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Instruction manual for miRNAssay™ qPCR RT Master Mix

Instruction manual for *SuperPrep*™ miRNAssay™ Cell Lysis & RT Kit for qPCR

F2184K

miRNAssay™ qPCR RT Master Mix

MIR-101 200 reactions
MIR-101T 40 reactions

Store at -20°C, protected from light

SuperPrep™ miRNAssay™ Cell Lysis & RT Kit for qPCR

MIR-201 100 reactions
MIR-201T 20 reactions

Store at -20°C, protected from light

Contents

CAUTION

All reagents in this kit are intended for research purposes only. not for use for diagnostic, clinical, food or cosmetic purposes. Please see SDS and observe general laboratory precautions and safety procedures while using this kit.

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[1] Introduction

Description

The miRNAssay™ qPCR RT Master Mix (Code No. MIR-101) is a reverse transcription reagent for real-time PCR dedicated to miRNA, developed using the highly efficient reverse transcriptase ReverTra Ace™. This product enables the specific and sensitive synthesis of miRNAs from purified total RNA into cDNA.

SuperPrep™ miRNAssay™ Cell Lysis & RT Kit for qPCR (Code No. MIR-201) consists of cell lysis reagents (Lysis Reagents) and reverse transcription reagents (RT Reagents) for gene expression analysis by real-time PCR. Using this product, lysates containing miRNAs, which can be used as templates for reverse transcription reactions, can be easily prepared from cells cultured in 96-well plates or purified extracellular vesicles.

This product is not supplied with miRNA-specific RT primers or real-time PCR reagents. For real-time PCR, we recommend using our highly efficient real-time PCR reagents THUNDERBIRD™ Next SYBR™ qPCR Mix (Code No. QPX-201) or THUNDERBIRD™ Next Probe qPCR Mix (Code No. QPX-101). (see [8] Related products).

Features

-Pre-mix reagents

The reverse transcription reagent (5×RT Master Mix) is a premix-type reagent that does not freeze at -20°C. Add a template RNA, an miRNA-specific RT primer, and water to synthesize cDNA easily and repeatedly. The no-RT Control, also of the premix type, is also included; hence, a control for the reverse transcription reaction (-) can also be easily prepared.

-High specificity

The specificity of the reverse transcription reaction has been improved by compositional optimization. The reduction of non-specific reactions improves the reliability of the reverse transcription reaction for low-concentration targets.

-Wide range of detection

Highly efficient, specific, and wide-range reverse transcription reactions are possible.

-Short and simple protocol

The reverse transcription reaction reagent uses a buffer optimized for cDNA synthesis for real-time PCR, enabling efficient reverse transcription reactions in just 35 min. The protocol is also simple, as no additional RNase H treatment is required to remove residual RNA, which can inhibit the real-time PCR.

-High-quality cDNA can be synthesized from cultured cells and extracellular vesicles

The *SuperPrep*™ miRNAssay™ Cell Lysis & RT Kit for qPCR (Code No. MIR-201) can be used to easily prepare lysates containing miRNAs that can be used as templates for reverse transcription reactions from cultured cells or purified extracellular vesicles. The lysate treated with the Lysis Solution can be used as a template for the reverse transcription reaction directly, thereby significantly shortening the analysis time. The optimized buffer composition effectively suppresses miRNA degradation through cellular components such as RNase. In addition, as cDNA synthesis is performed after the gDNA remover treatment, high-quality cDNA can be synthesized with minimal contamination of genomic DNA.

-Can be used with various real-time PCR reagents

This kit can be used in combination with a wide range of real-time PCR reagents, compatible with both SYBR™ Green I and TaqMan™ assays.

When used in combination with our THUNDERBIRD™ Next SYBR™ qPCR Mix (Code No. QPX-201) or THUNDERBIRD™ Next Probe qPCR Mix (Code No. QPX-101), this kit can be used to detect purified RNA, cultured cells, and purified extracellular vesicles easily and conveniently.

