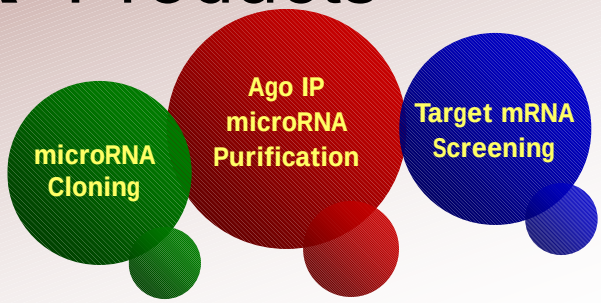
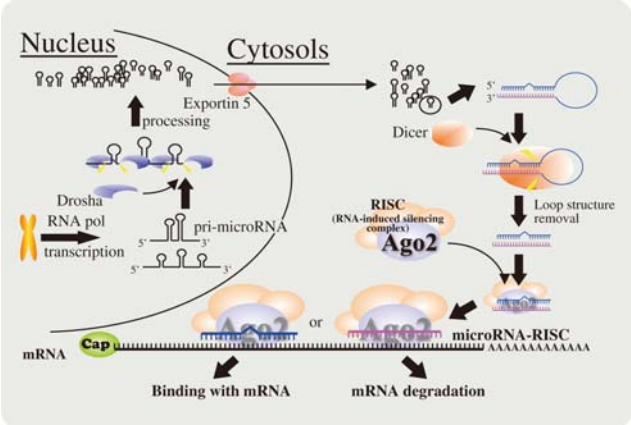
Wako microRNA Products

Wako Pure Chemical Industries, Ltd.





microRNAs (miRNAs) are endogenous RNAs, approximately 22 nucleotides in length, that can play important regulatory roles in animals and plants by targeting mRNAs for cleavage or translational repression. Since more than 1,000 kinds of miRNAs are suggested to exist in humans and mice, various researches such as identification of novel miRNAs with unknown functions and elucidation of the miRNA functions associated with specific diseases have been actively progressed around the world. Recently, the discovery of miRNAs in serum has opened up the possibility of using miRNAs as biomarkers of disease including cancer. microRNAs are considered to degrade the mRNA chains or inhibit the translation by the following manner: they are matured through multiple steps in cells and incorporated into a protein complex, RNA- induced silencing complex (RISC), they then bind to argonaute subfamily of proteins which are the main components of RISC, followed by binding to a target mRNA. Four kinds of Ago subfamilies (hAgo1 to hAgo4) exist in humans and are ubiquitously expressed even though the expression levels vary with types. Among a group of Ago subfamilies, Ago2 is the most expressed protein which is considered to play a central role in miRNA pathway because it only has a slicer activity for cleavage of the target RNAs. As RISC is immunoprecipitated by antibodies against these Ago proteins, the miRNAs can be recovered from the precipitated RISC, and furthermore, because the target mRNAs are found to be coprecipitated, immunoprecipitation is becoming an essential methodology to elucidate various functions of the miRNAs .
In these circumstances, Wako offers some analytical tools for the functional analysis of miRNAs, mainly the miRNA research tools using Anti Ago Antibodies. The combination use of these tools enables you to make a comprehensive analysis of the miRNAs and mRNAs contained in the RISC that exist in cells and tissues, which can be applied not only to miRNA analyses but to target mRNA analyses.

Product List

Description	Wako Catalog No.	Page #
microRNA Purification Kits based on Immunoprecipitation method using antibody against Argonaute protein		
microRNA Isolation Kit, Human/Mouse Ago1	291-70201 (10 reactions)	2
microRNA Isolation Kit, Human Ago2	292-66701 (10 reactions)	2
microRNA Isolation Kit, Mouse Ago2	292-67301 (10 reactions)	2
microRNA Isolation Kit, Human Ago3	297-70301 (10 reactions)	2
Preparation Kit of cDNA encoding microRNA		
microRNA Cloning Kit *Wako*	290-66501 (8 reactions)	5
Single Strand DNA Ligase, thermostable, recombinant, Solution	298-65103 (200 units); 292-65101 (500 units)	5
PCR Purification Kit *Wako*	298-67901 (30 reactions)	7
Target mRNA Cloning Kit *Wako*	298-68001 (10 reactions)	7
Antibodies		
Anti Ago1, Monoclonal Antibody (1F2) <for WB>	018-22401 (50 µL)	8
Anti Ago1, Monoclonal Antibody (2A7) <for IP>	015-22411 (50 µL)	8
Anti Human Ago2, Monoclonal Antibody (4G8)	011-22033 (50 µL); 015-22031 (100 µL)	8
Anti Mouse Ago2, Monoclonal Antibody (2D4)	014-22023 (50 µL); 018-22021 (100 µL)	8
Anti Human Ago3, Monoclonal Antibody	available soon!	-

microRNA Isolation Kit, Human Ago2; Mouse Ago2: patent pending (11, 30, 2007); microRNA Cloning Kit Wako: patent pending (1, 10, 2007)
All listed products are for research use only. Do not administer each to human..

microRNA Isolation Kit Series

microRNA (miRNA) Isolation Kit series can prepare high purity fractions of microRNA, which are bound with human or mouse Argonaute (Ago) protein, based on immunoprecipitation method by using a high affinity monoclonal antibody. The purified miRNA fraction contains very little contaminated degradation fragments of rRNA and tRNA. These kits will highly improve the miRNA cloning efficiency compared with conventional miRNA purification method. Furthermore, the purified miRNA are applicable to Analysis of target mRNA of miRNA.

