Cat. # PH017

For Research Use

TaKaRa

PrimerArray®
Hepatic Differentiation (Human)

Product Manual

v201902Da

Table of Contents

I. Description

PrimerArray Hepatic Differentiation (Human) is a set of real-time RT-PCR primers for the analysis of the expression of genes associated with hepatic differentiation. This array contains primer pairs optimized for real-time RT-PCR for the analysis of 88 genes associated with pluripotency, endoderm, and hepatic differentiation and 8 housekeeping genes. When comparing an unknown sample to a control sample, gene expression differences can be expressed using the relative quantification method. The PrimerArray Analysis Tool for Hepatic Differentiation＊1 and/or Multiplate RQ＊2 are useful for analyzing data obtained using this product.

＊1 Please download the tool from the PrimerArray product page. Using this tool, it is possible to compare a control sample and one unknown sample.

＊2 The Multiplate RQ software is used for relative quantification of gene expression data obtained with Thermal Cycler Dice™ Real Time Systems including the Thermal Cycler Dice Real Time System // MRQ (Cat. #TP960)＊3 and the Thermal Cycler Dice Real Time System III with PC/MRQ (Cat. #TP980)＊3. With Multiplate RQ, comparative analysis can be performed between numerous unknown samples and a control sample.

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

Procedural Overview:

Below is a flowchart, starting with RNA extraction and ending with data analysis. In parentheses is an estimate of the time required for one real-time PCR experiment; the entire process can be finished in approximately 2.5 to 3.5 hours. Multiple samples can be processed together through the reverse transcription step, and the synthesized cDNA should be stored at -20℃ for subsequent experiments.

RNA extraction (approx. 30 min):

Extract RNA from experimental materials (control sample and test sample) using NucleoSpin RNA (Cat #740955.10/.50/.250) or RNAiso Plus (Cat #9108) and treat with DNase I. Use approximately 2.5 μg of total RNA for one experiment.

↓

Reverse transcription (approx. 20 min):

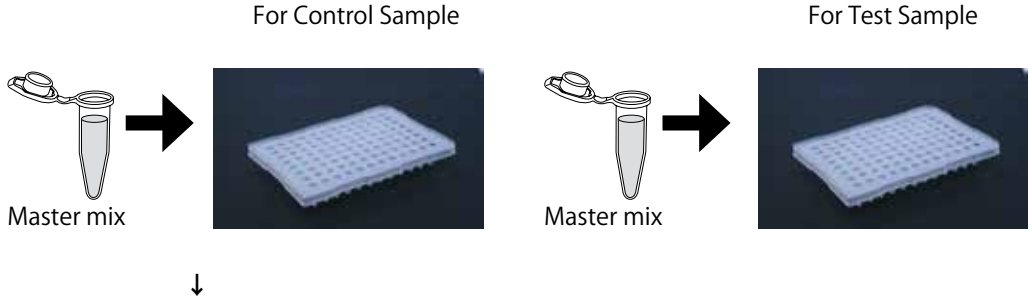
Synthesize cDNA from each of the total RNA samples. It is recommended that PrimeScript™ RT Master Mix (Perfect Real Time) (Cat. #RR036A)＊ be used.

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

↓

Dispense real-time PCR reaction solution (approx. 10 min):

Combine the synthesized cDNA and TB Green® *Premix Ex Taq*™ II (Tli RNase Plus) (Cat. #RR820A) to prepare a master mix solution for control and test samples, then dispense into wells of a 96-well real-time PCR plate (see illustration below).



