NIPPON Genetics EUROPE

Application

Evaluation of the FastGene® miRNA Enhancer kit with the FastGene® RNA Premium kit or other companie's RNA extraction kits

Product

FastGene® miRNA Enhancer kit（Cat.No. FG-RNAE-S, FG-RNAE-25）

FastGene® RNA Premium kit（Cat.No. FG-81006, FG-81050, FG-81250）

Manufacturer

FastGene®

The following data is kindly provided by Mr. Arizumi Kikuchi. Institute of Medical Science, General Hospital Daiyukai, Japan

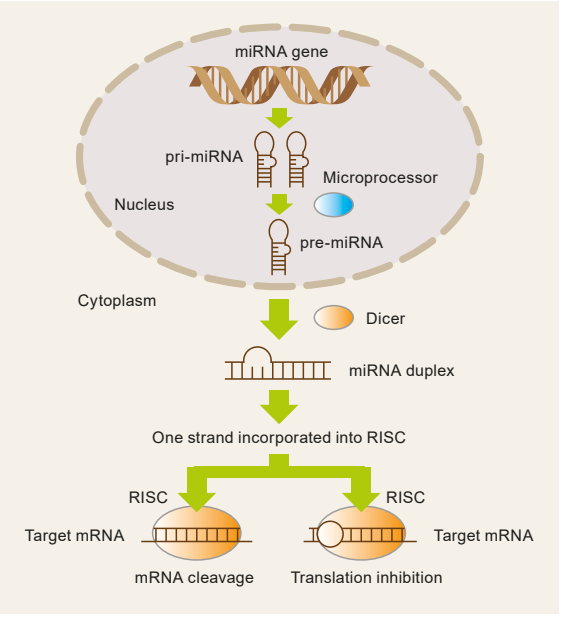
Summary

Analyzed samples are from clinical specimens such as tissue, blood, urine and cerebrospinal fluid. It is necessary to remove PCR reaction inhibitors to ensure a sufficient amount of highly purified RNA.
Extraction of nucleic acids from a biological sample in a genetic test is extremely important for maintaining the accuracy of the test. In particular, it is essential to construct an extraction method that enables stable sample preparation for clinical samples that are expected to have various conditions. In this application note, we combined the FastGene® miRNA Enhancer kit with the FastGene® RNA Premium kit or other supplier RNA extraction kit to extract miRNAs from various clinical samples. We evaluated the Cp value by real-time PCR after cDNA synthesis.

● What are miRNAs?

• Approximately 30% of human genes are expected to be regulated by miRNAs.
• After transcription from the genome, the precursor miRNA becomes a mature miRNA after sevaral enzymatic interactions
• mature miRNAs contain 21-23 nt single-stranded RNAs that interact with mRNAs.
• According to miRBase Release22 (2018), 38,589 are registered.

Protein translated by mRNA expression
↓
Suppresion of protein translation from mRNA due to increased expression of miRNA
↓
Decreased protein expression levels
↓
Involvement has been suggested for cancer, metabolic diseases, neurological and infectious diseases



Meltzer, P.S. Nature 435, 745–746（2005）.

Experimental conditions

Target sample and pretreatment method

• Cell line (K562)
→ Use pellets of 10^5 cells (n=3)

• human white blood cell (clinical specimen)
→ Blood was collected from 3 healthy subjects using a blood collection tube with EDTA・2Na anticoagulate.
Use white blood cell pellets after treatment with 500 µL of whole blood (n=3)

• Formalin-fixed paraffin-embedded (FFPE) tissue sample (clinical sample)
→ FFPE sample of colorectal tissue collected from 5 patients with colorectal cancer (＊for details)

• Bovine muscle
→ 10 µL of homogenized solution using the same amount of PBS (-) as the tissue (n=3)

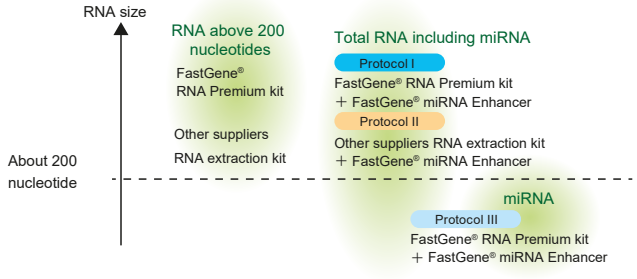
＊Details of FFPE pretreatment method

1） Take a sample from a 5 µm thick FFPE specimen
2） Add 800 µL xylene
3） Incubate for 5 min at room temperature
4） Add 400 µL absolute ethanol
5） Centrifuge for 2 min and discard the supernatant
6） Add 1000 µL absolute ethanol
7） Centrifuge for 2 min and discard the supernatant
8） Incubate at 56 ℃ for 20 min
9） Add 100 µL dissolution buffer (10 nmol/L Tris-HCl, 0.1 mmol/L EDTA, 5 g/L SDS)
10） Add 40 µL of proteinase K
11） Incubate at 56 ℃ for 30 min
12） Incubate at 85 ℃ for 30 min
13） Add lysis buffer to make the total volume of 150 µL