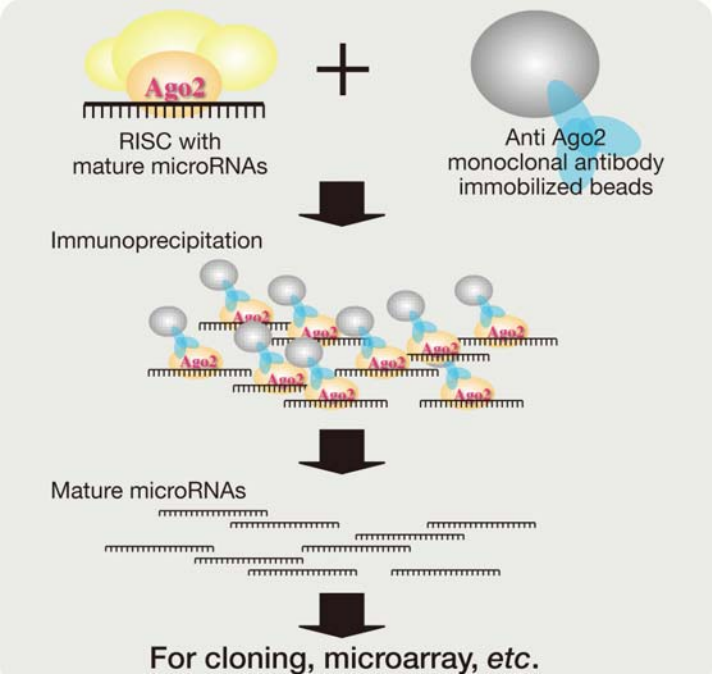
Sample		Platform			
Cultured Cells※	Tissues※	Microarray	Deep sequencer	Cloning	Quantitative RT-PCR
○	○	○	○	○	○

※Each isolation efficiency is depend on cell lines or tissues.

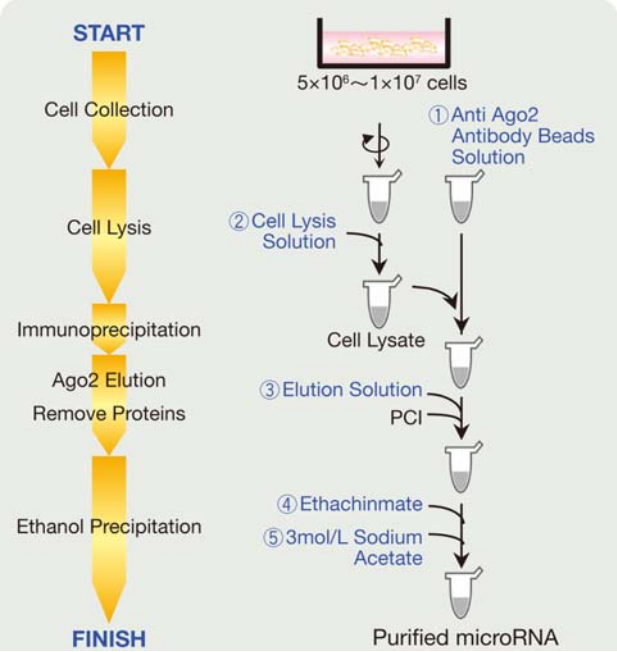
■ Features

1) Immunoprecipitation of endogenous Ago protein
2) High purification performance of miRNA bound with Ago protein
3) Little contamination of other RNAs such as degradation fragments of rRNA & RNA
4) Applicable to high efficiency of microRNA cloning and microarray with purified microRNA fraction

■ Principle



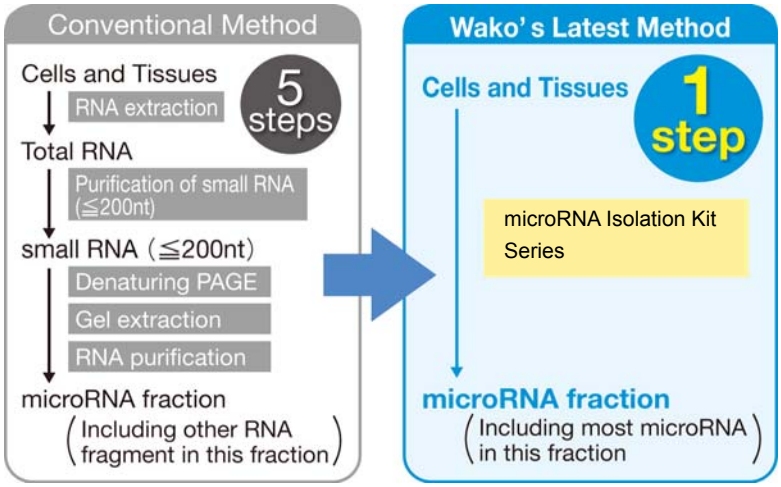
■ Procedure Outline



※: 2 x 10^7 cells are necessary for the procedure with miRNA Isolation

■ Comparison with conventional method

	conventional	Wako's microRNA Isolation Kits
Total RNA Extraction	necessary	unnecessary
small RNA (≦200 nt) Purification		
Denaturating PAGE		
Gel Extraction from denaturing PAGE		
contaminated degradation fragments of rRNA & tRNA in miRNA fraction	high	very low
Test duration	≧ 1 day	≧ 0.5 days



■ Kit Contents (for 10 reactions)

(1) Anti Ago Antibody Beads Solution	1 vial x 500 µL
(2) Cell Lysis Solution	1 bottle x 50 mL
(3) Elution Solution	1 vial x 500 µL
(4) Ethachinmate	1 vial x 30 µL
(5) 3 mol/L Sodium Acetate Solution	1 vial x 400 µL

■ Purification of microRNA fraction from various cell lines

microRNA Isolation Kit, **Human Ago2** (Wako Cat. #292-66701)

