

# **AccuPower<sup>®</sup> qPCR Array System: Human 5-plex Reference qPCR primer and probe set**

Cat. No. S-6043-SH0

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## **User Guide**

**S-6043-SH0**



**Version No.: 1 (2022-06-02)**

**Please read all the information in booklet before using the unit**



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## **Intended Use**

*AccuPower*<sup>®</sup> qPCR Array System: Human 5-plex Reference qPCR primer and probe set is developed and supplied for research purposes only. Certain applications possible with this kit may require special approval by appropriate local and/or national regulatory authorities in the country of use.

## **Safety Warning and Precaution**

Wear appropriate protection when handling any irritant or harmful reagents. The use of a laboratory coat, protective gloves and safety goggles are highly recommended. For more information, please consult the appropriate Material Safety Data Sheet (MSDS).

## **Warranty and Liability**

All BIONEER products undergo extensive Quality Control testing and validation. BIONEER guarantees quality during the warranty period as specified, when following the appropriate protocol as supplied with the product. It is the responsibility of the purchaser to determine the suitability of the product for its particular use. Liability is conditional upon the customer providing full details of the problem to BIONEER within 30 days.

## **Quality Management System ISO 9001 Certified**

Every aspect of our quality management system from product development, production to quality assurance and supplier qualification meets the world-class standards.

## **Trademark**

*AccuPower*<sup>®</sup> is a registered trademark of BIONEER Corporation.

## **Copyright**

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## **Notice**

BIONEER Corporation reserves the right to make corrections, modifications, improvements and other changes to its products, services, specifications or product descriptions at any time without notice.



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## Product Information

### Components

Components	Amount	
AccuPower® qPCR Array System: Human 5-plex Reference qPCR primer and probe set	A set	20 rxn x 1 ea
	B set	20 rxn x 1 ea
	C set	20 rxn x 1 ea

### Storage

This product is lyophilized and shipped at ambient temperature. Store at room temperature without direct sunlight for long term storage. Once dispensed, primers should be stored at -20°C and repeated freeze and thaw cycles (more than once) are not recommended.

### Specifications

Dye	Quencher	A set	B set	C set
FAM	EBQ	ACTB	GAPDH	TBP
TEXAS-RED	EBQ	B2M	TUBB	PGK1
Cyanine5	EBQ	TFRC	RPLP0	YWHAZ
TET	EBQ	GUSB	PPIA	ALAS1
TAMRA	EBQ	HPRT1	G6PD	RPL13A

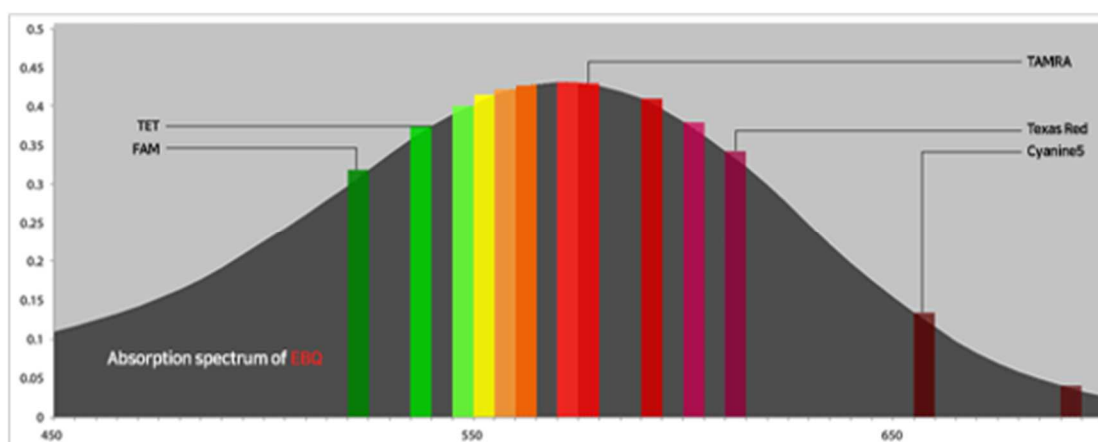


Figure 1. Reporter-dye type capable of fluorescence absorption (Quenching) with EBQ.

Gene Table

#	Gene symbol	Accession #	Description
<b>A set</b>	ACTB	NM_001101.3	actin, beta
	B2M	NM_004048.2	beta-2-microglobulin
	TFRC	NM_001128148.1, NM_003234.2	transferrin receptor
	GUSB	NM_000181.3, NM_001284290.1, NM_001293104.1, NM_001293105.1	glucuronidase, beta
	HPRT1	NM_000194.2	hypoxanthine phosphoribosyltransferase 1
<b>B set</b>	GAPDH	NM_001256799.2, NM_001289745.1, NM_001289746.1, NM_002046.5	glyceraldehyde-3-phosphate dehydrogenase
	TUBB	NM_001293212.1, NM_001293213.1, NM_001293214.1, NM_001293215.1, NM_001293216.1, NM_178014.3	tubulin, beta class I
	RPLP0	NM_001002.3, NM_053275.3	ribosomal protein, large, P0
	PPIA	NM_001300981.1, NM_021130.4	peptidylprolyl isomerase A (cyclophilin A)
	G6PD	NM_000402.4, NM_001042351.2	glucose-6-phosphate dehydrogenase
<b>C set</b>	TBP	NM_001172085.1, NM_003194.4	TATA box binding protein
	PGK1	NM_000291.3	phosphoglycerate kinase 1
	YWHAZ	NM_001135699.1, NM_001135700.1, NM_001135701.1, NM_001135702.1, NM_003406.3, NM_145690.2	tyrosine 3-monooxygenase/ tryptophan 5-monooxygenase activation protein, zeta
	ALAS1	NM_000688.5, NM_001304443.1, NM_001304444.1, NM_199166.2	5'-aminolevulinate synthase 1
	RPL13A	NM_001270491.1, NM_012423.3	ribosomal protein L13a

