AccuPower® qPCR Array System: Human Immune Checkpoint qPCR Panel Kit

Cat. No. S-6042-PH3

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AccuPower® qPCR Array System: Human Immune Checkpoint qPCR Panel Kit

User Guide

S-6042-PH3

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Please read all the information in booklet before using the unit

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Intended Use

AccuPower® qPCR Array System: Human Immune Checkpoint qPCR Panel Kit 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

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Product Information

Components

Components	Amount
AccuPower® qPCR Array System: Human Immune Checkpoint qPCR Panel Kit (96 well plate)	1 plate
Adhesive Optical Sealing Film	1 sheet per plate

Storage

This product is lyophilized and shipped at ambient temperature. Store at room temperature without direct sunlight for long term storage. If stored in the recommended temperature, this product will be stable for 2 years after the delivery date.

Plate Map

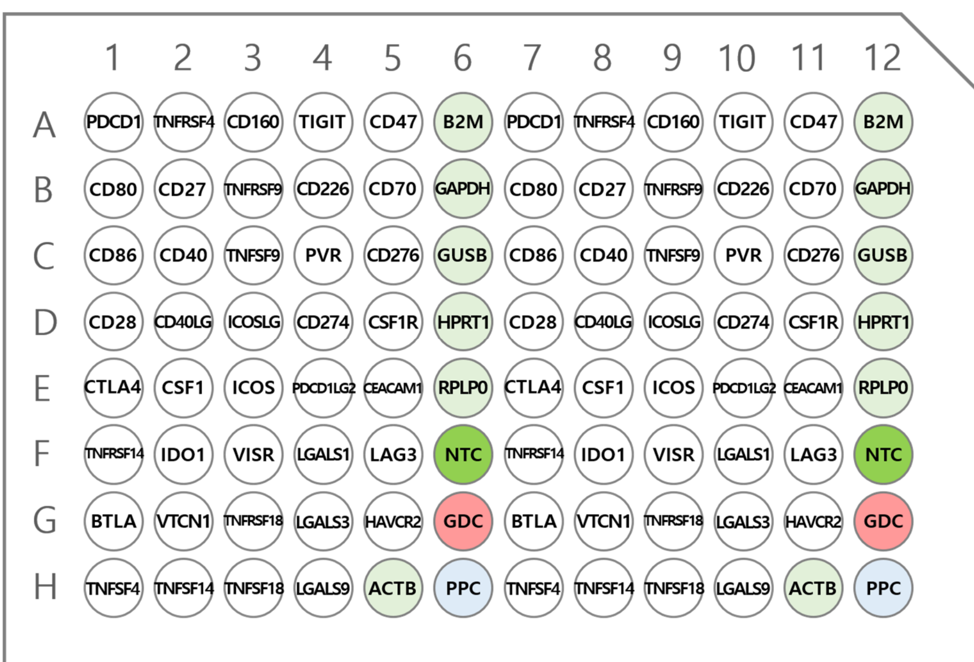

Figure 1. Layout of *AccuPower*® qPCR Array System: Human Immune Checkpoint qPCR Panel Kit. The panel is involved 39 target genes (A1-G5, A7-G11), 6 reference genes (H5-E6, H11-E12), and 3 control primers (F6-H6, F12-H12).

Control Primers

1. Non-Template Control (NTC)

- NTC is a negative control for checking on random or on reagent contamination.
- Just add the pre-mixture containing nuclease-free water but excluding the template into NTC well.
- If the value of Ct^{NTC} is less than 35, there is overall DNA contamination in your PCR system. In this case, clean up the equipment and replace all the reagents to new ones.

2. Genomic DNA Control (GDC)

- GDC primer is for the detection of non-transcribed genomic DNA contamination.
- In GDC well, primers which target genomic DNA are coated.
- Add pre-mixture (your template, 2X Master Mix, and nuclease free water) into the GDC well.
- If the value of Ct^{GDC} is less than 35, gDNA contamination might have occurred in your RNA samples. In this case, you ought to conduct an additional DNase treatment to clean up your samples.

