Cat. # 6167

For Research Use

TaKaRa

Provirus Copy Number Detection Primer Set, Human (for Real Time PCR)

Product Manual

v201808Da

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I. Description

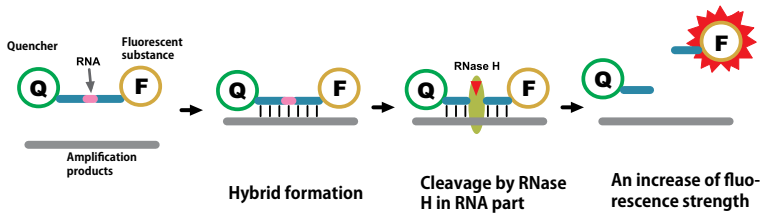
The Provirus Copy Number Detection Primer Set, Human (for Real Time PCR) is a kit for measuring provirus copy numbers of normal human cells transduced with a retroviral vector derived from MLV (murine leukemia virus)*1 using real-time PCR with cycling probe detection. A provirus is a retroviral genome that has been incorporated into the genome of a host cell.

This product is used in combination with the CycleavePCR™ Core Kit (Cat. #CY501) to measure the copy number of proviruses integrated into human genomic DNA. In addition, since the PCR amplification region in this kit is in the packaging signal of MLV, the provirus copy number can be measured in normal human cells transduced with MLV-based retroviral vectors.
Provirus copy numbers can be measured conventionally by performing Southern blotting using the cloned cells after transduction, and calculating the average provirus copy number. This product can used to easily obtain the average provirus copy number without cloning MLV-transduced cells.

The CycleavePCR Core Kit is used together with this kit and a real-time PCR kit that uses cycling probe technology*2, which excels in providing rapid and quantitative results and very high specificity.