● Kit and Protocol selection guide

FastGene® miRNA Enhancer can be used in combination with FastGene® RNA Basic kit, FastGene® RNA Premium kit or RNA extraction kits from other suppliers to recover small RNAs (miRNA) that are difficult to extract.
In this Application note, we used the protocol shown on the right that combines the FastGene® miRNA Enhancer kit with the FastGene® RNA Premium kit or RNA extraction kit from another supplier.
Furthermore, the Cq value was evaluated by performing real-time PCR after synthesizing miRNA with cDNA.

How to select the kit used in this app note.



When you want to collect total RNA with miRNA (in one tube)

Protocol I

FastGene® RNA Premium kit
＋ FastGene® miRNA Enhancer

< 1×10^5 cultured cells

350 µL buffer RL ※1
Sufficiently homogenized after addition

FastGene™ RNA filter column

≥ 10,000 x g (RT: 20 ～ 25°C) 1 min
Transfer the supernatant to a new collection tube

400 µL FastGene® miRNA enhancer solution
Mix by pipetting

FastGene™ RNA binding column

FastGene® RNA binding column
Add up to 700 µL of sample solution
≥ 10,000 x g (RT: 20 ～ 25°C) 1 min
Discard the flow through and place the column back in the tube (2.0 mL)

400 µL buffer RW2 ※1
≥ 10,000 x g (RT: 20 ～ 25°C) 30 s
Transfer column to a new collection tube (2.0 mL)

400 µL buffer RW2 ※1
≥ 10,000 x g (RT: 20 ～ 25°C) 30 s
Transfer column to a new collection tube (2.0 mL)

Centrifuge at full speed (RT: 20 ～ 25°C) 1 min
Transfer column to a new collection tube (1.5 mL)

50 µL buffer RE
(Added to the center of the membrane)
≥ 10,000 x g (RT: 20 ～ 25°C) 1 min
Collect the eluate after disposal of the FastGene™ RNA binding column

Protocol II

Other suppliers RNA kit
＋ FastGene® miRNA Enhancer

< 1×10^5 cultured cells

350 µL Buffer Lysis Buffer
Sufficiently homogenized after addition

14,000 rpm (RT: 20 ～ 25°C) 2 min
Transfer the supernatant to a new collection tube

350 µL FastGene® miRNA enhancer solution
Mix by pipetting

Add up to 700 µL of sample solution on the column
≥ 10,000 x g (RT: 20 ～ 25°C)1 min
Discard the flow through and place the column back in the tube (2.0 mL)

500 µL Wash Buffer
14,000 rpm (RT: 20 ～ 25°C) 1 min
Place column back to the collection tube (2.0 mL)

500 µL buffer RPE
14,000 rpm (RT: 20 ～ 25°C) 2 min
Transfer column to a new collection tube (2.0 mL)

Centrifuge at full speed (RT: 20 ～ 25°C) 1 min
Transfer column to a new collection tube (1.5 mL)

RNase free water 30~50 µL)
(Added to the center of the membrane)
14,000 rpm (RT: 20 ～ 25°C) 1 min
Collect eluate after disposal of the column

If you only want to collect miRNA separately

Protocol III

FastGene® RNA Premium kit
＋ FastGene® miRNA Enhancer

< 1×10^5 cultured cells

350 µL buffer RL※1
Sufficiently homogenized after addition

FastGene™ RNA filter column

Add Lysate to the
FastGene® RNA filter column
≥ 10,000 x g（RT: 20 ～ 25℃）1 min
Collect the filtrate and dispose the FastGene® RNA filter column

350 µL 70% ethanol
Mix by pipetting

FastGene™ RNA binding column

Add up to 700 µL of sample solution to the FastGene® RNA binding column
≥ 10,000 x g（RT: 20 ～ 25℃）1 min
Collect the filtrate

400 µL FastGene® miRNA enhancer solution tot the filtrate
Mix by pipetting

FastGene™ RNA mini-elute column

Add sample solution to the FastGene® RNA mini-elute column
≥ 10,000 x g（RT: 20 ～ 25℃）1 min
Transfer column to a new collection tube (2.0 mL)

400 µL buffer RW2 ※1
≥ 10,000 x g（RT: 20 ～ 25℃）30 s
Transfer column to a new collection tube (2.0 mL)

400 µL buffer RW2※1
≥ 10,000 x g（RT: 20 ～ 25℃）30 s
Transfer column to a new collection tube (2.0 mL)

