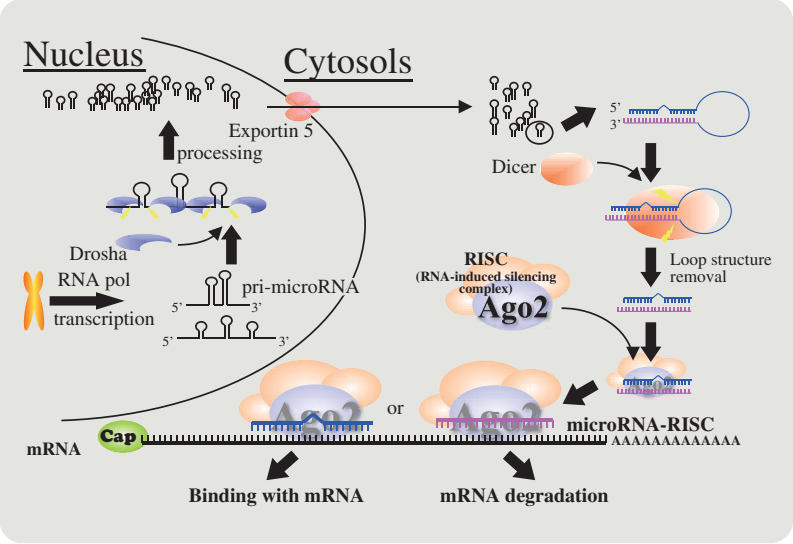
Wako

microRNA Research

The Pathway of Translational Silencing by microRNA



The microRNA matured in multiple steps in the cells is taken into a protein compound called an RNA-induced silencing complex (RISC), and it is known that it bonds with Argonaute (Ago) subfamily protein as its main component. Specific purification of RNA bound to protein of the Ago subfamily is considered to be an important technology for microRNA function determination, discrimination of new microRNA, and analysis of interaction between microRNA and mRNA.
Wako has developed and launched high-quality anti-Ago2 monoclonal antibodies, microRNA Isolation kits, and high-efficiency microRNA Cloning kits, and the line-up includes research reagents with powerful support for microRNA research.

for Highly Efficient Adapter Ligation

microRNA Cloning Kit *Wako*

Wako Cat. No. 290-66501

for Effective 65℃
Ligation of ssDNA, ssRNA and ssRNA-ssDNA

ssDNA Ligase, thermostable

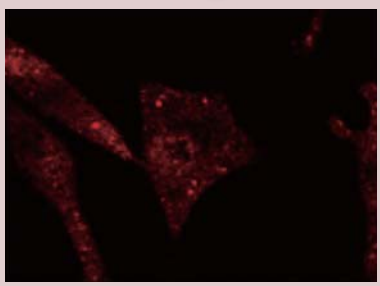
Wako Cat. No. 298-65103; 292-65101

Immunoprecipitation method with anti-Ago2 mAb

microRNA Isolation Kit, Human / Mouse Ago2
Anti Human / Mouse Ago2, Monoclonal Antibody

microRNA Isolation Kit, Human Ago2
(Wako Cat. No. 292-66701)

microRNA Isolation Kit, Mouse Ago2
(Wako Cat. No. 292-67301)



Anti Mouse Ago2, Monoclonal Antibody
(Clone No. 2D4), immunostained image

NEW METHOD!!

microRNA "Specific" Purification

microRNA Isolation Kit, Human Ago2 (Wako Cat. No. 292-66701), Mouse Ago2 (Wako Cat. No. 292-67301)

microRNA Isolation Kit, Human/Mouse Ago2 can prepare high purified fractions of microRNA, which binds with Argonaute2 (Ago2) protein, based on immunoprecipitation method by using a high affinity monoclonal antibody against Ago2.

The purified microRNA fraction will contain very little contaminated degradation fragments of rRNA and tRNA.