3. Positive PCR Control (PPC)

- PPC primer is for the PCR test.
- The PPC well contains positive template and primers, so just add 2X Master Mix and nuclease-free water into the PPC well.
- The value of Ct^{PPC} should be referred to the quick manual provided together.

Gene Table

#	Gene symbol	Description
1	PDCD1	Programmed cell death 1
2	CD80	CD80 molecule
3	CD86	CD86 molecule
4	CD28	CD28 molecule
5	CTLA4	Cytotoxic T-lymphocyte-associated protein 4
6	TNFRSF14	Tumor necrosis factor receptor superfamily, member 14
7	BTLA	B and T lymphocyte associated
8	TNFSF4	Tumor necrosis factor (ligand) superfamily, member 4
9	TNFRSF4	Tumor necrosis factor receptor superfamily, member 4
10	CD27	CD27 molecule
11	CD40	CD40 molecule, TNF receptor superfamily member 5
12	CD40LG	CD40 ligand
13	CSF1	Colony stimulating factor 1
14	IDO1	Indoleamine 2,3-dioxygenase 1
15	VTCN1	V-set domain containing T cell activation inhibitor 1
16	TNFSF14	tumor necrosis factor superfamily member 14 [Homo sapiens (human)]
17	CD160	CD160 molecule
18	TNFRSF9	tumor necrosis factor receptor superfamily member 9
19	TNFSF9	tumor necrosis factor superfamily member 9
20	ICOSLG	inducible T-cell co-stimulator ligand
21	ICOS	inducible T-cell co-stimulator
22	VISR	V-set immunoregulatory receptor
23	TNFRSF18	tumor necrosis factor receptor superfamily member 18
24	TNFSF18	tumor necrosis factor superfamily member 18

25	TIGIT	T-cell immunoreceptor with Ig and ITIM domains
26	CD226	CD226 molecule
27	PVR	Poliovirus receptor
28	CD274	CD274 molecule
29	PDCD1LG2	Programmed cell death 1 ligand 2
30	LGALS1	Lectin, galactoside-binding, soluble, 1
31	LGALS3	Lectin, galactoside-binding, soluble, 3
32	LGALS9	Lectin, galactoside-binding, soluble, 9
33	CD47	CD47 molecule
34	CD70	CD70 molecule
35	CD276	CD276 molecule
36	CSF1R	Colony stimulating factor 1 receptor
37	CEACAM1	Carcinoembryonic antigen-related cell adhesion molecule 1 (biliary glycoprotein
38	LAG3	Lymphocyte-activation gene 3
39	HAVCR2	Hepatitis A virus cellular receptor 2

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*® qPCR Array System: Human Immune Checkpoint qPCR Panel Kit 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.

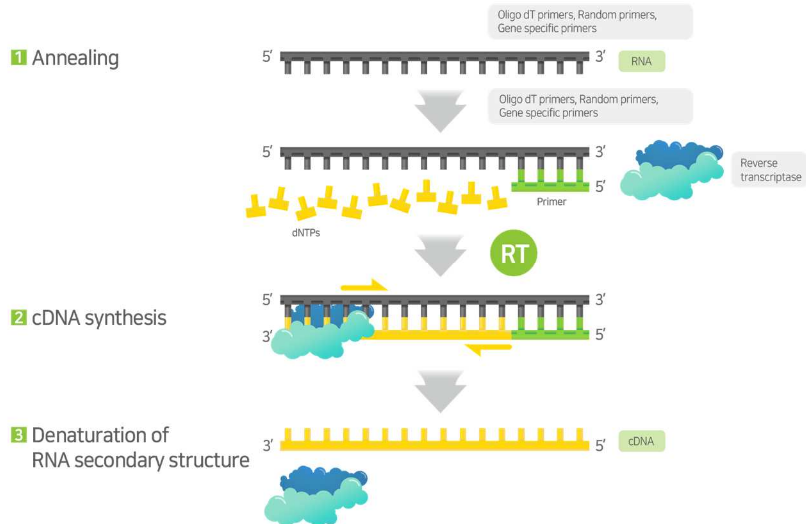

Figure 2. Procedures of reverse transcription.