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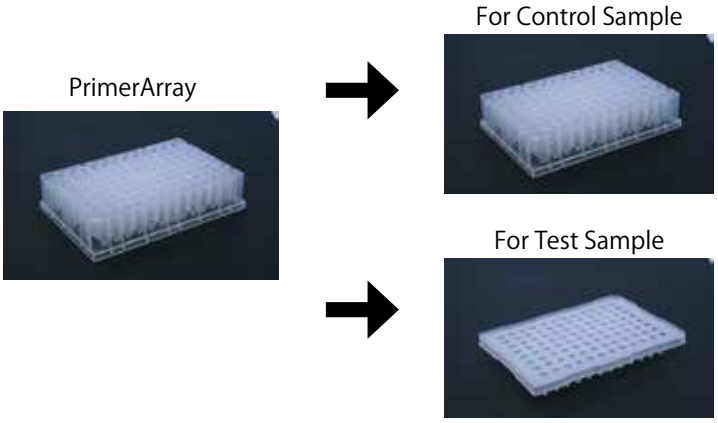
Add primers (approx. 5 min):

Add PrimerArray primers to the real-time PCR plate using 8-multichannel pipette, etc. (see illustration below).



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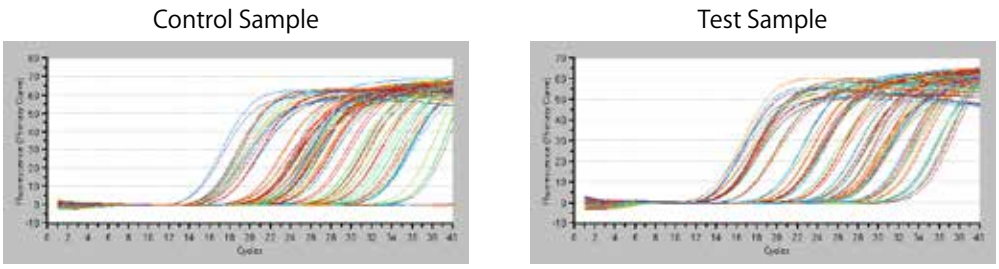
Real-time PCR (1 - 2 hours):

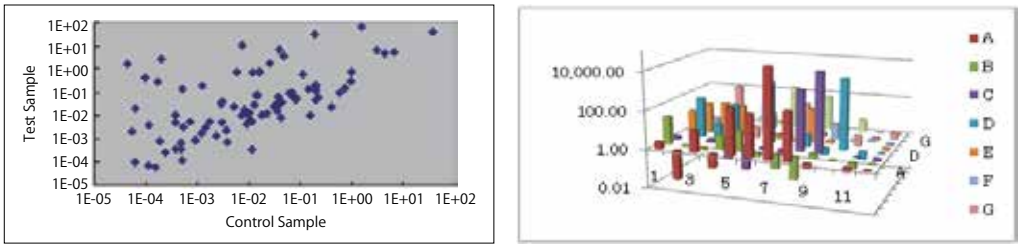
The reaction is carried out with real-time PCR instrument.

↓



Data analysis (about 30 min):

Relative quantification analysis is achieved by the ΔΔCt method.



II. Components (For 12 reactions, 25 μl volume per reaction)

PrimerArray Hepatic Differentiation (Human)

Primers for each marker gene 50 μl x 88 wells
Primers for housekeeping genes 50 μl x 8 wells

* Forward & Reverse primer mix (2.5 μM each) per well.

NOTE: The rubber mat (lid) is re-used repeatedly; therefore, do not discard. Primer information can be downloaded in excel spreadsheet format from the PrimerArray product page.

Primer layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	9	17	25	33	41	49	57	65	73	81	89
B	2	10	18	26	34	42	50	58	66	74	82	90
C	3	11	19	27	35	43	51	59	67	75	83	91
D	4	12	20	28	36	44	52	60	68	76	84	92
E	5	13	21	29	37	45	53	61	69	77	85	93
F	6	14	22	30	38	46	54	62	70	78	86	94
G	7	15	23	31	39	47	55	63	71	79	87	95
H	8	16	24	32	40	48	56	64	72	80	88	96

Primers for each marker gene related to Pluripotency, endoderm, hepatic differentiation, etc

