User's Guide

AccuPower® qPCR Array System: Human Cancer qPCR panel kit

REF S-6042-PH1

BIONEER
Innovation • Value • Discovery

AccuPower® qPCR Array System: Human Cancer qPCR panel kit

User's Guide

Version No.: 1.0 (2015-12)

Please read all the information in booklet before using the unit

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1. Overview

Description

The *AccuPower*® qPCR Array System: Human Cancer qPCR panel kit is constructed in a panel format to screen genes involved in various cancer pathways or a specific cancer disease at once. Since the data are generated based on MIQE guidelines, the results can be used for SCI paper publication.

The human cancer panel contains 88 target genes which are related with apoptosis invasion & metastasis, angiogenesis, oncogenes & tumor suppressor, signal transduction & transcription factors, and cell cycle & DNA damage repair. This panel also contains 5 human reference genes of ACTB, B2M, GAPDH, HPRT1 and RPLP0 for relative quantification, and control wells of PPC, GDC and NTC.

All validated PCR primers for target genes and reference genes are coated in each well of the panel plates. The panel is designed to provide reproducibility and higher sensitivity in experiments along with significantly reducing nonspecific reaction. Just add your template and SYBR® Green I into each well of Human cancer panel and you can get reliable data simply and conveniently.

Features and Benefits

- Get accurate, robust results based on the MIQE guidelines
- Save time and expenses on primer validation
- Multiple data obtained by a single experiment
- Easy to use

Applications

Applicable to Quantitative Real-Time PCR

2. Product Components

1) *AccuPower*® qPCR Array System: Human Cancer qPCR panel (96-well plate) ·····1 plate
2) Adhesive Optical Sealing Film (3111-4110, Bioneer) ·····1 sheet

* *AccuPower*® 2X GreenStar qPCR Master Mix solution (K-6251, Bioneer) is purchased separately.

3. Product Shipment and Storage

AccuPower® qPCR Array System: Human Cancer qPCR panel kit (S-6042-PH1, Bioneer) is shipped and stored away from UV and sunlight, and at room temperature, respectively. The product is guaranteed to be stable for a year after delivery when it's properly stored at room temperature and without opening. Repeated freeze/thaw cycles (more than once) should be avoided, because it may affect panel quality.

AccuPower® 2X GreenStar qPCR Master Mix solution (K-6251, 6252, 6253, 6254, Bioneer) is an optional product and shipped frozen. If the entire volume of solution is not used at all, aliquots can be stored at -20℃. Frequent freeze/thaw cycles make the solution efficiency falling off.

AccuPower® *RocketScript*™ Cycle RT Premix with oligo $(dT)_{20}$ (K-2201, 2202, 2203, 2204, Bioneer) is also an optional product and shipped frozen. *AccuPower*® *RocketScript*™ Cycle RT PreMix should be stored at -20°C upon receipt and is stable until the expiry date stated on the label.

4. Intended Use

AccuPower® qPCR Array System: Human cancer qPCR panel kit is intended for research use only. This product is not intended for human or veterinary diagnostics use.

5. Safety Warnings and Precautions

Please inquire BIONEER's Customer Service Center to obtain a copy of the Material Safety Data Sheet (MSDS) for this product.

Before, during and after use of this product as described in this User's Guide, all potentially hazardous materials (i.e. materials that may have come in contact with genetically recombinant samples) including tubes, tips and materials should be processed and disposed of according to applicable and appropriate regulations of the municipality/government in which this product is being used. A user must also be equipped with basic experimental techniques required for correct execution of the experiments described in this User's Guide.

Some applications that may be performed with this product may infringe upon existing patents in certain countries. The purchase of this product does not include or provide a license to perform patented applications. Users may be required to obtain a license depending on country and application. We do not condone nor recommend the unlicensed use of a patented application.

6. Warranty and Liability

All BIONEER products are manufactured and tested under strict quality control protocols. BIONEER guarantees the quality of all directly manufactured products during the warranty period of one (1) year from the date of purchase. If any issues are discovered relating to product quality, immediately contact the Gene Expression Analysis Team (qPCRarry@bioneer.com).

BIONEER does not assume liability for misuse of the product, i.e. usage of the product for any purposes other than its intended purpose as described in the User's Guide. BIONEER assumes liability under the condition that users disclose all information related to the problem to BIONEER in written form within 30 days of occurrence.

7. Technical Assistance

At Bioneer, we pride ourselves on being responsive to your needs. If you have any questions or would like to find out more information about *AccuPower*® qPCR Array System: Human Cancer qPCR panel kit, please contact us. We look forward to hearing from you!

Technical Support

For all technical questions and troubleshooting on *AccuPower*® qPCR Array System: Human Cancer qPCR panel kit.

Tel: +82-42-930-8673

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8. Quality Management

Every aspect of our quality management system from product development, production to quality assurance and supplier qualification meets the world-class standards. *AccuPower*® qPCR Array System: Human Cancer qPCR panel kit is carefully tested by the Gene Expression Analysis Team.

9. Product Specifications

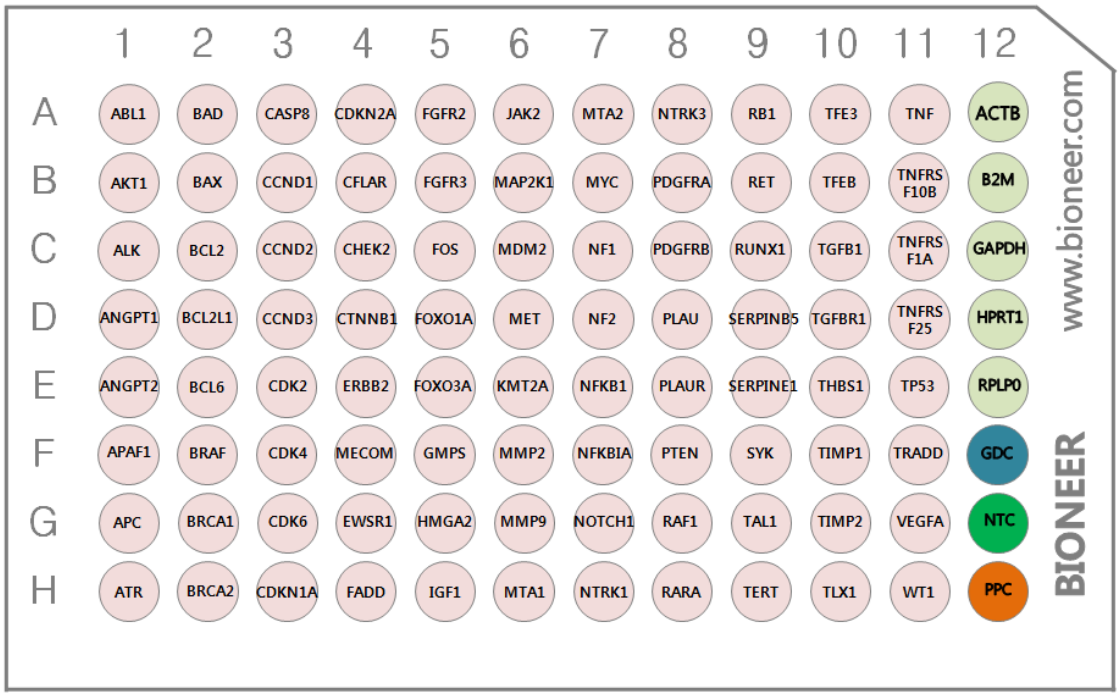


Figure 1. Example of layout of Human cancer panel containing target genes, reference genes and control primers.

