Cat. # RR290

For Research Use

TaKaRa

Mouse Feeder Cell Quantification Kit

Product Manual

v202203Da

Table of Contents

I. Description

Mouse feeder cells are used to support the growth and cultivation of stem cells including hESC (human embryonic stem cells) and hiPSC (human induced pluripotent stem cells). For certain stem cell applications such as regenerative medicine, removal of heterologous mouse feeder cells is critical. Therefore, a system to detect any remaining mouse cells is required to assess stem cell culture quality.

The Mouse Feeder Cell Quantification Kit is designed to facilitate highly sensitive detection and quantification of genomic DNA derived from residual mouse feeder cells using real-time PCR to analyze genomic DNA extracted from stem cell cultures (e.g., hESC, hiPSC).

The kit includes primers for real-time PCR that were designed to detect a mouse mitochondrial (mt) DNA sequence, allowing detection of mouse feeder cells with high sensitivity. The copy number of cellular mtDNA is known to vary depending on cell type, cell line, and differentiation state, and there is a possibility of fluctuation between mouse feeder cell lots. The primer set for quantification of mouse mtDNA copy number included in this kit can be used for lot-to-lot correction* of mouse feeder cells.

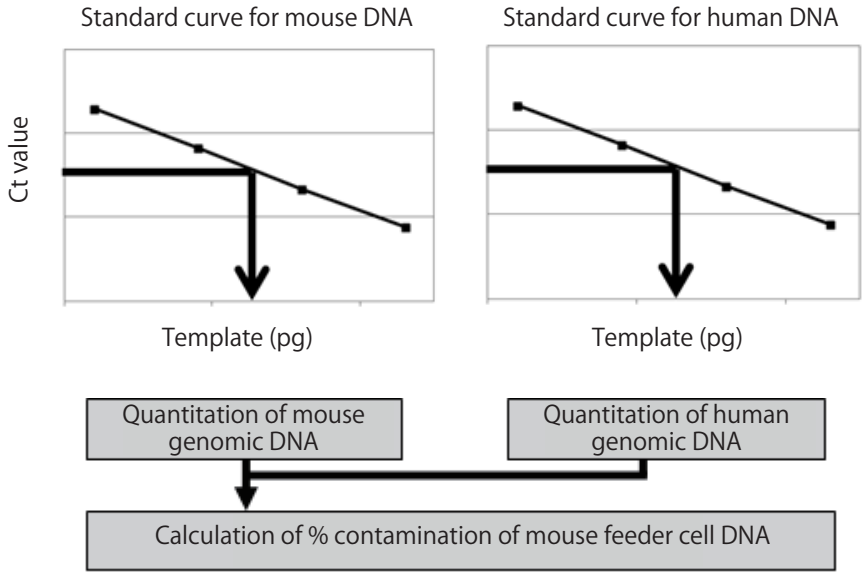
* When genomic DNA derived from different lots of mouse feeder cells are quantitated using the same standard curve, it is necessary to confirm that there is no difference in mtDNA copy number between each lot. When a standard curve is prepared for each lot of mouse feeder cells used, this correction is not necessary.

II. Principle

Quantitative analysis is performed as described below.

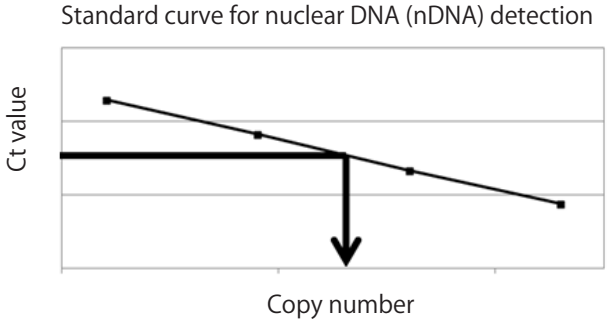
(A) Calculation of the percentage of mouse feeder cell genomic DNA contamination

The amount of mouse and human genomic DNA in a sample is assessed by real-time PCR analysis; the percentage of mouse feeder cell genomic DNA contamination is calculated.



(B, optional) Calculation of mouse mitochondrial DNA (mtDNA) copy number

Mouse mtDNA copy number is quantified by real-time PCR, using nuclear DNA (nDNA) for normalization. Real-time PCR analysis is performed for nDNA and mtDNA contained in the mouse genomic DNA sample. After preparation of a standard curve for nDNA (Y-axis: Ct value; X-axis: copy number), the relative copy number is calculated using the standard curve.