Primers for housekeeping genes

III. Materials Required but not Provided

- Thermal cycler for real-time PCR
  Thermal Cycler Dice Real Time System *II* (Cat. #TP900/TP960) *1
- Reaction plate and seal for real-time PCR
- 8-multi-channel pipette, micropipette, and tips
- Centrifuge for 96 well plate
- RNA extraction reagent
  NucleoSpin RNA (Cat. #740955.10/.50/.250); RNAiso Plus (Cat. #9108/9109)
- Recombinant DNase I (RNase-free) (Cat. #2270A/B)
- Reverse transcription reagents
  PrimeScript RT Master Mix (Perfect Real Time) (Cat. #RR036A) *1 is recommended.
- Real time PCR reagent
  TB Green *Premix Ex Taq* II (Tli RNaseH Plus) (Cat. #RR820A) *2 is recommended.
- PrimerArray Analysis Tool for Hepatic Differentiation or Multiplate RQ

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

*2 We have begun the process of changing the names for Takara Bio's intercalator-based real-time PCR (qPCR) products to the "TB Green series". These products can be used the same way as before, as only the names are changing. Catalog number and product performance are unaffected by this transition.

IV. Storage -20℃

Expires 1 year from date of receipt when kept under proper storage conditions.

If this product is used over a short time period (~1 month), it should be stored at 4℃. This product does not contain a preservative, therefore caution should be used when handling the product to prevent contamination.

NOTE: When stored at -20℃, thaw at room temperature, mix uniformly, and briefly centrifuge the plate before use. When stored at 4℃, mix uniformly and briefly centrifuge before use.

V. Protocol

Please refer to the operation manual for instruments and instructions for specific reagents.

1. Preparation:

When the PrimerArray plate is stored at -20℃, remove from the freezer and thaw at room temperature prior to use. After checking that the rubber mat (lid) is secure, gently shake the PrimerArray plate to uniformly mix the solution. Then briefly centrifuge the plate to collect the solution at the bottom of the well. When the PrimerArray plate is stored at 4℃, mix the plate gently and spin down briefly.

2. RNA extraction:

Please refer to "1. Preparation of RNA sample" in VI. Appendix for general considerations for RNA preparation.

2-1. RNA Isolation

It is recommended that RNA isolation kits such as NucleoSpin RNA (Cat. #740955.10/.50/.250) or RNAiso Plus (Cat. #9108/9109) be used for isolating high-purity total RNA. Please refer to each instruction manual for detailed protocols. After removing the genomic DNA (see step 2-2), dissolve the RNA sample in sterile purified water or TE buffer, and adjust the concentration to 250 ng/μl in preparation for reverse transcription.

2-2. Removal of genomic DNA

In some cases, total RNA samples may contain a small amount of genomic DNA, which could potentially be amplified by PCR and lead to inaccurate results. To avoid this situation, remove genomic DNA by DNase I treatment. When RNA is prepared using NucleoSpin RNA, DNase I treatment can be performed on the column according to the product manual. When RNA is extracted using another method, perform DNase I digestion following the protocol below.

Removal of genomic DNA by DNase I treatment

After extracting total RNA, the RNA sample is treated with Recombinant DNase I (RNase-free) (Cat. #2270A). After the treatment, DNase I should be inactivated by either heat treatment or phenol/chloroform extraction.

(1) Prepare the following reaction mixture:

Reagent	Volume	Final conc.
total RNA	x μl	20 - 50 μg
10X DNase I Buffer	5 μl	1X
RNase Inhibitor (40 U/μl)	0.5 μl	20 U
DNase I (RNase-free)	2 μl	10 U
DEPC-treated water	(42.5 - x) μl	up to 50 μl

(2) Incubate at 37℃ for 20 min.

(3) Perform one of the following procedures to inactivate DNase I:

A. Heat treatment:

i) Add 2.5 μl of 0.5 M EDTA, and incubate at 80℃ for 2 min.

ii) Bring the reaction volume to 100 μl with DEPC treated water.

B. Phenol/Chloroform extraction:

i) Add 50 μl of DEPC treated water and 100 μl of phenol/chloroform/isoamyl alcohol (25 : 24 : 1), and mix well.

ii) Centrifuge at 15,000 rpm for 5 min at room temperature, and transfer the upper layer to a new tube.

iii) Add equal amount of chloroform/isoamyl alcohol (24 : 1), and mix well.

iv) Centrifuge at 15,000 rpm for 5 min at room temperature, and transfer the upper layer to a new tube.