[2] Components

This product contains the following reagents.

miRNAssay™ qPCR RT Master Mix (Code No. MIR-101, MIR-101T)

Name of contents	storage	MIR-101 (200rxn／10 µL)	MIR-101T (40rxn／10 µL)
5×RT Master Mix	-20 °C	400 µL	80 µL
5×RT Master Mix no-RT Control	-20 °C	40 µL	10 µL
Nuclease-free Water	-20 °C	1.1 mL×2	440 µL

***SuperPrep*™ miRNAssay™ Cell Lysis & RT Kit for qPCR (Code No. MIR-201, MIR-201T)**

Name of contents	storage	MIR-201 (100rxn／40 µL)	MIR-201T (20rxn／40 µL)
5×RT Master Mix	-20 °C	800 µL	160 µL
5×RT Master Mix no-RT Control	-20 °C	80 µL	16 µL
Nuclease-free Water	-20 °C	1.7 mL×2	440 µL
Lysis Solution	-20 °C	6.5 mL	1.3 mL
gDNA Remover	-20 °C	33 µL	10 µL
RNase Inhibitor	-20 °C	110 µL	22 µL

5×RT Master Mix

This 5× concentration master mix contains the following: high-efficiency reverse transcriptase ReverTra Ace™; RNase Inhibitor, reaction buffer; $MgCl_2$; dNTPs; glycerol. Spin down and allow the liquid to bottom out before opening the lid. Furthermore, due to the viscous nature of the liquid, pipetting should be performed slowly.

5×RT Master Mix no-RT Control

This master mix is a 5×RT Master Mix minus ReverTra Ace™. It can be used for the preparation of reverse transcription (-) controls; as with the 5×RT Master Mix, spin down the liquid to the bottom before opening the lid. Moreover, due to the viscosity of the liquid, pipetting should be performed slowly.

Lysis Solution

The cell lysis solution is optimized for RT-qPCR, containing components to reduce RNase activity levels. For use with the supplied RNase Inhibitor and gDNA Remover. Simultaneously, lyses cells and degrades genomic DNA.

gDNA Remover

DNase I solution is optimized for this kit; added at a ratio of 0.3 to 58.7 µL of the lysis solution. Degrades genomic DNA that causes the background in the assay.

RNase Inhibitor

RNase Inhibitor solution is optimized for this kit; added at a ratio of 1 to 58.7 µL of the lysis solution. Reduces RNase activity in the sample.

[3] Materials required

In addition to this product, the following reagents and equipment should be prepared

1. Thermal cycler or incubator

Prepare equipment capable of maintaining the recommended temperatures (16 °C, 42 °C, 70 °C, and 95 °C) for the RT reaction of this product.

2. Nuclease-free Water

The miRNAssay™ qPCR RT Master Mix (Code No. MIR-101) comes with enough for 200 reactions and the *SuperPrep*™ miRNAssay™ Cell Lysis & RT Kit for qPCR (Code No. MIR-201) for 100 reactions. However, please prepare separately as required for template RNA dilution. We recommend the use of diethylpyrocarbonate (DEPC)-free nuclease-free water. DEPC-treated water can be used, but the reaction may be inhibited by residual DEPC, so autoclave the water thoroughly to remove DEPC completely before use. Nuclease-free water used for reverse transcription reactions and PCR should be stored separately from other experiments and should not be shared to prevent the contamination of nucleic acids.

3. miRNA-specific RT primers

Prepare RT primers with a stem-loop structure corresponding to the miRNA sequence of interest. Reverse transcription is performed using sequence-specific RT primers, so there is no need to prepare random primers or oligo(dT) primers.

The purity of the primers has a significant impact on reaction specificity. Generally, primers with low purification purity may have a greater variation in quality and are more prone to non-specific amplification. Where possible, use primers of a purification grade of HPLC purification, or at least is lightly stirred to make it homogeneous and then incubated at the following temperatures.

4. real-time PCR equipment and real-time PCR reagents

Follow the instructions for each device and reagent before use.

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[4] Protocol

1. When using purified RNA

(miRNAssay™ qPCR RT Master Mix (Code No. MIR-101))

(1) Preparation of reaction solution

Prepare the reaction solution on ice as follows.

Reagents	10 µL rxn *3	Final Conc.
5×RT Master Mix *1	2 µL	1×
RT Primer*4	X µL	50 nM *2
RNA template	Y µL	1 pg~100 ng
Nuclease-free Water	8-X-Yµ L	-
Total	10µL	

*1 If you take the reverse transcription (-) control here, instead of 5×RT Master Mix no-RT Control or instead of 5×RT Master Mix. By taking a control for reverse transcription (-), you can check whether the signal is derived from cDNA or not.

*2 If favorable results cannot be obtained at 50 nM, consider using an RT primer concentration of 20 to 100 nM.