Control wells

1) Genomic DNA Control (GDC): GDC primer is for detection of non-transcribed genomic DNA contamination.
- In GDC well, primers which target genomic DNA are coated.
- If the value of Ct^{GDC} is less than 35, there is gDNA contamination in your template. In this case, your RNA sample should be cleaned up before PCR running reaction.

2) Non Template Control (NTC): NTC is a negative control for checking on random or reagent contamination.
- Just add pre-mixture containing nuclease-free water except for 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 equipments and replace all the reagents to new one.

3) Positive PCR Control (PPC): PPC primer is for efficiency test of the PCR itself.
- PPC well is already coated with positive template and primers, so just add 2X Master Mix and nuclease-free water to the well.

10. Background Acknowledgement

10.1 MIQE guidelines

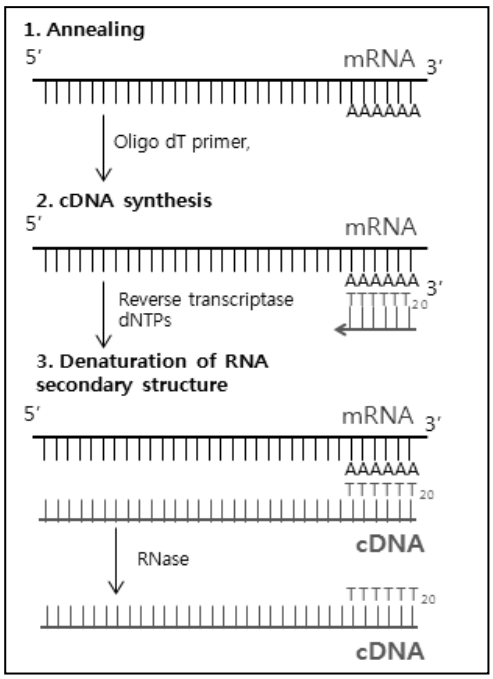
The experiment guidelines of quantitative PCR for publication, MIQE guidelines describe the minimum information required for publication of experimental results from quantitative PCR. The guidelines cover the entire processes of qPCR– experimental design, sample, nucleic acid extraction, reverse transcription, qPCR target information, qPCR oligonucleotides, qPCR protocol, qPCR validation and data analysis. The MIQE guildelines aim to improve the reliability and reproducibility of qPCR experiments and the results.

The human cancer panel has been developed by expertise following the MIQE guidelines and quickly provides accurate, reliable qPCR results of SCI publication grade.

The MIQE Guidelines

[Bustin, S.A., et al. 2009. The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. Clinical Chemistry 55:4, 611-622]

10.2 Reverse transcription



RT-PCR(Reverse Transcription – Polymerase Chain Reaction)

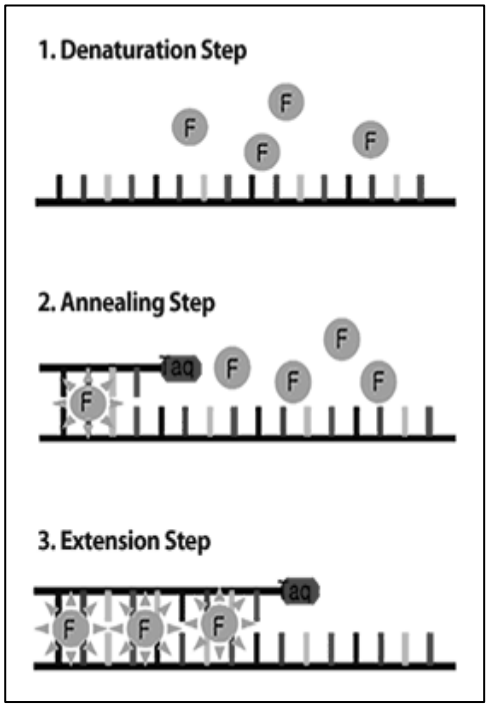
A RT-PCR must be carefully considered when optimizing the condition with specificity, sensitivity, reproducibility or fidelity of the reaction. Successful performance of the RT-PCR is dependent on a clear understanding of the primary aim of the assay. As RNA cannot serve as a template for PCR, the first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

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 reaction 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 reaction 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 reaction. 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 reaction using SYBR green due to its accuracy.

10.3 qPCR detection method



Dye-based detection

SYBR Green-based detection is popular detection method in real-time PCR. SYBR® Green I intercalating dye specifically binds to double-stranded DNA produced by DNA polymerase during PCR reaction 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.

Using intercalating dyes saves expenses on experiments because there is no need to purchase expensive probes such as TaqMan. And it is another merit that the primer design for the PCR reaction using SYBR green is relatively simple.

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 pirmer-dimer.

10.4 Primer design for qPCR

Primers of the human cancer panel 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 included in panel meet the followings.

