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StellARray™ Gene Expression System

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1.0 Introduction

Gene expression profiling is widely regarded to be integral for advancing life science research and clinical diagnosis in the 21st century. It is increasingly recognized that subsets of genes (hundreds rather than thousands) provide the most valuable gene profile definitions of any specific biological process. The StellARray™ Gene Expression System is a quantitative polymerase chain reaction (qPCR)-based method and provides reliable profiling of biologically focused gene sets. The StellARray™ Gene Expression System is comprised of three parts:

1. GeneSieve™ Gene Query
2. StellARray™ qPCR Arrays
3. Global Pattern Recognition™ (GPR) Data Analysis Tool

The GeneSieve™ Query merges databases containing gene-centric biological information in a new and innovative way. GeneSieve™ Query can filter or "sieve" thousands of genes for a custom population of real-time PCR arrays that have a unifying biological theme, such as a particular disease or pathway of interest.

Each StellARray™ qPCR Array is compiled to provide the highest information content with a scientifically relevant context. Each qPCR array is a 96- or 384-well formatted, wet lab validated group of gene primer sets, focused on specific biological processes. StellARray™ qPCR Arrays facilitate accurate gene expression analysis, as well as analysis of copy number variations (CNV). Therefore, it is possible to reveal a two-dimensional barcode of samples, such as tumors relating gene copy number with transcription levels.

StellARray™ qPCR Arrays can be used with most common qPCR thermal cyclers (for compatibility with your thermal cycler, please check table in "Plate Formats", page 5) and the SYBR® Green-based Reagents of your preference.

The Global Pattern Recognition™ (GPR) Analysis Tool allows gene expression analysis to be simple, fast and reliable. Data is simply uploaded to the GPR program and the software uniquely positions the expression level of each gene with respect to all genes within an experiment. This negates the assumption that a gene (normalizer) has an invariant expression level. Global Pattern Recognition™ Analysis Tool is unbiased in that it enables the experimental

data to define the invariant normalizer genes, not the experimenter. The use of any gene as a potential normalizer also maximizes the use of the limited real estate on a StellARray™ qPCR Array by eliminating the loss of wells used to contain housekeeping genes as potentially erroneously predefined normalizers.

2.0 Principles

The traditional approach to measure gene expression changes from real-time PCR data has been to normalize the results of a gene of interest with respect to one or a selection of housekeeping genes (e.g. reference or normalizer genes). The general assumption is that the level of expression of the normalizer gene(s) does not change in the context of the experiment and can be used to normalize the variability in RNA quantity between individual samples. By normalizing to a housekeeping gene, in theory, a magnitude of change can be calculated between groups of samples for a gene of interest.

However, this method of analysis is greatly complicated by the fact that housekeeping genes commonly used as normalizers (e.g., GAPDH, ß-actin, and HPRT) can change expression levels when comparing tissues or cells in different states (Bustin, 2000; Schmittgen *et al*., 2000; Goidin et al., 2001; Hamalainen *et al*., 2001). 18S rRNA is another normalizer that intuitively and experimentally appears to be more stable, but even 18S can vary in comparison to other genes when analyzed by sensitive real-time PCR techniques (Bustin, 2000, Akilesh *et al*., 2003). Any small variation in the normalizer amplification would therefore compromise the analysis of the complete real-time PCR data set.

Global Pattern Recognition™ Analysis Tool processes the data derived from groups of biological replicates and reveals the changes in groups of genes. Global Pattern Recognition™ Analysis Tool positions the expression level of each gene with respect to all genes within an experiment. This can be done without prior assumption that a gene (normalizer) has an invariant expression level. Global Pattern Recognition™ Analysis Tool is unbiased in that it enables the experimental data, not the experimenter, to define the invariant normalizer genes. The use of any gene as a potential normalizer also maximizes the use

of the limited real estate on a StellARray™ qPCR Array by eliminating the loss of wells used to contain potentially erroneously predefined normalizers.