*3 Scale up accordingly if necessary.

*4 Not included in this kit. You can design your own or use TaqMan™ MicroRNA Assays (Thermo Fisher Scientific™).

(2) Reverse transcription reaction

The reaction solution is lightly stirred to make it homogeneous and then incubated at the following temperatures.

16 °C, 30 min. ┐
42 °C, 5 min. ┘ Reverse transcription reaction
95 °C, 5 min. ・・・・ Enzyme inactivation reaction
4 °C, hold

After the completion of the reaction, store at 4 or -20°C. When performing real-time PCR, add directly or diluted to the reaction solution as a template*1.

*1 The maximum amount of reverse transcription reaction solution added to the real-time PCR reaction solution should be approximately 10%. Adding a large amount may reduce the reaction efficiency of PCR and may result in inaccurate quantification.

2. When using cultured cells

(*SuperPrep*™ miRNAssay™ Cell Lysis & RT Kit for qPCR
(Code No. MIR-201))

(1) Cell collection

① Measure the number of cells and centrifuge to remove the medium.

② Wash the cells with PBS(-).

③ Suspend the cells in PBS(-) so that the cells are reduced to 1×10^7 cells/mL or less. *1

④ Dispense 5 µL into microtubes.

*1 Too many cells may lead to inadequate lysis, RT-PCR inhibition, and insufficient degradation of genomic DNA. This reagent can process cell samples of 1×10^1 to 7×10^4 cells, but this may vary slightly depending on the cell type; 10^4 cells should be used as a guide, or it is recommended to check the upper limit in preliminary experiments.

(2) Cell lysis

① Add RNase Inhibitor and gDNA Remover to the required volume of Lysis Solution. Prepare a little extra than the required amount by referring to the table below. When using a multi-channel pipette, prepare an extra amount of about 10%.*2

	1 rxn	10 rxn	96 rxn
Lysis Solution	58.7 µL	587 µL	5635.2 µL
RNase Inhibitor	1 µL	10 µL	96 µL
gDNA Remover	0.3 µL	3 µL	28.8 µL

*2 The mixture of ① should be prepared for each experiment and Lysis Solution with RNase Inhibitor and gDNA Remover should not be stored.

② Add 60 µL of Lysis Solution (containing RNase Inhibitor and gDNA Remover) to the microtube in (1)-④.

③ Vortex at room temperature for 10 s and continue to incubate at room temperature for 5 min. The incubation time can be extended up to 10 min.

④ Incubate at 70 °C for 2 min.

⑤ Transfer the microtubes onto ice.

(3) Reverse transcription reaction

① Prepare the reaction solution on ice as follows.

Reagents	40 µL rxn [*4]	Final Conc.
5×RT Master Mix [*1]	8 µL	1×
RT Primer[*2]	X µL	50 nM [*3]
Lysate[*5]	8 µL	-
Nuclease-free Water	24-X-Y µL	-
Total	40 µL	

[*1] If you take the reverse transcription (-) control here, instead of 5×RT Master Mix no-RT Control or instead of 5× RT Master Mix. By taking a control for reverse transcription (-), you can check whether the signal is derived from cDNA or not.
[*2] Not supplied with this kit. Design your own or use TaqMan™ MicroRNA Assays (Thermo Fisher Scientific™).
[*3] If favorable results cannot be obtained at 50 nM, consider using an RT primer concentration of 20 to 100 nM as a guide.
[*4] Scale up as required.
[*5] For RT reactions, it is recommended to add 20% volume of the lysate (8 µL of the lysate for 40 µL) to a 20–40 µL reaction system, but quantification may be improved by adding 15% volume (6 µL of the lysate for 40 µL), depending on the cell type.

② The reaction solution is lightly stirred to make it homogeneous and then incubated at the following temperatures.

16 °C, 30 min.
42 °C, 5 min.] Reverse transcription reaction
95 °C, 5 min. ・・・・ Enzyme inactivation reaction
4 °C, hold

After the completion of the reaction, store at 4 °C or -20 °C. When performing real-time PCR, add directly or diluted to the reaction solution as a template[*1].

[*1] The maximum amount of reverse transcription reaction solution added to the real-time PCR reaction solution should be approximately 10%. Adding a large amount may reduce the reaction efficiency of PCR and may result in inaccurate quantification.

3. When using purified extracellular vesicles

(*SuperPrep*™ miRNAssay™ Cell Lysis & RT Kit for qPCR
(Code No. MIR-201))

(1) Template preparation.