## Introduction

### Background

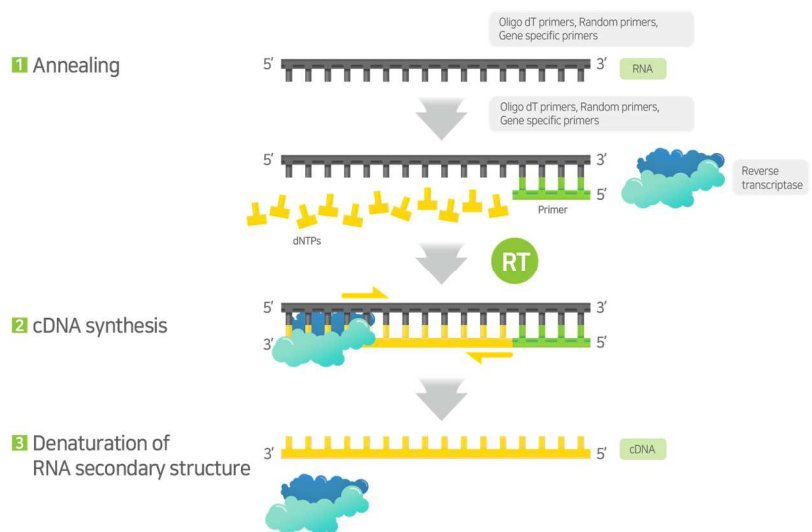
#### MIQE guidelines

MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines (Bustin et al., 2009) are standard of real-time PCR (qPCR, quantitative PCR) experiments. When performing qPCR, it is very important to ensure that the procedures such as experimental design, implementation, and data analysis comply with the MIQE guidelines. MIQE guidelines are classified into 9 different parts (experimental design, sample, nucleic acid extraction, reverse transcription, qPCR target information, qPCR oligonucleotides, qPCR protocol, qPCR validation, data analysis), suggesting guidelines for the overall qPCR experiment. They aim to improve the reliability and reproducibility of qPCR experiments and results.

*AccuPower*<sup>®</sup> qPCR Array System: Human 5-plex Reference qPCR primer and probe set has been developed expertise following the MIQE guidelines and provides accurate and reliable qPCR results of SCI publication grade.

#### Reverse transcription

RT-PCR must be carefully considered when optimizing the condition with specificity, sensitivity, reproducibility or fidelity of the reaction. Successful performance of the RT-PCR is dependent on a clear understanding of the primary aim of the assay. As RNA cannot serve as a template for PCR, the first step in the RT-PCR is the reverse transcription of the RNA template into cDNA, followed by its exponential amplification in the PCR.



**Figure 2. Procedures of reverse transcription.**

The quantification of mRNA expression can be carried out by one-step or two-step RT-PCR. One-step RT-PCR in which RT reaction for cDNA synthesis and PCR for DNA amplification occur in a single test tube allows easy, fast result analysis with many samples. However, this

method is generally lower in accuracy and specificity than two-step RT-PCR and is not recommended for PCR with SYBR green that binds double stranded DNA and emits green light because of the possibility of primer dimer formation.

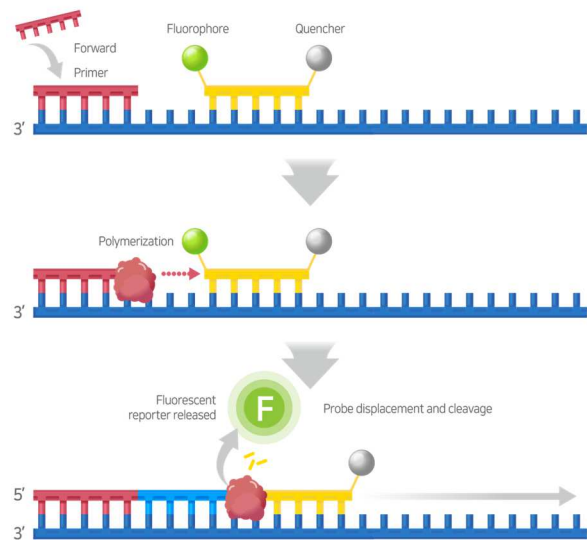
Two-step RT-PCR occurs in two steps of separate RT reaction and PCR. This method is more sensitive, and is useful in analyzing the expression of different target genes from a single sample by adjusting the amount of cDNA for the following reaction. It is also favored in the PCR using SYBR green due to its accuracy.

### qPCR detection method

#### Hydrolysis probe method

Human 5-plex Reference qPCR primer and probe set is based on 5' exonuclease reaction using a fluorogenic probe. The probe enables the detection of the PCR product specifically as it is being accumulated during PCR cycle. The Figure 3 describes real-time PCR process utilizing a hydrolysis probe.

1. After the first denaturation step, lowered temperature leads to specific annealing of primers and probes. During these steps fluorescent signal from fluorophore on 5' end of probe is quenched by EBQ (Excellent BIONEER Quencher), on 3' end.
2. Utilizing primers, HotStart DNA polymerase starts to synthesize a new DNA strand according to its template.
3. When the polymerase reaches the probe attached to template, it cleaves the probe by its 5' endogenous nuclease activity. During the cleavage separation of fluorophore from quencher, it emits a fluorescence signal from the freed fluorophore. Intensity of that fluorescence signal is proportional to the amount of freed fluorophore.



**Figure 3. Overview of real-time PCR using hydrolysis probe method.**

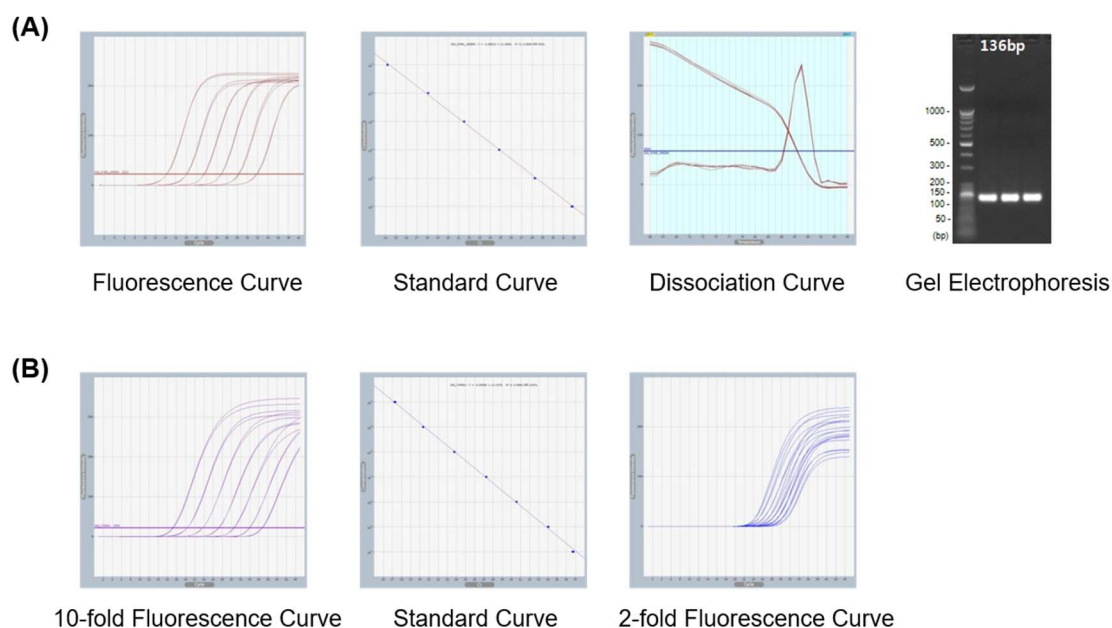


## Primer design for qPCR

Primer & probe sets of Human 5-plex Reference qPCR primer and probe set are the product of BIONEER, which is one of the world's leading suppliers of synthetic oligonucleotides. There are several important points to design primers and probes for qPCR on the basis of MIQE guidelines and all primers included in panel meet the followings.