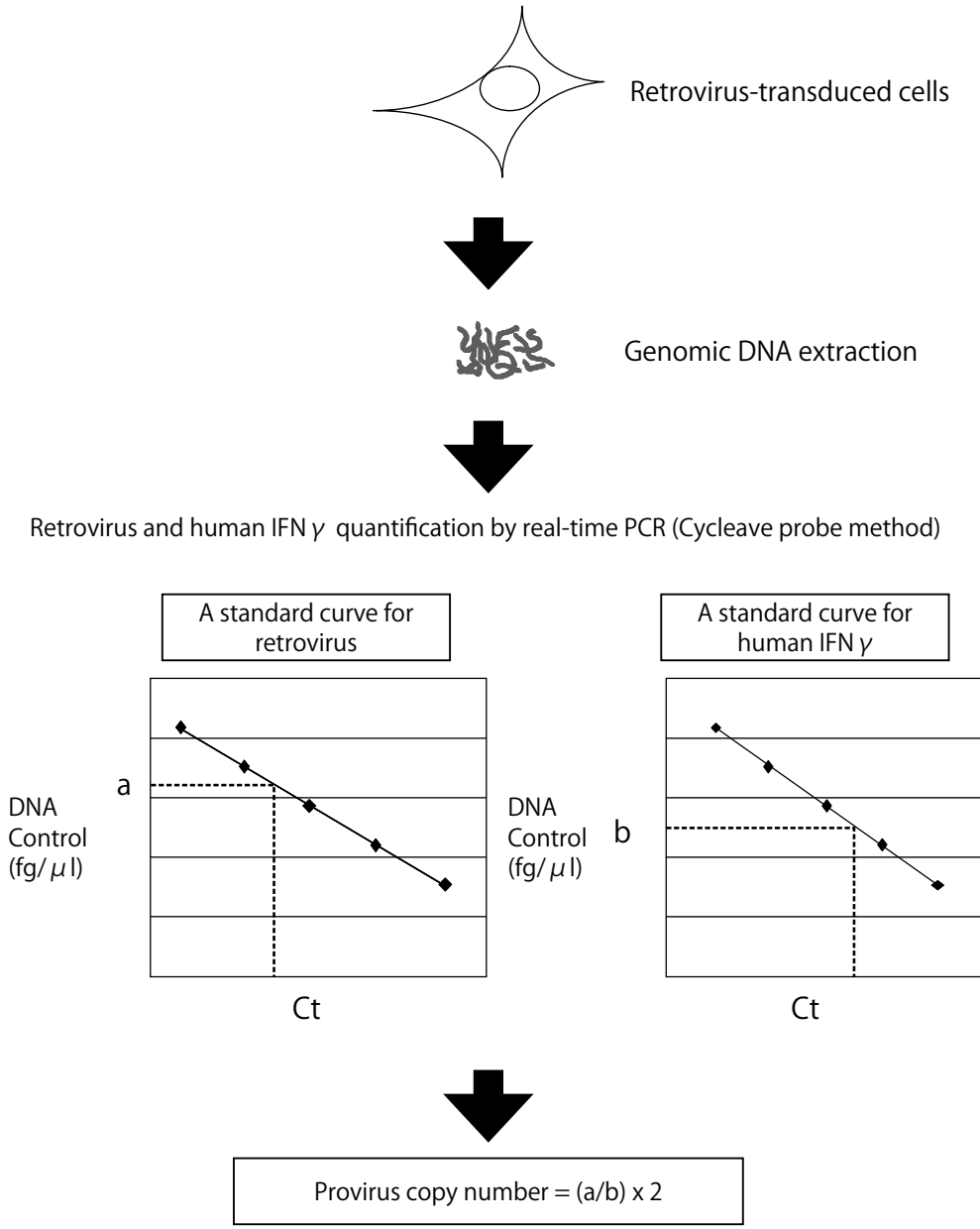
* 1 This product should only be used with human cells transduced with MLV retrovirus. In addition, its use is limited to cells whose autosomal chromosome is diploid, such as normal cells. For analysis of cancer cells and cultured cells whose autosomal chromosome is not diploid, refer to Section VI-5.
When transduced cells are assayed immediately after infection with a viral vector, this product will detect not only provirus but also double-stranded DNA and circular DNA (2LTR and 1LTR) that was not integrated into genomic DNA. Since this error diminishes over time after infection, we recommend measuring the provirus copy number several days after viral infection. A DNA extraction kit designed for isolating genomic DNA, such as NucleoSpin Tissue, is recommended for preparing genomic DNA from virus-transduced cells.

* 2 Cycling Probe Technology is a highly sensitive detection method utilizing a combination of a chimeric probe composed of RNA and DNA, and RNase H. The specific sequence of the target gene to be amplified can be detected efficiently during or after amplification by this method.



Principle of cycling probe technology

As long as this probe remains intact, no strong fluorescence can be emitted because of the quenching function. When the probe forms a hybrid with the complementary sequence of amplified product, RNase H specifically cuts the RNA region of this probe, resulting in emission of strong fluorescence. By measuring the intensity of emitted fluorescence, the amount of amplified product can be monitored. A probe including a mismatch in its probe region is not cut by RNase H, therefore Cycling Probe Technology allows highly specific detection and can recognize even an SNP.



Two standard curves (retrovirus and human IFN γ) are prepared using DNA Control Template for Provirus, Human into which is cloned a copy of a corresponding region of retrovirus and of human IFN γ (interferon γ). These curves are used to quantify retrovirus and human IFN copies in the genomic DNA of a target sample.
As the human IFN γ is present in one copy/genomic chromosome, the copy ratio of retrovirus to human IFN γ is calculated by a/b. Since autosomal chromosomes are diploid in normal cells, the provirus copy number is calculated by (a/b) x 2.

Figure 1. Principle of provirus copy number measurement using this kit.