3.0 Procedure Overview

Statistical data analysis requires the use of at least 3 biological replicates. Therefore, GPR uses a minimum of 3 'Control' samples versus 3 'Experimental' samples in order to facilitate a statistical analysis of the gene expression data. (Figure 1)

A typical experiment would utilize biological replicates. Biological replicates are defined as samples collected from separate and closely matched biological samples. They are processed individually under closely matched conditions. We recommend using a minimum of 3 biological replicates in each of two groups, for example '3 sick vs. 3 healthy' or '3 treated vs. 3 untreated' groups.

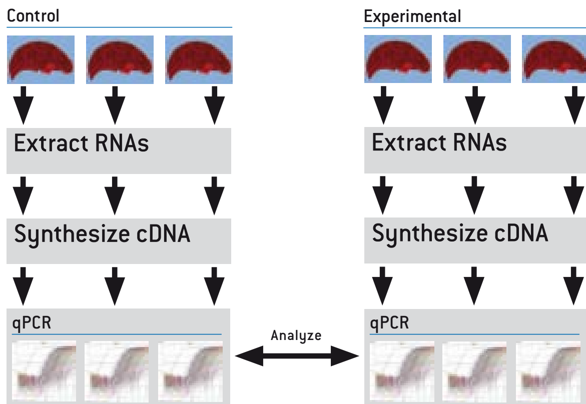
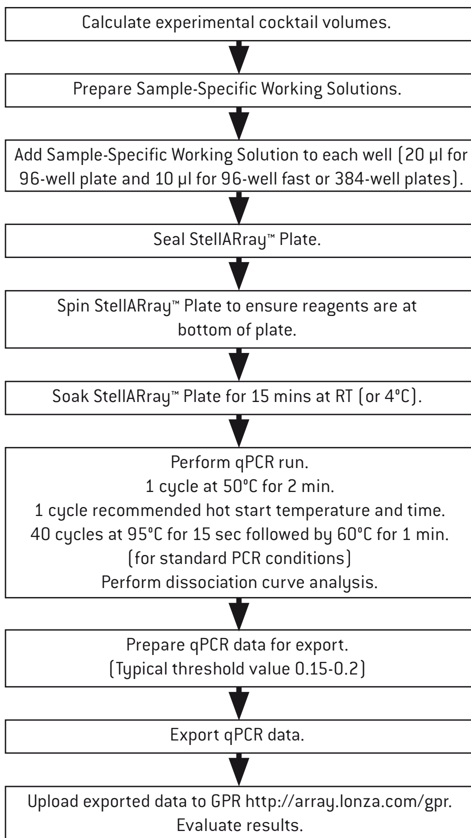


Figure 1. Example of experimental design to produce statistically correct data.

4.0 StellARray™ qPCR Protocol at a Glance



5.0 Plate Format

StellARray™ Plates are available in four different plate formats, each tailored to a specific subset of real-time PCR instruments and associated blocks.

Product type	For Real-Time Instruments
96-well Plate	Most thermal cyclers (non-ABI®, non-Roche®, non-low profile), such as Eppendorf®, Bio-Rad®, iCycler®, iQ5®, myiQ® Stratagene™, MX3000P® and MX3005P®
FAST 96-well Plate	ABI® FAST 96-well blocks
AB 96-well Plate	All ABI® "standard" blocks (7000, 7300, 7500, 7700, 7900)
384-well Plate	All common 384-well thermal cyclers, such as ABI®, Eppendorf®, Bio-Rad®

If you are using the Roche® LightCycler® 480 with 96-well block or 384-well block or a device requiring a low-profile plate such as the BIO-RAD® CFX96® or Stratagene™ MX4000®, please contact us to inquire about a StellARray™ qPCR Array in compatible plate format.

Note

Be sure that you have the correct PCR array format for your instrument before starting the experiment.