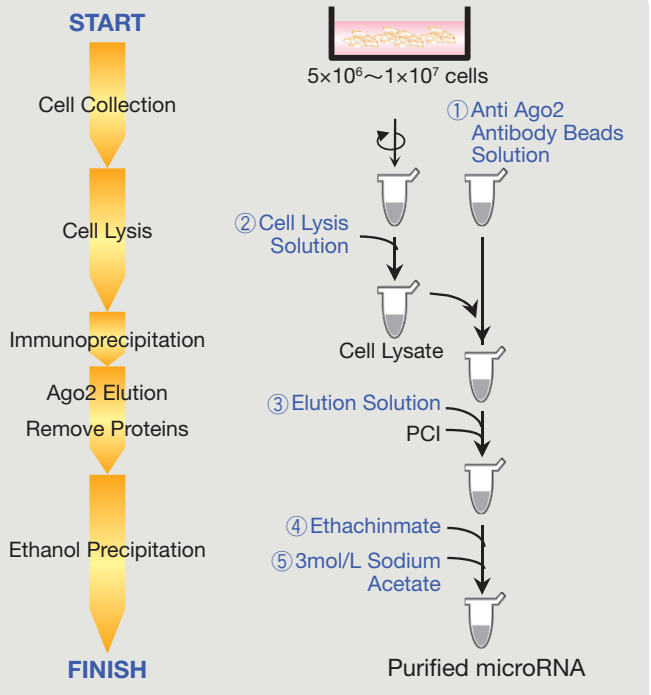
These kits will highly improve the microRNA cloning efficiency compared with that by using conventional microRNA purification method.

Features

1. Achieve Immunoprecipitation of internal Ago2 from human, mouse, rat, and hamster. Note)
2. microRNA bound to Ago2 protein can be isolated with high purity.
3. Few rRNA or tRNA decomposition products or impurities like smallRNA etc.
4. The isolated microRNA fraction can be used for cloning and micro arrays.

Note) Wako Cat. #292-66701 is for human cells and tissues and #292-67301 is for cells and tissues of mice and rats.

Outline of procedure

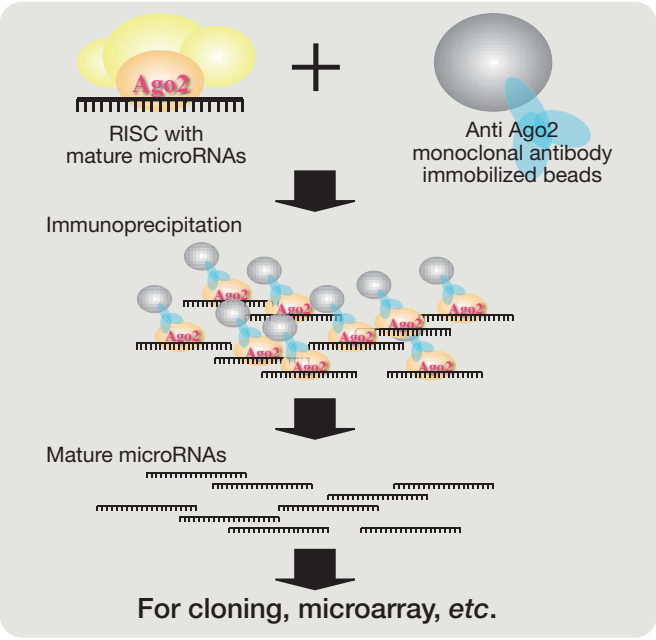


(①～⑤ show numbers of each kit content)

Kit contents (10 reactions)