Note: Each primer for nDNA and mtDNA corresponds to a single copy gene. Each primer set has been designed to have the same amplification efficiency (E) in real-time PCR.

III. Components (100 reactions, 25 μl volume)

1.	TB Green® *Premix Ex Taq*™ II (Tli RNaseH Plus)＊1 (2X Conc.)	625 μl x 4
2.	Mouse_Mt_primer (10 μM each)	100 μl
3.	Mouse_genome_primer (10 μM each)	100 μl
4.	Human_primer_Type I (CHR3) (10 μM each)	100 μl
5.	Human_primer_Type II (CHR7) (10 μM each)	100 μl
6.	Human_primer_Type III (CHR15) (10 μM each)	100 μl
7.	dH_2O	1 ml x 2
8.	EASY Dilution (for Real Time PCR)	1 ml x 2
9.	ROX Reference Dye (50X Conc.)＊2	50 μl
10.	ROX Reference Dye II (50X Conc.)＊2	50 μl
11.	Human Genomic DNA (100 ng/μl)	20 μl

＊1 The name of intercalator-based qPCR reagent has been changed.

＊2 ROX Reference Dye and ROX Reference Dye II are intended to be used with instruments that correct for between-well fluorescent signal, such as the real-time PCR devices by Applied Biosystems.

◆ Use the ROX Reference Dye
- Applied Biosystems StepOnePlus Real-Time PCR System (Thermo Fisher Scientific)

◆ Use the ROX Reference Dye II
- Applied Biosystems 7500 Fast Real-Time PCR System (Thermo Fisher Scientific)

◆ Do not use ROX Reference Dye
- Thermal Cycler Dice™ Real Time System (Cat. #TP900/TP960: discontinued) etc.

IV. Materials Required but not Provided

- Genomic DNA for preparing standard curve [refer to section VII. Protocol, part (A)]
- Reagents for genomic DNA preparation
  - NucleoSpin Tissue (Cat. #740952.10/.50/.250)*
  - NucleoSpin Tissue XS (Cat. #740901.10/.50/.250)* etc.
- Real-time PCR instrument
  - Thermal Cycler Dice Real Time System *II* (Cat. #TP900/TP960: discontinued)
  - Thermal Cycler Dice Real Time System *Lite* (Cat. #TP700/TP760: discontinued)
  - StepOnePlus Real-Time PCR System (Thermo Fisher Scientific)
  - Applied Biosystems 7500 Fast Real-Time PCR System (Thermo Fisher Scientific)
- Microcentrifuge
- Tubes or plates for use in real-time PCR
  - 0.2 ml Hi-8-Tube (Cat. #NJ300)
  - 0.2 ml Hi-8-Flat Cap (Cat. #NJ302)
  - 96well Hi-Plate for Real Time (Cat. #NJ400)
  - Sealing Film for Real Time (Cat. #NJ500)
- Micropipettes and sterile tips

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

V. Storage

TB Green *Premix Ex Taq* II (Tli RNaseH Plus)

Store at 4℃ for up to 6 months.
Protect from light and avoid contamination.

Note: Keep at -20℃ for long term storage. Once thawed, store at 4℃ and use within 6 months.

Others

-20℃

VI. Primers

VI-1. Primer information

The primers in this kit are designed based on the following nuclear genes or mtDNA:

Primer name	Gene	Chromosome	Accession No.
Mouse_Mt_primer	COXII	M*1	-
Mouse_genome_primer	Rplp1	9	NM_018853
Human_primer_Type I (CHR3)	ALAS1	3	NM_000688
Human_primer_Type II (CHR7)	PPIA	7	NM_021130
Human_primer_Type III (CHR15)	B2M	15	NM_004048

* 1 Mitochondrial gene

VI-2. Primer selection

(A) Calculation of % mouse feeder cell DNA contamination

Detection of mouse (mtDNA)	Detection of human (nuclear DNA)
Use Mouse_Mt_primer	Select from Human_primer_Type I, II, or III＊2

＊2 Three different human primers are included in the kit to accommodate known chromosomal mutations or copy number variation (CNV). Use primer Type I for Chromosome 3, primer Type II for Chromosome 7, and primer Type III for Chromosome 15 based on the known CNV and/or mutational status of those regions of the cultured cells being analyzed.