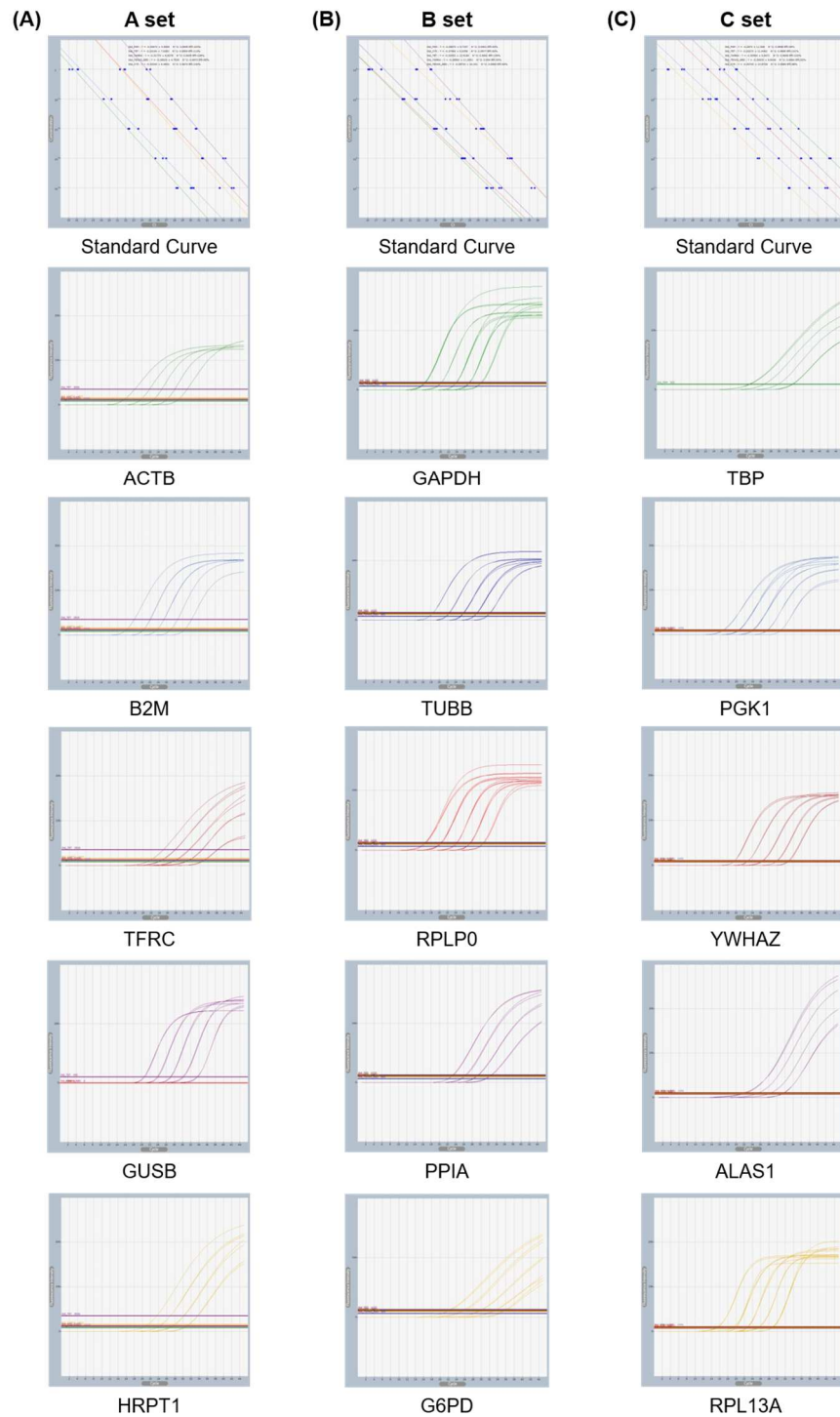
Specific primer designing using primer Blast (NCBI) and BIONEER's bioinformatics tool.

- Designing primers longer than 19 bases.
- Specific dissociation curves.
- Short amplicon size (80-160 bp).
- 90-110% optimal qPCR efficiency.
- Probe with higher T<sub>m</sub> value compared to the given primer.
- Detection of expected size with single band via gel electrophoresis.



**Figure 4. Validation data of primer and hydrolysis probe. (A)** Confirmation of target specificity and high amplification efficiency using *AccuPower*<sup>®</sup> 2X *GreenStar*<sup>™</sup> qPCR Master Mix (Cat. No. K-6253). Dynamic range: 10<sup>7</sup>-10<sup>2</sup>, R<sup>2</sup>=0.99, qPCR efficiency: 93%. **(B)** Confirmation of high amplification efficiency using 10 and 2-fold amplification efficiency test using hydrolysis probe method with *AccuPower*<sup>®</sup> Plus *DualStar*<sup>™</sup> qPCR Master Mix (Cat. No. K-6603). Dynamic range: 10<sup>7</sup>-10<sup>1</sup>, R<sup>2</sup>=0.99, qPCR efficiency: 100%.