① Add RNase Inhibitor and gDNA Remover to the required volume of Lysis Solution. Refer to the table below and prepare a little extra than the required amount. When using a multi-channel pipette, prepare an extra amount of about 10%.*1

	Mixing ratio
Lysis Solution	58.7
RNase Inhibitor	1
gDNA Remover	0.3

*1 The mixture of ① should be prepared for each experiment and Lysis Solution with RNase Inhibitor and gDNA Remover should not be stored.

② Mix 10 µL of Lysis Solution (containing RNase Inhibitor and gDNA Remover) with 5 µL of purified extracellular vesicle fraction. *2

*2 Mix extracellular vesicle fraction: Lysis Solution = 1:2. If favorable results are not obtained, consider a ratio of 1:1 to 1:10.

③ Pipet or vortex and incubate as is for 5 min at room temperature. Incubation time can be extended up to 10 min.

④ Incubate at 70°C for 2 min.

⑤ Transfer the microtubes onto ice.

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(2) Reverse transcription reaction

① Prepare the reaction solution on ice as follows.

Reagents	20 µL rxn [*4]	Final Conc.
5×RT Master Mix [*1]	4 µL	1×
RT Primer[*2]	X µL	50 nM [*3]
Lysate	4 µL	-
Nuclease-free Water	12-X-Y µL	-
Total	20 µL	

[*1] If you take the reverse transcription (-) control here, instead of 5×RT Master Mix no-RT Control instead of 5× RT Master Mix. By taking a control for reverse transcription (-), you can check whether the signal is derived from cDNA or not.
[*2] Not supplied with this kit. Design your own or use TaqMan™ MicroRNA Assays (Thermo Fisher Scientific™).
[*3] If good results cannot be obtained at 50 nM, consider using an RT primer concentration of 20 nM to 100 nM as a guide.
[*4] Scale up as required.

② The reaction solution is lightly stirred to make it homogeneous and then incubated at the following temperatures.

16 °C, 30 min. ┐
42 °C, 5 min. ┘ Reverse transcription reaction
95 °C, 5 min. ・・・・ Enzyme inactivation reaction
4 °C, hold

After completion of the reaction, store at 4°C or -20°C. When carrying out real-time PCR, add directly or diluted to the reaction solution as a template[*1].

[*1] The maximum amount of reverse transcription reaction solution added to the real-time PCR reaction solution should be approximately 10%. Adding a large amount may reduce the reaction efficiency of PCR and may result in inaccurate quantification.

[5] Real-time PCR

For real-time PCR, please refer to the instruction manual of the reagents and equipment used. Generally, a highly reproducible real-time PCR reaction can be achieved by using cDNA at a volume of 10%. Depending on the real-time PCR reagent used, the optimum template volume may differ; hence, we recommend that you carry out preliminary experiments. Examples using our reagents are shown below.

1. THUNDERBIRD™ Next Probe qPCR Mix (Code No. QPX-101)

For more information, please refer to the THUNDERBIRD™ Next Probe qPCR Mix (Code No. QPX-101) instruction manual.

(1) Preparation of reaction solutions

The following is an example of preparation for 20- and 50-µL reactions using the TaqMan™ Probe. Increase or decrease the volume of reaction solution according to the characteristics of the real-time PCR system used.

Reagents	20 µL rxn	50 µL rxn	Final Conc.
Nuclease-free Water	X µL	X µL	
THUNDERBIRD™ Next Probe qPCR Mix	10 µL	25 µL	1×
Forward Primer	6 pmol	15 pmol	0.3 µM[*1]
Reverse Primer	6 pmol	15 pmol	0.3 µM[*1]
TaqMan™ probe	4 pmol	10 pmol	0.2 µM[*2]
50×ROX Reference dye	0.4 /0.04µL[*3]	1/0.1 µL[*3]	1×/0.1×[*3]
(Uracil-N-Glycosylase)	0.4 unit[*4]	1 unit[*4]	
cDNA template	~2 µL	~5 µL	
Total	20 µL	50 µL	

[*1] If good results are not obtained at 0.3 µM, consider 0.2 to 0.5 µM as a guide.

[*2] If good results are not obtained with 0.2 µM, consider 0.2 to 0.4 µM as a guide.