(4) Add 10 μl of 3 M sodium acetate, 250 μl of cold ethanol (>99%), and mix well. Then incubate on ice for 10 min.

(5) Centrifuge at 15,000 rpm for 15 min at 4℃, and remove the supernatant.

(6) Wash the pellet with 70% ethanol, centrifuge at 15,000 rpm for 5 min at 4℃, and remove the supernatant.

(7) Dry the pellet.

(8) Dissolve the pellet in an appropriate amount of DEPC treated water.

3. Reverse Transcription:

Perform reverse transcription using the total RNA prepared above as a template. PrimeScript RT Master Mix (Perfect Real Time) (Cat. #RR036A)＊ is recommended for reverse transcription.

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

(1) Prepare the reverse transcription mixture on ice.

<For 1 reaction>

Reagent	Volume	Final conc.
5X PrimeScript RT Master Mix	10 μl	1X
total RNA (250 ng/μl)	10 μl	2.5 μg
RNase Free dH_2O	30 μl	
Total	50 μl	

＊ It is possible to scale up the RT reaction as needed.

(2) Incubate the reaction mixture under the following conditions

37℃	15 min	(Reverse transcription reaction)
85℃	5 sec	(Heat inactivation of reverse transcriptase)
4℃		

NOTE: The subsequent protocol depends on the specific real-time PCR instrument used. In this section, the protocol for the Thermal Cycler Dice Real Time System is described. For other instruments, please refer to the section, "2. How to use with various real-time PCR instruments" in VI. Appendix.

4. Preparation of Real-time PCR Reaction plates:

The preparation protocol shown in this section is for real-time PCR reaction plates, one control sample and one unknown sample. Please scale up the number of reaction plates according to the number of experimental samples.

4-1. Preparation and dispensing of Master Mix

The cDNA prepared in "3. Reverse Transcription" and TB Green *Premix Ex Taq* II (Tli RNaseH Plus) (Cat. #RR820A) are combined to prepare a master mix, then the mixture is dispensed to real-time PCR plates.

(1) Using cDNA of the control sample or of the unknown sample, prepare each master mix (PCR reaction) as outlined below.

Reagent	For 1 well	For 110 wells
TB Green *Premix Ex Taq* II (Tli RNaseH Plus) (2X)	12.5 μl	1,375 μl
cDNA (50 ng/μl)*	0.4 μl	44 μl
Sterile purified water	8.1 μl	891 μl
Total	21 μl	2,310 μl

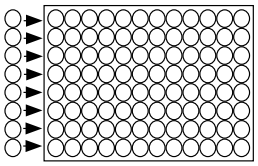
* total RNA equivalent

(2) Dispense 21 μl of the master mix into each well of the plate that will be used for the real-time reaction.

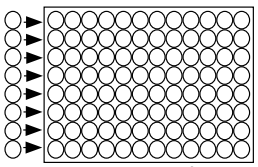
Example using an 8-multichannel pipette:

1) Dispense 273 μl (sufficient for 13 wells) of the master mix into an appropriate 8-multichannel container.
2) Dispense 21 μl of the mix to each well of the plate for real-time PCR with an 8-multichannel pipette.

4-2. Addition of Primers:



For control sample



For test sample

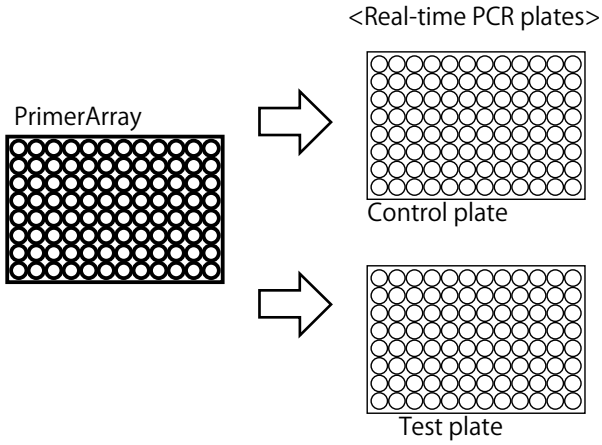
The PrimerArray primers are added into the plate for real-time PCR that contains the master mix.