6.0 Materials Needed (not provided)

- Optical adhesive plate covers
- 2 x SYBR® Green Master Mix, with hot start *Taq* DNA polymerase

Note

Depending on the requirements of your thermal cycler, StellARray™ Products can be used with SYBR® Green Master Mixes containing ROX™, fluorescein or no passive reference dye. Check with your instrument manufacturer for the requirements regarding the passive reference dyes used in the Master Mix. Be sure to use the correct Master Mix for your thermal cycler before continuing with this protocol.

- Nuclease-Free dH_2O
- cDNA samples for gene expression analysis or gDNA samples for copy number variation (CNV) analysis.

Note

Ensure you have read the recommendations for sample preparation and quality control.

7.0 Recommendations for RNA Sample Preparation and Quality Control

Note

High quality RNA is ESSENTIAL for obtaining accurate gene expression results. The most important prerequisite for any gene expression analysis experiment is consistent, high-quality RNA from every experimental sample. Therefore, the sample handling and RNA isolation procedures are critical to the success of the experiment. Residual traces of proteins, salts and especially genomic DNA, will negatively impact the accuracy of gene expression experiments.

The use of high quality products for RNA purification is strongly recommended (please contact scientific.support@lonza.com for specific recommendations).

A DNase treatment step (on-column or in separate tube) must be performed.

RNA Quality Control

Remove an aliquot of the DNase-treated RNA for quantification and qualification. Quantification and check of purity should be done by UV spectrophotometry.

- A_{260}:A_{230} ratio should be greater than 1.7

– A_{260}:A_{280} ratio should be greater than 2.0
– Concentration by A_{260} should be greater than 4 µg / ml total RNA

Determine the quality of your RNA samples by denaturing agarose gel electrophoresis (e.g. by using the FlashGel® System for RNA; Lonza)

Note

There should be a sharp distinction at the small side of both the 18S and 28S ribosomal RNA (rRNA) bands or peaks. Any smearing or shoulder to the rRNA bands or peaks indicates that degradation has occurred in the RNA sample.

8.0 cDNA Synthesis

A cDNA generation kit that works with random decamers (hexamers are acceptable) or oligo-dT primers or a mixture of both should be used for the cDNA synthesis step.

9.0 Protocol for qPCR

1. Calculate experimental cocktail volumes using the Reagent Calculator. (http://array.lonza.com/reagentcalculator)

Super Mix Recipe (per plate)

Note

At least 3 replicates are required per experimental condition

	96-well plate	96-well fast plate	384-well plate
2xSYBR® Green Master Mix	1056 µl	528 µl	2112 µl
dH_2O	998 µl	499 µl	1996 µl

Sample-Specific Working Solutions (per plate)

	96-well plate	96-well fast plate	384-well plate
Super Mix	2006 µl	1003 µl	4013 µl
Template*	106 µl	53 µl	211 µl

* Typically a 1:10 or 1:20 dilution of cDNA in nuclease-free water; gDNA 400 ng per 96-well plate; 1500 ng 384-well plate.

2. Remove foil seal from StellARray™ Plate.
3. **Either:** Add 20 µl Sample-Specific Working Solution to each well (96-well plate)
   Or: Add 10 µl Sample-Specific Working Solution to each well for 384-well or 96-well fast plates.
4. Firmly seal the StellARray™ Plate.
5. Centrifuge (hand-spin) the StellARray™ Plate to ensure all reagents are at bottom of well.
6. "Soak" StellARray™ Plate by incubating at room temperature (or 4°C) for 15 minutes prior to loading into qPCR machine.
7. Load plate into preprogrammed machine.