[*3] Applied Biosystems™ instruments and Agilent Technologies™ instruments use a passive reference to correct for fluorescence intensity and dispensing errors between wells. When reacting on these instruments, ROX Reference dye should be added. The optimal amount to add depends on the instrument. Addition amounts for the main instruments are listed in Table 1. It does not need to be added for instruments that do not perform correction.

[*4] When performing Uracil-N-Glycosylase (UNG) treatment, use heat-labile UNG.
Uracil-DNA Glycosylase (UNG), Heat-labile (Code No. UNG-101), sold separately, can be used.

Table 1: Optimum ROX Reference dye concentrations for main instruments

Real-time cycler	Optimal dye concentration (dilution ratio)
Applied Biosystems™ 7000, 7300, 7700, 7900HT, StepOne™, StepOnePlus™ etc.	1×(50:1)
Applied Biosystems™ 7500, 7500Fast, QuantStudio™, Agilent Technologies™ cyclers (Optional) etc.	0.1×(500:1)
Roche's cyclers, Bio-Rad's cyclers, Qiagen's cyclers, etc.	Not required

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(2) PCR cycling conditions (example)

Step	Temperature	Time	Ramp
(UNG reaction)	(20–25 °C*[1])	(10 min.*[1])	(Maximum)
Pre-denaturation	95 °C	20 s	Maximum
Denaturation	95 °C	5 s	Maximum
Extension	60 °C	30 s	Maximum
(40–45 cycles)*[2]	(Data collection should be set at the extension step)		

*[1] For UNG processing (UNG is not included in this product. Uracil-DNA Glycosylase (UNG), Heat-labile (Code No. UNG-101), sold separately, can be used). Before initial denaturation, set the UNG reaction step. The general temperature conditions and reaction times are shown in the table above, but the preparation should be carried out according to the recommended conditions of the respective companies.

*[2] The number of cycles should be carried out at 40 cycles, increasing to 45 cycles if amplification is insufficient.

2. THUNDERBIRD™ Next SYBR™ qPCR Mix (Code No. QPX-201)

For more information, please refer to the THUNDERBIRD™ Next SYBR™ qPCR Mix (Code No. QPX-201) instruction manual.

(1) Preparation of reaction solutions

The following is an example of preparation for 20 and 50 µL reactions using the TaqMan™ Probe. Increase or decrease the volume of reaction solution according to the characteristics of the real-time PCR system used.

Regents	20 µL rxn	50 µL rxn	Final Conc.
Nuclease-free Water	X µL	X µL	
THUNDERBIRD™ Next SYBR™ qPCR Mix	10 µL	25 µL	1×
Forward Primer	6 pmol	15 pmol	0.3 µM*[1]
Reverse Primer	6 pmol	15 pmol	0.3 µM*[1]
(Uracil-N-Glycosylase)	0.4 unit*[2]	1 unit*[2]	
cDNA template	~2 µL	~5 µL	
Total	20 µL	50 µL	

*[1] If amplification efficiency is insufficient, increasing the primer concentration or reducing the primer concentration if a non-specific reaction occurs may improve reaction results. Please consider a final primer concentration of 0.2 to 0.6 µM as a guide.

*[2] Use heat-sensitive (heat-labile) UNG when performing Uracil-N-Glycosylase treatment. Uracil-DNA Glycosylase (UNG), Heat-labile (Code No. UNG-101), sold separately, can be used.

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(2) PCR cycling conditions (example)

Step	Temperature	Time	Ramp
(UNG reaction)	(20–25℃*[1])	(10 min.*[1])	(Maximum)
Pre-denaturation	95 ℃	30 s	Maximum
Denaturation	95 ℃	5 s	Maximum
Extension	60 ℃	30 s	Maximum
(40～45cycles)*[2]	(Data collection should be set at the extension step)		

*[1] For UNG processing (UNG is not included in this product. Uracil-DNA Glycosylase (UNG), Heat-labile (Code No. UNG-101), sold separately, can be used). Before initial denaturation, set the UNG reaction step. The general temperature conditions and reaction times are shown in the table above, but the preparation should be carried out according to the recommended conditions of the respective companies.

*[2] The number of cycles should be 40, and increased to 45 if amplification is insufficient.

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[6] Application data

1. Principle

This reagent uses RT primers with a looped structure, and when miRNAs are reverse transcribed, the chain length is extended by the addition of an adaptor, enabling detection by real-time PCR (reference 1). The RT primers are miRNA-specific and therefore highly specific compared to other principles (Fig. 1).