(1) Remove the rubber mat (lid) of PrimerArray.

The rubber mat should be removed carefully, ensuring that primer solutions do not leak into adjacent wells. Please pay close attention to this as contamination will occur if primer solutions mix. (Do not throw away the rubber mat.)

* Mix the primer solution well, and spin down briefly prior to use.
* After using the PrimerArray, it should be covered with the rubber mat again and stored appropriately.

(2) Dispense primers.
Add 4 μl of PrimerArray primers to each well of the real-time PCR plates.



(3) Cover the plate with Sealing Film for real-time PCR.

Firmly attach the Sealing Film to the plate using a special spatula (Plate Sealing Pad, Cat. #9090). If it is not attached firmly, the reaction mixture will evaporate in the unsealed areas of the plate during real-time PCR reaction, possibly leading to inaccurate results .

NOTE: Preparation of real-time PCR reaction plate is complete. If real-time PCR will not be started immediately, store the plate at 4℃ protected from light. Start the reaction within 24 hours after plate preparation.

(4) Cover the PrimerArray with the rubber mat.

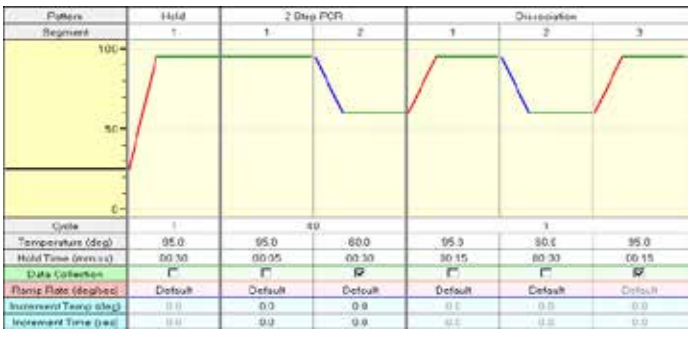
The rubber mat should be firmly reattached to the plate. If it is not attached firmly, evaporation or contamination of the primer solution may occur during storage.

5. Real-time PCR reaction:

(1) Centrifuge the real-time PCR plate briefly to collect the reaction mixture at the bottom of well.

(2) Set the plate in the real-time PCR instrument, and start the reaction.

(3) Perform PCR using the conditions below.



Hoid (Initial denaturation)
Cycle: 1
95℃ 30 sec
2 Step PCR
Cycles: 40
95℃ 5 sec
60℃ 30 sec
Dissociation (Melting curve analysis)

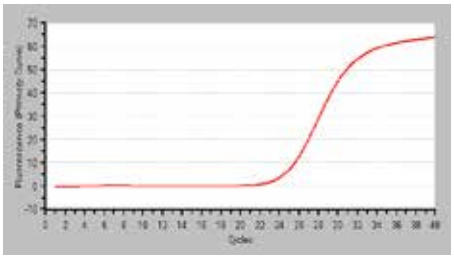
6. Data Analysis:

Ct values are calculated by the real-time PCR instrument's analysis software after the reaction. Relative quantification analysis is performed using PrimerArray Analysis Tool for Hepatic Differentiation (Human) and/or Multiplate RQ.

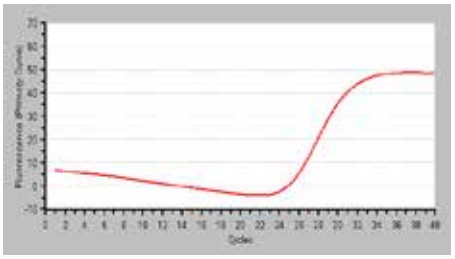
(1) Setting analysis parameters: For most real-time PCR software, the analysis parameters are set automatically. Check that the setting parameters are correct, and reset them manually if necessary.

- Baseline region: Set the flat region of the curve on the left side of the graph as a baseline region. If the baseline is set incorrectly, the amplification curve will not be flat, but will tilt.