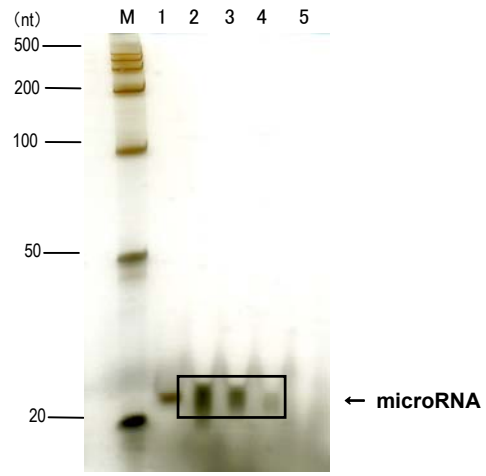


Figure: Purification of microRNA fraction by using microRNA Isolation Kit, Human Ago2
The purified microRNA fraction from human cultured cell lines (HeLa, HepG2, HEK293) were specifically detected by Urea-PAGE and silver staining (Wako Cat. #311-03961; CLEAR STAIN Ag). Cell number of each cell line is approximately 5 x 10^6. The applied volume per lane is half of 10μL of final solution prepared with an IP by this kit.

microRNA Isolation Kit, **Mouse Ago2** (Wako Cat. #292-67301)

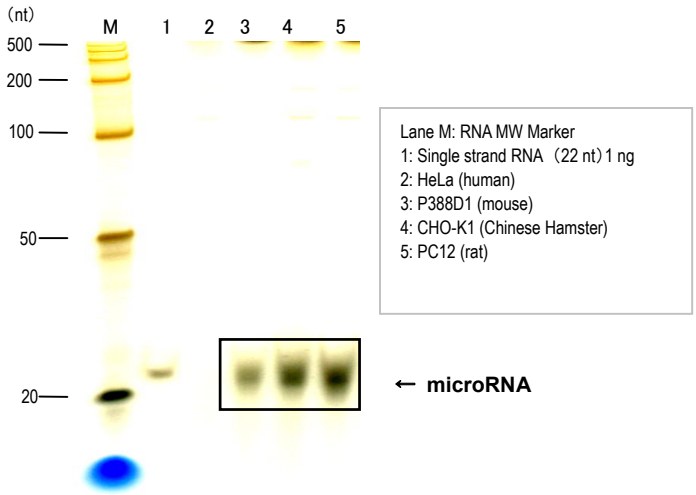


Figure: Purification of microRNA fraction by using microRNA Isolation Kit, Mouse Ago2
The purified microRNA fractions from rodent cultured cell line (P388D1, CHO, PC-12) were specifically detected by Urea-PAGE and silver staining (#311-03961; CLEAR STAIN Ag). Cell number of each cell line is approximately 5 x 10^6.

microRNA Isolation Kit, **Human/Mouse Ago1** (Wako Cat. #291-70201)

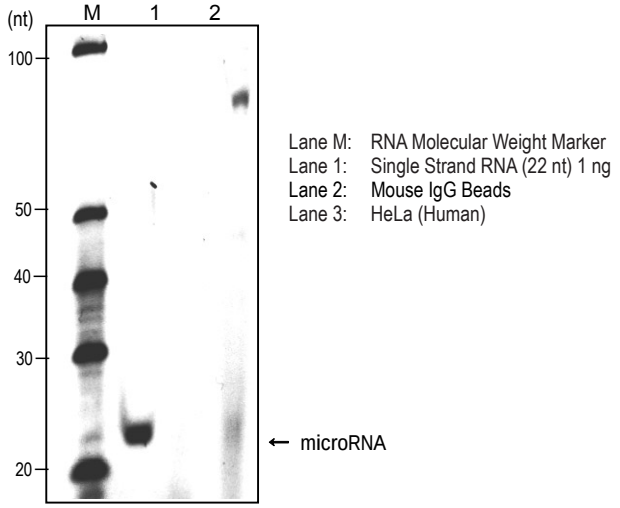


Figure: Purification of microRNA fraction by using microRNA Isolation Kit, Human/Mouse Ago1.
The purified microRNA fraction from human cultured cell line (HeLa) was specifically detected by Urea-PAGE and silver staining (Wako Cat #311-03961; CLEAR STAIN Ag). Cell number is 2×10^7 .

microRNA Isolation Kit, **Human Ago3** (Wako Cat. #297-70301)

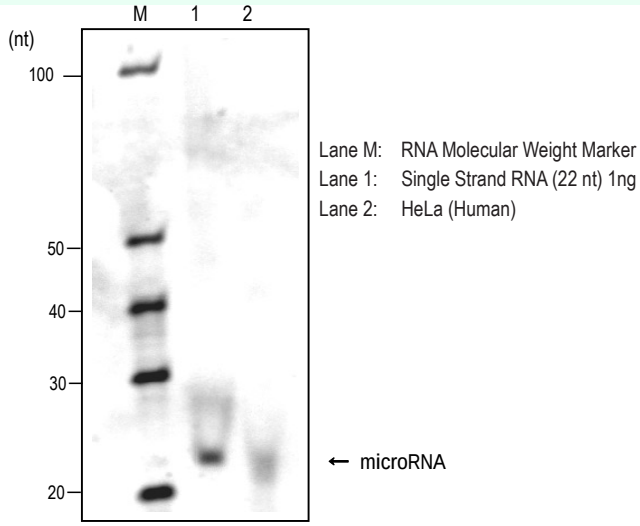


Figure: The purified microRNA fraction by using microRNA Isolation Kit, Human Ago3.
The purified microRNA fraction from human cultured cell line (HeLa) was specifically detected by Urea-PAGE and silver staining (Wako Cat. #311-03961、CLEAR STAIN Ag). Cell number is 5×10^6.