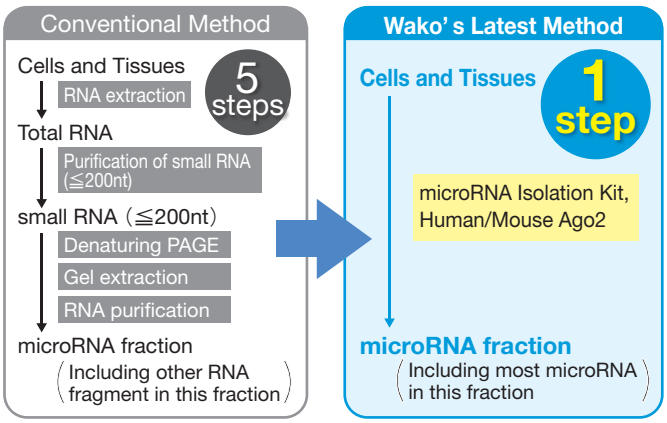
①Anti Human Ago2 Antibody Beads Solution ····· 500 µL×1 vial
②Cell Lysis Solution ····· 50 mL×1 vial
③Elution Solution ····· 500 µL×1 vial
④Ethachinmate ····· 30 µL×1 vial
⑤3mol/L Sodium Acetate ····· 400 µL×1 vial

Simple procedure



Comparison with conventional method

	Conventional method	Wako's Latest Method
Total RNA extraction	Necessary	Unnecessary
small RNA (≦200nt) purification		
Denaturing PAGE		
Gel extraction from denaturing PAGE		
Gel purification from denaturing PAGE		
Contamination ratio of rRNA fragments	High	Low
Contamination ratio of tRNA fragments	High	Low
Handling time	≦12hr	≦4hr



Specific Purification of microRNA fraction from several cell lines

microRNA Isolation Kit, **Human** Ago2
(Wako Cat. No. 292-66701)

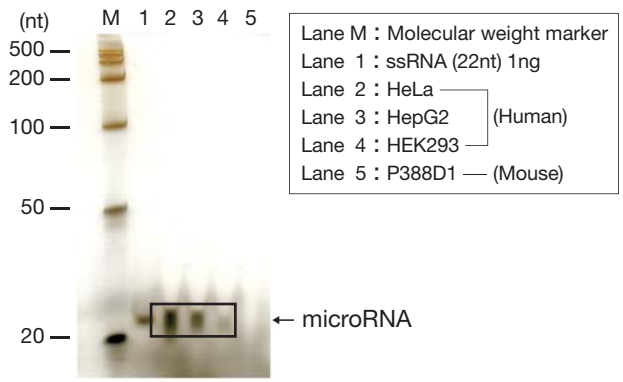


Figure. 1 **Purification of microRNA fraction by using microRNA Isolation Kit, Human Ago2 (Wako catalog #292-66701).**
The purified microRNA fraction from human cultured cell lines (HeLa, HepG2, HEK293) were specifically detected by Urea-PAGE. Cell number of each cell line is approximately 5×10^6. The 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. No. 292-67301)

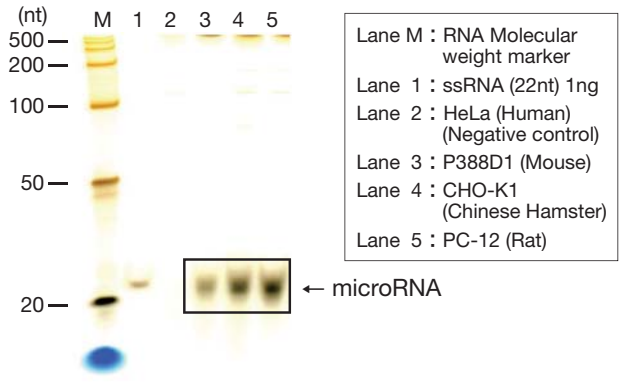
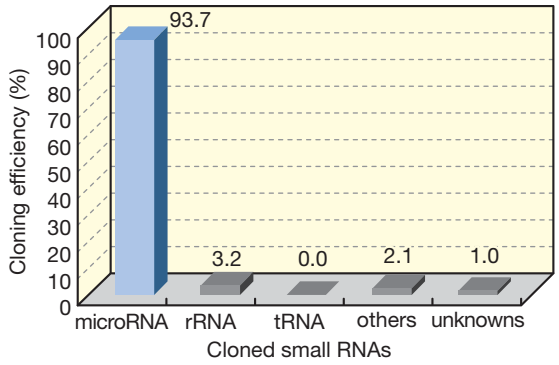


Figure. 2 **Urea-PAGE pattern of purified RNA by using microRNA Isolation Kit, Mouse Ago2 (Wako catalog #292-67301).**
The purified microRNA fractions from cultured rodent cell lines (P388D1, CHO-K1, PC-12) were detected by silver stain. Cell number of each cell line is approximately 1×10^7. The applied volume per lane is half of isolated sample by this kit.