NOTE: The baseline correction will not work well if the region is narrow, and if the region is too wide, the curve will progressively slope right-down. (see below)

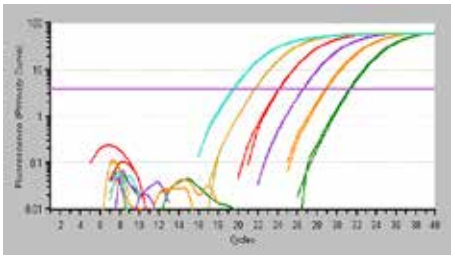


Baseline is set correctly



Baseline region is too wide

- Threshold: Set the threshold in the exponential region of the primary curve. The exponentially amplified region is the range where the primary curve becomes a straight line when the fluorescence intensity (vertical axis) is plotted is on a log scale.



Correctly set threshold

(2) Calculation of Ct value and Tm value: Ct and Tm value are automatically calculated by the real-time PCR software.

(3) Output data: Export Ct values in either the Excel format or CSV format to perform relative quantification analysis using the PrimerArray Analysis Tool for Hepatic Differentiation (Human)*. When using the Thermal Cycler Dice Real Time System, you do not need to obtain Ct values for performing analysis with Multiplate RQ. For data obtained with other instruments, Multiplate RQ can not be used for analysis.

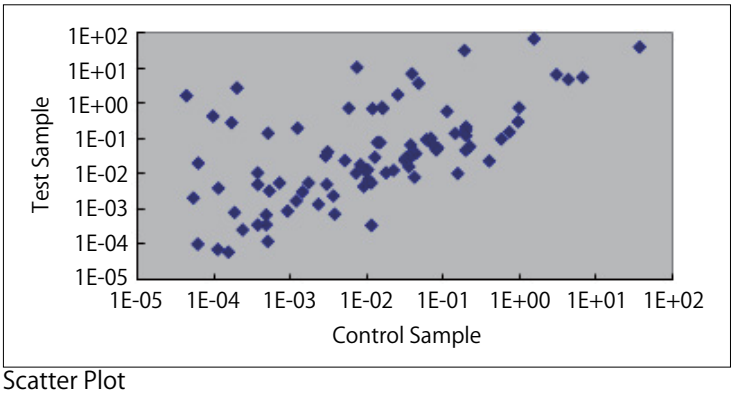
＊ For some types of real-time PCR software, data generated from wells without sample information are omitted from analysis and may not be exported. This can lead to errors when the data is imported into PrimerArray Analysis Tool for Hepatic Differentiation (Human). Therefore, the data should be reviewed carefully to make sure that all of the well information is correct when imported into the Tool.

(4) Relative quantification analysis: Expression of each gene in the unknown sample is relative to the expression in control sample. Please refer to the Multiplate RQ manual and/or PrimerArray Analysis Tool for Hepatic Differentiation manual for details of the analysis procedure.

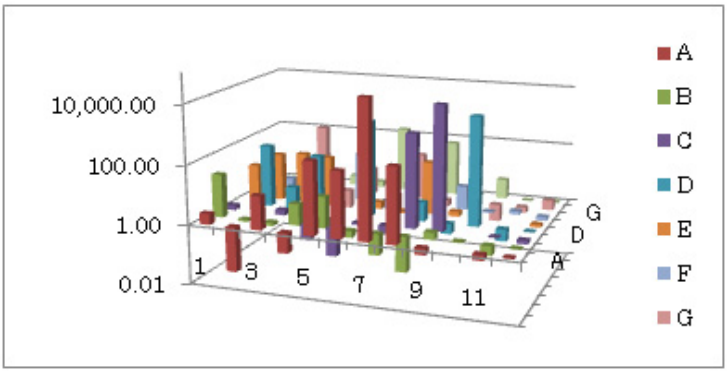
Analysis using PrimerArray Analysis Tool for Hepatic Differentiation

The results comparing the control sample with one unknown sample are shown graphically as a Scatter Plot and a 3D Profile of Fold Difference.