■ Purification of microRNA fraction from tissues

microRNA Isolation Kit, **Human Ago2** (Wako Cat. #292-66701)

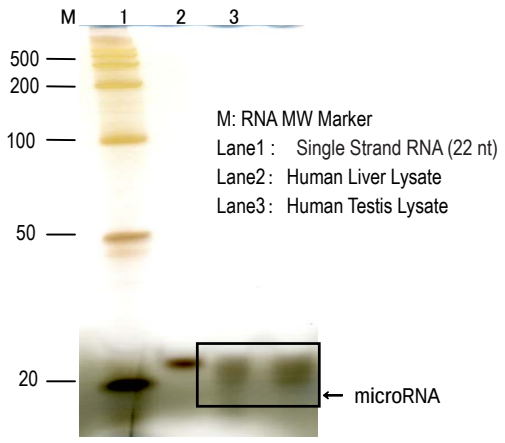


Figure: microRNA fractions, which were isolated by microRNA Isolation Kit, Human Ago2 from human liver and testis Lysate, were specifically detected by Urea-PAGE and silver stain (Wako Cat #311-03961; CLEAR STAIN Ag) (50 mg Tissues→2 mL Total tissue lysate→Take the 1 mL→ Immunoprecipitation→10 μL Total RNA soln.→Take the 5 μL→Urea-PAGE)

microRNA Isolation Kit, **Mouse Ago2** (Wako Cat. #292-67301)

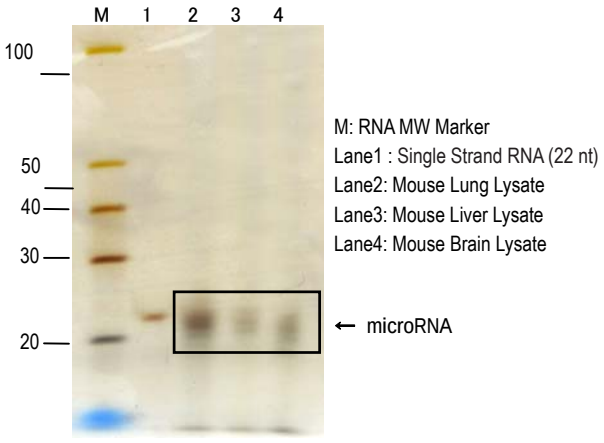
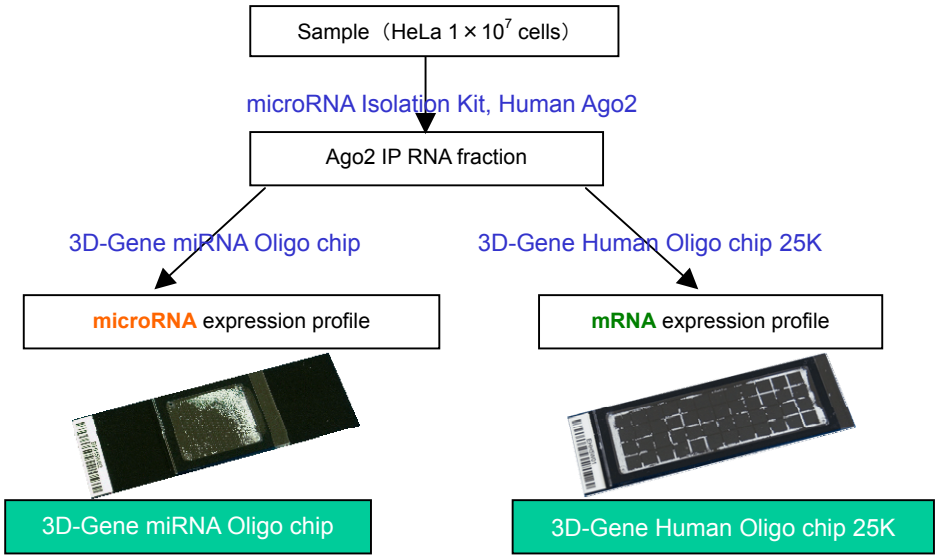


Figure: microRNA fractions, which were isolated by microRNA Isolation Kit, Mouse Ago2 from mouse lung, liver and brain Lysate, were specifically detected by Urea-PAGE and silver stain (Wako Cat #311-03961; CLEAR STAIN Ag) (50 mg Tissues→2 mL Total tissue lysate→Take the 1 mL→ Immunoprecipitation→10 μL Total RNA soln.→Take the 5 μL→Urea-PAGE)

■ Microarray analysis of Ago2 IP RNA fraction

Expression analysis of the miRNAs and mRNAs contained in the Ago2 IP RNA fractions can be carried out by using concomitantly the microRNA Isolation Kit, Human Ago2, and 3D-Gene miRNA Oligo chip and Human Oligo chip 25K (both available from Toray Industries, Inc.).

[Expression Analysis of miRNA using Ago2 IP RNA]



[Expression analysis of microRNA using Ago2 IP RNA]

■ Features

1) High-sensitivity and low-background expression analysis is possible due to highly-concentrated microRNA
2) Capable of exclusively profiling the microRNAs bound to RISC
3) Microarray analysis is possible from 1x10^6 cells

Sample		Ago2 IP (1x10^5 cells)	Ago2 IP (1x10^6 cells)	Ago2 IP (1x10^5 cells)	Total RNA
Amount of RNA, average		Total IP RNA	Total IP RNA	Total IP RNA	0.5μg
Back ground	Average	88.41	89.18	91.75	99.18
	SD	0.73	0.70	0.80	1.35
	Baseline value (BG+2SD)	89.87	90.58	90.58	101.87
CV		0.008	0.008	0.009	0.014
Number of active spots (over BG baseline value)		312	466	522	420

↑
Number of Active Spot was increased.

[Expression analysis of mRNA using Ago2 IP RNA]

■ Features

1) The signal of mRNAs in RISC is detectable from Ago2 IP RNA of 1x10^7 cells.
2) Usable for target mRNA analyses, since most of the mRNAs highly concentrated in Ago2 IP RNA have the seed sequences of the miRNA detected by the miRNA array.

(Please see the figure on the page # 7.)