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

1) Designing primers longer than 19 bases
2) Specific dissociation curves
3) Detection of expected size with single band via gel electrophoresis
4) Short amplicon size (70~150 bp)

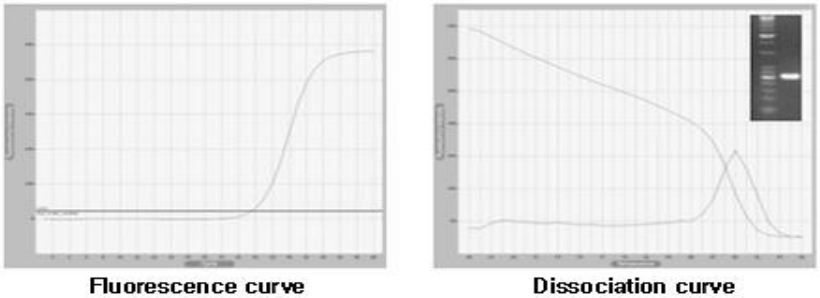


Figure 2. Primer validation result (Example. CCND3 gene)

11. Materials and Equipment Needed But Not Provided

- Real-time PCR instrument such as *Exicycler*™ 96 Real-Time Quantitative Thermal Block
- PCR/Microtiter Plate Centrifuge
- SYBR® Green I reagent such as AccuPower™ 2X Greenstar qPCR Master Mix
- Nuclease-free water

12. Recommended Protocol

Start with isolating RNA from your experimental samples using *AccuZol*™ Total RNA Extraction Reagent (K-3090, Bioneer) or *MagListo*™ 5M Cell Total RNA Extraction Kit (K-3610, K-3611, Bioneer). 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 with oligo $(dT)_{20}$ (K-2201, Bioneer). Add equal volume of the synthesized cDNA and *AccuPower*® 2X Greenstar qPCR Master Mix (K-6251, Bioneer) into each well of the sample PCR array plate containing the pre-dispensed gene-specific primer sets. And perform PCR with *Exicycler*™ 96 (A-2060, Bioneer). Use Analysis ExiCycler3 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.

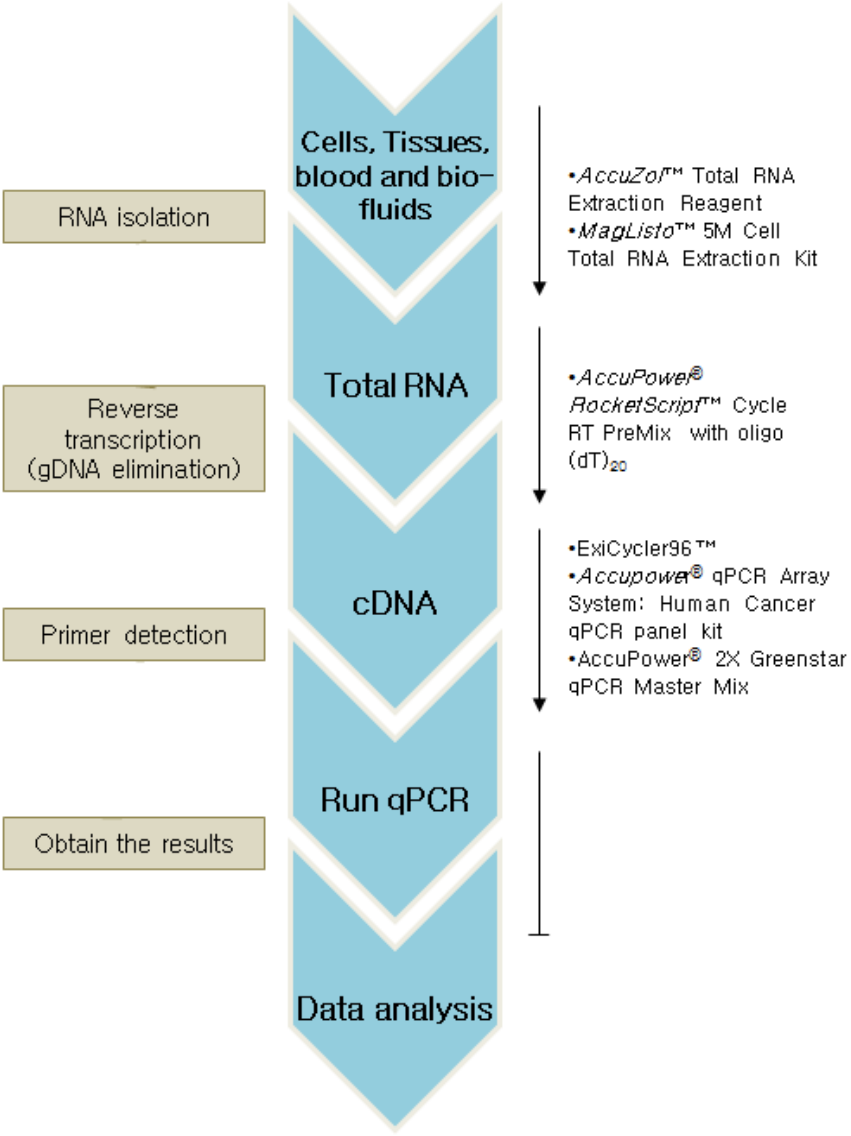


Figure 3. Total process of qPCR following the MIQE guideline.

12.1 Isolation of RNA from experimental samples

*Related product: *AccuZol*™ Total RNA Extraction Reagent (K-3090, Bioneer)

The *AccuZol*™ Total RNA Extraction Reagent (K-3090, Bioneer) is a ready-to-use reagent for the isolation of total RNA from various sample materials. It is a monophasic solution of phenol and guanidine-salt inhibiting RNase activity, and so the reagent maintains integrity of the RNA during sample homogenization or lysis. *AccuZol*™ allows you to extract total RNA with high-yield from even little amount of starting materials.

BEFORE YOU BEGIN

1) DNA contamination

It is important to prevent genomic DNA contamination for accurate and reproducible results of qPCR. Genomic DNA contamination will distort the SYBR® Green I signal in gene expression analysis. To Keep a working environment with DNA contamination free, follow as:

- The samples must be treated with DNase I before reverse transcription.
- Carefully open and close all sample tubes or reaction plates.
- Wear a clean lab coat and clean gloves when handling samples.
- Reagent should be divided and stored in aliquots as little as possible.

2) Ribonuclease (RNase)

RNase is very stable and extremely active enzyme even without co-factors. RNases are quite difficult to be inactivated and small amounts are enough to destroy RNA. To minimize RNase contamination, the following guidelines should be followed when working with RNA.

- Always wear latex or vinyl gloves and change gloves often. RNases arise readily from bacteria and molds present in dust, on skin and clothing.
- Use sterile, autoclaved disposable plastic ware to prevent cross-contamination with RNases from shared equipment.
- Non-disposable plastic ware or glassware can be washed with detergent, and rinsed several times with sterile distilled water, followed by thorough rinse with 0.1 N NaOH, 1 mM EDTA and finally, RNase-free sterile distilled water.
- Alternatively, glassware can be washed with detergent, well rinsed and baked in a dry oven at 240°C for at least 4 hours. Please note that autoclaving will not completely inactivate all RNases. Glassware can be treated with DEPC (diethyl pyrocarbonate) by filling with 0.1% DEPC, left to stand 12 hrs at 37°C or more hours, and then autoclaved to eliminate the DEPC.
- Electrophoresis tanks are cleaned with detergent and rinses several times with RNase-free water, rinsed with ethanol and allowed to dry.