Cloning of purified microRNA

High efficiency of microRNA cloning by using these kits followed by using microRNA Cloning Kit *Wako*.

microRNA Isolation Kit, **Human** Ago2
(Wako Cat. No. 292-66701)



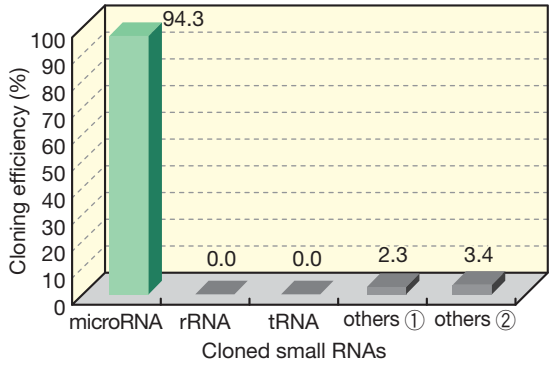
rRNA : rRNA fragment
tRNA : tRNA fragment
others : cDNAs found in genome sequence, but not listed in miRBase
unknowns : cDNAs not found in genome sequence

Table 1. The contents of cloned microRNA species

microRNA	The number of clone
hsa-miR-23a	36
hsa-miR-22	31
hsa-miR-92a	18
hsa-miR-23b	2
hsa-miR-21	1
hsa-miR-125b	1
Total	89

Figure. 3 **Cloning efficiency of microRNA from HeLa cell lysate**
The microRNA fraction was prepared by microRNA Isolation Kit, Human Ago2 (#292-66701) from 5×10^6 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%.
The contents of cloned microRNA species were indicated on **Table 1**.

microRNA Isolation Kit, **Mouse** Ago2
(Wako Cat. No. 292-67301)



rRNA : rRNA fragment
tRNA : tRNA fragment
others ① : cDNAs listed in miRBase of other organism species
others ② : cDNAs found in genome sequence, but not listed in miRBase

Table 2. The contents of cloned microRNA species

microRNA	The number of clone
mmu-miR-92a	40
mmu-miR-23a	21
mmu-miR-25	5
mmu-miR-315	2
mmu-miR-31	2
Other species	12
Total	82

Figure. 4 **Cloning efficiency of microRNA from P388D1 cell lysate**
The microRNA fraction was prepared by microRNA Isolation Kit, Mouse Ago2 (#292-67301) from 5×10^6 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%.
The contents of cloned microRNA are indicated on **Table 2**.

Purification of microRNA fraction from tissues

microRNA Isolation Kit, **Human** Ago2
(Wako Cat. No. 292-66701)

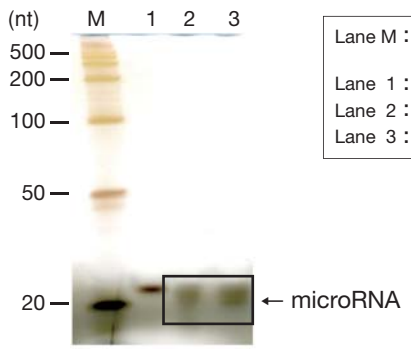


Figure. 5 microRNA fractions, which were isolated by this kit from human liver and testis lysate, were detected by silver stain after Urea-PAGE.

