USER GUIDE

AccuPower® qPCR Array System: Mouse Reference qPCR primer A set

Cat. No. S-6042-TM0A

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User Guide

S-6042-TM0A

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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: Mouse Reference qPCR primer A 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

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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.

Contents

Product Information

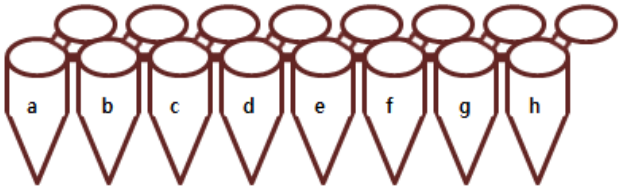
Components

Components		Amount
AccuPower® qPCR Array System: Mouse Reference qPCR primer A set	8-tube strip x 2	0.3 nmol

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.

Gene Table



#	Gene symbol	Accession #	Description	Also known as
a	Actb	NM_007393	Actin, beta	Actx, beta-actin, E430023M04Rik
b	B2m	NM_009735	Beta-2 microglobulin	Ly-m11, beta2m, beta2-m
c	Gapdh	NM_001289726 NM_008084	Glyceraldehyde-3-phosphate dehydrogenase	Gapd
d	Gusb	NM_010368	Glucuronidase, beta	Gur, Gus, Gut, asd, Gus-r, Gus-s, Gus-t, Gus-u, AI747421
e	Hprt	NM_013556	Hypoxanthine guanine phosphoribosyl transferase	HPGRT, Hprt1, C81579
f	Ppia	NM_008907	Peptidylprolyl isomerase A	Cphn, CypA, CyP-18, 2700098C05
g	Rpl13a	NM_009438	Ribosomal protein L13A	Tstap198-7, tum-antigen, 1810026N22Rik
h	Tbp	NM_013684	TATA box binding protein	Gtf2d, SCA17, TFIID, GTF2D1

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: Mouse Reference qPCR primer A 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.

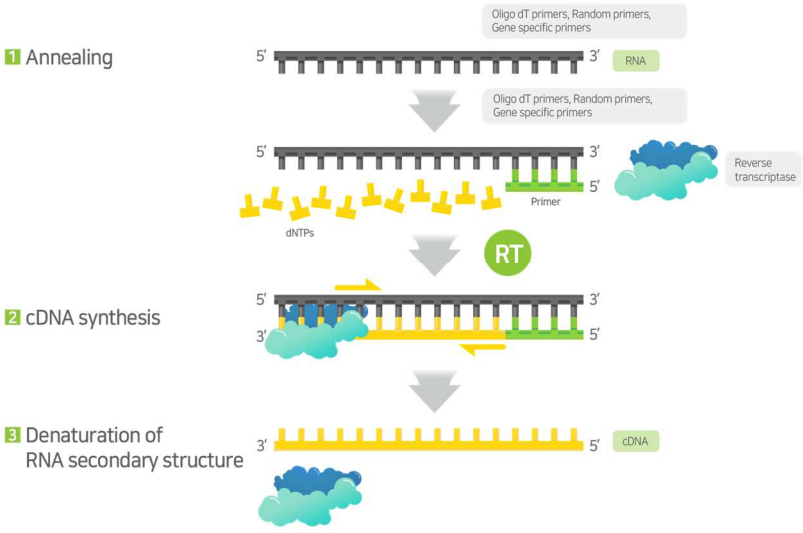


Figure 1. Procedures of reverse transcription.

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

Intercalating dye-based method (dsDNA binding dye type)

Intercalating dye-base method is popular detection method in real-time PCR. Intercalating dye (ex. SYBR® Green) specifically binds to double-stranded DNA produced by DNA polymerase during PCR and emits fluorescence, resulting in an increase in fluorescence intensity up to a thousand times proportional to the amount of PCR products, which provides the basis for the mRNA quantification of target genes.