Multiplex qPCR efficiency



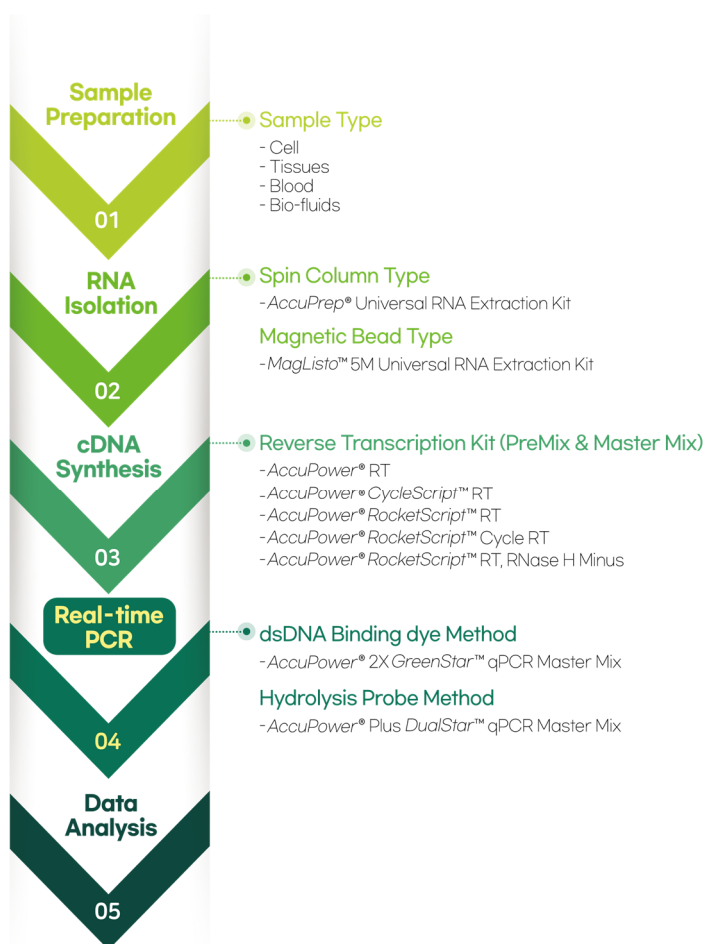
**Figure 5. Efficiency test of multiplex qPCR.** To determine the amplification efficiency of Human 5-plex Reference qPCR primer and probe set, cDNA stock of HeLa cell total RNA (500 ng/ 20  $\mu$ l) was serially diluted by 10 times and the efficiency test was performed.  $R^2=0.99$ , qPCR efficiency: 100% ( $\pm 10\%$ ).

## Product Description

*AccuPower*<sup>®</sup> qPCR Array System: Human 5-plex Reference qPCR primer and probe set is a mixture containing validated primers and probes, which can perform 5-reference gene screening in a single reaction. This product consists of 3 tubes – each for set A, B, and C. All primer and probe sets are designed in accordance with MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines, and the results can be used for SCI paper publication. Each set contains 5 dyes - FAM, TEXAS-RED, CY5, TET, and TAMRA. With our complete set, 15 typical/most used reference genes can be detected, which are provided in 3 qPCR tubes each with 5 genes. Then, the user can select most appropriate reference gene with low amount of template genes. Reference gene screening can be performed easily and quickly with the 3 sets and no additional primer/probe setting for multiplex is required. This product is optimized for utilization of *AccuPower*<sup>®</sup> Plus *DualStar*<sup>™</sup> qPCR Master Mix (Cat. No. K-6603) and *Exicycler*<sup>™</sup> 96 (Cat. No. A-2060-1) that gives the best result.

## Experimental Procedures

Start with isolating RNA from your experimental samples using *AccuPrep*<sup>®</sup> Universal RNA Extraction Kit (Cat. No. K-3141) or *MagListo*<sup>™</sup> 5M Universal RNA Extraction Kit (Cat. No. K-3613). It is necessary to treat RNase-free DNase I to all qPCR samples. Then, convert the isolated RNA to cDNA template with *AccuPower*<sup>®</sup> *RocketScript*<sup>™</sup> Cycle RT PreMix (dT<sub>20</sub>) (Cat. No. K-2201). Add equal volume of the synthesized cDNA and *AccuPower*<sup>®</sup> Plus *DualStar*<sup>™</sup> qPCR Master Mix (Cat. No. K-6603) into each well of the sample PCR array plate containing the pre-dispensed gene-specific primer sets. And perform PCR with *Exicycler*<sup>™</sup> 96 (Cat. No. A-2060-1). Use Analysis Exicycler4 software to calculate the threshold cycle (Ct) values for all the genes on each PCR array. Finally, calculate fold-changes in gene expression for pair-wise comparison using  $2^{-\Delta\Delta Ct}$  method. It is the proper normalization method that the reference gene has consistency of Ct value on samples.



**Figure 6. Schematic diagram of experimental procedures from sample preparation to data analysis.**

## Materials to be Prepared by Users

- *AccuPrep*<sup>®</sup> Universal RNA Extraction Kit (Cat. No. K-3141) or *MagListo*<sup>™</sup> 5M Universal RNA Extraction Kit (Cat. No. K-3613)
- *AccuPower*<sup>®</sup> *RocketScript*<sup>™</sup> Cycle RT PreMix (dT<sub>20</sub>) (Cat. No. K-2201)
- *AccuPower*<sup>®</sup> Plus *DualStar*<sup>™</sup> qPCR Master Mix (Cat. No. K-6603)
- Real-time PCR instrument [*Exicycler*<sup>™</sup> 96 (Cat. No. A-2060-1)]
- Nuclease-free water
- 96-100% ethanol

## RNA Isolation with Spin Column Type

*AccuPrep*<sup>®</sup> Universal RNA Extraction Kit is designed for extraction of highly purified RNA from cultured cells, plant and animal tissues within 20 min. The kit employs *AccuPrep*<sup>®</sup> Binding Column-III with silica membrane for nucleic acid binding in the presence of chaotropic salts. This silica membrane has enough surface area to bind up to 120 µg of RNA. The process does not require phenol/chloroform extraction, ethanol precipitation, or other time-consuming steps. RNA extracted through this kit can be used for a variety of applications, including reverse transcription PCR (RT-PCR), reverse transcription quantitative PCR (RT-qPCR), Northern blot analysis, and cDNA synthesis. We recommend DNase treatment for only RNA quantitation.

## Specifications

<i>AccuPrep</i> <sup>®</sup> Universal RNA Extraction Kit		
Amount of Starting Sample	Cultured cells	10 <sup>4</sup> -10 <sup>8</sup> cells
	Animal tissue	25-50 mg
Typical RNA Yield	Cultured cells	15-20 µg
	Animal tissue	10-60 µg
Column Binding Capacity		Up to 120 µg
Column Loading Volume		800 µl
Elution Volume		30-100 µl
RNA Purity		$A_{260}/A_{280} > 2.0$ , $A_{260}/A_{230} > 1.7$
Isolation Technology		Silica Column

\* **Note:** There may be differences in measured values depending on the type of samples.