Centrifuge at full speed（RT: 20 ～ 25℃）1 min
Transfer column to a new collection tube (1.5 mL)

50 µL buffer RE
（Added to the center of the membrane）
≥ 10,000 x g（RT: 20 ～ 25℃）1 min
Collect the eluate after disposal of the FastGene® RNA binding column

Real-time PCR conditions

Stem-loop RT primer and cDNA synthesis used for miR-21 analysis（used in Cell line・human white blood cell・FFPE sample）

- Stem- Loop RT-primer sequences 5'→3'

  miR-21

  GTCAGAGGAGGTGCAGGGTCCGAGGTATTCGCACCTCCTCTGACTCAACA

- Reverse Transcription → cDNA

Transcriptor RT Reaction Buffer（5×）（Roche）	2	μL
Stem-Loop RT-primer（10 μM）	0.2	μL
Transcriptor Reverse Transcriptase（20 U/μL）（Roche）	0.25	μL
Protector Rnase Inhibitor（40 U/μL）（Roche）	0.25	μL
Deoxynucleotide Mix（10 mM）（Roche）	1	μL
RNA solution	2.5	μL
Water	3.8	μL

- Reaction condition

16 ℃	30 min	
30 ℃	30 s	60 cycles
42 ℃	30 s	
50 ℃	1 s	
85 ℃	5 min	

Dilute the reaction product 5-fold with TE buffer, and used for subsequent reactions

Primer sequence, probe, reaction conditions and reaction solution during real-time PCR used for miR-21 analysis

- Primer sequences（5'→3'）and Probe

  miR-21

  Forward primer GATCGGTAGCTTATCAGACTGATG

  Reverse primer GTGCAGGGTCCGGGTAAT

  Universal ProbeLibrary Probe（Roche） #82

- Reaction conditions

95 ℃	10 min	
95 ℃	10 sec	40 cycles
60 ℃	30 sec	

- Reaction mixtures

  2.5 μL of cDNA solution

  5 μL of Essential Probe Master（Roche）

  0.4 μM of each primer

  0.4 μM of UPL probe（Roche）

  （in a final volume of 10 μL）

The reaction was performed with LightCycler 96（Roche). The average value of the double meassurement was used as the measured value.

Stem-loop RT primer and cDNA synthesis used for bta-miR-23a analysis (used in Bovine muscle)

- Stem- Loop RT-primer sequences 5'→3'

  bta-miR-23a

  CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGTGGAAATC *

  *Guan L et. al.：Sci Rep, 7: 43716, 2017

- Reverse Transcript → cDNA

Transcriptor RT Reaction Buffer（5×）（Roche）	2	μL
Stem-Loop RT-primer（10μM）	0.2	μL
Transcriptor Reverse Transcriptase（20 U/μL）（Roche）	0.25	μL
Protector Rnase Inhibitor（40 U/μL）（Roche）	0.25	μL
Deoxynucleotide Mix（10 mM）（Roche）	1	μL
RNA solution	2.5	μL
Water	3.8	μL

- Reaction condition

16 ℃	30 min	
30 ℃	30 s	60 cycles
42 ℃	30 s	
50 ℃	1 s	
85 ℃	5 min	

Dilute the reaction product 5-fold with TE buffer, and used for subsequent reactions

Primer sequence, reaction conditions and reaction solution used for bta-miR-23a analysis