II. Components (100 reactions for 25 μl reaction system)

1.	Retrovirus Primer Mix for Provirus (each 10 pmol/μl)	50 μl
2.	Retrovirus Probe for Provirus (10 pmol/μl)*	50 μl
3.	hIFNg Primer Mix for Provirus (each 10 pmol/μl)	50 μl
4.	hIFNg Probe for Provirus (10 pmol/μl)*	50 μl
5.	DNA Control Template for Provirus, Human (200 pg/μl)	15 μl
6.	EASY Dilution (for Real Time PCR)	1 ml x 4

* Store these components protected from light, since they contain a fluorescently labeled probe (FAM).

III. Storage -20℃

IV. Materials Required but not Provided

CycleavePCR Core Kit (Cat. #CY501)

[Materials]
- 1,000 μl, 200 μl, 20 μl, and 10 μl micropipettes
- Micropipette tips (with hydrophobic filter)
- Microtubes
- Refrigerated microcentrifuge (with 4℃ setting)

[Instruments]
- Heat block (capable of temperature settings up to 95℃) or thermal cycler

V. Precautions for Use

Make sure to read the following guidelines before using this product.

1. It is useful to prepare a Master Mix (a mixture of dH_2O, buffer, and enzyme etc.) to reduce the loss of reaction mixture through pipetting and the number of steps needed to mix and dispense reagent. Using a Master Mix makes it possible to dispense reagents accurately, providing consistent results between experiments.
2. Briefly centrifuge the *TaKaRa Ex Taq*® HS and Tli RNase H II included in the CycleavePCR Core Kit (Cat. #CY501) before use to collect the liquid at the bottom of the tube. Pipet these enzymes slowly and carefully, since they are in a highly viscous 50% glycerol solution. Store them at -20℃ until just before use and immediately after use.
3. The 10X CycleavePCR Buffer included in the CycleavePCR Core Kit (Cat. #CY501) may contain some insoluble material after thawing. Vortex the tube to dissolve this material completely before use.
4. When you dispense reagents, always use new disposable tips and make certain to avoid contamination between samples.

VI. Protocol

1. Genomic DNA preparation
   1) Purify genomic DNA from normal human cells transduced by an MLV-based retroviral vector.*
   2) Prepare 20–100 ng/μl purified genomic DNA with sterilized water and transfer the required amount of the DNA solution into microtubes. Then perform heat denaturation at 95℃ for 5 minutes and immediately cool the tubes on ice.
      * Using a NucleoSpin Tissue (Cat. #740952.10/.50/.250) makes it possible to quickly and easily extract high-purity genomic DNA.

2. Sample preparation for standard curves

   Perform real-time PCR using the "DNA Control Template for Provirus, Human" in this product as a template, then generate standard curves for retrovirus and human IFN γ . Calculate the provirus copy number based on both standard curves.

   Prepare stepwise dilutions of DNA Control Template for Provirus, Human as follows:

   1) Dilute the Control Template 100-fold by adding 99 μl of EASY Dilution (for Real Time PCR) to 1 μl of DNA Control Template for Provirus, Human (200 pg/μl) in order to prepare a 2 pg/μl DNA Control Template solution (about 5.7 x 10^5 copies/μl) (Tube # 1).
   2) Add 45 μl of EASY Dilution (for Real Time PCR) to each of four new tubes (Tube # 2–5).
   3) After mixing Tube # 1 well, transfer 5 μl of 2 pg/μl DNA Control Template from Tube # 1 to Tube # 2, and mix.
   4) Transfer 5 μl from Tube # 2 to Tube # 3, and mix.
   5) Repeat Step 4 to obtain the desired dilutions in Tubes # 4 and # 5.

Tube #	Amount of DNA (μl)	EASY Dilution (μl)	DNA Concentration
1	1	99	2 pg/μl
2	5	45	200 fg/μl
3	5	45	20 fg/μl
4	5	45	2 fg/μl
5	5	45	0.2 fg/μl

   6) Perform heat denaturation on all tubes at 95℃ for 5 minutes and cool immediately on ice.