Standard PCR conditions

One (1) cycle at 50°C for 2 minutes
One (1) cycle of recommended Master Mix Specific Hot Start Temperature and Time
Forty (40) cycles at 95°C for 15 seconds followed by 60°C 1 for minute
Dissociation (Melt) Curve – refer to instrument specifications

Fast PCR conditions

One (1) cycle at 50°C for 1 minute
One (1) cycle of recommended Master Mix Specific Hot Start Temperature and Time
Forty (40) cycles at 95°C for 1 second followed by 60°C at 20 seconds
Dissociation (Melt) Curve – refer to instrument specifications

Perform Global Pattern Recognition™ Data Analysis

8. Prepare qPCR data for export to GPR. On threshold based instruments, set common threshold value (typically 0.15-0.2) after evaluating amplification curves.
9. Export data produced to a CSV (Comma Separated Value) or Microsoft® Excel® file spreadsheet. The format of the file should adhere to the following guidelines (Fig. 2):

– The first field should contain well positions
– The next group of fields should be the CT values from the control
– The next group of fields should be the CT values from the experiment

Figure 2. Example of layout for uploading data to Global Pattern Recognition™ Analysis Tool.

10. Upload data to Global Pattern Recognition™ Analysis Tool (http://array.lonza.com/gpr):

– Enter the Lonza product identifier (For Example: 00188162)
– Please select either a CSV (Comma Separated Value) or Microsoft® Excel® file to upload
– Indicate the number of control samples and experiment samples

10.0 Interpretation of Results

The results obtained from the GPR analysis are not ranked strictly by fold change. When a single gene normalizer is selected, gene expression changes are typically ranked by their magnitude of change using the $\Delta\Delta Ct$ method, with those genes showing the largest fold changes ranked as most significant. Unfortunately, these large changes in gene expression may mask small but biologically important changes in gene expression, such as master regulator genes (e.g., transcription factors).

In biological systems, however, large is not always synonymous with importance. The Global Pattern Recognition™ Analysis Tool algorithm generates a ranked list of significantly changed genes within a real-time PCR dataset. The unique algorithm and accompanying software overcomes the problem of identifying invariant normalizers and the pitfalls of producing faulty statistics based merely on magnitude of change. Only after the genes are statistically ranked is the magnitude of the change calculated. The Global

Rank	Gene Name	p-value	Fold Change
1	FOS	0.0001	29.7582
2	EGR1	0.0004	20.6085
3	JUNB	0.0005	7.9065
4	GADD45B	0.0015	4.0261
5	CYP1A1	0.0023	-3.5846
6	EDN1	0.0046	-2.4949
7	HMOX1	0.0049	-2.6288
8	MYC	0.0051	-2.6534
9	JUN	0.0056	2.3803
10	DNAJB1	0.0062	2.3285
11	HBEGF	0.0067	3.7693
12	SERPINE1	0.0081	3.0827
13	CYP3A4	0.0091	-2.4592
14	IGFBP3	0.0131	2.0451
15	NOS2A	0.0137	2.7647
16	MCL1	0.0140	-1.8457
17	GAP43	0.0153	3.1655
18	MT3	0.0160	2.5987
19	CYR61	0.0177	1.8896

Rank	Gene Name	p-value	Fold Change
20	SLC16A3	0.0240	1.6755
21	FADD	0.0242	-1.8505
22	ABCC2	0.0259	-1.5363
23	SLC22A6	0.0263	5.1732
24	TXNRD1	0.0270	-1.5292
25	SLCO1C1	0.0276	3.6201
26	MMP9	0.0280	2.6985
27	TNF	0.0308	1.9693
28	NAT2	0.0347	-1.5462
29	CCNG2	0.0378	1.6557
30	ABCB9	0.0381	-1.6587
31	VEGFA	0.0390	1.6334
32	BCL6	0.0396	1.6786
33	ESR1	0.0398	1.8955
34	BCL2L1	0.0400	-1.4412
35	FOSB	0.0404	2.4147
36	CYP7A1	0.0405	2.6760
37	SLC2A1	0.0427	1.6791

Figure 3. GPR results derived from Acetaminophen treatment of Clonetics® ReadyHeps™ Primary Human Hepatocytes. Results shown represent genes whose expression levels are statistically different between the treated and untreated groups and Fold Change values are displayed with respect to the treated group.