## Before You Begin

Before proceeding, please check the following:

1. Add 10 µl of β-mercaptoethanol per 1 ml of RB Buffer.
2. g-force can be calculated as follows:  $rcf = 1.12 \times r \times (rpm/1,000)^2$   
\* **Note:** Where 'rcf' is the relative centrifugal force (in g), 'r' is the radius of the rotor in centimeters, and rpm is the speed of the centrifuge in revolutions per minute.

## Preparing Lysates from Animal Tissue

1. **(Lysis & homogenization)** Grind (or homogenize) 20-30 mg of fresh or thawed animal tissue sample with a mortar and pestle (or homogenizer) and place it into a clean 1.5 ml tube.
2. Add 500 µl of RB Buffer to the sample and mix thoroughly by vortexing.
3. Centrifuge at full speed for 3 minutes and carefully transfer the supernatant to a new 1.5 ml tube.  
\* **Note: (Optional)** Centrifugation through the *AccuPrep*<sup>®</sup> Filtering Column (Cat. No. KA-1160) removes debris effectively.
4. **(RNA precipitation)** Add 200 µl of absolute ethanol (not provided) to the lysate and mix immediately by pipetting.  
\* **Note:** Do not centrifuge.
5. Proceed immediately to “Purification Procedure Using Spin Columns” on page 13.

### Preparing Lysates from Cultured Cells

1. **(Cell harvesting)** Harvest cells according to step 1-A or 1-B.

1-A. Suspension cell culture:

Harvest cultured cells ( $10^4$ - $10^6$  cells) by centrifugation at 300 x g for 5 minutes to pellet cells. Discard the supernatant carefully without disturbing a cell pellet and go to step 2.

1-B. Monolayer cell culture:

There are two different methods to collect cells grown in monolayer culture.

a. Direct cell harvesting on the culture dishes:

Completely discard the cell culture medium and go to step 2.

\* **Note:** You should completely remove the cell culture medium because it may inhibit the RNA extraction.

b. Cell harvesting with trypsin:

Remove the cell culture medium and wash the cell monolayer with DPBS. Add 0.1-0.25% trypsin to the washed cell monolayer to detach the cells. After the cells detach, add cell culture medium. Transfer the cells to an RNase-free tube (not provided) and centrifuge at 300 x g for 5 minutes. Discard the supernatant carefully and go to step 2.

2. **(Lysis & homogenization)** Resuspend the cell pellet from step 1 in 400 µl of RB Buffer by vortexing.

\* **Note:** You should completely resuspend the sample to achieve maximum lysis efficiency.

3. **(RNA precipitation)** Add 300 µl of 80% ethanol<sup>†</sup> to the lysate and mix immediately by pipetting.

\* **Note:** Do not centrifuge.

<sup>†</sup> When purifying RNA from certain cell lines, precipitates may be visible after addition of ethanol. This does not affect the procedure.

4. Proceed immediately to “Purification Procedure Using Spin Columns” on page 13.



## Purification Procedure Using Spin Columns

1. **(RNA binding)** Transfer up to 700 µl of sample to the *AccuPrep*<sup>®</sup> Binding Column-III fit in a 2 ml collection tube. Close the lid gently and centrifuge at ≥14,000 rpm for 20 seconds. Discard the flow through<sup>†</sup>. Reuse the collection tube in step 2.

\* **Note:** If the sample volume exceeds 700 µl, centrifuge successive aliquots in the same *AccuPrep*<sup>®</sup> Binding Column-III and discard the flow through.

<sup>†</sup> Discard the flow through after each centrifugation.

2. **(1<sup>st</sup> Washing)** Wash the *AccuPrep*<sup>®</sup> Binding Column-III by adding 700 µl of RWA1 Buffer.

3. Close the lid gently and centrifuge at 14,000 rpm for 20 seconds. Discard the flow through. Reuse the collection tube in step 4.

\* **Note:** After centrifugation, carefully remove the *AccuPrep*<sup>®</sup> Binding Column-III from the collection tube so that the column does not contact the flow through.

4. **(2<sup>nd</sup> Washing)** Wash the *AccuPrep*<sup>®</sup> Binding Column-III by adding 500 µl of RWA2 Buffer.

5. Close the lid gently and centrifuge at 14,000 rpm for 20 seconds. Discard the flow through. Reuse the collection tube in step 6.

6. Wash the *AccuPrep*<sup>®</sup> Binding Column-III by adding 500 µl of RWA2 Buffer.

7. Close the lid gently and centrifuge at 14,000 rpm for 2 minutes. Discard the flow through. Reuse the collection tube in step 8. The long centrifugation dries the spin column membrane, ensuring that no ethanol is carried over during RNA elution.

\* **Note:** Residual ethanol may interfere with downstream reactions. After centrifugation, carefully remove the *AccuPrep*<sup>®</sup> Binding Column-III from the collection tube so that the column does not contact the flow through.

8. Centrifuge once more at 14,000 rpm for 1 minute to remove residual ethanol completely and check whether there is no droplet clinging to the bottom of the binding column.

9. **(Elution)** Place the *AccuPrep*<sup>®</sup> Binding Column-III in a clean 1.5 ml Tube (supplied with a kit). Add 50-200 µl of ER Buffer or RNase-free water to elute RNA.

10. Incubate at room temperature for 1 minute to be absorbed the ER Buffer completely into the glass fiber of the binding column.
11. Close the lid gently and centrifuge at 10,000 rpm for 1 minute.
12. To recover more RNA (>30 µg), repeat once more elution step using the eluate from step 11.
13. Close the lid gently and centrifuge at 10,000 rpm for 1 minute.
14. **(Note)** DNase treatment must be performed on the sample to confirm accurate mRNA expression.