(B) Calculation of mouse mtDNA copy number (optional)

Nuclear DNA detection	Mitochondrial DNA detection
Mouse_genome_primer	Mouse_Mt_primer

VII. Protocol

(A) Calculation of the % mouse feeder cell DNA contamination

Genomic DNA for standard curve

	Genomic DNA preparation	Concentration to Use	Volume
Analysis sample	Genomic DNA from cultured human stem cell sample	10 ng/μl	Each 2 μl
Mouse genomic DNA for standard curve	Genomic DNA from mouse feeder cells used while culturing human stem cells[*1]	100 pg/μl, 10 pg/μl, 1 pg/μl, 0.1 pg/μl	
Human genomic DNA for standard curve	Genomic DNA from feeder-free hES/hiPS cells, or Human Genomic DNA[*2] included in this kit	10 ng/μl, 2 ng/μl, 400 pg/μl, 80 pg/μl	

＊1 In this kit, a primer set for mouse mtDNA is used for highly sensitive detection. The copy number of mtDNA varies depending on the cell type, cell line, and differentiation state. Therefore, the mouse genomic DNA used for the standard curve must be prepared from the same mouse feeder cell line used during stem cell culturing. Mouse genomic DNA is not included in this kit.

＊2 If feeder-free hES/hiPS cells are not available, use the Human Genomic DNA included with this kit. It is high quality genomic DNA prepared from pooled whole blood obtained from healthy subjects.

Procedure

1. Prepare genomic DNA from 1 x 10^6 - 1 x 10^7 cells.*3

   *3 Highly pure genomic DNA can be prepared easily using NucleoSpin Tissue.

2. Dilution of genomic DNA
   a. Dilute genomic DNA sample using EASY Dilution (for Real Time PCR) to a concentration of 10 ng/μl.
   b. Dilute genomic DNAs for the standard curves using EASY Dilution (for Real Time PCR):
      - Mouse genomic DNA for standard curve:
        Prepare a 10 - fold dilution series: 100 pg/μl, 10 pg/μl, 1 pg/μl, 0.1 pg/μl.
      - Human genomic DNA for standard curve:
        Prepare a 5 - fold dilution series: 10 ng/μl, 2 ng/μl, 400 pg/μl, 80 pg/μl.

3. Preparation of reaction mixture for real time PCR

   Prepare the reaction mixture for mouse and human DNA detection according to the following table. Make sufficient master mix for the number of samples to be analyzed, plus a few extra to account for pipetting error.*4

	Mouse detection (mtDNA)	Human detection (nuclear DNA)
primer	Mouse_Mt_primer	Select from Human_primer_ Type I, II, or III*5
Genomic DNA for standard curve	Mouse genomic DNA (4 concentrations)	Human genomic DNA (4 concentrations)
Analysis sample	Sample genomic DNA (2 μl of 10 ng/μl for each)	

   *4 Include negative controls for the standard curve and analysis sample. To account for pipetting error, prepare a larger volume of reaction master mix than the number of samples to be analyzed.

   *5 Three different human primers are included in the kit; use primer Type I for Chromosome 3, primer Type II for Chromosome 7, and primer Type III for Chromosome 15 based.

[Example using a Thermal Cycler Dice Real Time System]

● Reaction mixture for detecting mouse DNA (Per reaction)

Reagent	Volume
TB Green *Premix Ex Taq* II (Tli RNaseH Plus) (2X conc.)	12.5 μl
Mouse_Mt_primer (10 μM each)	1.0 μl
dH_2O	9.5 μl
Total	23 μl

● Reaction mixture for detecting human DNA (Per reaction)

Reagent	Volume
TB Green *Premix Ex Taq* II (Tli RNaseH Plus) (2X conc.)	12.5 μl
Human_primer Type I, II, or III (10 μM each)	1.0 μl
dH_2O	9.5 μl
Total	23 μl

[Example using an Applied Biosystems real-time PCR instrument]

● Reaction mixture for detecting mouse DNA (Per reaction)

Reagent	Volume
TB Green *Premix Ex Taq* II (Tli RNaseH Plus) (2X conc.)	12.5 μl
Mouse_Mt_primer (10 μM each)	1.0 μl
ROX Reference Dye or ROX Reference Dye II＊6	0.5 μl
dH_2O	9.0 μl
Total	23 μl