(50mg Tissues→2mL Total tissue lysate→1mL Tissue lysate →Immunoprecipitation→10μL Total RNA solution→5μL RNA solution→Urea-PAGE.)

microRNA Isolation Kit, **Mouse** Ago2
(Wako Cat. No. 292-67301)

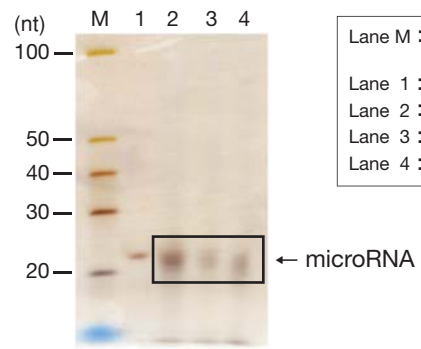


Figure. 6 microRNA fractions, which were isolated by this kit from mouse lung, liver and brain lysate, were detected by silver stain after Urea-PAGE.

(50mg Tissues→2mL Total tissue lysate→1mL Tissue lysate →Immunoprecipitation→10μL Total RNA solution→5μL RNA solution→Urea-PAGE.)

GOOD METHOD!

Achieve Highly Efficient, Accurate & Easy-to-Use Adapter Ligation

microRNA Cloning Kit *Wako* (Wako Cat. No. 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), single strand DNA Ligase, thermostable (Wako Cat. No. 298-65103; 292-65101) (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. Highly efficient and accurate adapter ligation by thermostable ligase .
2. Suitable for cloning of microRNA forming secondary structures.
3. Simple operation achieves forming of cDNA coding microRNA in 1.5 days.

Kit contents (8 reactions)

① SAP	16 μL	⑩ 1mol/L Tris-HCl, pH7.5	160 μL
② 5×SAP Buffer	64 μL	⑪ Ethachinmate	24 μL
③ 40×Ligation Buffer	16 μL	⑫ 10mol/L Ammonium Acetate	960 μL
④ RNase Inhibitor	16 μL	⑬ 3' Adaptor (50pmol/μL)	8 μL
⑤ 10mmol/L $MnCl_2$	16 μL	⑭ 5' Adaptor (50pmol/μL)	8 μL
⑥ Reverse Transcriptase	8 μL	⑮ RT Primer (50pmol/μL)	8 μL
⑦ 10×RT Buffer	16 μL	⑯ 5' PCR Primer (50pmol/μL)	16 μL
⑧ dNTP Mixture	112 μL	⑰ 3' PCR Primer (50pmol/μL)	16 μL
⑨ 0.5mol/L EDTA, pH8.0	16 μL	⑱ Control RNA (30ng/μL)	8 μL

Thermostable ligase and DNA polymerase are not included in this kit.

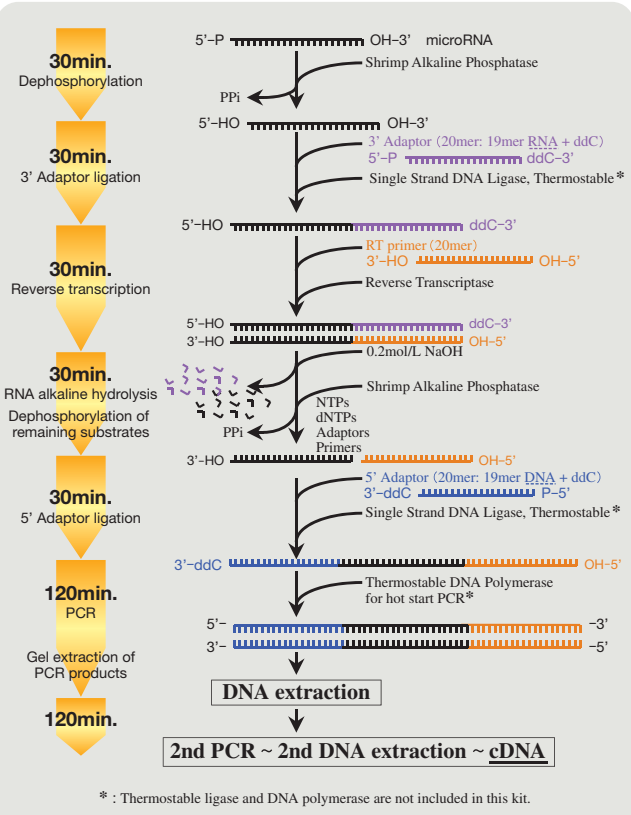
IMPORTANT

microRNA Cloning Kit ***Wako*** must be used together with Single Strand DNA Ligase, Thermostable (Wako Cat. No. 298-65103; 292-65101), which is sold separately as a single product.

