3 Schematic diagram of RT - PCR of HL60 total RNA with 3' - RACE System

A. Reverse Transcription

1. Prepare the reaction mixture in a tube by combining the reagents in the proportions shown as below.

Reagents	Volume	Final concentration
$MgCl_2$	2 μl	5 mM
10x RNA PCR Buffer	1 μl	1 X
RNase Free distilled H_2O	4.25 μl	
dNTP Mixture	1 μl	1 mM
RNase Inhibitor	0.25 μl	1 unit / μl
Reverse Transcriptase	0.5 μl	0.25 units / μl
Oligo dT - Adaptor primer	0.5 μl	0.125 μM
HL60 total RNA (1 μg / μl)	0.5 μl	0.25 μg / 10 μl RT
Total volume	10 μl per sample	

2. Place all tubes in a Thermal Cycler and set the parameters by the following condition.

30 ℃ ,	10 min.	1 cycle
50 ℃ ,	30 min.	
95 ℃ ,	2 min.	
5 ℃ ,	5 min.	

B. PCR

1. Prepare reaction mixture for PCR by combining the following reagents.

Reagents	Volume	Final concentration (per 50 μl mixture)
$MgCl_2$	3 μl	2.5 mM
10x LA PCR Buffer II (Mg^{2+} free)	4 μl	1x
Sterilized distilled water	31.75 μl	
TaKaRa LA Taq ™	0.25 μl	1.25 U / 50 μl
M13 Primer M4	0.5 μl	0.2 μM
TFR - 1 Primer	0.5 μl	0.2 μM
Total volume	40 μl per sample	

2. Add 40 μl of the mixture into a tube containing the cDNA obtained at A.

3. Place the tubes in a Thermal Cycler and perform PCR under the following condition.

94 ℃ ,	30 sec.	30 cycles
55 ℃ ,	30 sec.	
72 ℃ ,	5 min.	

4. After the amplification is completed, apply 5 μl of the reactant for agarose gel electrohporesis to verify the amplified DNA fragments. The target cDNA is verified with the amplified fragment of 1105 bp.

XII. Related Products：

Reverse Transcriptase XL (AMV) for RT - PCR (Cat.#2630A)
Ribonuclease Inhibitor (Cat.#2311A / B)
Takara LA Taq ™ (Cat.#RR002A / RR002M)
Random Primer (pd (N) 9) (Cat.#3802)
TaKaRa PCR Thermal Cycler Dice ™ Gradient / Standard (Cat.#TP600 / TP650)
Mupid® - 2plus (Cat.#AD110)

XIII. Note：

This product is intended to be used for research purpose only. They are not to be used for drug or diagnostic purposes, nor are they intended for human use. They shall not to be used products as food, cosmetics, or utensils, etc.
Takara products may not be resold or transfered, modified for resale or transfer, or used to manufacture commercial products without written approval from TAKARA BIO INC.
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[P1] PCR Notice
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[M57] LA Technology
This product is covered by the claims 6-16 of U.S. Patent No. 5,436,149 and its foreign counterpart patent claims.