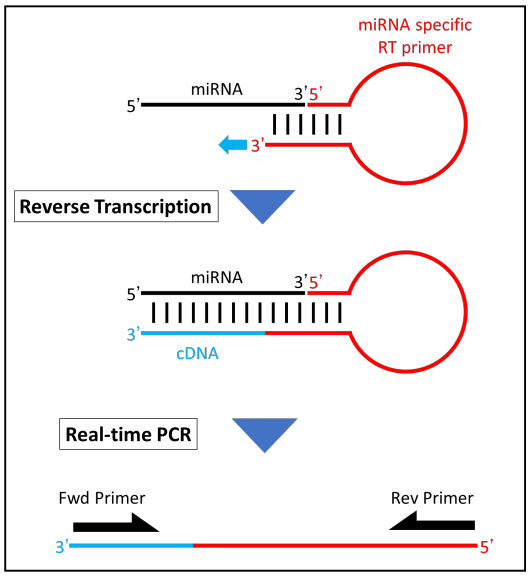

Fig. 1 Detection principle of this reagent

2. High specificity and wide dynamic range

＜Method＞

Using miRNAssay™ qPCR RT Master Mix (Code No. MIR-101), a cDNA synthesis reaction (10 µL rxn) was performed for let-7a, a type of miRNA, in 1 pg to 100 ng of HeLa Total RNA. Real-time PCR analysis was carried out using THUNDERBIRD™ Next SYBR™ qPCR Mix (Code No. QPX-201), our real-time PCR reagent (bring-in volume: 5%). Similarly, a cDNA synthesis reaction was performed for let-7a using the Competitor A Reverse Transcription Reagent and real-time PCR analysis was performed. The primer sequences used in the experiments are shown below (reference 2).

RT Primer
CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGAACTATAC

Real-time PCR, Forward Primer
CCAGCTGGGTGAGGTAGTAGGTTGT

Real-time PCR, Reverse Primer
CTGGTGTCGTGGAGTCGGCAATT

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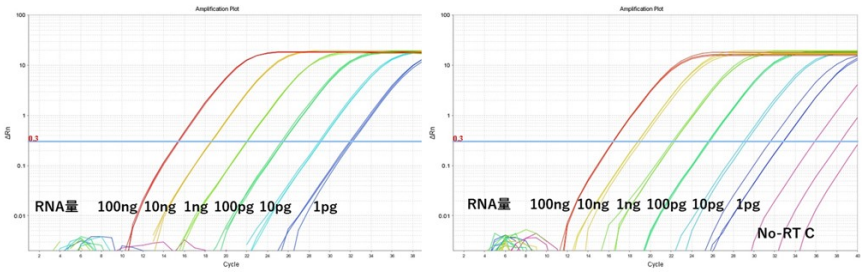
＜Resalt＞

miRNAssayTM qPCR RT Master Mix (Code No. MIR-101)

Competitor A



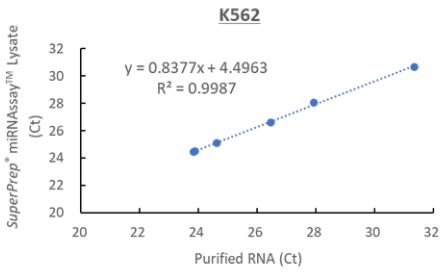
It was found that miRNAssay™ qPCR RT Master Mix (Code No. MIR-101) has higher specificity than other companies' reagents, as no amplification signal was detected in the No-RT Control, which is the RT(-) control. In addition, the amplification signal interval was uniform from 1 pg to 100 ng depending on the amount of template, indicating that reverse transcription is possible in a wide range.

3. Comparison between cultured cells and purified RNA

＜Method＞

Using *SuperPrep*™ miRNAssay™ Cell Lysis & RT Kit for qPCR(Code No. MIR-201), cell lysates were prepared from K562 (human chronic myeloid leukemia cell line), HepG2 (human hepatoma-derived cell line), A431 (human epithelioid cell carcinoma-derived cell line), Jurkat (human Leukemia T cell-derived cell line) and HeLa (human cervical carcinoma-derived cell line). cDNA synthesis reactions were performed after cell lysates were prepared from 5×10^4 cells. Simultaneously, RNA was purified from each cell type and cDNA synthesis reactions were performed targeting six types of miRNAs using miRNAssay™ qPCR RT Master Mix (Code No. MIR-101) from 10 ng of total RNA. Using each cDNA as a template, real-time PCR analysis was performed on the six synthesized cDNAs using THUNDERBIRD™ Next SYBR™ qPCR Mix (Code No. QPX-201) and compared.