Specifications:

Samples		Starting amount	Elution volume (μl)	Average yield (μg)
Animal Tissue	Liver Kidney Spleen Lung Brain	10 mg	50	50 – 70 25 – 40 40 – 60 15 – 25 8 – 12
Cultured Cell	HeLa A549 PC3	1 x 10^6 cells	50	15 – 30 15 – 25 15 – 25
Bacterial Cell	*E. coli*	1 x 10^7 cells	50	10 – 15
Plant Tissue	Bean leaf	100 mg	50	30 – 50
Blood	Human whole blood	250 ul	50	2 – 3

1) Required reagents
- *AccuZol™* Total RNA Extraction Reagent
- Chloroform
- Isopropyl alcohol
- 80% Ethanol (in DEPC-treated water)
- RNase-free water or 0.5% SDS solution

2) Procedures

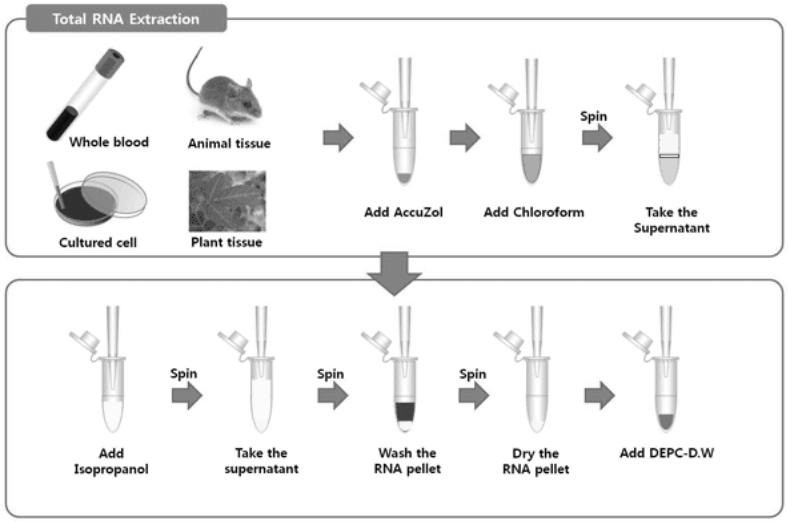


Figure 4. Procedure of RNA isolation using *AccuZol™*

⚠Caution: In all steps, we recommend experimenter use autoclaved and unused tips

① Sample preparation

Mammalian Tissue	fresh	After dissection, homogenize immediately in *AccuZol*™ or freeze rapidly in liquid nitrogen.
	frozen	Weigh frozen tissues, break into suitably sized pieces, and homogenize directly in *AccuZol*™.
Plant	Weigh tissues, immediately place it in liquid nitrogen, and grind with a mortar and pestle. Fresh tissues can be homogenized directly in *AccuZol*™ with a mortar and pestle.	
Blood	Whole blood should be collected in the presence of an anti-coagulant. For optimal results, blood samples should be processed within a few hours after collection.	

② Homogenize prepared samples

Tissue	Homogenize tissue samples in 10-20 volumes of *AccuZol*™ (e.g., 1ml of *AccuZol*™ per 50-100mg of tissue) using a homogenizer or equivalent. The sample volume should not exceed 10% of the volume of *AccuZol*™ used for homogenization.	
Plant	Homogenize plant samples in 1ml of *AccuZol*™ per 10-100mg of plant tissues using a homogenizer or equivalent. The sample volume should not exceed 10% of the volume of *AccuZol*™ used for homogenization.	
Blood	Add 750ul of *AccuZol*™ per each 250ul of sample. Lyse cells in the sample suspension by passing the suspension several times through a pipette or vortexing.	
Cell	Adherent	Lyse cells directly in the culture dish by adding 1ml of *AccuZol*™ per 10㎠ of culture dish area, and passing the cell lysate through a pipette several times. The amount of reagent added is based on the area of culture dish and not on the number of cells present.
	Suspension	Pellet cells, then lyse in 1ml of *AccuZol*™ per 5-10x10^6 animal, plant, or yeast cells, or per 1x10^7 bacterial cells, by several pipetting or vortexing. Disruption of some yeast and bacterial cells may require the use of a homogenizer.

③ Add 200ul of chloroform per 1ml of *AccuZol*™, and vortex vigorously for 15 seconds.

④ Incubate the mixture on ice for 5 minutes.

⑤ Centrifuge at 12,000 rpm for 15 minutes at 4℃.

⚠Caution: Following centrifugation, the mixture separates into a lower organic phase (green color), an inter-phase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase.

⑥ Transfer the aqueous phase to a new 1.5 ml tube and add equal volume of isopropyl alcohol.

⚠Caution: When pipetting the upper layer, pay attention not to take any white sediment.

⑦ Mix by inverting the tube 4-5 times and incubate at -20℃ for 10 minutes.

⑧ Centrifuge at 12,000 rpm for 10 minutes at 4℃, and then carefully remove the supernatant.

⑨ Add 1ml 0f 80% ethanol and mix well by inverting or vortexing briefly.

⑩ Centrifuge at 12,000 rpm for 5 minutes at 4℃, and then carefully remove the supernatant.

⑪ Dry the pellet.
⚠Caution: Do not dry the pellet by centrifugation under vacuum. Do not completely dry the RNA pellet, because this will greatly decrease its solubility.

⑫ Dissolve RNA pellet in RNase-free water or 0.5% SDS solution by passing the solution a few times through a pipette tip, and Incubate for 10 minutes at 55 to 60℃ (0.5% SDS should not be used).
⚠Caution: RNA can also be in 100% formamide (deionized) and store at −70℃.

⑬ It is important to treat RNase-free DNase I to all RNA samples. Representation of DNA will cause distortion SYBR® Green I signal.

⑭ Measure RNA concentration for next steps, reverse transcription.
⚠Caution: Use Spectrophotometer to measure RNA concentration at 260nm, and purity at $A_{260}/_{280}$ ratio (1.8-2.0), such as Nanodrop (Thermo)

CHECK POINTS

1) For desirable results outcome, checking quality of RNA is highly recommended as:

- RNA purity: $A_{260}/_{280}$ ratio should be 1.8 to 2.0
- Comparing intensity of the 28S and 18S ribosomal RNA bands (28S:18S=2:1) on a gel electrophoresis

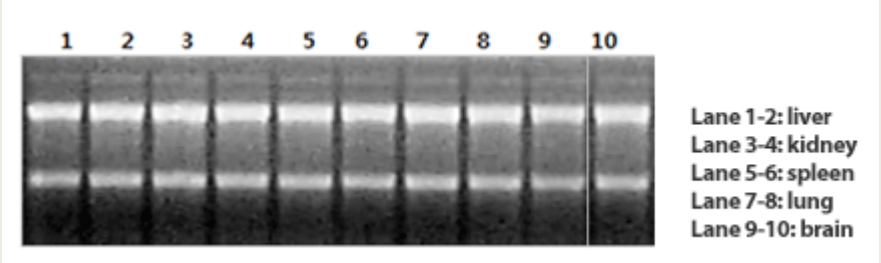


Figure 5. Electrophoresis pattern of total RNA extracted from animal tissues by *AccuZol*™: Total RNA was isolated from 10-20 mg of rat tissues and 2 μg of total RNA was loaded per lane on a 1% denaturing agarose gel.