● Reaction mixture for detecting human DNA (Per reaction)

Reagent	Volume
TB Green *Premix Ex Taq* II (Tli RNaseH Plus) (2X conc.)	12.5 μl
Human_primer Type I, II, or III (10 μ M each)	1.0 μl
ROX Reference Dye or ROX Reference Dye II＊6	0.5 μl
dH_2O	9.0 μl
Total	23 μl

＊6 ROX Reference Dye is used with StepOnePlus, and ROX Reference Dye II is used with Applied Biosystems 7500 Fast Real-Time PCR System.

4. Dispense 23 μl of the prepared master mix into each 0.2 ml tube.
5. Add 2 μl of the diluted DNA sample for analysis or of the standard.
6. Centrifuge the tubes briefly to collect liquid at the bottom of the tubes.
7. Set each tube in the real-time PCR instrument and start the reaction according the following conditions:

   Note: Refer to the user manual for your real-time PCR system for operating instructions. (Refer to section VIII. Appendix for the Thermal Cycler Dice Real Time System *II* (discontinued).)

   Initial denaturation (Hold)
   Number of cycle: 1
   95℃ 30 sec

   2 step PCR
   Number of cycles: 40
   95℃ 5 sec
   60℃ 30 sec (detection: FAM)

   Melting curve analysis

8. After the reaction is complete, perform data analysis.

Calculating % mouse feeder cell DNA contamination

After the real-time PCR reaction is complete, confirm the amplification curve for the FAM filter. Then, prepare a standard curve and calculate the % of mouse feeder cell DNA contamination according to the following calculation method.

1. Based on a mouse standard curve, calculate the amount of mouse genomic DNA contained in the analysis sample.
2. Based on a human standard curve, calculate the amount of the human genomic DNA contained in the analysis sample.
3. Based on the DNA amount obtained from both mouse and human DNA quantifications, calculate the % mouse feeder cell DNA contamination.

% mouse genomic DNA contamination
= Mouse genomic DNA / Total DNA (Mouse + Human) x 100

[Example]

DNA was extracted from 3 types of hES cells (approximately 1 x 10^6 cells) cultured in the absence of feeder cells for several passages, and the % mouse feeder cell DNA contamination was analyzed.

(a) Standard curve (mouse)

Template (pg)	Ct value
200 pg	21.62
20 pg	25.21
2 pg	28.88
0.2 pg	32.79

Equation: Y= - 3.718 X + 30.103
R^2= 0.9996

(b) Standard curve (human)

Template (pg)	Ct value
20,000 pg	24.43
4,000 pg	26.69
800 pg	29.16
160 pg	31.45

Equation: Y= - 3.366 X + 38.882
R^2 = 0.9997

(c) Calculation of genomic DNA amount from Ct value of analysis sample

	Mouse genomic DNA		Human genomic DNA		Total amount (pg)	% Contamination of mouse DNA
	Ct value	Detected amount (pg)	Ct value	Detected amount (pg)		
A	19.51	707	24.32	21,168	21,875	3.230%
B	22.52	110	24.21	22,822	22,932	0.478%
C	26.01	13	24.3	21,460	21,472	0.059%

[Result] % mouse feeder cell DNA were A: 3.23%, B: 0.478%, and C: 0.059%.

(B) Calculation of mouse mitochondrial DNA (mtDNA) copy number (optional)

1. Extract genomic DNA from the mouse feeder cells.＊1

   ＊1 Highly pure genomic DNA can be extracted easily using NucleoSpin Tissue.

2. Dilute the genomic DNA sample using EASY Dilution (for Real Time PCR) as follows.

   • For nuclear DNA (nDNA) detection
   Prepare a 10-fold dilution series (10 ng/μl, 1 ng/μl, 100 pg/μl, 10 pg/μl)
   • For mitochondrial DNA (mtDNA) detection
   Prepare a 10 pg/μl concentration

3. Preparation of reaction mixture for real time PCR:

   Prepare master mixes for nDNA and mtDNA detection according to the following table. Make sufficient master mix for the number of samples to be analyzed, plus a few extra to account for pipetting error.＊2

   ＊2 Include negative controls for the standard curve and analysis sample. To accommodate pipetting error, prepare a larger volume of reaction master mix than the number of samples to be analyzed.