- 1 Primer sequences（5'→3'）*

  bta-miR-23a

  Forward primer CCGAGTCAGATCACATTGCCAGG

  Reverse primer CTCAACTGGTGTCGTGGAGTCG

  *Guan L et. al.：Sci Rep, 7: 43716, 2017

- Reaction conditions

95 ℃	10 min	
95 ℃	10 sec	40 cycles
60 ℃	30 sec	

- Reaction mixtures

  2.5 μL of cDNA solution

  5 μL of KAPA SYBR Fast qPCR（KAPA）

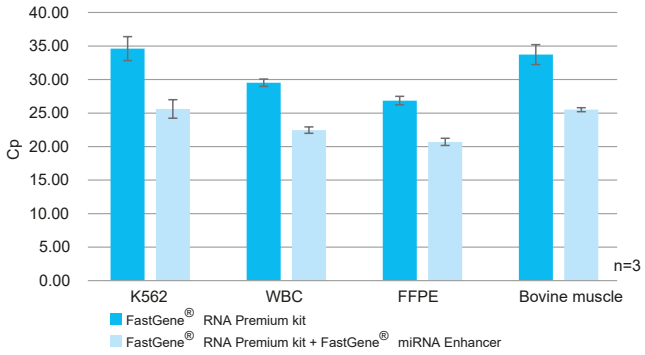
  0.4 μM of each primer

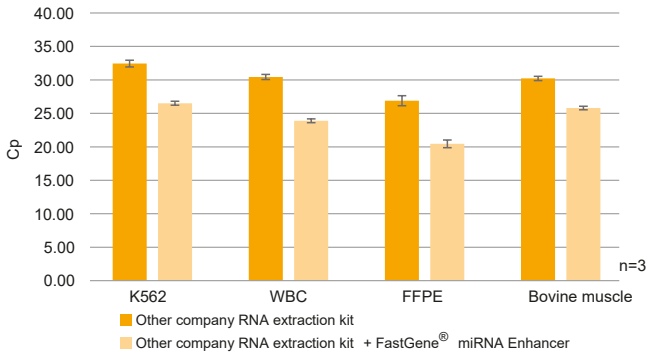
  （in a final volume of 10 μL）

The reaction was performed with LightCycler 96（Roche). The average value of the double meassurement was used as the measured value.

Result

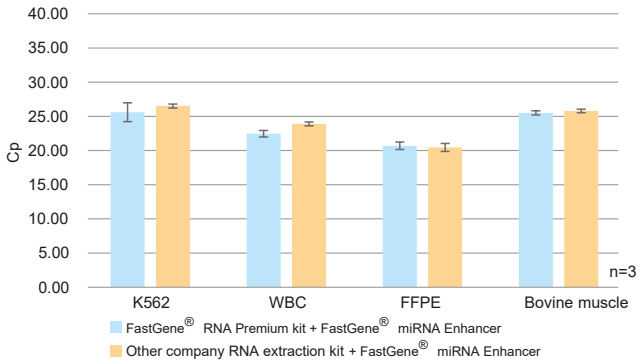
● Using Protocol Ⅰ or Ⅱ：Evaluation of Cp value by real-time PCR while using FastGene® miRNA Enhancer or not





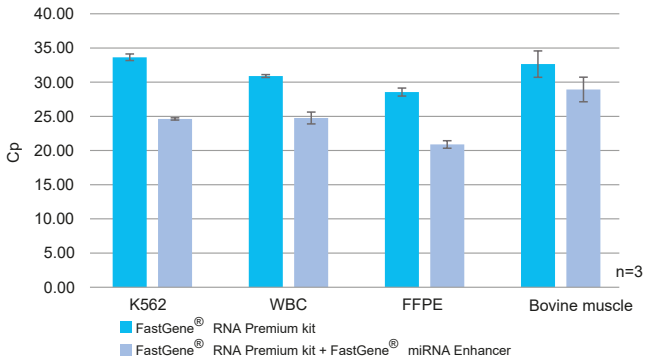
Conclusion: The addition of FastGene® miRNA Enhancer improved miRNA yields in all kits

● Usage of Protocol Ⅰ and Ⅱ:
Real-time PCR when using FastGene® miRNA Enhancer by Evaluation of Cp value



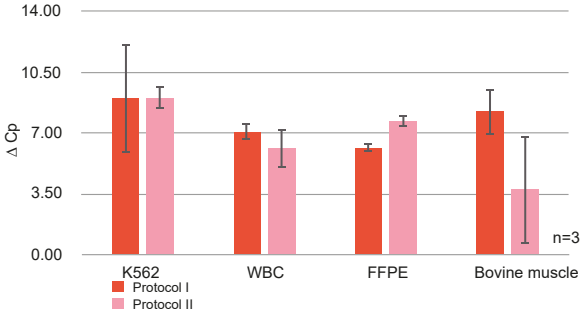
Conclusion: The Cp values are the same when using protocol Ⅰ and Ⅱ

● Usage of protocol Ⅲ:
Evaluation of Cp value by real-time PCR when using various samples



Conclusion: The yield of miRNA was improved by using protocol Ⅲ

● Using protocol Ⅰ and Ⅲ: Evaluation of ΔCp



The amount of extracted miRNA was similar, even if you use protocol Ⅰ or Ⅲ

Calculation method of ΔCp:
ΔCp＝(Cp value when FastGene® miRNA Enhancer is not used) - (Cp value when FastGene® miRNA Enhancer is used)

●Summary The addition of FastGene® miRNA Enhancer improved miRNA yields with either kit.
It was also found that the amount of extracted miRNA was about the same even if the number of times the column was passed was different.

Customer's comment

In our laboratory, we have experience in extracting miRNA using FFPE and peritoneal dialysate drainage as samples, but there were still cases where it was difficult to extract a sufficient amount for analysis. In such a case, I thought that the use of this reagent would be effective.
Since the FastGene® series RNA extraction reagents have excellent purity and yield, we believe that combining them with these reagents can be expected to be as effective as or better than the miRNA extraction reagents of other companies.

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