This ligase will be used for the high efficient adaptor ligation and reaction buffers of this kit are optimized with this ligase.

This ligase is ATP-dependent and used for ligation of ssDNA-ssDNA and ssRNA-ssRNA. Reaction of this ligase at 55-65℃, which is the optimal temperature of enzymatic reaction, will promote the ligation efficiency of single strand nucleic acids.

Outline of procedure



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
BioAnalzer (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)

ssDNA Ligase, thermostable (Wako Cat. No. 298-65103; 292-65101)

1. High thermal stability
2. Optimum temperature: 55~65℃
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: 10mmol/L Tris-HCl (pH 8.0), 50mmol/L KCl, 0.1mmol/L EDTA, 1mmol/L DTT and 50% Glycerol

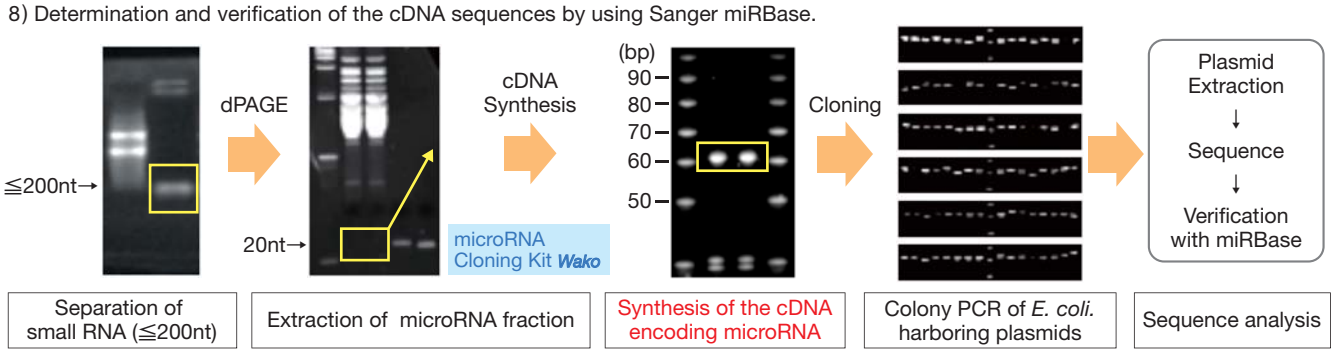
Activity: Shown on each label. (approx. 10units/μL)

Application with the microRNA Cloning Kit *Wako* is described in the package insert of the kit.

Cloning of microRNA from HeLa cells

Procedure

1) Preparation of total RNA from HeLa cells (1×10^7 cells) by ISOGEN (Nippon Gene #315-02504, 10mL).
2) Preparation of small RNA fraction, less than 200nt, from total RNA by microRNA Isolation Kit (Bio Chain Institute Inc. catalog #KS341025).
3) Separation of microRNA fraction by denaturing PAGE.
4) Collection of the gels of 20~23nt region after electrophoresis.
5) Cloning by using microRNA Cloning Kit *Wako* (Wako catalog #290-66501).
6) Construction of the plasmids harboring cDNA encoding microRNA and transformation of *E. coli.*
7) Random selection of the 96 transformed *E. coli* from selection LB agar medium.
8) Determination and verification of the cDNA sequences by using Sanger miRBase.