- Recently, automated capillary electrophoresis system, such as the LabChip GX (Calipers) has become a popular tool to assess the quality of RNA.

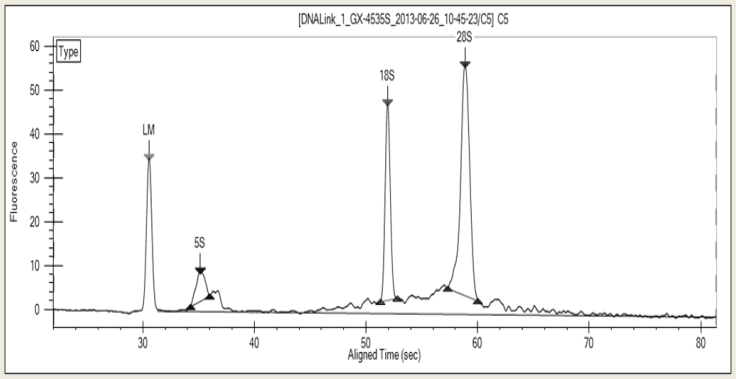


Figure 6. LabChip GX (Caliper) electropherogram for typical RNA samples showing sharp peaks of 18S and 28S ribosomal RNA.

2) All samples must be stored at −80℃.

*Related product: *MagListo*™ 5M Cell Total RNA Extraction Kit (K-3610, K-3611, Bioneer)

MagListo™ 5M Cell Total RNA Extraction Kit is an innovative product to extract total RNA from a wide range of cell types. This kit is designed to be applicable to variable scales of total RNA extraction (micro/mini/midi Extraction) by adding proper volume of each solution suggested in this User's Guide. *MagListo*™ 5M Cell Total RNA Extraction Kit guarantees RNA of high purity in unprecedented short period of time. *MagListo*™ Magnetic Separation Rack, which is also available from Bioneer (TM-1000~1020), will greatly enhance the user's convenience and save the processing time by removing the necessity of the centrifugation.

BEFORE YOU BEGIN

① Buffer 1 (Binding) and Buffer 2 (1st Washing) contains chaotropic salt. You should take the appropriate laboratory safety precautions and wear gloves and lab goggle when handling.
② The relative centrifugal force (RCF) is calculated in g as follows:
RCF = 1.12 x r x $(rpm/1,000)^2$
Where 'r' is the radius of a rotor in cm, and 'rpm' is the speed of the rotor in revolutions per minute.
③ Buffer 1 (1st Washing) and Buffer 3 (2nd Washing) are supplied as concentrated solutions. Before using for the first time, add absolute ethanol as indicated on the bottles.
④ To inhibit RNase activity, we recommend adding β-mercaptoethanol to Buffer 1 (Binding) before use. Add 10 µl β-mercaptoehanol(>99%) per 1 ml Buffer 1 (Binding).

Typical Yields of Total RNA

Cell type (1x 10^6)	Yield
Hela	10~15 ug
293T	20~30 ug
Balb/c 3T3	20~30 ug
Huh7	10~15 ug

(Note) We recommend using our *MagListo*™ Magnetic Separation Rack for guaranteed results

Protocol for Micro(~10^4)/Mini(1x10^5~5X10^6)/Midi (5x10^6~2x10^7) Extraction

Harvest of Cells

① Cells grown in suspension:
Count the cell number and centrifuge the proper number of cells at 300 x g for 5 min. Discard supernatant carefully and go to Lysis & Homogenization (step 3).

② Cells grown in a monolayer: There are 2 different ways to collect cells grown in a monolayer.
A. Direct cell lysis on the Culture Dish:
Completely remove cell culture medium and go to Lysis & Homogenization (step 3). (Remaining medium may inhibit the RNA extraction)
B. Harvesting cells with trypsin:
Remove Cell Culture Medium and wash the monolayer with DPBS. Add 0.1%-0.25% typsin to the washed cell monolayer. When the cells are detached, add Cell Culture Medium to inactivate the typsin. Transfer the cells into a RNase-free tube and centrifuge at 300 x g for 5 min. Discard supernatant carefully and go to Lysis & Homogenization (step 3).

Lysis & homogenization

③ Add 50 µl (micro)/ 400 µl (mini)/ 2 ml(midi) of Buffer 1 (Binding) to the Cell culture Dish or the collected cell pellet and completely homogenize the cells by pipetting or vortexing for at least 1 min.

(Note) Insufficient homogenization can decrease the RNA purification yield, and also cause clogging of Magnetic Nano beads in the following steps. For the sufficient homogenization of the lysate, make the lysate passed through blunt 20-gauge needle (0.9 mm diameter) 5 to 10 times.

A. For (micro/mini) scale extraction, please transfer the Lysate to a 2 ml (or 1.5 ml) tube.
B. For (midi) scale extraction, please transfer the Lysate to a 15 ml tube.

RNA binding

④Add 50 µl for (micro) 400 µl (mini)/ 2 ml (midi) scale extraction of Absolute Ethanol to the tube and mix by vortexing or shaking.

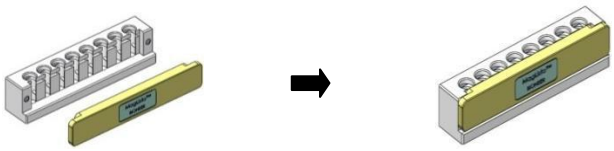
⑤Add 50 µl for (micro)/ 100 µl (mini)/ 400 µl (midi) scale extraction of Magnetic Nano Bead solution to the tube and mix by vortexing or shaking.

(Note) Magnetic Nano Bead solution contains magnetic nano beads. Please vortex well before use.

⑥ Place the tube in *MagListo*™-2 (micro/Mini)/ *MagListo*™-15 (midi) scale separation rack with the magnet plate attached and invert the tube 3~4 times gently until the beads tightly bind to magnet.