With intercalating dye-based method, there is no need to purchase expensive probes (e.g., TaqMan®), which reduces experimental costs and has the advantage of relatively simple primer design.

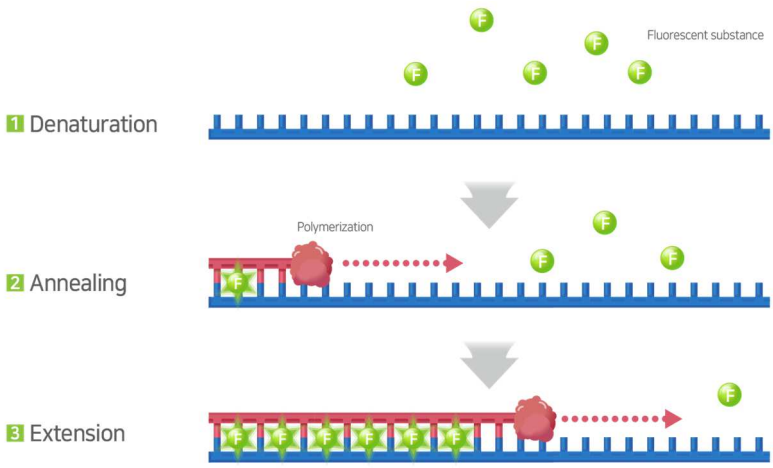


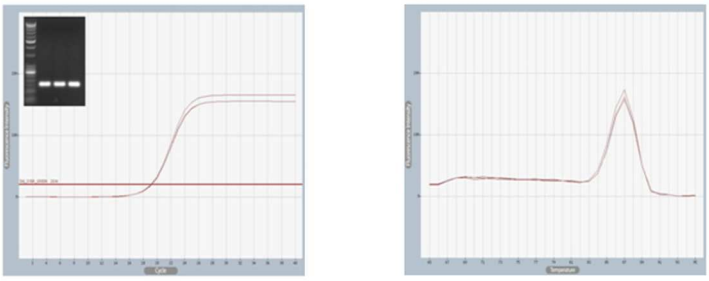
Figure 2. Overview of real-time PCR using intercalating dye-based method.

On the other hand, the greatest issue with intercalating dyes is their non-specificity. The dyes indiscriminately bind to all double-stranded DNA molecules not only target specific amplified PCR products but also non-specific artifacts (such as non-specific target or primer-dimer). Thus, well designed primers are necessary to avoid non-specific binding and primer dimerization. And melting curve analysis should be performed to exclude primer-dimer.

Primer design for qPCR

Primers of the Mouse Reference qPCR primer A 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 for qPCR on the basis of MIQE guidelines and all primers meet the followings. Specific primer designing using primer Blast (NCBI) and BIONEER's bioinformatics tool.

- Designing primers longer than 19 bases.
- Specific dissociation curves.
- Detection of expected size with single band via gel electrophoresis.
- Short amplicon size (70-150 bp).



Fluorescence curve Standard Curve

Figure 3. Primer validation result (e.g., RPL13A gene).