- Scatter Plot: The expression level of the control sample is plotted on the horizontal axis, and the expression level of the unknown sample is plotted on the vertical axis.
- 3D Profile (Fold Difference): The expression level (Fold Difference) of the unknown sample is normalized to the expression level of the control sample.



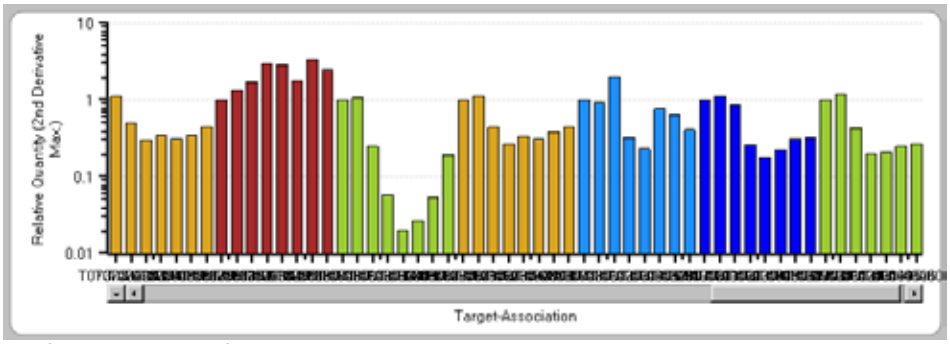
Scatter Plot



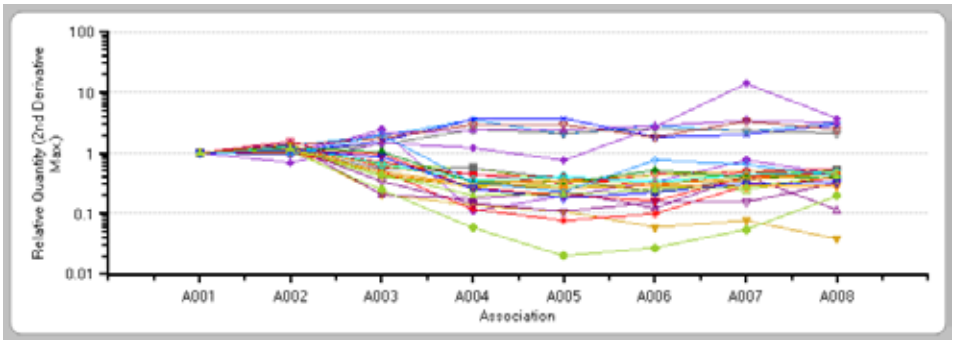
3D Profile (Fold Change)

Analysis using Multiplate RQ

The results comparing numerous unknown samples and a control sample (relative quantification) are shown graphically as a bar chart (Relative Quantity Chart) or a line graph (Gene Profile).



Relative Quantity Chart



Gene Profile

VI. Appendix

1. Preparation of RNA sample

It is important to prepare RNA samples with high purity for better cDNA yields. In addition, it is essential to inhibit cellular RNase activity and to prevent contamination with RNase from the equipment and solutions used. Extra precautions should be taken during the sample preparation to prevent the RNase contamination from operators' sweat or saliva, including using disposable gloves and a workspace dedicated exclusively for RNA preparation, and avoiding unnecessary speaking during sample preparation.

Equipment

Disposable plastic equipment should be used. Glass tools should be treated with either of the below protocols prior to use.

(1) Dry-heat sterilization (180℃, 60 min).

(2) Treat with 0.1% Diethylpyrocarbonate (DEPC) at 37℃ for 1 hour, then autoclave at 120℃ for 30 min to remove DEPC. It is recommended that this equipment be used exclusively for RNA preparation.

Reagents

All reagents used in this experiment must be prepared using tools that were treated as described in previous section (dry-heat sterilization or DEPC treatment), and purified water must be treated with 0.1% DEPC and autoclaved. All reagents and purified water should be used exclusively for RNA experiments.

2. How to use with various real-time PCR instruments

Please operate according to the instruction manual of each instrument. Please refer to "V. Protocol" for details and precautions for the experimental procedure.