	nDNA detection	mtDNA detection
Primer	Mouse_genome_primer	Mouse_Mt_primer
Sample	Mouse genomic DNA	
Template	10 ng/μl, 1 ng/μl, 100 pg/μl, 10 pg/μl	10 pg/μl

[Example using a Thermal Cycler Dice Real Time System]

● Reaction mixture for nDNA detection (Per reaction)

Reagent	Volume
TB Green *Premix Ex Taq* II (Tli RNaseH Plus)	12.5 μl
Mouse_genome_primer (10 μM)	1.0 μl
dH_2O	9.5 μl
Total	23 μl

● Reaction mixture for mtDNA detection (Per reaction)

Reagent	Volume
TB Green *Premix Ex Taq* II (Tli RNaseH Plus)	12.5 μl
Mouse_Mt_primer (10 μM)	1.0 μl
dH_2O	9.5 μl
Total	23 μl

[Example using an Applied Biosystems real time PCR instrument]

● Reaction composition for nDNA detection (Per reaction)

Reagent	Volume
TB Green *Premix Ex Taq* II (Tli RNaseH Plus)	12.5 μl
Mouse_genome_primer (10 μ M)	1.0 μl
ROX Reference Dye or ROX Reference Dye II＊3	0.5 μl
dH_2O	9.0 μl
Total	23 μl

● Reaction composition for mtDNA detection (Per reaction)

Reagent	Volume
TB Green *Premix Ex Taq* II (Tli RNaseH Plus)	12.5 μl
Mouse_Mt_primer (10 μM)	1.0 μl
ROX Reference Dye or ROX Reference Dye II＊3	0.5 μl
dH_2O	9.0 μl
Total	23 μl

＊3 Use ROX Reference Dye is with StepOnePlus. Use ROX Reference Dye II with the Applied Biosystems 7500 Fast Real-Time PCR System.

4. Dispense 23 μl of the prepared master mix into each 0.2 ml tube.
5. Add 2 μl of the diluted samples for analysis or of the standards.
6. Centrifuge the tubes briefly to collect liquid at the bottom of the tubes.
7. Set each tube in the real-time PCR instrument and start the reaction according the following conditions:

   Note: Refer to the user manual for your real time PCR system for operating instructions. (Refer to section VIII. Appendix for the Thermal Cycler Dice Real Time System *II* (discontinued).)

   Initial denaturation (Hold)
   Number of cycle: 1
   95℃ 30 sec

   2 step PCR
   Number of cycles: 40
   95℃ 5 sec
   60℃ 30 sec (detection: FAM)

   Melting curve analysis

8. After the reaction is complete, perform data analysis.

Calculating mouse mtDNA copy number

After the real-time PCR reaction is complete, confirm the amplification curve for the FAM filter. Then, generate a mouse nDNA standard curve and calculate the relative copy number of mtDNA using nDNA as a standard by applying the mouse mtDNA Ct value to the standard curve.

1. Calculating mouse nDNA copy number

   Convert the mouse nDNA amount to copy number using the following formula.

   Mouse nDNA copy number
   = Avogadro' s constant x Template amount (g)/(bp genome size x average molecular weight)

   - Avogadro' s constant : 6.02×10^{23}
   - Mouse genome size (bp) : 3.3×10^{9}
   - Average molecular weight : 660

nDNA*	nDNA copy number
20,000 pg	5.528×10^{3}
2,000 pg	5.528×10^{2}
200 pg	5.528×10^{1}
20 pg	5.528

   * Totsal amount of nDNA used as a template.

2. Produce a standard curve using nDNA copy number (X-axis) and nDNA Ct value (Y-axis).
3. Use the standard curve to calculate the corresponding mtDNA copy number based on the Ct value obtained for mtDNA real-time PCR detection.
4. Calculate the ratio of mtDNA copy number by dividing the mtDNA copy number by nDNA copy number at an equivalent template amount.

   mtDNA copy number ratio = mtDNA copy number/nDNA copy number

   Note: Use mtDNA and nDNA copy numbers obtained from the same amount of template.

[Example: mouse cell mtDNA copy number]

Genomic DNA was extracted from approximately 1×10^{6} MEF cells, and the ratio of mtDNA copy number was examined in comparison with mouse nuclear DNA (nDNA).