Achieves Highly Efficient, Accurate & East-to-Use Adapter Ligation

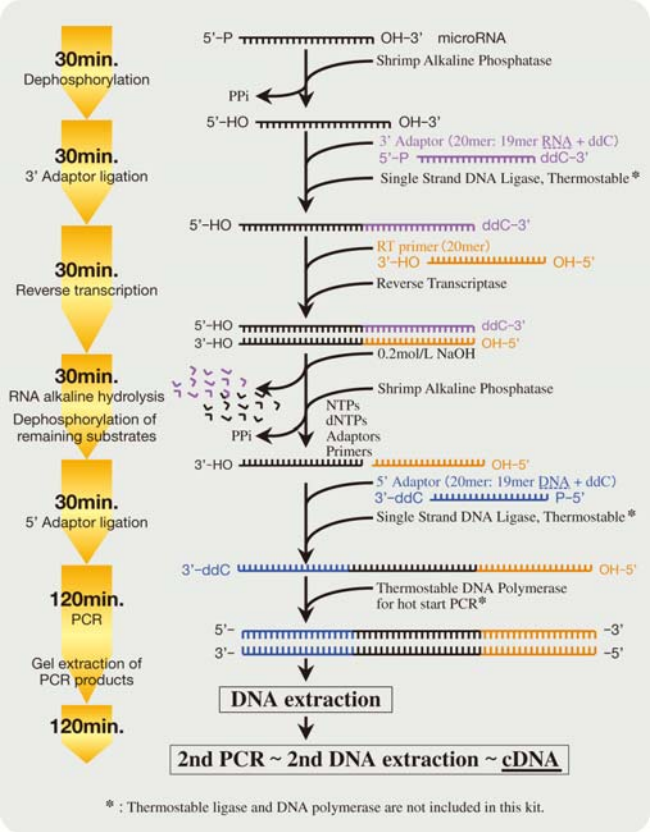
microRNA Cloning Kit *Wako* (Wako Cat. #290-66501)

The microRNA Cloning Kit *Wako* can prepare the cDNA encoding microRNA. The cloning procedure will be completed within 1.5 days after preparation of microRNA fraction. This kit is supported by shrimp alkaline phosphatase (SAP), thermostable single strand DNA ligase (selling separately), and original modified adaptors. The cloning efficiency using this kit is improved higher than that of the conventional methods, which used bacterial alkaline phosphatase and T4 RNA ligase.

■ Features

1) High cloning efficient and accurate adapter ligation by thermostable ligase
2) Suitable for cloning of microRNA forming secondary structure
3) Simple operation achieves forming of cDNA coding microRNA in 1.5 days

■ Outline of Procedure



■ Kit Contents (for 8 reactions)

(1) SAP	16 µL	(10) 1 mol/L Tris-HCl (pH 7.5)	160 µL
(2) 5 × SAP Buffer	64 µL	(11) Ethachinmate	24 µL
(3) 40 × Ligation Buffer	16 µL	(12) 10 mol/L Ammonium Acetate	960 µL
(4) RNase Inhibitor	16 µL	(13) 3' Adaptor (50 pmol/µL)	8 µL
(5) 10mmol/L $MnCl_2$	16 µL	(14) 5' Adaptor (50 pmol/µL)	8 µL
(6) Reverse Transcriptase	8 µL	(15) RT Primer (50 pmol/µL)	8 µL
(7) 10 × RT Buffer	16 µL	(16) 5' PCR Primer (50 pmol/µL)	16 µL
(8) dNTP Mixture	112 µL	(17) 3' PCR Primer (50 pmol/µL)	16 µL
(9) 0.5 mol/L EDTA (pH 8.0)	16 µL	(18) Control RNA (30 ng/µL)	8 µL

■ Single Strand DNA Ligase, thermostable, recombinant, solution (Wako Cat. #298-65103; 292-65101): ※

Features:

1. High thermal stability
2. Optimum temperature: 55 ~ 65°C
3. High ligation efficiency
4. Ligation of ssRNA, ssDNA, and ssRNA-ssDNA is applicable.

Source: *E. coli* expressed thermophilic phage TS2126 single strand DNA ligase
Appearance: 10 mmol/L Tris-HCl (pH 8.0), 50 mmol/L KCl, 0.1 mmol/L EDTA, 1 mmol/L DTT and 50 % Glycerol
Activity: Shown on each label. (approx. 10units/µL)

■ Comparison with conventional method

	Conventional method	microRNA Cloning kit *Wako*
Number of adaptor ligation steps	8	**3**
Phosphorylation of adaptors	Necessary	**Unnecessary**
Complete inactivation of Alkaline Phosphatase	Impossible	**Possible**
Apparatus for magnet beads	Necessary	**Unnecessary**
RI	Necessary	**Unnecessary**
BioAnalyzer (Agilent)	Necessary	**Unnecessary**
Adaptor ligation time	≧ 8 hr	**2.5 hr**
Handling time	2.5 days	**1.5 days**
Reproducibility of adaptor ligation time	Low	**High**
Efficiency of microRNA cloning	< 10 %	**> 70 %**
Detection by EtBr	Impossible	**Possible**
Cloning efficiency of secondary structured microRNA	Low (by T4 RNA Ligase)	**High** (by Single Strand DNA Ligase, thermostable)

■ High efficiency of microRNA cloning

microRNA Isolation Kit, **Human Ago2** (Wako Cat. #292-66701)

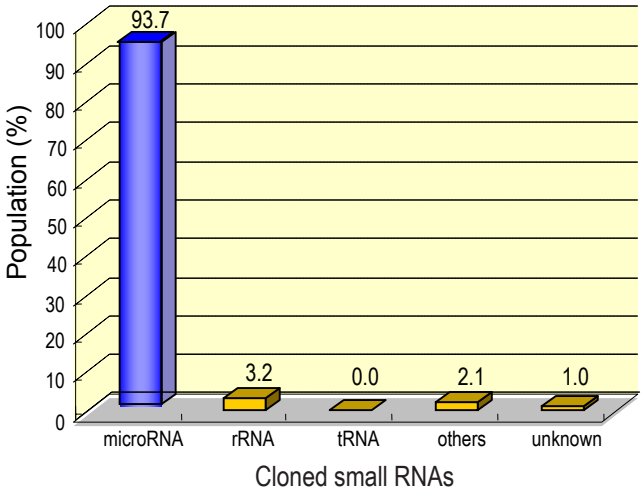


Figure: Cloning effi ciency of microRNA from HeLa cell lysate. The microRNA fraction was prepared by microRNA Isolation Kit, Human Ago2 (#292-66701) from 5 × 106 HeLa cells. The cDNA encoding microRNA was synthesized by microRNA Cloning Kit Wako (#290-66501) and was inserted into T-vector. The presence ratio of microRNA was more than 90%.