## RNA Clean-Up

1. Adjust the sample to a volume of 100 µl with RNase-free water. Add 400 µl RB Buffer and mix well.
2. Add 300 µl of 80% ethanol to the diluted RNA and mix well by pipetting.  
\* **Note:** Do not centrifuge.
3. Transfer the sample to the *AccuPrep*<sup>®</sup> Binding Column-III fit in a 2 ml collection tube. Close the lid gently and centrifuge at  $\geq 14,000$  rpm for 20 seconds. Discard the flow through<sup>†</sup>. Reuse the collection tube in step 4.  
\* **Note:** After centrifugation, carefully remove the *AccuPrep*<sup>®</sup> Binding Column-III from the collection tube so that the column does not contact the flow through.  
<sup>†</sup> Discard the flow through after each centrifugation.
4. Wash the *AccuPrep*<sup>®</sup> Binding Column-III by adding 500 µl of RWA2 Buffer.
5. Close the lid gently and centrifuge at 14,000 rpm for 2 seconds. Discard the flow through. Reuse the collection tube in step 6.
6. Wash the *AccuPrep*<sup>®</sup> Binding Column-III by adding 500 µl of RWA2 Buffer.
7. Close the lid gently and centrifuge at 14,000 rpm for 2 minutes. Discard the flow through. Reuse the collection tube in step 8. The long centrifugation dries the spin column membrane, ensuring that no ethanol is carried over during RNA elution.
8. Centrifuge once more at 14,000 rpm for 1 minute to remove residual ethanol completely and check whether there is no droplet clinging to the bottom of the binding column.
9. Place the *AccuPrep*<sup>®</sup> Binding Column-III in a clean 1.5 ml Tube (supplied with a kit). Add 50-200 µl of ER Buffer or RNase-free water to elute RNA.
10. Incubate at room temperature for 1 minute to be absorbed the ER Buffer completely into the glass fiber of the binding column.
11. Close the lid gently and centrifuge at 10,000 rpm for 1 minute.

## RNA Isolation with Magnetic Bead Type

*MagListo*<sup>™</sup> 5M Universal RNA Extraction Kit is designed for extraction of highly purified RNA from cultured cells, plant and animal tissues. The kit employs Magnetic Nano Beads to extract total RNA with the aid of *MagListo*<sup>™</sup> Magnetic Separation Rack and *ExiPrep*<sup>™</sup> 96 Lite (Cat. No. A-5250). The use of *MagListo*<sup>™</sup> Magnetic Separation Rack along with the kit greatly increases user convenience by shortening the extraction time without centrifugation. On the same principle, *ExiPrep*<sup>™</sup> 96 Lite is designed for rapid extraction of nucleic acids delivering up to 96 extracted samples (including controls).

RNA extracted through this kit can be used for a variety of applications, including: reverse transcription PCR (RT-PCR), reverse transcription quantitative PCR (RT-qPCR), northern blot analysis, and cDNA synthesis.

## Specifications

<i>MagListo</i> <sup>™</sup> 5M Universal RNA Extraction kit			
Amount of Starting Sample	Cultured cells		10 <sup>4</sup> -10 <sup>8</sup> cells
	Liver		25-50 mg
	Spleen		100 mg
Typical RNA Yield	Cultured cells		15-20 µg
	Liver		10-60 µg
	Spleen		30-60 µg
Typical RNA Yield	Scale	Mini	up to 100 µg
		Midi	up to 500 µg
Turnaround Time		Mini	< 10 min
		Midi	< 15 min
Elution Volume	Mini	50 µl	
	Midi	500 µl	
RNA Purity			$A_{260}/A_{280} > 2.0$ , $A_{260}/A_{230} > 1.7$
Isolation Technology			Magnetic Nano Bead

\* **Note:** There may be differences in measured values depending on the type of samples.

## Before You Begin

Before proceeding, please check the following:

1. Add 10 µl of β-mercaptoethanol per 1 ml of RD Buffer.
2. g-force can be calculated as follows:  $rcf = 1.12 \times r \times (rpm/1,000)^2$   
\* **Note:** Where 'rcf' is the relative centrifugal force (in g), 'r' is the radius of the rotor in centimeters, and rpm is the speed of the centrifuge in revolutions per min.

## Preparing Lysates from Animal Tissue

1. **(Lysis & homogenization)** Grind (or homogenize) 20-30 mg (mini)\* of fresh or thawed animal tissue sample with a mortar and pestle (or homogenizer) and place it into appropriate tubes.  
\* **Note:** The amount of sample required may vary depending on the extraction scale.
2. Add 500 µl (mini) / 5 ml (midi) of RD Buffer to the sample and mix thoroughly by vortexing.
3. Centrifuge at full speed for 3 minutes and carefully transfer the supernatant to new 1.5 ml tubes (mini) or 50 ml tubes (midi) with a pipette.
4. **(RNA precipitation)** Add 300 µl (mini) / 3 ml (midi) of absolute ethanol (not provided) to the lysate and mix immediately by pipetting.  
\* **Note:** Do not centrifuge.
5. Proceed immediately to “Purification Procedure Using Magnetic Nano Beads” on page 19.

### Preparing Lysates from Cultured Cells

1. **(Cell harvesting)** Harvest cells according to step 1-A or 1-B.

1-A. Suspension cell culture:

Harvest cultured cells ( $10^4$ - $10^6$  cells, mini)\* by centrifugation at  $300 \times g$  for 5 minutes to pellet cells. Discard the supernatant carefully without disturbing a cell pellet and go to step 2.

\* **Note:** The amount of sample required may vary depending on the extraction scale.

1-B. Monolayer cell culture:

There are two different methods to collect cells grown in monolayer culture.

a. Direct cell harvesting on the culture dishes:

Completely discard the cell culture medium and go to step 2.

\* **Note:** You should completely remove the cell culture medium because it may inhibit the RNA extraction.

b. Cell harvesting with trypsin:

Remove the cell culture medium and wash the cell monolayer with DPBS. Add 0.1-0.25% trypsin to the washed cell monolayer to detach the cells. After the cells detach, add cell culture medium. Transfer the cells to an RNase-free tube (not provided) and centrifuge at  $300 \times g$  for 5 minutes. Discard the supernatant carefully and go to step 2.

2. **(Lysis & homogenization)** Resuspend the cell pellet from step 1 in 500  $\mu$ l (mini) / 5 ml (midi) of RD Buffer by vortexing.

\* **Note:** You should completely resuspend the sample to achieve maximum lysis efficiency.

3. **(RNA precipitation)** Add 300  $\mu$ l (mini) / 3 ml (midi) of absolute ethanol<sup>†</sup> (not provided) to the lysate and mix immediately by pipetting.

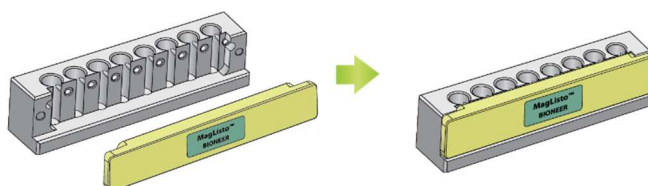
\* **Note:** Do not centrifuge.

<sup>†</sup> When purifying RNA from certain cell lines, precipitates may be visible after addition of ethanol. This does not affect the procedure.