(a) Calibration curve for nDNA

Template (pg)	nDNA copy number	Ct value
20,000 pg	5.528×10^{3}	24.64
2,000 pg	5.528×10^{2}	27.81
200 pg	5.528×10^{1}	30.38
20 pg	5.528	33.93

Equation: $Y = -3.044 X + 36.016$
$R^2 = 0.9965$

(b) Calculating mtDNA copy number ratio based on the Ct value of mtDNA detection

Template (pg)	Ct Value	mtDNA copy number	nDNA copy number	Copy number ratio
20	25.40	3,074	5.528	556

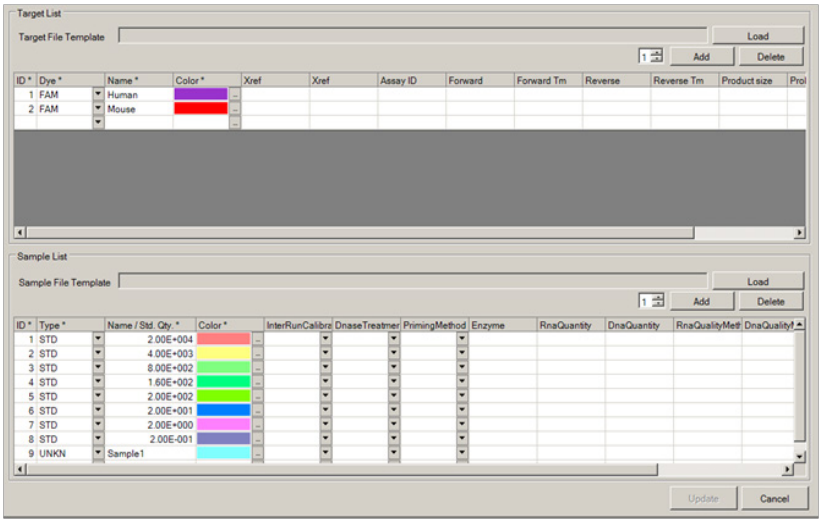
[Result] For the MEF cells used for hES cell culture, the ratio of mitochondrial DNA copy number was 556 times higher than nuclear DNA (nDNA) copy number.

Note: This data is an experimental example. Please note that mtDNA copy number can vary depending on mouse cell type, cell line, and differentiation state.

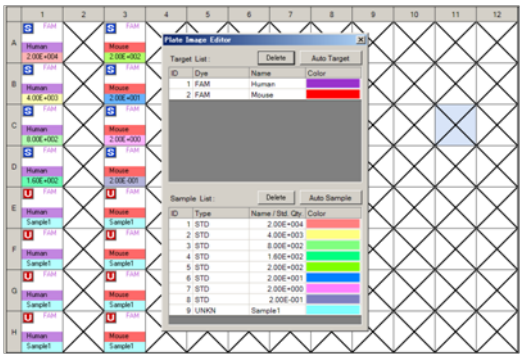
VIII. Appendix

[Operating methods for the Thermal Cycler Dice Real Time System // (discontinued)]

1. Create a new run file, and select AQ(S) Absolute Quantification Single.
2. Enter FAM as the Dye to be detected and standard (STD) and sample (UNKN) in Sample List in Target & Sample Setting under Plate Setup. Then select the Update button.



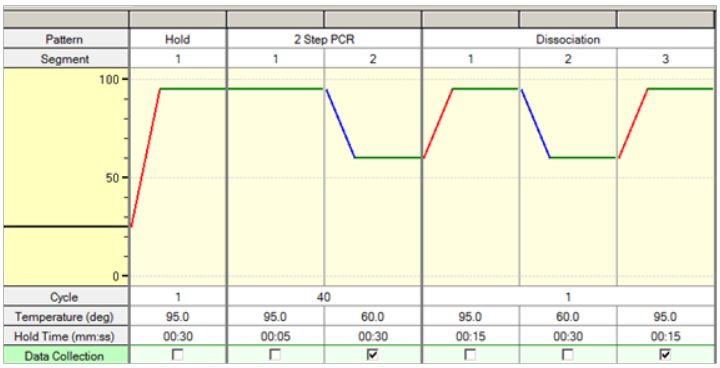
Enter the contents of the wells in the Plate Image window under Plate Setup.



