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I. Description: PCR(Polymerase Chain Reaction) process is a simple and powerful method which allows *in vitro* amplification of DNA fragments through a succession of three incubation steps at different temperatures. In principle, PCR is a method to amplify DNA segments, and not directly amplify RNA. However, synthesis of cDNA from RNA using reverse transcriptase enables to apply PCR process to the RNA analysis. Many reports of various fields have been made by applying this method, such as of structual analysis of RNA, efficient cDNA cloning, analysis of gene expression at the RNA level, etc. TaKaRa RNA PCR Kit(AMV) Ver.3.0 is designed to perform the reverse transcription of RNA to cDNA using AMV (Avian Myeloblastosis Virus) Reverse Transcriptase and subsequent amplification using *TaKaRa Ex Taq*™ HS all in a single tube. By including all reagents necessary for the reverse transcription and for subsequent cDNA amplification and 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 amplificatoin of unknown 3'-termini utilizing 3'-RACE System. As this kit uses Takara's PCR enzyme efficient for Hot Start PCR, *Takara Ex Taq*™ HS (#RR006), non-specific amplification deriving from mispriming or from primer-dimers before thermal cycling can be avoided.

II. Kit components (100 reactions): One reaction means 10 μl RT followed by 50 μl PCR.

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

*Manufactured by Life Science Co.

**Primers Sequence

- Random 9 mers: dp (5'-NNNNNNNNN-3')
- Oligo dT-Adaptor primer:
  This original primer includes dT and the 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 from pBR322, 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.

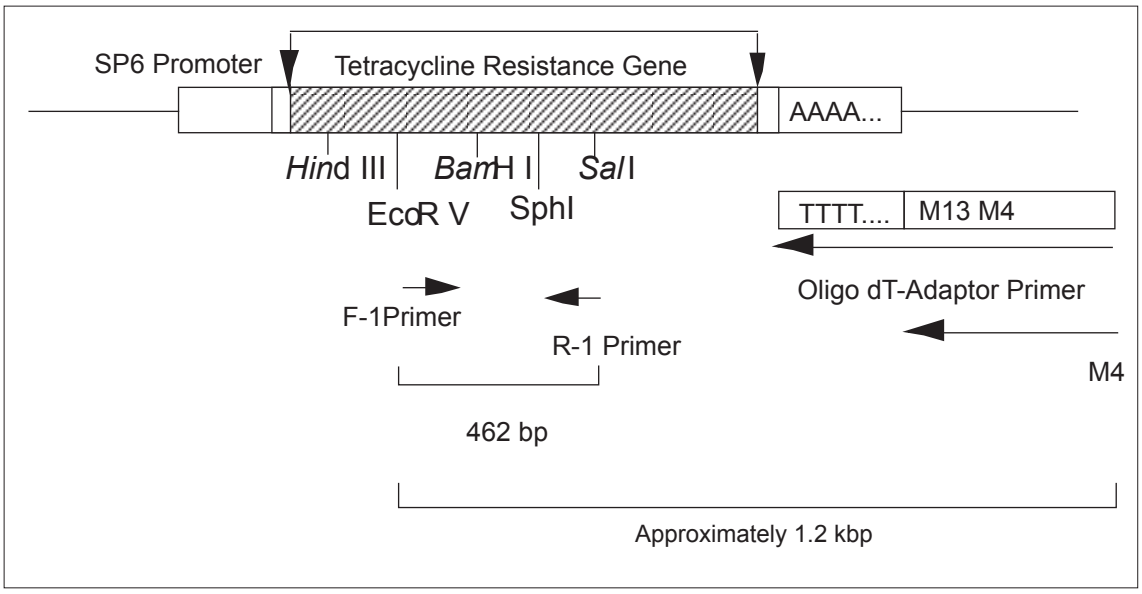

Fig. 1 Amplified DNA fragments using control RNA and several primers

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 instruments for PCR
   ex. TaKaRa PCR Thermal Cycler Dice™ (Takara Cat.#TP600/TP650)
   TaKaRa PCR Thermal Cycler Dice™ mini (Takara Cat.#TP100)
2. Agarose gel electrophoresis apparatus
   ex. Mupid®-2plus (Takara Cat.#AD110)
3. Microcentrifuge
4. 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:

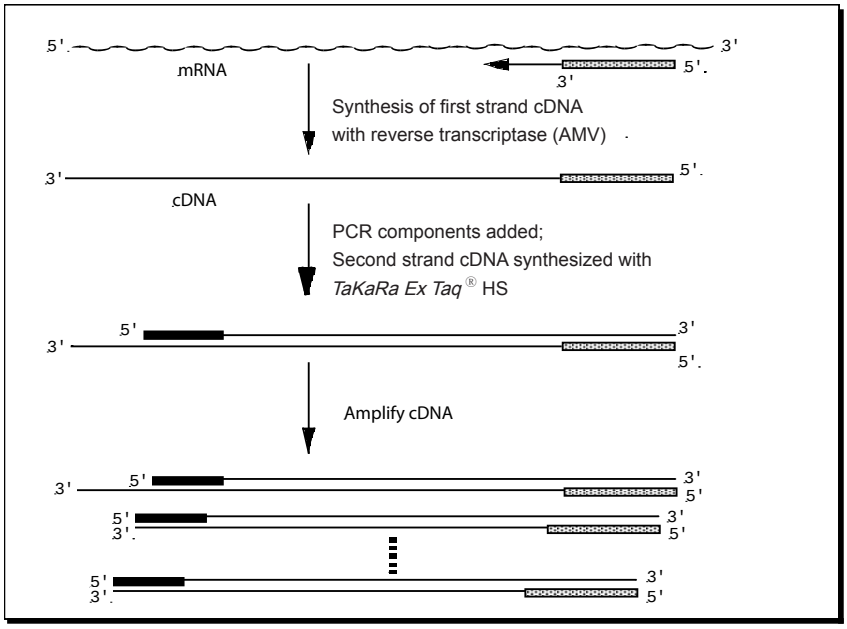

Fig.2 Schematic diagram of RNA PCR

mRNA

mRNA
Prepare reaction mixture by combining AMV RTase and other reagents necessary for 1st strand cDNA synthesis.
Condition of thermal cycling:

(30℃	10 min.)*	
42℃ - 60℃	15-30 min.	
95℃	5 min.	1 cycle
5℃	5 min.	

*Random 9 mers should be reverse transcribed at 30℃, 10 min., prior to cDNA sysnthesis to have enough length to be used for annealing at 42-60℃.

Into the same tube, add *TaKaRa Ex Taq*™ HS and other reagents necessary for 2nd strand cDNA synthesis.
Condition of thermal cycling:

94℃	30 sec.	
55 - 65℃	30 sec.	25-30 cycles
72℃	1 min./kb	

cDNA

Perform agarose gel electrophoresis for the analysis of the amplified products.

TaKaRa RNA PCR Kit (AMV) Ver.3.0 allows reverse transcription from RNA to cDNA using AMV RTase and subsequent amplification in the same tube utilizing *TaKaRa Ex Taq*™ HS DNA Polymerase. 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 ≦ 5.0 kbp
Reverse Transcriptase	AMV Reverse Transpriptase XL (in the range of 42~60℃)
DNA Polymerase	*TaKaRa Ex Taq*™ HS
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 PCR Kit (AMV) Ver.3.0 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□C, 12 hours.

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

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

B. Reagent

Reagents for RNA preparation, including distilled water, shall be prepared with heat sterilized glass tools (180□C, 60 min.), or if possible those treated with 0.1% DEPC solution and autolalved. 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 of the each reagent. This helps to minimize variation of the data among the experiments.

2) Enzymes such as RTase, *TaKaRa Ex Taq*™ HS, 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, 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 the 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. It will allow 3'-RACE method utilizing M13 primer M4 which is complementary to Adaptor region after reverse transcription.