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 Immune Checkpoint qPCR Panel Kit 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.

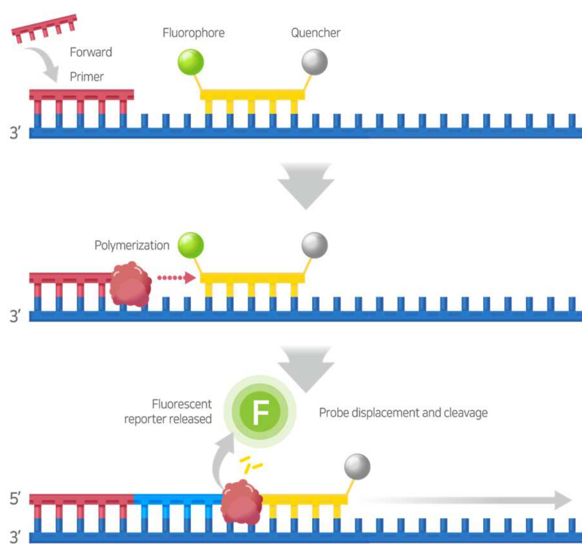

Figure 3. Overview of real-time PCR using hydrolysis probe method.

Primer design for qPCR

Primer & probe sets of Human Immune Checkpoint qPCR Panel Kit 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 (70-150 bp).
- 90-110% optimal qPCR efficiency.
- Probe with higher Tm value compared to the given primer.
- Detection of expected size with single band via gel electrophoresis.

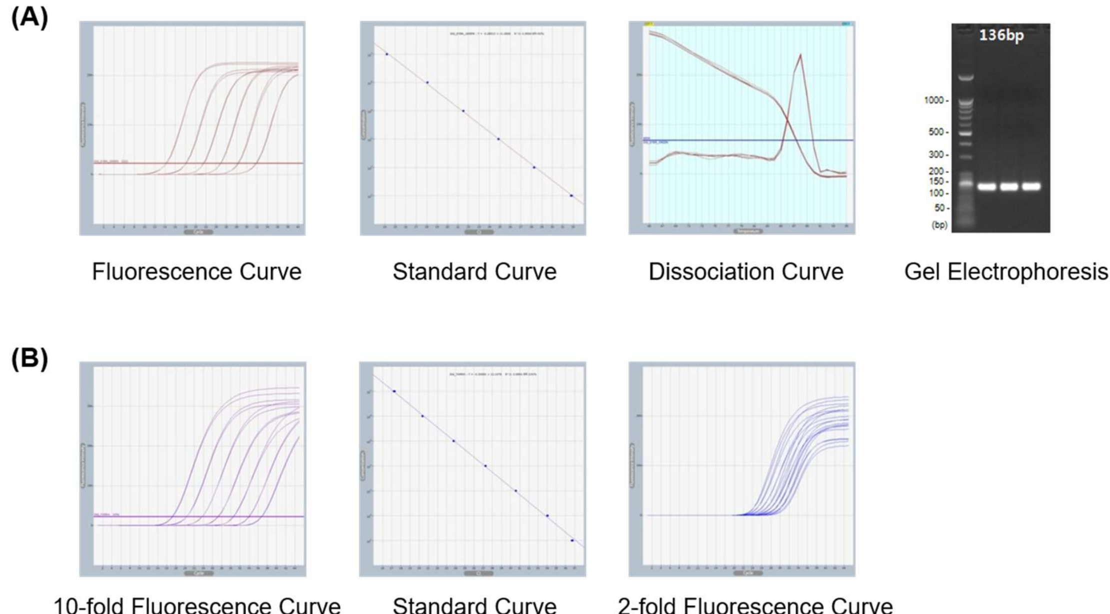

Figure 4. Validation data of primer and hydrolysis probe. (A) Confirmation of target specificity and high amplification efficiency using *AccuPower*® 2X *GreenStar*™ qPCR Master Mix (Cat. No. K-6253). Dynamic range: 10^7-10^2, R^2=0.99, qPCR efficiency: 93%. **(B)** Confirmation of high amplification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