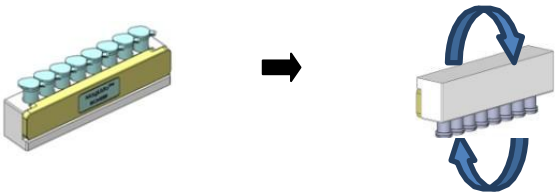
* How to Use

- Attachment



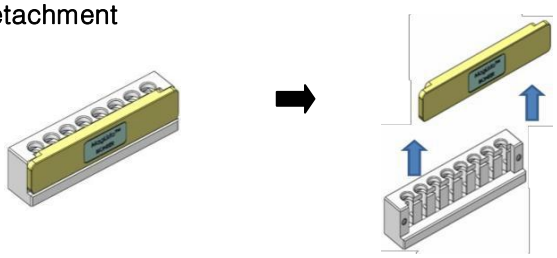
Combine the magnet plate to the stand.

- Discard solution



Discard solution by inverting the *MagListo*™ rack. The silicone immobilizer inside the stand holds the tubes from falling in an upside down position. When discard solution, invert the rack completely for the solution not to smear on the rack.

- Detachment



Push up the magnet plate gently.

⑦ Keeping the tubes from *MagListo*™-rack, discard the supernatant and remove the remaining supernatant using a paper towel by blotting.

Washing

⑧Detach the magnet plate from *MagListo*™-rack. Add 350 µl for (micro)/ 700 µl (midi)/ 3.5 ml (midi) scale extraction of Buffer 2 (1st Washing) to the tube. Close the cap and mix by vortexing or shaking until the bead are fully resuspended.

⑨Place the tubes in *MagListo*™-rack with the magnet plate attached and invert the tube 3~4 times gently until the beads tightly bind to the magnet.

⑩ Maintaining the tubes in the *MagListo*™-rack, discard the supernatant and completely remove the remaining supernatant using a paper towel by blotting.

⑪ Detach the magnet plate from *MagListo*™-rack. Add **350 µl for (micro)/ 700 µl (midi)/ 3.5 ml (midi) scale extraction** of Buffer 3 (2nd Washing) to the tube. Close the cap and mix by vortexing or vigorous shaking until the bead are fully resuspended.

⑫ Repeat the above step 9~10 for additional washing.

⑬ Detach the magnet plate from *MagListo*™-rack. Add **350 µl (micro)/ 700 µl (mini)/ 3.5 ml (midi)** of absolute ethanol to the tube. Close the cap and mix by vortexing or shaking until the bead are fully resuspended.

⑭ Repeat the above step 9~10 for additional washing.

Drying

⑮ With a heat gun or a blow dryer, completely dry the beads with the tube open 3 cm away from the top of the tube. (micro/mini: >1 min , midi: >3 min)

(Note) Alternatively, the beads can be dried with a dry oven at 65℃ for current times. (micro/mini: >5 min, midi: >25 min) Please use the Clean Bench during the drying procedure is recommended to prevent RNase or other aerosol contamination.

Elution

⑯ Add for **80 µl (micro/mini), or 250 µl (midi) scale extraction** of Buffer 4 (Elution) to the tube with the magnet plate detached and resuspend by vortexing or pipetting

⑰ Incubate the tube at 50℃~65℃ for 2 min.(Note) Warm up the tube with a heat block, a heat gun or a blow dryer.

⑱ Vortex the tube for 15 sec.

⑲ Attach the magnet plate to *MagListo*™-rack and invert the tube 3~4 times gently until the beads tightly bind to the magnet.

⑳ Without removing the tube from *MagListo*™-rack, carefully take the supernatant containing RNA to a sterile microcentrifuge tube.

㉑ Discard the used Magnetic Nano Beads. Do not reuse the beads

DNase treatment

㉒ After finishing total RNA preparation, treat DNase I to all RNA samples.
DNase treatment of RNA samples is an essential step for real-time PCR experiment.

Summary of reagent volumes required in each step of Cell Total RNA Extraction

Step	Buffer	Micro	Mini	Midi	Page
Cell Lysis	Buffer 1 (Binding)	50 µl	400 µl	2 ml	P. 16
Add EtOH	Absolute Ethanol	50 µl	400 µl	2 ml	P. 16
RNA Binding	Magnetic Nano Bead – RNA	50 µl	100 µl	400 µl	P. 16
1st Washing	Buffer 2 (1st Washing)	350 µl	700 µl	3.5 ml	P. 17
2nd Washing	Buffer 3 (2nd Washing)	350 µl	700 µl	3.5 ml	P. 18
3rd Washing	Absolute Ethanol	350 µl	700 µl	3.5 ml	P. 18
Elution	Buffer 4 (Elution)	80 µl	80 µl	250 ul	P. 18

RNA Cleanup Protocol

This protocol is to remove enzymes, buffers, or chemical inhibitors and concentrate RNA for use in certain applications.

1. Transfer RNase-free water into RNA sample to make a total volume of **100 µl**.

2. Add **50 µl** of Buffer 1 (Binding) and mix thoroughly.

3. Add **200 µl** of Absolute Ethanol and mix well.

4. Add **100 µl** of Magnetic Nano Bead solution to the tube and mix by vortexing or shaking
(Note) Magnetic Nano Bead solution contains magnetic nano beads. Please vortex well before use.

5. Place the tube in ***MagListo*™-2** with the magnet plate attached and inverts the tube 3~4 times gently until the beads tightly bind to the magnet.

6. Without removing the tube from *MagListo*™-rack, discard the supernatant and remove the remaining supernatant using a paper towel by blotting.

7. Go to **Washing step 11 (page number 9.)** and follow the instruction described above for the remaining steps

12.2 Reverse transcription

*Related product: *AccuPower® RocketScript™* Cycle RT Premix with oligo(dT)$_{20}$ (K-2201)

BEFORE YOU BEGIN
1) For desirable results outcome, we recommend to use total RNA 0.4~1㎍ per 1 tube(20μl) for cDNA synthesis. 2) It is important to use a consistent amount of total RNA for all samples in a single experiment. 3) Avoid DNA and RNase contamination. (Refer to the above)

1) Components

Component	Final concentration
RocketScript™ Reverse Transcriptase	200U
Oligo (dT)$_{20}$	100pmole
5 X Reaction Buffer	1 X
DTT	0.25mM
dNTP	250μM each
RNase Inhibitor	1U

2) Procedures

① Thaw total RNA and nuclease-free water just before use.

② Add proper volume of total RNA into the *AccuPower® RocketScript* Cycle RT PreMix tubes.

③ Add nuclease-free water into the *AccuPower® RocketScript* Cycle RT PreMix tubes to a total volume of 20μl (K-2201, K-2202) or 50μl (K-2203, K-2204). Do not consider a volume of the dried pellet.