4. Proceed immediately to “Purification Procedure Using Magnetic Nano Beads” on page 19.

## Purification Procedure Using Magnetic Nano Beads

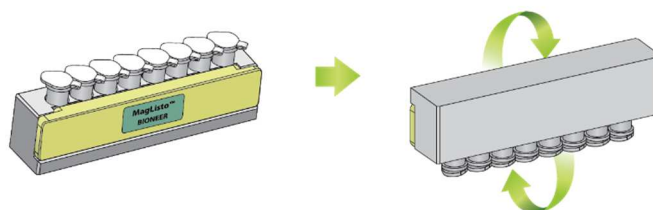
1. **(RNA binding)** Add 100 µl (mini) / 1 ml (midi) of Magnetic Nano Bead solution to each tube and mix thoroughly by vortexing until the beads are fully resuspended.
2. Place the tube in *MagListo*<sup>TM</sup>-2 (mini) or *MagListo*<sup>TM</sup>-50 (midi) Magnetic Separation Rack with the magnet plate attached and invert the rack gently 3-4 times until the beads bind tightly to the magnet.



**Figure 7. Attachment of the magnet plate.** Combine the magnet plate to the stand.

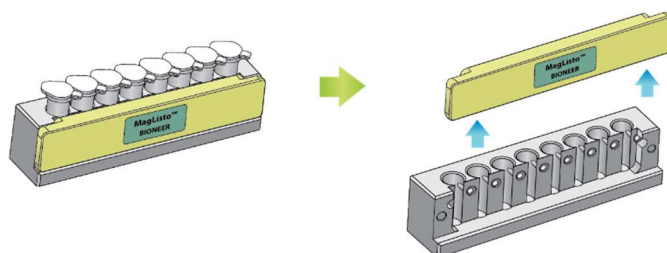
3. Without removing the tube from *MagListo*<sup>TM</sup> Magnetic Separation Rack, discard the supernatant carefully and completely remove the remaining supernatant using a paper towel by blotting. During this process, the beads containing RNA remain attached to the side of the tube.

\* **Note:** If you want to perform the optional RNA Clean-Up, follow the steps on page 22 after performing this step.



**Figure 8. How to discard the supernatant.** Discard the supernatant by inverting the *MagListo*<sup>TM</sup> Magnetic Separation Rack. The silicone immobilizer inside the stand prevents the tubes from falling. When discarding the supernatant, invert the rack completely so that the solution does not spill on the rack.

4. **(1<sup>st</sup> Washing)** Detach the magnet plate from *MagListo*<sup>TM</sup> Magnetic Separation Rack. Add 800 µl (mini) or 8 ml (midi) of RWM1 Buffer to each tube and close the cap. Mix by vortexing until the beads are fully resuspended.

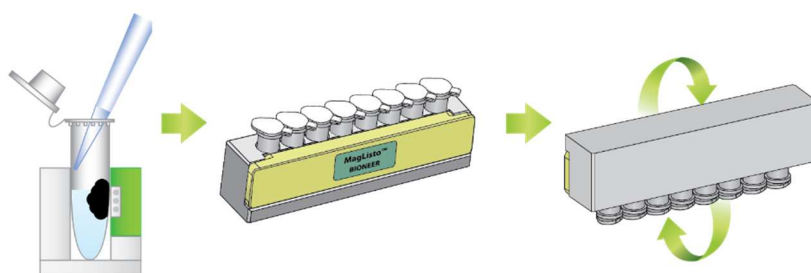


**Figure 9. Detachment of the magnet plate.** Pull the magnet plate up and gently separate it.

5. Attach the magnet plate to stand and invert the rack gently 3-4 times until the beads bind tightly to the magnet.
6. Without removing the tubes from *MagListo*<sup>™</sup> Magnetic Separation Rack, discard the supernatant carefully and completely remove the remaining supernatant using a paper towel by blotting.
7. (**2<sup>nd</sup> Washing**) Repeat steps 4-6 by adding 800  $\mu$ l (mini) or 8 ml (midi) of RWA2 Buffer for additional washing. Repeat steps 5-6 once more.
8. (**3<sup>rd</sup> Washing**) Remove residual ethanol according to step 8-A or 8-B.

### 8-A. Washing beads:

Without removing the tube from *MagListo*<sup>™</sup> Magnetic Separation Rack, add 700  $\mu$ l (mini) or 10 ml (midi) of WE Buffer to the opposite side of beads. Close the cap and invert the rack twice in order to remove ethanol from the sample. Discard the supernatant carefully and completely remove the remaining supernatant using a paper towel by blotting.



**Figure 10. Washing the beads to remove residual ethanol.** Please refer to the information above.

\* **Note:** Direct pipetting of WE Buffer onto the beads, vortexing and/or vigorous shaking of the tubes may



release nucleic acid from the beads, which may result in lower RNA yield.

8-B. Drying beads:

Add 800 µl of 80% ethanol, mix thoroughly by vortexing, and repeat the steps 5-6.

Completely dry the beads with the tube open at 60°C for at least 5 minutes. Remove the remaining supernatant with a pipette.

9. **(Elution)** Detach the magnet plate from *MagListo*<sup>™</sup> Magnetic Separation Rack. Add 50-100 µl (mini) or 500 µl-1 ml (midi) of ER Buffer to each tube and resuspend RNA by vortexing or pipetting.

10. Incubate at 55-65°C for 1 minute.

11. Attach the magnet plate to *MagListo*<sup>™</sup> Magnetic Separation Rack and invert the rack gently 3-4 times until the beads bind tightly to the magnet.

12. Without removing the tube from *MagListo*<sup>™</sup> Magnetic Separation Rack, transfer supernatant containing RNA carefully to a new tube.

13. Discard the tubes with the remaining beads.

\* **Note:** Do not reuse the beads.

14. **(Note)** DNase treatment must be performed on the sample to confirm accurate mRNA expression.

### RNA Clean-Up

1. Adjust the sample to a volume of 100  $\mu$ l with RNase-free water.
  - \* **Note:** If DNA-free RNA is required, add RNase-free DNase and DNase reaction buffer to each tube and adjust the volume up to 100  $\mu$ l with RNase-free water. Incubate at room temperature for 10 min.
2. Add 100  $\mu$ l of RD Buffer and mix well by pipetting.
3. Add 200  $\mu$ l of absolute ethanol and mix well by pipetting.
4. Add 100  $\mu$ l of Magnetic Nano Beads and mix well by pipetting until the beads are fully resuspended.
  - \* **Note:** Please mix well Magnetic Nano Beads by vortexing before use.
5. Place the tube in *MagListo*<sup>TM</sup>-2 (mini) Magnetic Separation Rack with the magnet plate attached and invert the rack gently 3-4 times until the beads bind tightly to the magnet.
6. Without removing the tube from *MagListo*<sup>TM</sup> Magnetic Separation Rack, discard the supernatant carefully and completely remove the remaining supernatant using a paper towel by blotting.
7. Go to step 4 of Purification Procedure Using Magnetic Nano Beads on page 19.