XI. Protocol:

General RT-PCR

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 RT Buffer		1 μl	1X
RNase Free dH_2O		3.75 μl	
dNTP Mixture		1 μl	1 mM
RNase Inhibitor		0.25 μl	1 unit/μl
AMV Reverse Transcriptase XL[*3]		0.5 μl	0.25 units/ μl
Random 9 mers or Oligo dT-Adaptor primer or Specific downstream PCR primer (R-1 primer)	0.5 μl		2.5 μM or 0.125 μM or 1.0 μM
Positive control RNA or Experimental Sample	1 μl		[2×10^5 copies] or [≦ 500 μg total RNA]
Total volume		10 μl per sample	

2. Ovelay mineral oil* (50~100 μl) 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.	
95℃	5 min.[*1]	
5℃	5 min.	

*1) AMV RTase binds to cDNA and inhibits PCR amplification. Heat treatment of 95℃, 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 (≧ 2 kb), it is advisable to perform reverse transcription at around 42℃. When positive control RNA is used as template, reverse transcription at 50℃ is recommended.

B. PCR

1. Prepare reaction mixture by combining the following reagents.
   When the reaction mixture is added into the RT reactant for the subsequent 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 conc. in PCR reaction (per 50 μl mixture)
5x PCR Buffer	10 μl	1x
Sterilized distilled water	28.75 μl	
TaKaRa Ex Taq™ HS	0.25 μl	1.25 units/50 μl
Upstream PCR Primer (F-1 Primer for Control RNA)	0.5 μl	0.2 μM (20 pmol)
Downstream PCR Primer* 「For Control RNA, R-1 Primer or M13 primer M4 (when Oligo dT Adaptor primer is used in reverse transcription)」	0.5 μl	0.2 μM (20 pmol)
Total volume	40 μl per sample	

*When downstream PCR primer is used in reverse transcription, add 1 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.**

General Condition

94℃ 30 sec.
55 - 65℃ 30 sec.] 25-30 cycles
72℃ 1 min./kb

Positive Control RNA

94℃ 30 sec.
60℃ 30 sec.] 30 cycles
72℃ 1 min.

**Conditions for PCR

- Annraling Temperature
  60℃ is optimal for amplification of the Control RNA; however, is necessary to change the annealing temperature (55 - 65℃) depending on the targets.
  It may be necessary to determine the optimal annealing temperature experimentally in the range of 45 - 65℃ .

- Extension time
  The extension time depends on the target length.
  Usually, *TaKaRa Ex Taq*™ HS extends DNA at 1kb per minute at 72℃ .

- Number of cycle
  40 - 50 cycles are recommended if the cDNA amount is small.

- Most of the PCR products amplified using this kit have a 3' A overhang.
  Therefore, it is possible to clone the PCR product directly into a T-Vector.
  In addition, it is possible to clone into a blunt end vector by blunting the ends or phosphorylation. This can be done using the Reagent Set Mighty Cloning Kit (Blunt End) (Cat.#6027) for blunt end vector cloning.
  * Reagent Set for Mighty Cloning Kit (Blunt End) should be used in combination with Micropure™-EZ (Milipore).

5. After the amplification is completed, apply 5~10 µl 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.

In case of a reaction with Positive control RNA

Primer for reverse transcription	PCR Primer	Amplified fragment
Oligo dT-Adaptor Primer	F1 and M13 Primer M4	Approximately 1.2 kbp
	or F-1 and R-1	462 bp
Random 9 mers	F-1 and R-1	462 bp
R-1 Primer	F-1 and R-1	462 bp