④ Dissolve the air dried pellet completely by vigorous voltexing and spin down. Bioneer's *ExiSpin*™ has both vortex and centrifuge functions (A-7040).

⑤ Perform the reaction under the following conditions:

Step	Temperature	Time	No. of Cycles
Primer annealing	37°C	30 sec	12
cDNA synthesis	48°C	4 min	
Denaturation of RNA secondary structure and cDNA synthesis	55°C	30 sec	
Heat inactivation	95°C	5 min	1

⑥ Maintain the reaction product at 4°C after amplification.
The product should be stored at −20°C until use.

12.3 qPCR reaction

*Related product – *AccuPower*® qPCR Array System: Human Cancer qPCR panel kit (Cat.No. S-6041-PH1)

BEFORE YOU BEGIN

1) There are already PPC (Positive PCR control) as a positive template included, so you don't need to load additional template in the PPC well.

2) 15pmole primers mix (forward and reverse) are contained in each well.

3) Do not open any previously processed qPCR array. Removing the film from plate allows release of PCR product into the air, and it may contaminate and affect the result of further qPCR experiment.

4) Use only high quality, nuclease-free water, not DEPC-treated water.

5) Avoid gDNA and RNase contamination. (Refer to the above)

1) Required supplies

- Qualified template
- 2X Greenstar qPCR Master Mix
- qPCR instrument
  (Recommend using *Exicycler*™ 96 Real-Time Quantitative Thermal Block (A-2060, Bioneer))
- High quality, nuclease-free water
- Pipetter, Nuclease-free pipette tips and tubes

2) Procedures

① Prepare the PCR components mixture in a tube as described in following table.

Components	Amount
Template	Variable
2X Greenstar qPCR Master Mix	25μl
Nuclease-free water	Adjust to 50μl
Total volume	50μl

⚠Caution: Do not vortex for mixing, but use pipetting to avoid bubbles.

② Carefully remove the film covered the panel.

③ Dispense 50μl of the PCR components mixture into each well on *AccuPower*® qPCR Array System: Human Cancer panel kit.

⚠Caution: Change pipette tips following each pipetting step to avoid cross-contamination among the wells.

④ Carefully and tightly seal the plate with Adhesive Optical Sealing Film (3111-4110, Bioneer) supplied.

⑤ Spin down the plates, and then completely mix by vortexing for resuspension of lyophilized primers.

⑥ Spin down the plates again.

⚠Caution: Check carefully if there are residues on the film.

⑦ Turn on the qPCR instrument and load the plate.

⚠Caution: Before start, please wipe surface of film to eliminate dust.

⑧ Start program. The qPCR program setting is below:

PCR Steps	Condition		Cycle
	temperature	time	
Pre-denaturation	95℃	10 min	1
Denaturation	95℃	5 sec	40
Annealing	58℃	25 sec	
Extension	72℃	30 sec	
Detection	Scan		
Final Extension	65℃	5 min	1
Melting	65 to 95℃	1 sec	-

⑨ When reaction is completed, perform data analysis.

12.4 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. For our Human cancer panel is suitable for analysis by the $2^{-\Delta\Delta Ct}$ method.

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

- ΔCt: the difference between Ct value of target gene and Ct value of reference gene.
- ΔΔCt: 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.

13. Better qPCR Array System

13.1 *AccuPower*® *RocketScript*™ Cycle RT PreMix with olido(dT)$_{20}$

The *AccuPower*® *RocketScript*™ Cycle RT Premix with oligo(dT)$_{20}$ is a ready-to-use lyophilized PreMix containing all components for first-strand cDNA synthesis from purified total RNA template. Oligo (dT)$_{20}$ is also included in the PreMix for convenience. Simply add your RNA template and nuclease-free water to begin your reaction.

The *AccuPower*® *RocketScript*™ Cycle RT PreMix contains *RocketScript*™ Reverse Transcriptase. This enzyme is a new *M-MLV* originated Reverse Transcriptase that has been engineered to provide increased thermal stability for more efficient full-length first-strand cDNA synthesis. The amount of starting material can be various from 1 pg to more than 1 μg of total RNA. RNA targets from 100 bp to more than 10 kb can be amplified with the *AccuPower*® *RocketScript*™ Cycle RT PreMix.

1) Experimental data

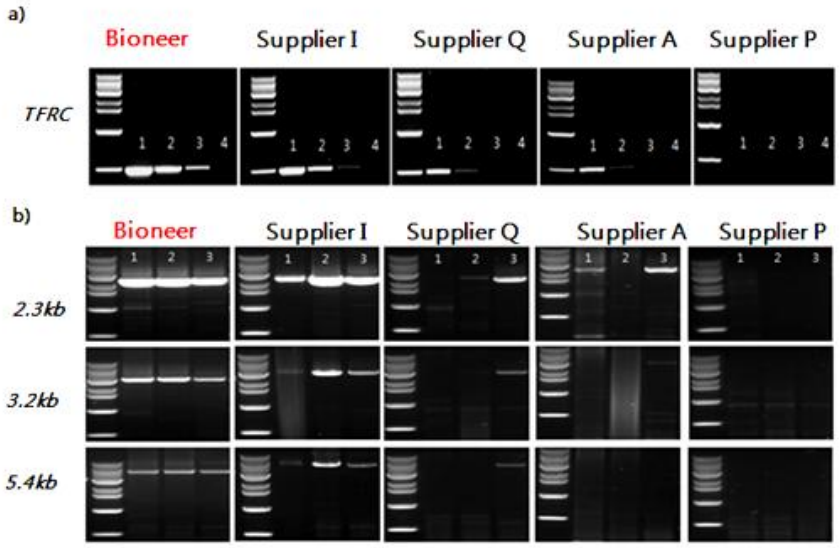


Figure 7. Comparison of amplification efficiency between Bioneer *AccuPower*® *RocketScript*™ Cycle RT PreMix and competitors' RTases (Primer: Human transferrin receptor set)
a) Sensitivity test
Lane M: 1 kb DNA Ladder
Lane 1: 100 ng Human total RNA from HeLa cell
Lane 2: 10 ng Human total RNA from HeLa cell
Lane 3: 1 ng Human total RNA from HeLa cell
Lane 4: 100 pg Human total RNA from HeLa cell

b) Full-length cDNA synthesis capability
Lane 1: 1 μg Human total RNA from HeLa cell
Lane 2: 100 ng Human total RNA from HeLa cell
Lane 3: 10 ng Human total RNA from HeLa cell