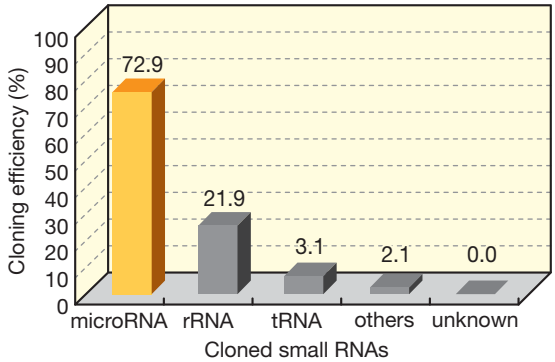


Table 3. Contents of cloned microRNA species

microRNA species	The number of clone
hsa-miR-23a	38
hsa-miR-92a	16
hsa-miR-22	5
hsa-miR-25	5
hsa-miR-23b	3
hsa-miR-19b	1
hsa-miR-21	1
hsa-miR-210	1
Total	70

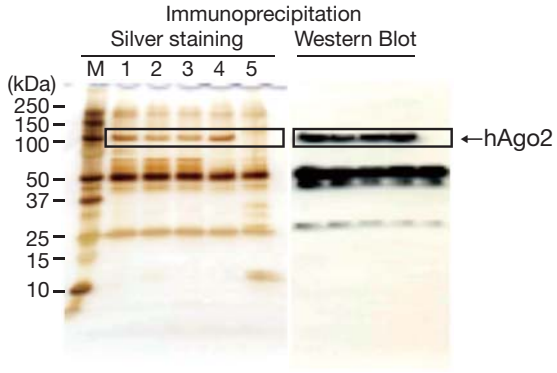
Figure. 7 Cloning efficiency of microRNA from HeLa cell lysate
The cloning efficiency of microRNA was more than 70%. Others indicate that isolated cDNA sequences were not matched miRBase. Unknowns indicate that isolated cDNA sequences were not matched human genome sequence. The contents of cloned microRNA species are indicated on **Table 3**.

HIGH QUALITY!!

Anti Ago2, Monoclonal Antibody

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)

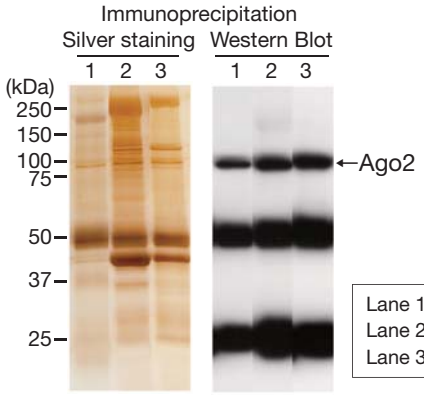
Application	Working Dilution
Western Blot	1 : 100 - 1 : 200
Immunoprecipitation	1 : 50
Immunocytochemical staining	1 : 20 - 1 : 50

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

Figure. 8 Immunoprecipitation of hAgo2 protein from human cultured cell lines (HeLa, HepG2, HEK293, THP-1) and mouse cultured cell line (P388D1) by using 20µL 10% Protein G slurry immobilized with 10µg of this antibody. The bands of hAgo2 protein were detected in approximately 100kDa 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. 9 Immunoprecipitation of Ago2 protein from NIH-3T3(Mouse), SCC-131(Rat) and CHO(Hamster) cell line by using 20µL of 10% Protein G slurry immobilized with 5µg of this antibody (2D4). The bands of endogenous Ago2 protein were detected in approximately 100kDa 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.

Product List

	Description	Wako Cat. No. (Package Size)
NEW!!	microRNA Isolation Kit, Mouse Ago2	292-67301 (10 reactions)
	microRNA Isolation Kit, Human Ago2	292-66701 (10 reactions)
NEW!!	Anti Mouse Ago2, Monoclonal Antibody	014-22023 (50 µL)
NEW!!		018-22021 (100 µL)
NEW!!	Anti Human Ago2, Monoclonal Antibody	011-22033 (50 µL)
NEW!!		015-22031 (100 µL)
	microRNA Cloning Kit ***Wako***	290-66501 (8 reactions)
	Single Strand DNA Ligase, thermostable, recombinant, Solution	298-65103 (200 units)
		292-65101 (500 units)

・microRNA Isolation Kit, Human/Mouse Ago2 is patent pending (11, 30, 2007).
・microRNA Cloning Kit ***Wako*** is patent pending (1, 10, 2007).
・All products shown on the leaflet are for research use only.

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