Product Description

The *AccuPower*® qPCR Array System: Human Immune Checkpoint qPCR Panel Kit is designed to screen human immune checkpoint relevant genes in real-time PCR. BIONEER offers 39 Human Immune Checkpoint relevant genes, 6 reference genes and 3 control primers, and 39 primer and probe set in panel cover the majority of human immune checkpoint genes. Since all primers are designed and validated in accordance with the MIQE guidelines, the results can be used for SCI paper publication and can precisely detect and quantify all the gene expression of your samples.

Human Immune Checkpoint qPCR Panel Kit is easy-to-use product as it simplifies preparation of real-time PCR mixture by making the user add the template DNA, 2X Master Mix (hydrolysis probe type), and nuclease-free water into the 96 well plate. Therefore, users can get a reliable data in a simple and convenient way.

Experimental Procedures

Start with isolating RNA from your experimental samples using *AccuPrep*® Universal RNA Extraction Kit (Cat. No. K-3141) or *MagListo*™ 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*® *RocketScript*™ Cycle RT PreMix (dT_{20}) (Cat. No. K-2201). Add equal volume of the synthesized cDNA and *AccuPower*® Plus *DualStar*™ 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*™ 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.

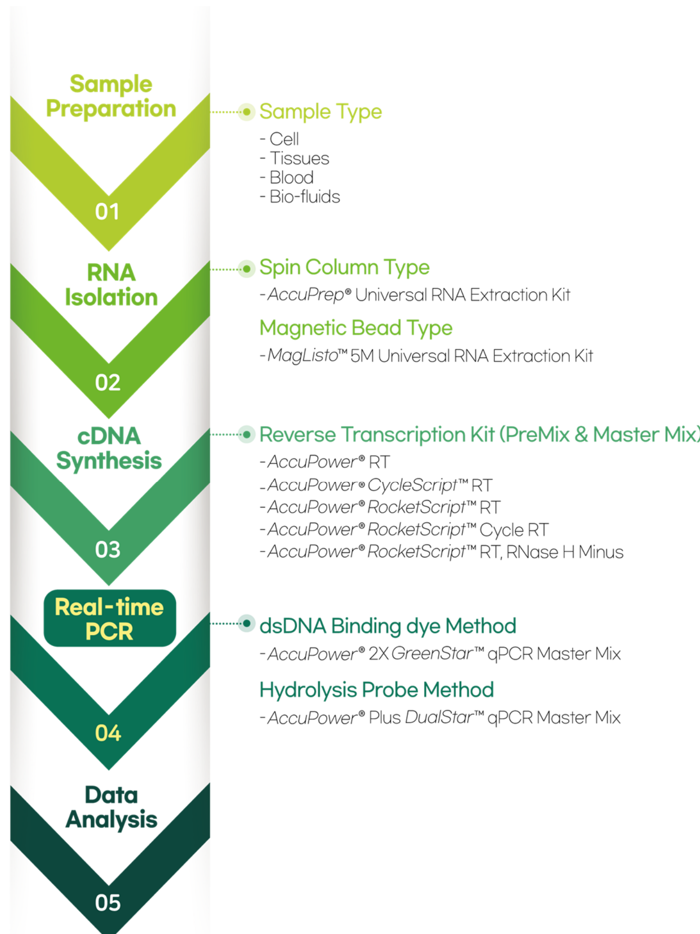

Figure 5. Schematic diagram of experimental procedures from sample preparation to data analysis.

Materials to be Prepared by Users

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

RNA Isolation with Spin Column Type

AccuPrep® 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®* 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

***AccuPrep®* Universal RNA Extraction Kit**		
Amount of Starting Sample	Cultured cells	10^4-10^8 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 x r x $(rpm/1,000)^2$
   * **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®* 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 14.

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† to the lysate and mix immediately by pipetting.