3. Reaction mixture preparation for real-time PCR

   (Follow the instructions in the CycleavePCR Core Kit.)
   Prepare the following reaction mixtures on ice for each retrovirus and human IFN γ *1

<For retrovirus: for 1 reaction>

Reagents	Amount	Final conc.
10X CycleavePCR Buffer*2	2.5 μl	1X
dNTP Mixture (2.5 mM each)*2	3 μl	0.3 mM
Mg^{2+} solution (25 mM)*2	5 μl	5 mM
TaKaRa Ex Taq HS (5 U/μl)*2	0.25 μl	
Tli RNase H II (200 U/μl)*2	0.5 μl	
Retrovirus Primer Mix for Provirus (10 pmol/μl each)	0.5 μl	0.2 μM each
Retrovirus Probe for Provirus (10 pmol/μl)	0.5 μl	0.2 μM
Genomic DNA or standard curve sample	5 μl	
dH_2O*2	7.75 μl	
Total	25 μl	

<For human IFN γ : for 1 reaction>

Reagents	Amount	Final conc.
10X CycleavePCR Buffer*2	2.5 μl	1X
dNTP Mixture (2.5 mM each)*2	3 μl	0.3 mM
Mg^{2+} solution (25 mM)*2	5 μl	5 mM
TaKaRa Ex Taq HS (5 U/μl)*2	0.25 μl	
Tli RNase H II (200 U/μl)*2	0.5 μl	
hIFN γ Primer Mix for Provirus (10 pmol/μl each)	0.5 μl	0.2 μM each
hIFN γ Probe for Provirus (10 pmol/μl)	0.5 μl	0.2 μM
Genomic DNA or standard curve sample	5 μl	
dH_2O*2	7.75 μl	
Total	25 μl	

* 1 Use these reaction mixtures with the Thermal Cycler Dice™ Real Time System *II* (Cat. #TP900)*3. If you are using another instrument, follow the instruction manual for that model.

* 2 Components in CycleavePCR Core Kit (Cat. #CY501).

* 3 Not available in all geographic locations. Check for availability in your region.

4. Start reaction. (Protocol for Thermal Cycler Dice Real Time System *II*)*

   After briefly centrifuging the reaction tubes or plate, insert them into the instrument and start the reaction.
   On the Thermal Profile Setup screen, check FAM in Collect Data.

Step		Temp	Time	Detection
Initial denaturation		95℃	30 sec	OFF
PCR reaction	Denaturation	95℃	5 sec	OFF
	Annealing	55℃	15 sec	OFF
	Extension	72℃	15 sec	ON
Number of cycles		40 cycles		

   * The cycling conditions are for the Thermal Cycler Dice Real Time System *II* (Cat. #TP900). For another instrument, follow the instruction manual for that model.

5. After the reaction is complete, perform an analysis.

   After the end of the reaction, view the amplification curve for the FAM filter, prepare a standard curve, and calculate the provirus copy number of the sample being tested according to the following calculation method.

   <Provirus copy number calculation method>
   Based on the standard curves for retrovirus and for human IFN γ , calculate the concentration of retrovirus and human IFN γ in a sample.

   Provirus copy number =
   (Retrovirus concentration in a sample/ Human IFN γ concentration in a sample) x 2*

   * Since autosomal chromosomes are diploid in normal cells, multiply by 2

   Note: Provirus copy number calculation of transduced cells whose autosomal chromosome is not diploid:

   Provirus copy number is calculated based on the human IFN γ copy number, which is present at one copy/genome. In normal cells with an autosomal chromosome that is diploid, use the equation in Step 5 to multiply by 2. In non-diploid cells such as cancer cells, the approximate provirus copy number can be calculated by determining the number of chromosomes beforehand and multiplying by the ploidy of the cells instead of 2 in the equation in Step 5.