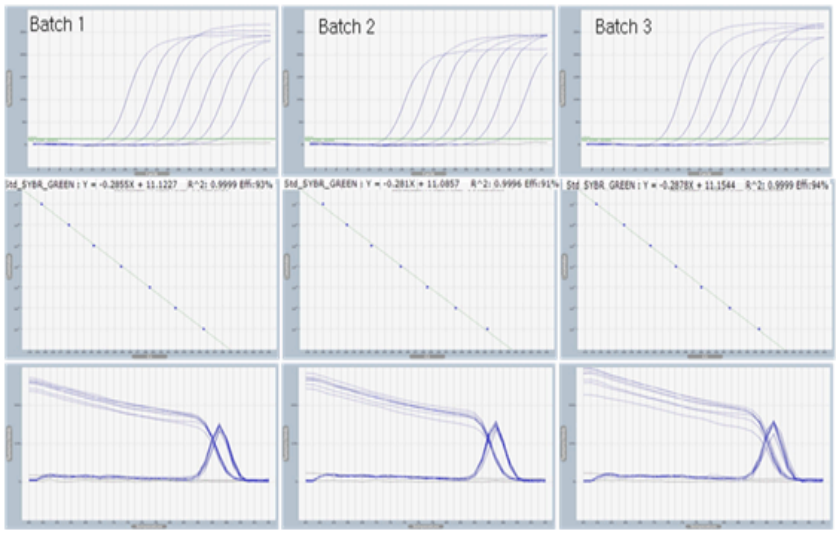
13.2 *AccuPower*® 2X GreenStar qPCR Master Mix

AccuPower® 2X GreenStar qPCR Master Mix is a ready-to-use cocktail containing all components except for primers. It is for the amplification and detection of DNA in real-time quantitative PCR(qPCR). It combines the automatic "Hot-Start" technology of Taq DNA polymerase and SYBR® Green I fluorescent dye to provide excellent sensitivity in the quantification of target sequences, with a linear dose response over a wide range of target concentration. Provided reagent volumes are enough for 100 or 200 amplification reactions with 50 µl reaction each.

This product can be used in real-time PCR experiments for the amplification and detection of genomic DNA and cDNA targets, differential gene expression profiling, and microbial & viral pathogen detection. This product provides the reproducible results with the superior specificity, high sensitivity, wide dynamic range and accurate quantification

- **High Specificity:** *AccuPower*® 2X GreenStar qPCR Master Mix generates more accurate real-time PCR result due to Hot-start method.
- **Stability:** The chemical stabilizer maintains reliable enzyme activity for 2 years when stored at -20°C.
- **Simplicity:** *AccuPower*® 2X Greenstar qPCR Master Mix contains all required components for real-time PCR. So it is ready to use. Just add primer, template and RNase-free water. Also, it contains 50 X ROX dye with no extra charge.
- *Rox dye: The control dye for the ABI instrument.
- **Reproducibility:** Bioneer's strictly quality controlled production system guarantees your reproducible results.

1) Experimental data



Step	Conditions	Cycle
Pre-Denaturation	95℃, 15min	1
Denaturation	95℃, 15sec	45
Annealing Extension	60℃, 30sec	
Detection(Scan)		
Melting	65℃-90℃, every 1℃, 1sec	1

PCR reaction mixture	Per reaction
2X Greenstar Master Mix	25㎕
PCR F-primer (10pmole)	1.5㎕
PCR R-primer (10pmole)	1.5㎕
Template	5㎕
RNase free water	Adjust to 50㎕

Copy	C(t) Value			
	Batch 1	Batch 2	Batch 3	Error Rate
NTC	UD	UD	UD	0.5 C(t)
10	36.33	36.74	35.54	
100	32.22	31.77	31.73	
1000	28.51	28.7	28.04	
10000	25.09	25.12	24.73	
100000	21.27	21.42	21.47	
1000000	18.12	18.13	18.04	
10000000	14.99	14.43	14.45	

Figure 8. Highly reproducible Ct values

Amplification of 90 bp target gene was detected using serially diluted LP(*Legionella Pneumoniae*) genomic DNA (from 10^6 to 10^1 copies) with *AccuPower*® 2X Greenstar qPCR Master Mix. As shown in **Figure 7**, it is reproducible among different Lots. Data value is average of triplicate.

14. Ordering Information

Cat. No.	Product Description
S-6042-**PH1**	*AccuPower*® qPCR Array System: Human Cancer qPCR Panel kit
K-6251	*AccuPower*® 2X Greenstar qPCR Master Mix with 50X ROX Dye / 100 Rxn, 50 µL reaction
K-6252	*AccuPower*® 2X Greenstar qPCR Master Mix with 50X ROX Dye / 200 Rxn, 50 µL reaction
K-6253	*AccuPower*® 2X Greenstar qPCR Master Mix / 100 Rxn, 50 µL reaction
K-6254	*AccuPower*® 2X Greenstar qPCR Master Mix / 200 Rxn, 50 µL reaction
3111-4110	Adhesive Optical Sealing Film, 100 sheets
A-2060	*Exicycler*™ 96 Real-Time Quantitative Thermal Block

Cat. No.	Related Product Description
K-3090	*AccuZol*™ Total RNA Extraction Reagent, 100 ml
K-3610	*MagListo*™ 5M Cell Total RNA Extraction Kit, 8 rxn in mini
K-3611	*MagListo*™ 5M Cell total RNA Extraction Kit, 100 rxn in mini
K-2201	*AccuPower*® *RocketScript*™ Cycle RT PreMix with oligo $(dT)_{20}$, 20 µl, 0.2 ml thin-wall 8-strip tubes with attached cap, 96 tubes
K-2202	*AccuPower*® *RocketScript*™ Cycle RT PreMix with oligo $(dT)_{20}$, 20 µl, 0.2 ml thin-wall 8-strip tubes with attached cap, 480 tubes
K-2203	*AccuPower*® *RocketScript*™ Cycle RT PreMix with oligo $(dT)_{20}$, 50 µl, 0.2 ml thin-wall 8-strip tubes with attached cap, 96 tubes
K-2204	*AccuPower*® *RocketScript*™ Cycle RT PreMix with oligo $(dT)_{20}$, 50 µl, 0.2 ml thin-wall 8-strip tubes with attached cap, 480 tubes

15. References

1. Bustin, S.A., et al. 2009. **The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments**, Clinical Chemistry 55:4, 611-622
2. Kenneth J. Livak and Thomas D. Schmittgen, 2001, **Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{-\Delta\Delta Ct}$ Method**, METHODS. 25, 402-408
3. Bustin, S.A., 2000. **Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays**, Journal of Molecular Endocrinology. 25, 169-193

16. Explanation Symbols

	**Consult Instruction For Use**		**Caution, Consult accompanying documents**		**DO NAT REUSE**		**USE BY**

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