PCR efficiency test

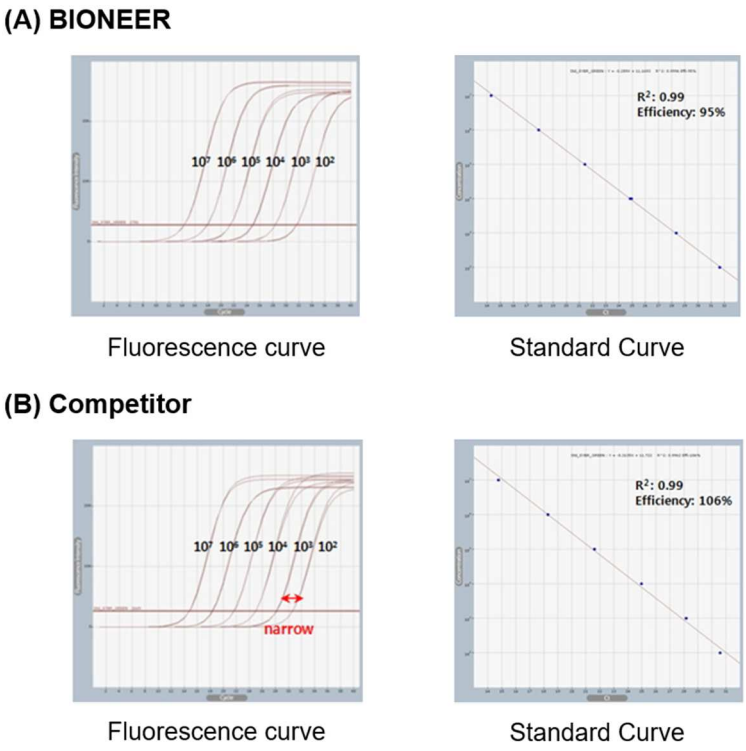


Figure 4. PCR efficiency test of a purified qPCR product with 10-fold dilutions. (A) Dynamic range & PCR efficiency (e.g. GAPDH gene). It corresponds to the qPCR efficiency range recommended by the MIQE guideline (100% ±10%). Limit of detection (LOD) is found at 100 copies or less. **(B)** Dynamic range & PCR efficiency (e.g. GAPDH gene). It corresponds to the qPCR efficiency range recommended by the MIQE guideline (100% ±10%). An irregular gap was observed between 10^3 and 10^2 copies, showing limit of detection (LOD) at 103 copies or less. This implies that its detection efficiency is lower than our company's products.

Target specificity test

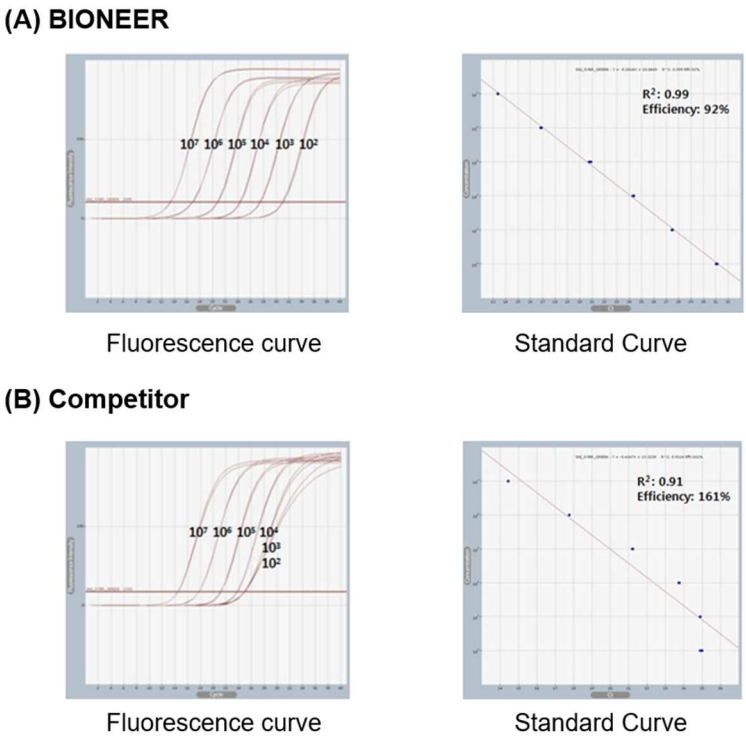


Figure 5. Target specificity test with the 10-fold serial dilution of purified qPCR product and efficiency test of reference mouse cDNA (1 µg mouse total RNA/20 µl). (A) Target specificity test using Mouse template (e.g. ACTB gene). It can be seen that specificity for the target gene and qPCR efficiency are within the range recommended by the MIQE guideline (100% ±10%). Limit of detection (LOD) is found at 100 copies or less. **(B)** Target specificity test using Mouse template (e.g. ACTB gene). Aggregation is found in the efficiency fluorescence curve from 10^4 copy. The efficiency is 161%, which is out of the qPCR efficiency range recommended by the MIQE guideline (100% ±10%). This implies that its target specificity is lower than that of our company's.