microRNA Isolation Kit, **Mouse Ago2** (Wako Cat. #292-67301)

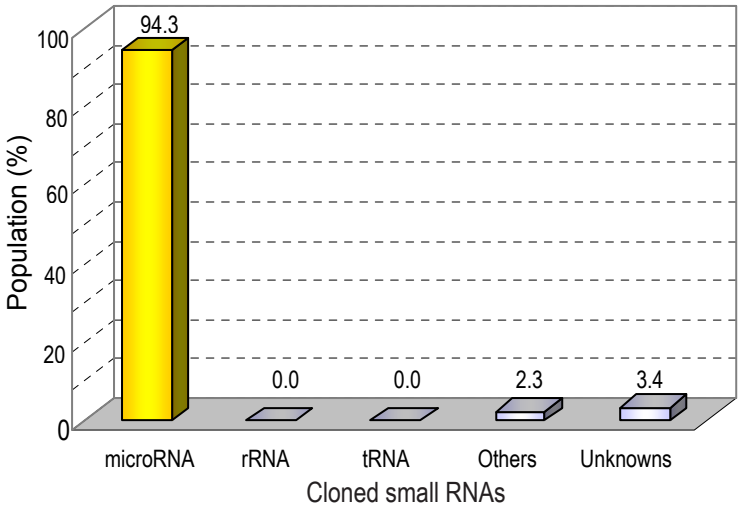


Figure: Cloning efficiency of microRNA from P388D1 cell lysate. The microRNA fraction was prepared by microRNA Isolation Kit, Mouse Ago2 (#292-67301) from 5 × 106 P388D1 cells. The cDNA encoding microRNA was synthesized by microRNA Cloning Kit Wako (#290-66501) and was inserted into T-vector. The presence ratio of microRNA was more than 90%.

microRNA Isolation Kit, **Human/Mouse Ago1** (Wako Cat. #291-70201)

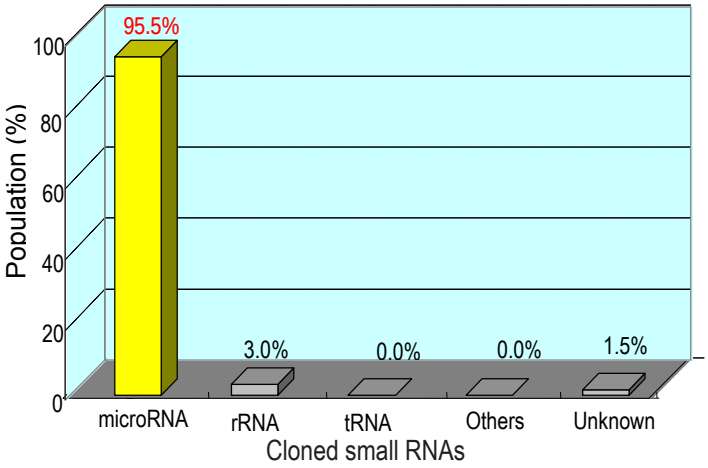


Figure: Cloning efficiency of microRNA from HeLa cell lysate. The presence ratio of microRNA was more than 90%. Others indicated cDNAs which were listed in miRBase of other organism species. Unknowns indicated cDNAs which were found in genome sequence, but not listed in miRBase.

microRNA Isolation Kit, **Human Ago3** (Wako Cat. #297-70301)

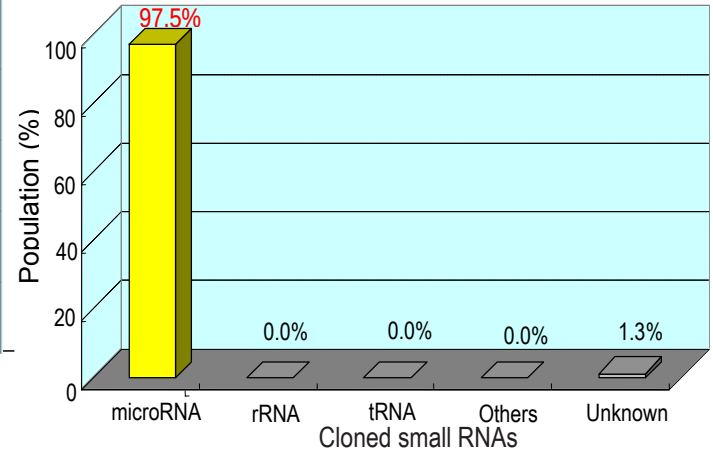


Figure: Cloning efficiency of microRNA from HeLa cell lysate. The presence ratio of microRNA was more than 90%. Others indicated cDNAs which were listed in miRBase of other organism species. Unknowns indicated cDNAs which were found in genome sequence, but not listed in miRBase.