VII. Experimental Example

1. Method
   Normal human cells (autosomal diploid) were transduced with an MLV-based retroviral vector, then cloned. At first, the provirus copy number was measured by Southern blotting analysis using the genomic DNA purified from each clone. By using 100 ng of genomic DNA with a provirus copy number between 1 and 6 copies/cell determined using Southern blotting, the provirus copy number was also measured with this product. These results were compared to the results of Southern blotting.

2. Results
   Standard curves for retrovirus and human IFN γ are shown in Figures 2A and B, respectively. A comparison of the provirus copy numbers obtained using this product and Southern blotting are shown in Table 1.

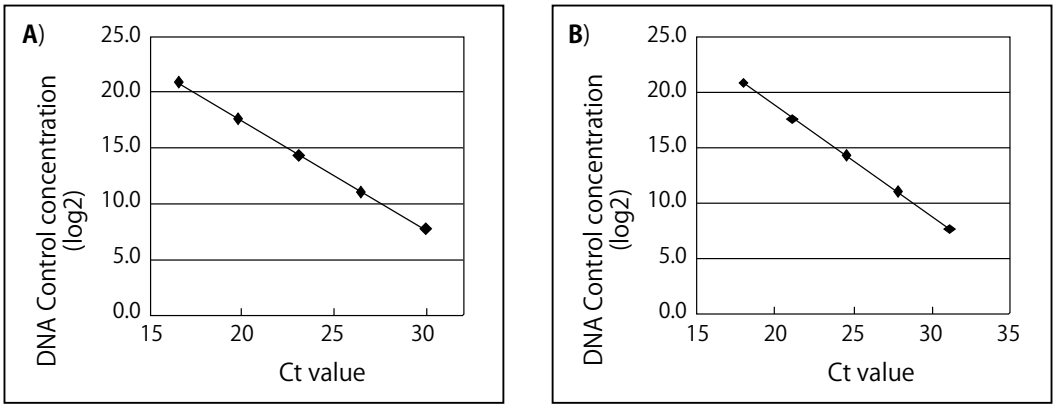


Figure 2. Standard curves of this product
A: For retrovirus, B: For human IFN γ

Table 1. Comparison of provirus copy numbers obtained using this product and Southern blotting

Provirus copy number from this product	Provirus copy number from Southern blotting
0	0
0.93	1
2.01	2
2.88	3
3.01	3
6.25	6

VIII. Related Products

Use this product together with the CycleavePCR™ Core Kit (Cat. #CY501).

[Real Time PCR Machine]
Thermal Cycler Dice™ Real Time System // (Cat. #TP900)*

[8-tube strip and cap]
0.2 ml Hi-8-Tube (Cat. #NJ300)
0.2 ml Hi-8-Flat Cap (Cat. #NJ302)

[High efficiency gene transfection reagent for retroviral vector]
RetroNectin® (Recombinant Human Fibronectin Fragment) (Cat. #T100A/B)

[Cells for retrovirus production]
Cells for retrovirus production, G3T-hi cells (Cat. #6163)*

[Transient retroviral vector production kit]
Retrovirus Constructive System Eco (Cat. #6164)*
Retrovirus Constructive System Ampho (Cat. #6165)*

[Retroviral vector plasmid]
pDON-AI-2 Neo DNA (Cat. #3653)
pDON-AI-2 DNA (Cat. #3654)
pMEI-5 Neo DNA (Cat. #3655)
pMEI-5 DNA (Cat. #3656)
pDON-5 Neo DNA (Cat. #3657)
pDON-5 DNA (Cat. #3658)

[Retroviral vector for siRNA expression]
pSINsi-hH1 DNA (Cat. #3660)*
pSINsi-hU6 DNA (Cat. #3661)*
pSINsi-mU6 DNA (Cat. #3662)*

[For measuring retrovirus titer]
Retrovirus Titer Set (for Real Time PCR) (Cat. #6166)

* Not available in all geographic locations. Check for availability in your region.