Product Description

The *AccuPower*® qPCR Array System: Mouse Reference qPCR primer A set is constructed in 8-tube strip format to screen various reference genes for several times. Since all primers are designed and validated in accordance with the MIQE guidelines, the results can be used for SCI paper publication.

The Mouse Reference qPCR primer A set contains 8 reference genes. *AccuPower*® qPCR Array System: Mouse Reference qPCR primer set is composed of two types of set, A and B. Type A set contains reference genes' primers which are commonly used. Reference genes in Type B set are relatively not commonly used than Type A set. If the screening result from A set is poor, try to use B set to select more suitable reference genes.

The primer set is designed to provide high reproducibility and better sensitivity in experiments, along with significantly reducing nonspecific reactions. Add your template DNA, 2X Master Mix (intercalating dye type), and the primer from qPCR primer set into the 96 well plate and you 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*® 2X *GreenStar*™ qPCR Master Mix (Cat. No. K-6251) 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.

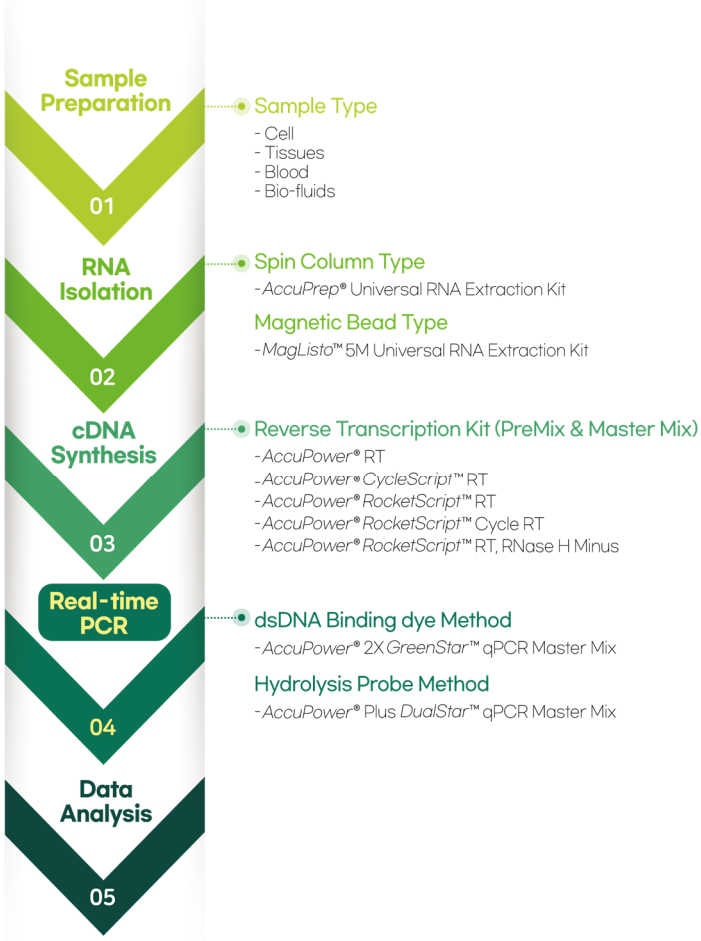


Figure 6. Schematic diagram of experimental procedures from sample preparation to data analysis.

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*® 2X *GreenStar*™ qPCR Master Mix (Cat. No. K-6251)
- 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.