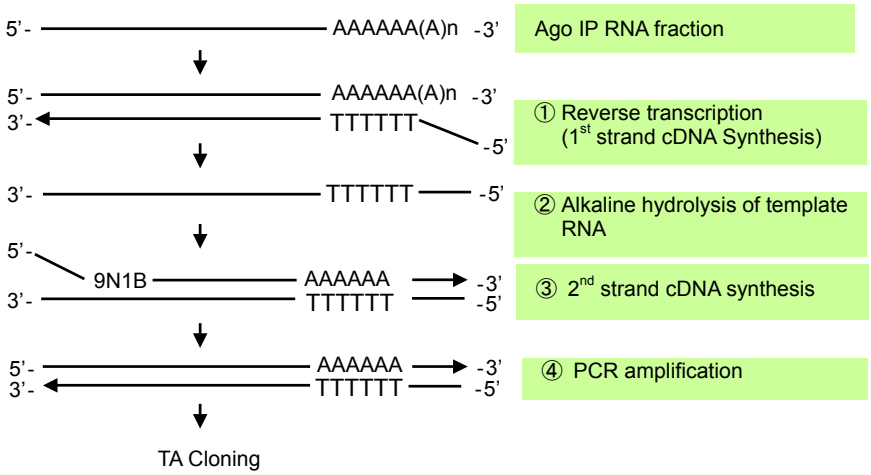
rRNA: rRNA fragment; tRNA: tRNA fragment; Others: cDNAs which were listed in miRBase of other organism species; Unknowns: cDNAs which were found in genome sequence, but not listed in miRBase

Target mRNA Cloning Kit Wako is used for the cDNA amplification of low population mRNA. Especially, this product will be used for the cloning of microRNA-targeted mRNA from Ago IP RNA fraction. The amplified cDNA will be the target mRNA of microRNA.

■ Features

1) Simple Protocol
2) Amplification of low population mRNA
3) Independent cDNA amplification to the length of mRNAs
4) Investigation of microRNA targeted mRNA

■ Principle



■ Kit Contents (10 reactions)

(1) dT (20)-RT Primer (20 pmol/μL) 1 tube x 10 μL
(2) 10x RT Buffer 1 tube x 20 μL
(3) dNTP Mixture Solution (2.5 mmol/L each) 1 tube x 20 μL
(4) RNase Inhibitor(20 U/μL) 1 tube x 1 0μL
(5) Reverse Transcriptase(200 U/μL) 1 tube x 10 μL
(6) 0.5 mol/L EDTA Solution 1 tube x 20 μL
(7) 1 mol/L Tris-HCl Solution 1 tube x 200 μL
(8) Ethachinmate 1 tube x 30 μL
(9) 10 mol/L Ammonium Acetate Solution 1 tube x 480 μL
(10) 2nd Strand Synthesis Primer (20 pmol/μL) 1 tube x 10 μL
(11) 5' PCR Primer(20 pmol/μL) 1 tube x 10 μL
(12) 3' PCR Primer(20 pmol/μL) 1 tube x 10 μL
(13) 5' Colony PCR Primer (5 pmol/μL) 1 tube x 960 μL
(14) 3' Colony PCR Primer (5 pmol/μL) 1 tube x 960 μL

■ Identification of amplified cDNA and categories of cDNAs isolated from HeLa cells

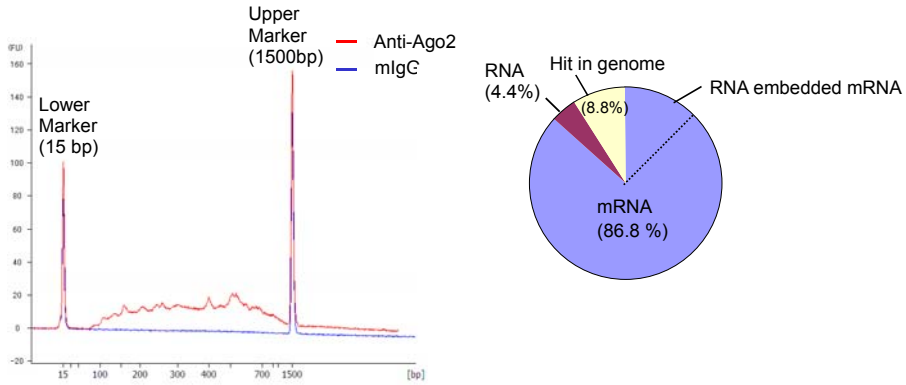


Figure: Size distribution of synthesized cDNA and categories of cDNAs isolated from HeLa cells.

[Advantages of Target mRNA Cloning Kit *Wako*]

There is a possibility that RNA molecules other than mRNAs, which can not be identified by the RISC-trapped microarray, can be identified by cloning of the cDNAs synthesized using this kit. According to *BMC Research Notes*, **2**, 169 (2009), free Alu RNAs besides mRNAs are contained in the RISC.

[Additional materials required in addition to Target mRNA Cloning Kit *Wako*]

(1) **THUNDER Taq Gold DNA Polymerase** (Mg^{2+} free buffer) (Wako Cat. #317-07081; 250 U): Thermostable DNA polymerase for PCR
We recommend THUNDER Taq Gold DNA Polymerase (Mg^{2+} free buffer) for suppression of non-specific amplification. It is used for hot start PCR and activated by 10 minute incubation at 95℃ before PCR cycle. DNA fragments, which were amplified by THUNDER Taq Gold DNA Polymerase (Mg^{2+} free buffer), are 3' protruding end of adenine base. These fragments therefore are able to directly insert into T-vectors.

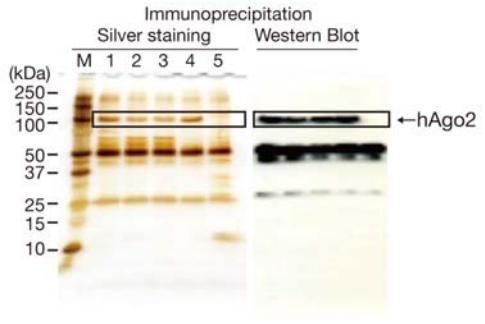
(2) **PCR Purification Kit *Wako*** (Wako Cat. No. 298-67901)
PCR Purification Kit *Wako* is used for removal of primers, primer dimmers, dNTPs, enzymes such as DNA polymerase and restriction enzyme from reaction buffer of various molecular biology experiments in only 10 minutes after DNA synthesis by RT-PCR or PCR.