## Reverse Transcription

1. Add template RNA (0.4-1 µg for total RNA) and nuclease-free water into *AccuPower*<sup>®</sup> *RocketScript*<sup>™</sup> Cycle RT PreMix (dT<sub>20</sub>) (Cat. No. K-2201, not provided) tubes to make a total volume of 20 µl or 50 µl. Do not include the dried pellet.
2. Dissolve the vacuum-dried pellet by vortexing or pipetting, and briefly spin down.
3. Perform the reaction under the following conditions.

### 1) CTRT reaction (Example 1)

Step	Temperature	Time	Cycles
Primer annealing	37°C	10-30 sec	10 cycles or more
cDNA synthesis	50°C	4 min	
Melting secondary structure & cDNA synthesis	55-60°C	30 sec	
Heat inactivation	95°C	5 min	1 cycle

### 2) CTRT reaction (Example 2)

Step	Temperature	Time	Cycles
Primer annealing	37°C	1 min	10 cycles or more
Melting secondary structure & cDNA synthesis	42-70°C	4 min	
Heat inactivation	95°C	5 min	1 cycle

### 3) Single temperature reaction (Example 3)

Step	Temperature	Time	Cycles
cDNA synthesis	22-55°C*	30-60 min	1 cycle
Heat inactivation	95°C	5 min	1 cycle

\* **Note:** Recommended temperature is range of 42-48°C.

4. After the reaction, maintain the reaction mixture at 4°C. The samples can be stored at -20°C until use.

### Real-time PCR

1. Add 100 µl of nuclease-free water into *AccuPower*<sup>®</sup> qPCR Array System: Human 5-plex Reference qPCR primer and probe set tubes (A, B, and C), which can be used for 20 reactions.

\* **Note:** This product can be diluted according to the user's purpose.

2. Prepare template DNA, *AccuPower*<sup>®</sup> Plus *DualStar*<sup>™</sup> qPCR Master Mix (K-6603, not provided), primer and probe set, 50X ROX dye (optional), and nuclease-free water into real-time PCR plate to make a total volume of 50 µl as described in following table.

Components	50 µl reaction
<i>AccuPower</i> <sup>®</sup> Plus <i>DualStar</i> <sup>™</sup> qPCR Master Mix	25 µl
Template DNA	10 pg-100 ng
Human 5-plex Reference qPCR primer and probe set	5 µl
(Optional) 50X ROX dye*	1 µl
Nuclease-free water	Variable
Total volume	50 µl

\* **Note:** ROX dye is used for normalization of intensity by background subtraction. The use of ROX dye is recommended for Applied Biosystems 7500 Real-Time PCR System, but not required for BIONEER *Exicycler*<sup>™</sup> 96 Real-Time PCR System.

3. Seal the plate with adhesive optical sealing film (Cat. No. 3111-4110, not provided) and briefly spin down.

4. Perform the reaction under the following conditions.

Step	Temperature	Time	Cycles
Pre-denaturation	95°C	10 min	1 cycle
Denaturation	95°C	5 sec	45 cycles
Annealing/Extension	58°C	15 sec	
Detection	Scan		

5. After the reaction, perform data analysis.

## Data Analysis

Two most commonly used methods to analyze data from qPCR are absolute quantification and relative quantification. Absolute quantification determines the input copy number, usually by calculating the PCR signal on the basis of a standard curve. Relative quantification relates the PCR signal of the target transcript in a treatment sample to that of an untreated control sample. The  $2^{-\Delta\Delta Ct}$  method is a reasonable way to analyze the relative changes in gene expression from real-time quantitative PCR (qPCR) experiments.

<ul style="list-style-type: none"><li>- <math>\Delta Ct = Ct_{[\text{target gene}]} - Ct_{[\text{reference gene}]}</math></li><li>- <math>\Delta\Delta Ct = \Delta Ct_{[\text{treated sample}]} - \Delta Ct_{[\text{control sample}]}</math></li><li>- Fold Change = <math>2^{-\Delta\Delta Ct}</math></li></ul>
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- $\Delta Ct$ : the difference between Ct value of target gene and Ct value of reference gene.
- $\Delta\Delta Ct$ : the difference between average Ct value of treated sample and average of Ct value of control sample.
- $2^{-\Delta\Delta Ct}$ : fold change in gene expression of the treated sample compared to the untreated control sample.

### References

Bustin, S. A. (2000). Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *Journal of molecular endocrinology*, 25(2), 169-193.

Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., ... & Wittwer, C. T. (2009). The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments.

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the  $2^{-\Delta\Delta CT}$  method. *methods*, 25(4), 402-408.





## Ordering Information

Description		Cat. No
<i>AccuPower</i> <sup>®</sup> qPCR Array System: Human 5-plex Reference qPCR primer and probe set	20 rxn	S-6043-SH0

## Related Products

Description		Cat. No
<i>AccuPrep</i> <sup>®</sup> Universal RNA Extraction Kit		K-3141
<i>MagListo</i> <sup>™</sup> 5M Universal RNA Extraction Kit		K-3613
<i>MagListo</i> <sup>™</sup> -2 Magnetic Separation Rack		TM-1010
<i>MagListo</i> <sup>™</sup> -50 Magnetic Separation Rack		TM-1030
<i>AccuPower</i> <sup>®</sup> <i>RocketScript</i> <sup>™</sup> Cycle RT PreMix	dT <sub>20</sub>	K-2201
	dN <sub>6</sub>	K-2205
	dN <sub>12</sub>	K-2208
<i>AccuPower</i> <sup>®</sup> Plus <i>DualStar</i> <sup>™</sup> qPCR Master Mix		K-6603
<i>ExiPrep</i> <sup>™</sup> 96 Lite		A-5250
<i>AllInOneCycler</i> <sup>™</sup> PCR system		A-2041
<i>Exicycler</i> <sup>™</sup> 96		A-2060-1
<i>AccuPower</i> <sup>®</sup> qPCR Array System: Single gene qPCR Primer Set		S-6042-S200

### Explanation of Symbols

 Caution	 Consult Instructions For Use	 Do not Re-use	 Use-by Date
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