   * **Note:** Do not centrifuge.

   † 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 14.

Purification Procedure Using Spin Columns

1. **(RNA binding)** Transfer up to 700 μl of sample to the *AccuPrep®* 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†. Reuse the collection tube in step 2.

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

   † Discard the flow through after each centrifugation.

2. **(1st Washing)** Wash the *AccuPrep®* 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®* Binding Column-III from the collection tube so that the column does not contact the flow through.

4. **(2nd Washing)** Wash the *AccuPrep®* 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®* 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®* 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®* 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*® 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†. Reuse the collection tube in step 4.
   * **Note:** After centrifugation, carefully remove the *AccuPrep*® Binding Column-III from the collection tube so that the column does not contact the flow through.
   † Discard the flow through after each centrifugation.

4. Wash the *AccuPrep*® 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*® 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*® 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™ 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*™ Magnetic Separation Rack and *ExiPrep*™ 96 Lite (Cat. No. A-5250). The use of *MagListo*™ Magnetic Separation Rack along with the kit greatly increases user convenience by shortening the extraction time without centrifugation. On the same principle, *ExiPrep*™ 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

***MagListo*™ 5M Universal RNA Extraction kit**			
Amount of Starting Sample	Cultured cells		10^4-10^8 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 x r x $(rpm/1,000)^2$
   * **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 20.

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 x *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 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 500 µl (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 µl (mini) / 3 ml (midi) of absolute ethanol† (not provided) to the lysate and mix immediately by pipetting.

* **Note:** Do not centrifuge.

† 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 20.

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*™-2 (mini) or *MagListo*™-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.

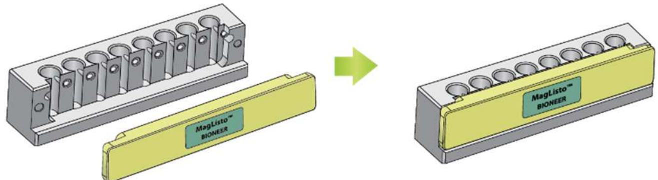

Figure 6. Attachment of the magnet plate. Combine the magnet plate to the stand.

3. Without removing the tube from *MagListo*™ 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 23 after performing this step.

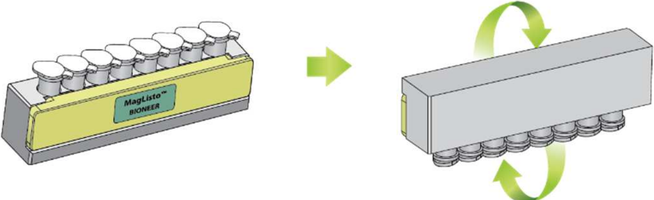

Figure 7. How to discard the supernatant. Discard the supernatant by inverting the *MagListo*™ Magnetic Separation Rack. The silicone immobilizer inside the stand prevents the tubes from falling. When discarding the supernatant, invert the rack completely so that the solution does not spill on the rack.

4. **(1st Washing)** Detach the magnet plate from *MagListo*™ 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.

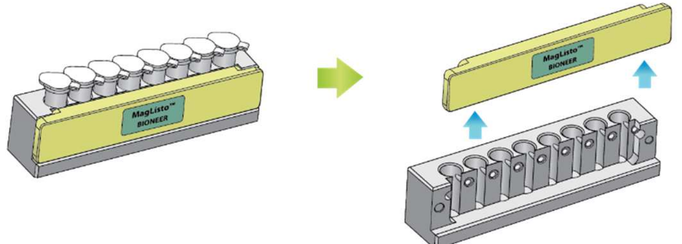

Figure 8. Detachment of the magnet plate. Pull the magnet plate up and gently separate it.

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*™ Magnetic Separation Rack, discard the supernatant carefully and completely remove the remaining supernatant using a paper towel by blotting.

7. **(2nd Washing)** Repeat steps 4-6 by adding 800 μl (mini) or 8 ml (midi) of RWA2 Buffer for additional washing. Repeat steps 5-6 once more.