＜Resalt＞



Cell Line	Correlation
K562	0.99
HepG2	0.99
A431	0.99
Jurkat	0.97
HeLa	0.99

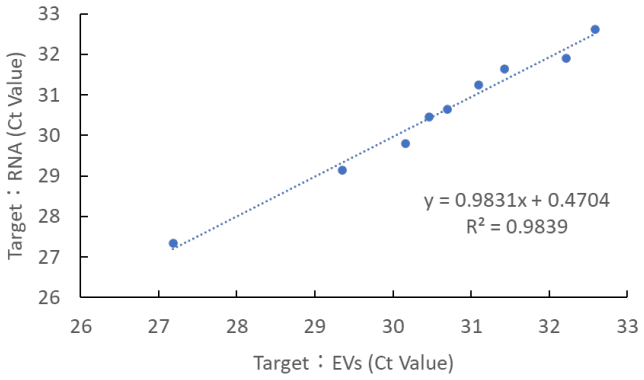
observed between cDNA prepared from lysates and cDNA prepared from purified RNA for six targets and five cell types. This indicates that this kit can be used for miRNA expression analysis by real-time PCR without the need for complicated RNA purification.

4. Comparison between extracellular vesicles and purified RNA

＜Method＞

Twenty milliliters of culture supernatant of HeLa cells cultured for 48 h was concentrated by ultrafiltration to 1 mL using a centrifugal concentrator (Sartorius) and the extracellular vesicle fraction (EVs) was isolated using the MagCapture™ Exosome Isolation Kit PS Ver. 2 (Wako). RNA containing miRNAs was purified from the isolated extracellular vesicle fraction using the microRNA Extractor™ SP Kit (Wako). Using the purified RNA, cDNA synthesis was performed from nine different miRNAs and real-time PCR analysis was performed using the THUNDERBIRD™ Probe qPCR Mix (Code No. QPS-101). Additionally, lysates were prepared from the extracellular vesicle fraction using the *SuperPrep*™ miRNAssay™ Cell Lysis & RT Kit for qPCR (Code No. MIR-201), followed by cDNA synthesis reactions for nine different miRNAs and real-time PCR analysis using THUNDERBIRD™ Probe qPCR Mix (Code No. QPS-101) was performed.

＜Resalt＞



For nine targets, a high correlation was observed between cDNA prepared directly from the extracellular vesicle fraction and cDNA prepared from purified RNA. This indicates that this kit can be used for miRNA expression analysis by real-time PCR without the need for complicated RNA purification.

A high correlation was

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[7] Troubleshooting

miRNAssay™ qPCR RT Master Mix(Code No. MIR-101)

Symptom	Cause	Solution
No signal or delayed detection in real-time PCR	Low RNA purity.	Reverse transcription reaction may be inhibited by impurities left over from RNA preparation. Re-purify the template RNA.
	RNA degraded.	RNA may have been degraded by contamination with RNase; repurify the RNA. If RNA is stored at low concentrations, it is more susceptible to degradation by RNase and adsorption to the reaction vessel may reduce the actual amount of RNA. It is recommended that diluted RNA should not be frozen and reused after use but prepared from a high-concentration storage solution at each use.
	Insufficient or excess RNA.	The product has been confirmed to be capable of performing reverse transcription with stable efficiency when approximately 1 pg to 100 ng of RNA (10 µL reaction) is used, but the amount of RNA that can be reacted may vary depending on the type and quality of RNA. Increase or decrease the amount of template RNA added.
	Target expression too low or too high.	The product only reverse transcribes the target miRNA during the reverse transcription reaction. Therefore, the optimal amount of RNA may vary depending on the expression level of the target. Perform a preliminary study by increasing or decreasing the amount of template RNA.
	Excess reverse transcription reaction solution added.	The product has confirmed that adding up to 10% of the reverse transcription reaction solution to the real-time PCR reaction solution does not cause any problems with linearity, but this value may decrease depending on the nature of the real-time PCR reagent used. Reduce the amount of reverse transcription reaction solution added.
	RT primers are not appropriate	The RT primers may not have been designed properly. It is recommended to refer to references 3, 4 for design or use commercially available TaqMan™ MicroRNA Assays (Thermo Fisher Scientific™).
Amplification is seen in the reaction solution with no-RT Control in real-time PCR.	Occurrence of primer dimers.	If the no-template control peak is on the cooler side of the melting curve analysis than the target sequence, the occurrence of primer dimers is suspected. Primer dimers may be caused by poor primer quality as well as primer sequence. First, review the PCR reaction conditions, and if no improvement is observed, consider redesigning or re-synthesizing the primers. When re-synthesizing, the purification grade should be HPLC or higher.