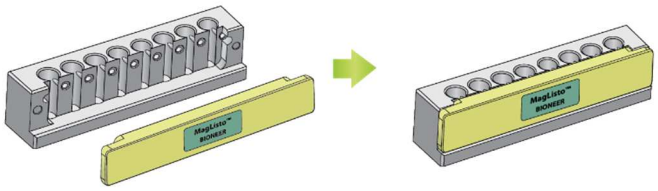


Figure 7. Attachment of the magnet plate. Combine the magnet plate to the stand.

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.

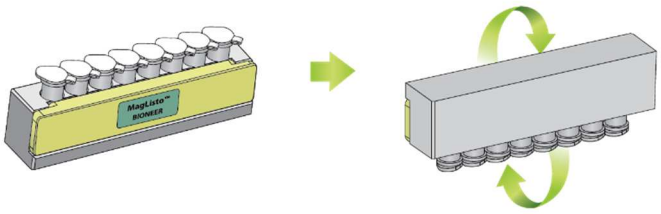


Figure 8. How to discard the supernatant. Discard the supernatant by inverting the *MagListo*™ Magnetic Separation Rack. The silicone immobilizer inside the stand prevents the tubes from falling. When discarding the supernatant, invert the rack completely so that the solution does not spill on the rack.

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.

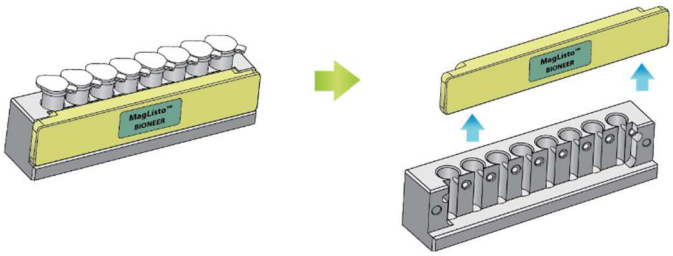


Figure 9. Detachment of the magnet plate. Pull the magnet plate up and gently separate it.

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*™ 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.

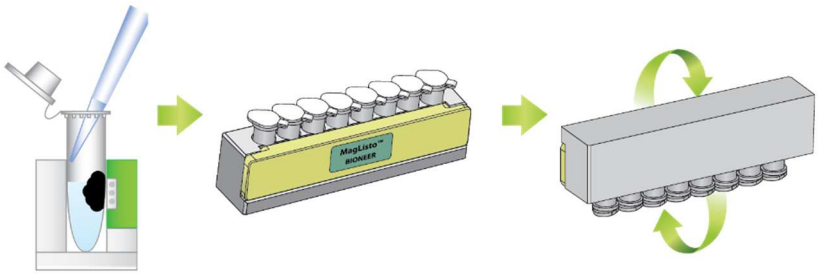


Figure 10. Washing the beads to remove residual ethanol. Please refer to the information above.

*** 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*™ 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. Dissolve primers in 50 µl of nuclease-free water to make a concentration of 3 pmol/µl.

2. Add template DNA, primers, nuclease-free water, and *AccuPower*® 2X *GreenStar*™ qPCR Master Mix (K-6251, not provided) into real-time PCR plate to make a total volume of 50 µl.

Components	50 µl reaction
AccuPower® 2X *GreenStar*™ qPCR Master Mix	25 µl
Template DNA	5 pg-100 ng
qPCR primers (3 pmol/µl)	5 µl
(Optional) 80X ROX dye	0.625-5 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.

3. Seal real-time PCR 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	40 cycles
Annealing	58°C	25 sec	
Extension	72°C	30 sec	
Detection	Scan		
Final extension	65°C	5 min	1 cycle
Melting	65-95°C	1 sec	-

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 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− ΔΔCT method. *methods*, *25*(4), 402-408.

Ordering Information

Description		Cat. No
AccuPower® qPCR Array System: Mouse Reference qPCR primer A set	20 rxn	S-6042-TM0A

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® 2X *Greenstar*™ qPCR Master Mix		K-6251
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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