8. **(3rd Washing)** Remove residual ethanol according to step 8-A or 8-B.

8-A. Washing beads:

Without removing the tube from *MagListo*™ Magnetic Separation Rack, add 700 μl (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.

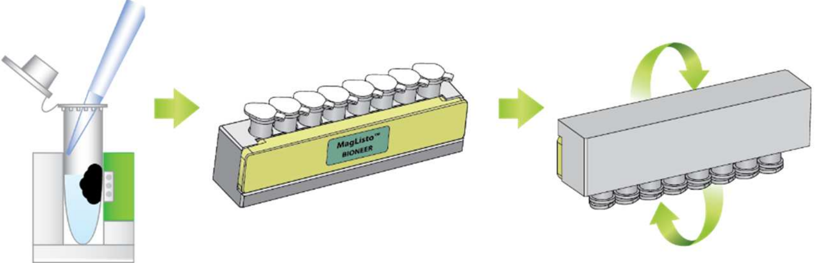

Figure 9. Washing the beads to remove residual ethanol. Please refer to the information above.

*** 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*™ 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*™ 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*™ 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 µl 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 µl with RNase-free water. Incubate at room temperature for 10 min.

2. Add 100 µl of RD Buffer and mix well by pipetting.

3. Add 200 µl of absolute ethanol and mix well by pipetting.

4. Add 100 µl 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*™-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*™ 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 20.

Reverse Transcription

1. Add template RNA (0.4-1 μg for total RNA) and nuclease-free water into *AccuPower® RocketScript*™ Cycle RT PreMix (dT_{20}) (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. Prepare template DNA, *AccuPower*® Plus *DualStar*™ qPCR Master Mix (K-6603, not provided), 50X ROX dye, and nuclease-free water in a tube to make a total volume of 50 μl as described in following table.

Components	50 μl reaction
AccuPower® Plus *DualStar*™ qPCR Master Mix	25 μl
Template DNA	5 pg-100 ng
(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*™ 96 Real-Time PCR System.

2. Carefully remove the covered film of panel and dispense 50 μl of reaction mixture into each well of *AccuPower*® qPCR Array System: Human Immune Checkpoint qPCR Panel Kit.
   * **Note:** Change pipette tips following each pipetting step to avoid cross-contamination among the wells.

3. Seal the plate with adhesive optical sealing film (Cat. No. 3111-4110) and briefly spin down.

4. Then, completely mix by vortexing to resuspend lyophilized primers and spin down again.
   * **Note:** Before start, check carefully if there are residues on the film.

5. Perform the reaction under the following conditions.

Step	Temperature	Time	Cycles
Pre-denaturation	95°C	5 min	1 cycle
Denaturation	95°C	5 sec	45 cycles
Annealing/Extension	58°C	15 sec	
Detection	Scan		
Melting	20°C	1 min	1 cycle

6. 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 basic 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.

- $\Delta Ct = Ct_{[target\ gene]} - Ct_{[reference\ gene]}$
- $\Delta\Delta Ct = \Delta Ct_{[treated\ sample]} - \Delta Ct_{[control\ sample]}$
- Fold Change = $2^{-\Delta\Delta Ct}$

- ΔCt: 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
AccuPower® qPCR Array System: Immune Checkpoint qPCR Panel Kit	39 genes	S-6042-PH3

Related Products

Description		Cat. No
AccuPrep® Universal RNA Extraction Kit		K-3141
MagListo™ 5M Universal RNA Extraction Kit		K-3613
MagListo™-2 Magnetic Separation Rack		TM-1010
MagListo™-50 Magnetic Separation Rack		TM-1030
AccuPower® *RocketScript*™ Cycle RT PreMix	dT_{20}	K-2201
	dN_6	K-2205
	dN_{12}	K-2208
AccuPower® Plus *DualStar*™ qPCR Master Mix		K-6603
ExiPrep™ 96 Lite		A-5250
AllInOneCycler™ PCR system		A-2041
Exicycler™ 96		A-2060-1
AccuPower® 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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