Pattern Recognition™ Tool provides a true statistical analysis of results based on consistency in the data, making the Global Pattern Recognition™ Analysis Tool optimally suited to detect small, but reproducible changes.

11.0 References

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5. Chen YG, Choisy-Rossi CM, Holl TM, Chapman HD, Besra GS, Porcelli SA,Shaffer DJ, Roopenian D, Wilson SB, Serreze DV. Activated NKT cells inhibit autoimmune diabetes through tolerogenic recruitment of dendritic cells to pancreatic lymph nodes. *J Immunol*. 2005 Feb 1;174(3):1196-204.

12.0 FAQs

What type of Master Mix should we use with the StellARray™ Gene Expression System?

The Master Mix of choice is a 2x SYBR® Green Master Mix with a hotstart *taq* polymerase. Check with the instrument manufacturer for the requirements regarding the passive reference dyes used in the Master Mix. StellARray™ qPCR Arrays are compatible with Master Mixes containing ROX™ (e.g. for ABI® and Stratagene® Thermal Cyclers), fluorescein (e.g. for Bio-Rad® iCycler®, MyiQ™, and iQ™5 Thermal Cyclers) or without (e.g. Roche®, Bio-Rad® Opticon™, Opticon™ 2, and Chromo4™ Thermal Cyclers) passive reference dye.

How are genes ranked in the GeneSieve™ Query?

All mammalian genes tagged with the search term are filtered through the GeneSieve™ Query modules to look for relationships. The result is a hit list of genes ranked by their relevance associated with the particular search term. GeneSieve™ Query then filters genes of interest using a variety of parameters such as overall score, the type of data used, literature reference, and microarray fold change.

For gene expression, are the primers designed to detect 3'-end or 5'-end mRNA sequences?

The primer designs are not usually directed to any specific region of a message as the aim is for primer candidates that will produce highly efficient and specific PCRs. Because the use of a mixture of oligo-DT and random decamers is recommended, the reverse transcription is not effected by the location of the amplicon.

If the same assay can detect both gDNA and mRNA, how do I know that your plate won't detect genomic DNA when I am trying to measure changes in mRNA expression?

As for any method for gene expression analysis, quality of RNA sample prep is critical. StellARray™ Products can be used to detect genomic DNA or cDNA (synthesized from mRNA). If you are evaluating mRNA expression, genomic DNA must be removed by DNase digestion before cDNA synthesis. StellARray™ Plates include a control that checks for the absence of genomic DNA.

Does the StellARray™ Process and GPR analysis use normalizers?

Yes, but not in the "conventional" sense. GPR finds the genes that are invariant and sets those as "normalizers". The researcher no longer has to guess which housekeeping gene is invariant before conducting the experiment

How much RNA is needed per well in a 96-well plate?

250 to 500 ng total RNA is recommended per cDNA reaction. A dilution of the cDNA is made (typically 1:20 in dH_2O) and the required volume of cDNA

added to the Sample-Specific Working Solutions. In some cases successful experiments have been conducted using as little as 25 ng total RNA per cDNA reaction and applied to a 384-well StellARray™ qPCR Array.

How are the primers validated?

Each primer pair undergoes extensive *in silico* and wet lab validations, ensuring specificity and high sensitivity. Primer design parameters were selected to enhance:

- Gene specificity
- Amplicon efficiency
- Uniformity of primer Tm's
- A/T-rich 3'-ends of primers
- Elimination of primer-dimer artifacts
- Generation of single-peak dissociation curves
- Detection of single bands of expected size via gel electrophoresis

By utilizing stringent criteria for both design and performance, accurate, gene-specific results are ensured.

If technical support is required please contact scientific.support@lonza.com

13.0 Ordering Information

Please find the ideal StellARray™ qPCR Array for your research area at www.lonza.com/arrays.

14.0 Related Products

FlashGel® System for RNA

Lonza

Contact Information

North America
Customer Service: 800 638 8174 (toll free)
Scientific Support: 800 521 0390 (toll-free)
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