(A) When calculating the % contamination from genomic DNA derived from mouse feeder cells:
- Create a Target List for mouse and human.
- Enter the total template amount (pg) for the standards (STD).
  (Example: 100 pg 2 μl → 200 pg)

(B) When calculating mtDNA copy number in mouse cells:
- Create a Target List for only mouse.
- Enter the nDNA copy number that has been calculated from the total amount of template (pg), for the standards (STD).
  (Example: 100 pg 2 μl → 5.528 x 10^{1})

3. Enter the following reaction parameters.



Initial denaturation (Hold)
Cycle : 1
95℃ 30 sec

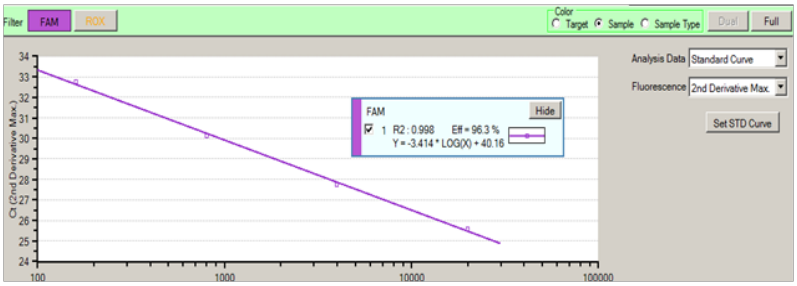
2 step PCR
Cycles : 40
95℃ 5 sec
60℃ 30 sec (detection: FAM)

Melting curve analysis

4. Click the "Start Run" button at the lower right side, name the Run File as desired, and start the reaction.

5. Data analysis

- After the reaction is complete, select the FAM detection filter. In the Result/Analysis window, select "Standard Curve" under Analysis Data and "2nd Derivative Max" under Fluorescence, and confirm that the R^2 of the standard curve is >0.97. A typical standard curve is shown below.



- In the Result/Analysis window, select "Text Report" under Analysis Data, "Data Set of Each Well" or "Data Set of Replicate" under Fluorescence.

(A) When the % mouse feeder cell DNA contamination is calculated:
- Qty (SDM) indicates detected amount.
- The % contamination is calculated based on the sum total of the mouse and human amounts.

Mouse DNA contamination (%)
= Mouse genome DNA / Total DNA (Mouse + Human) x 100

(B) When mtDNA copy number in mouse cells is calculated
- Qty (SDM) indicates copy number.
- The mtDNA copy number ratio is calculated by dividing the mtDNA copy number by nDNA copy number at an equivalent template amount, using nDNA as a standard.

mtDNA copy number ratio = mtDNA copy number/nDNA copy number

IX. Precautions for Data Analysis

1. Confirm that no amplification occurred in the negative controls with each primer set.
   → When amplification is detected in the negative controls and the melting curve temperature (Tm value) is different from that of the target gene, nonspecific amplification has occurred. Nonspecific amplification with Ct values >35 can occur with the human primers.
2. Confirm that the correlation coefficient (R^2) of the calibration curve is >0.97.
   → When R^2 is less than 0.97, re-analysis is recommended.

X. Troubleshooting

- The standard curve is not produced.
  → When products for the standard curve samples are not amplified, confirm that the correct templates were used.
  → Confirm the quality of genomic DNA used to produce the standard curve, and confirm that dilutions were prepared properly.

  Note:
  Using low quality genomic DNA can affect the accuracy of quantification.

- When calculating % contamination, the Ct value of mouse mtDNA detection is not shown.
  → When contamination is below than the detection limit, the Ct value is below the detection range and a value is not shown.

- There is no quantification.
  → Confirm whether the dilution of the standard curve is correct.
  → Confirm the quality of the template genomic DNA. Impure genomic DNA can inhibit real-time PCR. If endogenous PCR inhibitors are present in the sample, purify or prepare another genomic DNA sample.

XI. Related Products

NucleoSpin Tissue (Cat. #740952.10/.50/.250)*
NucleoSpin Tissue XS (Cat. #740901.10/.50/.250)*
Human Genomic DNA (Cat. #636401)
0.2 ml Hi-8-Tube (Cat. #NJ300)
0.2 ml Hi-8-Flat Cap (Cat. #NJ302)
96well Hi-Plate for Real Time (Cat. #NJ400)
Sealing Film for Real Time (Cat. #NJ500)

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