Symptom	Cause	Solution
No signal or delayed detection in real-time PCR	Too many cells.	Excess cell-derived components may inhibit RT and qPCR reactions. Reduce the number of cells to be lysed.
	RNA degraded.	-Some cell types have strong RNase activity and the RNase in the lysate may not be completely inactivated. For such cells, it is recommended to add Lysis Solution, incubate at room temperature/70°C, transfer the lysate to ice and immediately perform RT reaction to convert to cDNA. -The lysate should be frozen at -80°C and thawed as little as possible. -Cells should be prepared for use. When storing cells for assays, remove PBS(-) from cells washed with PBS(-) after incubation and freeze at -80°C.
	Too much reverse transcription reaction solution added.	-We have confirmed that adding up to 10% of the product's RT Reaction Solution to the qPCR Reaction Solution does not cause any problems with linearity; However, depending on the nature of the qPCR reagent used, this tolerance may be reduced. Reduce the amount of reverse transcription reaction solution added. -When using RT reagents other than this product, the allowable amount carried into the real-time PCR reagent may change. Perform preliminary experiments before use.
	RT primers are not appropriate	The RT primer design may not be appropriate. It is recommended to refer to references 3, 4 for design or use commercially available TaqMan™ MicroRNA Assays (Thermo Fisher Scientific™).
Low quantitatively.	Insufficient cell lysis	After adding Lysis Solution, mix thoroughly to avoid uneven mixing.

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[8] Related products

Product name	Package	Code No.
Highly efficient real-time PCR master mix **THUNDERBIRD™ Next SYBR™ qPCR Mix**	1 mL × 1 (100 rxns) 1.67 mL × 3 (500 rxns)	QPX-201T QPX-201
Highly efficient real-time PCR master mix **THUNDERBIRD™ Next Probe qPCR Mix**	1 mL × 1 (100 rxns) 1.67 mL × 3 (500 rxns)	QPX-101T QPX-101
Heat-labile Uracil-DNA Glycosylase **Uracil-DNA Glycosylase (UNG), Heat-labile**	200 U	UNG-101
Cell lysis & cDNA synthesis kit for real-time PCR (for cultured cells) ***SuperPrep*™ Cell Lysis & RT Kit for qPCR**	100 rxns	SCQ-101
Cell lysate for real-time PCR (for cultured cells) ***SuperPrep*™ Cell Lysis Kit for qPCR**	100 rxns	SCQ-201
Cell lysis & cDNA synthesis kit for real-time PCR (for cultured cells) ***SuperPrep*™ II Cell Lysis & RT Kit for qPCR**	100 rxns	SCQ-401
Cell lysate for real-time PCR (for cultured cells) ***SuperPrep*™ II Cell Lysis Kit for qPCR**	100 rxns	SCQ--501

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「Real-time quantification of microRNAs by stem-loop RT-PCR」

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Reference 2

Yilin Wang, Jianwen Zhou, Yanlian Chen, Chunhua Wang, Enyin Wu, Liang Fu and Chen Xie

「Quantification of distinct let-7 microRNA family members by a modified stem-loop RT-qPCR」

Molecular Medicine Reports 17: 3690-3696, 2018

Reference 3

Martha F. Kramer

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Curr Protoc Mol Biol. 2011 July ; CHAPTER: Unit15.10.

Reference 4

Zsolt Czimmerer, Julianna Hulvely, Zoltan Simandi, Eva Varallyay, Zoltan Havelda, Erzsebet Szabo, Attila Varga, Balazs Dezso, Maria Balogh, Attila Horvath, Balint Domokos, Zsolt Torok, Laszlo Nagy, Balint L. Balint

「A Versatile Method to Design Stem-Loop Primer-Based Quantitative PCR Assays for Detecting Small Regulatory RNA Molecules」

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