2. 3'-RACE System

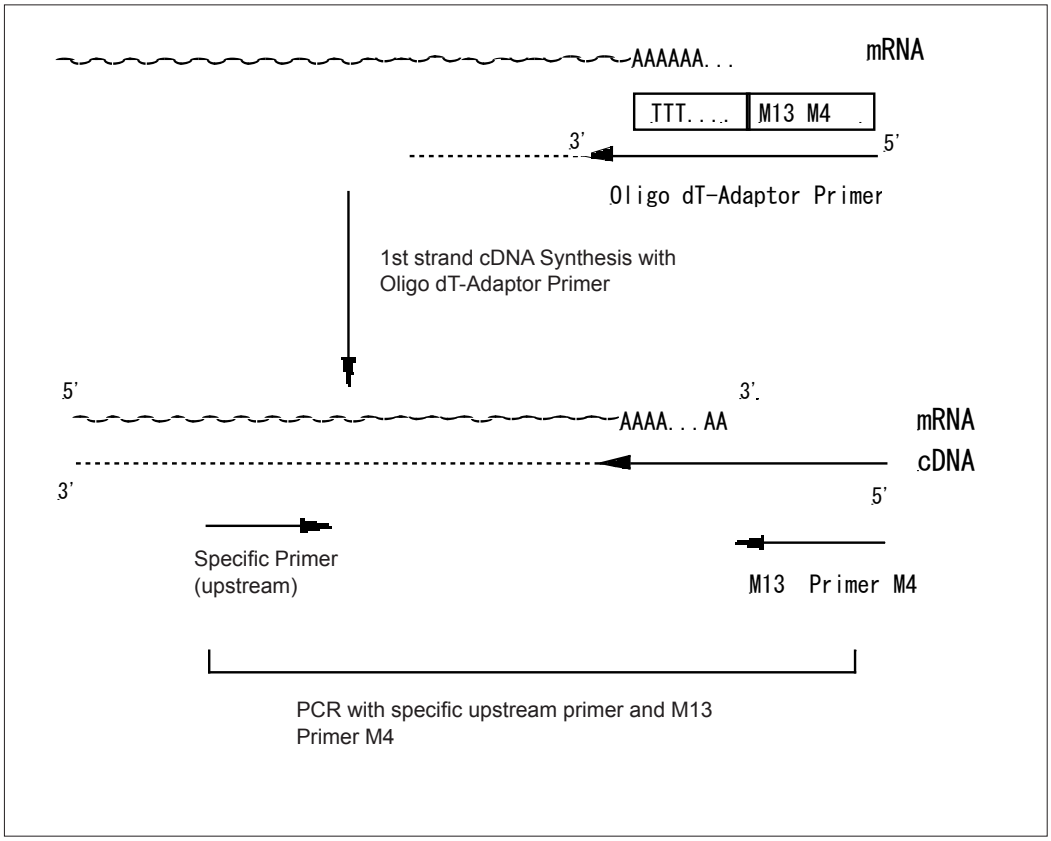

Fig.3 Schematic diagram RT-PCR with 3'-RACE System

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 RT Buffer	1 µl	1X
RNase Free dH_2O	3.75 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
total RNA (500 ng/ µl)	1 µl	500 ng/10 µl
Total volume	10 µl	

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

42 - 60℃	30 min.	┐
95℃	5 min.	├ 1 cycle
5℃	5 min.	┘

B. PCR

1. Prepare reaction mixture by combining the following reagents.

Reagents	Volume	Final conc. in PCR reaction (per 50 µl mixture)
5x PCR Buffer	10 µl	1x
Sterilized distilled water	28.75 µl	
TaKaRa Ex Taq™ HS	0.25 µl	1.25 units/50 µl
M13 Primer M4 (20 µM)	0.5 µl	0.2 µM
Specific upstream Primer (20 µM)	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 amplification by the following condition.

94℃	30 sec.	┐
55℃	30 sec.	├ 30 cycles
72℃	0.5 - 5 min.	┘

4. After the amplification is completed, apply 5 µl of the reactant for agarose gel electrophoresis to verify the amplified DNA fragments.
Target cDNA can be verified by the amplified fragment.

XII. Related products:

Reverse Transcriptase XL (AMV) for RT-PCR (TaKaRa Cat.#2630A)
Ribonuclease Inhibitor (TaKaRa Cat.#2310A/2310B)
TaKaRa Ex Taq™ Hot Start Version (TaKaRa Cat.#RR006A/RR006B)
Random Primer (pd$(N)_9$) (TaKaRa Cat.#3802)
Reagent Set for Mighty Cloning Kit (Blunt End) (TaKaRa Cat.#6027)
TaKaRa Thermal Cycler Dice (TaKaRa Cat.#TP600/TP650)
TaKaRa Thermal Cycler Dice mini (TaKaRa Cat.#TP100)
Mupid® -2plus (TaKaRa Cat.#AD110)