For functional RNA Research

Anti Ago2, Monoclonal Antibodies

Anti **Human** Ago2, Monoclonal Antibody

(Clone No.4G8) (Wako Cat. No. 011-22033; 015-22031)



Lane M : Molecular weight marker
Lane 1 : HeLa (Human)
Lane 2 : HepG2 (Human)
Lane 3 : HEK293 (Human)
Lane 4 : THP-1 (Human)
Lane 5 : P388D1 (Mouse)

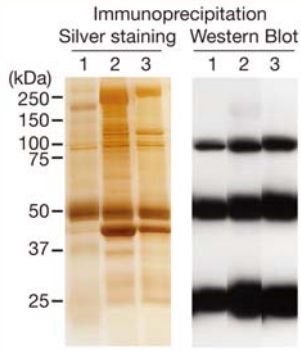
Application	Working Dilution
Western Blot	1 : 100 - 1 : 200
Immunoprecipitation	1 : 50
Immunocytochemical staining	1 : 20 - 1 : 50

Species	Human	Mouse	Hamster	Rat
Cell	HeLa	P388D1	CHO	SCC-131
Western Blot	○	×	×	×
Immunoprecipitation	○	×	×	×
Immunocytochemistry	○	×	×	×
microRNA Purification	○	×	×	×

Figure: Immunoprecipitation of human Ago2 protein from human cultured cell lines (HeLa, HepG2, HEK293, THP-1) and mouse cultured cell line (P388D1) by using 20 µL 10% Protein G slurry immobilized with 10 µg of this antibody (4G8). The bands of hAgo2 protein were detected in approximately 100 kDa by using silver staining and western blot. Cell number was 5×10^6 cells.

Anti **Mouse** Ago2, Monoclonal Antibody

(Clone No.2D4) (Wako Cat. No. 014-22023; 018-22021)



Lane 1 : NIH-3T3 (Mouse)
Lane 2 : SCC-131 (Rat)
Lane 3 : CHO (Hamster)

Application	Working Dilution
Western Blot	1 : 200 - 1 : 1,000
Immunoprecipitation	10µg / IP
Immunocytochemical staining	1 : 100 - 1 : 500

Species	Mouse	Hamster	Rat	Human
Cell	P388D1 NIH-3T3	CHO	SCC-131	NCI-H460
Western Blot	○	○	○	×
Immunoprecipitation	○	○	○	×
Immunocytochemistry	○ (NIH-3T3)	NT	NT	×
microRNA Purification	○ (P388D1)	○	○	×

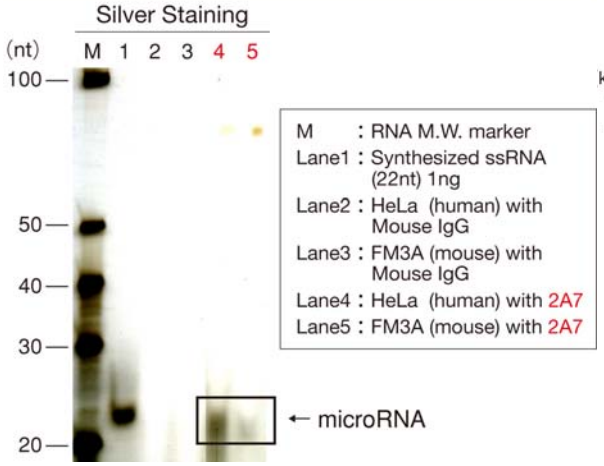
NT : Not Tested

Figure: Immunoprecipitation of Ago2 protein from NIH-3T3 (Mouse), SCC-131(Rat) and CHO (Hamster) cell line by using 20 µL of 10 % Protein G slurry immobilized with 5 µg of this antibody (2D4). The bands of endogenous Ago2 protein were detected in approximately 100 kDa by using silver staining and western blot. The 1/1,000 diluted antibody was used as the 1st antibody for western blot. Cell number was 5×10^6 cells.

For functional RNA Research

Anti Ago1, Monoclonal Antibodies (Clone #1F2: Wako Cat. #018-22401; 2A7: #015-22411)

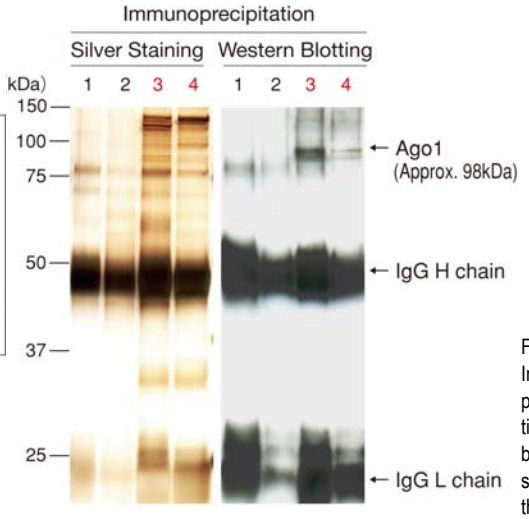
microRNA purification from endogenous Ago1 IP fraction (Cell)



M : RNA M.W. marker
Lane1 : Synthesized ssRNA (22nt) 1ng
Lane2 : HeLa (human) with Mouse IgG
Lane3 : FM3A (mouse) with Mouse IgG
Lane4 : HeLa (human) with 2A7
Lane5 : FM3A (mouse) with 2A7

Figure: microRNA purification from Ago1 IP fraction of HeLa and FM3A cell line by using 20 µL 10 % Protein G slurry immobilized with 5 µg this antibody (2A7). The purified microRNA fraction was specifically detected by silver stain after Urea-PAGE. Cell number was 2×10^7 cells.

Immunoprecipitation of endogenous Ago1 (Mouse Tissue)



Lane1 : Lung with Mouse IgG
Lane2 : Brain with Mouse IgG
Lane3 : Lung with Anti Ago1 (2A7)
Lane4 : Brain with Anti Ago1 (2A7)

Figure: Immunoprecipitation of Ago1 protein from lung and brain tissue (15 mg each) of mouse by using 20 µL 10 % Protein G slurry immobilized with 5 µg this antibody (2A7). The band of endogenous Ago1 protein was detected in approximately 98 kDa by western blot analysis with this antibody (1F2).

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