[Applied Biosystems 7300/7500 Real Time PCR System (Thermo Fisher Scientific)]

(1) Prepare a master mix (PCR reaction) using each cDNA as shown below.

Reagent	For 1 well	For 110 wells
TB Green *Premix Ex Taq* II (Tli RNaseH Plus) (2X)	25 μl	2,750 μl
ROX Reference Dye or Dye II (50X)＊1	1 μl	110 μl
cDNA (50 ng/μl)＊2	0.8 μl	88 μl
Sterile purified water	15.2 μl	1,672 μl
Total	42 μl	4,620 μl

＊1 ROX Reference Dye II is used for Applied Biosystems 7500 Real Time PCR System. ROX Reference Dye is used for 7300 Real Time PCR System.

＊2 total RNA equivalent

(2) Dispense 42 μl of the mixture into each well of a real-time PCR plate.

(3) Add 8 μl of each PrimerArray primer to wells of the real-time PCR plate.

(4) Perform real-time PCR reaction using the following conditions.

Stage 1: Initial denaturation
Reps: 1
95℃ 30 sec
Stage 2: PCR reaction
Reps: 40
95℃ 5 sec
60℃ 31 or 34 sec ＊3
Stage 3: Melt Curve (Melting curve analysis)

＊3 For the Applied Biosystems 7300, set to 31 sec; for the Applied Biosystems 7500, set to 34 sec.

[Applied Biosystems 7500 Fast Real Time PCR System (Thermo Fisher Scientific)]

(1) Prepare a master mix (PCR reaction) using each cDNA as shown below.

Reagent	For 1 well	For 110 wells
TB Green *Premix Ex Taq* II (Tli RNaseH Plus) (2X)	10 μl	1,100 μl
ROX Reference Dye II (50X)	0.4 μl	44 μl
cDNA (50 ng/μl)*	0.4 μl	44 μl
Sterile purified water	6 μl	660 μl
Total	16.8 μl	1,848 μl

＊ total RNA equivalent

(2) Dispense 16.8 μl of the mixture into each well of a real-time PCR plate.
(3) Add 3.2 μl of each PrimerArray primer to wells of the real-time PCR plate.
(4) Perform real-time PCR reaction using the following conditions.

Holding Stage
Number of cycle: 1
95℃ 30 sec
Cycling Stage
Number of cycles: 40
95℃ 3 sec
60℃ 30 sec
Melt Curve Stage (Melting curve analysis)

VII. Related Products

PrimeScript™ RT Master Mix (Perfect Real Time) (Cat. #RR036A/B)*
TB Green® *Premix Ex Taq*™ II (Tli RNaseH Plus) (Cat. #RR820A/B)

[Real-time PCR systems]
- Thermal Cycler Dice™ Real Time System III (Cat. #TP950)*
- Thermal Cycler Dice™ Real Time System III with PC/MRQ (Cat. #TP980)*
- FrameStar® 0.1ml 96 well qPCR plate (Cat. #NJ904)
- Thermal Cycler Dice™ Real Time System II (Cat. #TP900)*
- Thermal Cycler Dice™ Real Time System II MRQ (Cat. #TP960)*
- 96well Hi-Plate for Real Time (Cat. #NJ400)
- Sealing Film for Real Time (Cat. #NJ500)
- Sealing Film for Real Time (Adhesive) (Cat. #NJ501)*
- Plate Sealing Pads (Cat. #9090)

NucleoSpin RNA (Cat. #740955.10/.50/.250)
RNAiso Plus (Cat. #9108/9109)
Recombinant DNase I (RNase-free) (Cat. #2270A/B)

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

VIII. References

1) Cai J, *et al*. *Hepatology.* (2007) **45**(5): 1229-1239.
2) Takahashi K, *et al*. *Cell.* (2007) **131**: 861-872.
3) DeLaForest A, *et al*. *Development.* (2011) **138**: 4143-4153.
4) Takayama K, *et al*. *Journal of Hepatology.* (2012) **57**: 628-636.
5) Yu Y, *et al*. *Stem Cell Research.* (2012) **9**: 196-207.