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A Simple and Reliable Method For Prokaryotic mRNA Amplification for Gene Expression Analysis with Affymetrix GeneChip® Arrays and QPCR

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ABSTRACT

Gene expression analysis for prokaryotes has gained significant importance in biodefense, biological research, and drug development. One major challenge, however, has been obtaining enough RNA for gene expression profiling from small biologically significant samples. Moreover, current RNA amplification methods designed for eukaryotes are not applicable for prokaryotic mRNA amplification. Therefore, there is a need for a robust and reliable method to globally amplify prokaryotic mRNA. To meet this requirement for transcription profiling from small samples, NuGEN Technologies, Inc. has developed a novel, isothermal linear amplification method known as Ribo-SPIA™. Ribo-SPIA™ is rapid, robust and efficient, generating labeled targets for array analysis within a single day. Ribo-SPIA™ reproducibly achieves highly efficient mRNA amplification from very small (5 to 100ng) total RNA samples. Ribo-SPIA™ generates single-stranded DNA products that are homologous to the first strand cDNA, while maintaining consistent representation of transcripts within the population. Products that incorporate the Ribo-SPIA™ amplification have been commercialized under the product family name Ovation™. In addition, we have developed novel methods for fragmenting and biotin labeling the amplified cDNA products, and have successfully applied this novel target preparation method for expression profiling analysis using Affymetrix GeneChip® technology. We will now present a new method called WT-SPIA™ (Whole Transcript-SPIA™) for rapid, efficient and robust prokaryotic mRNA amplification. WT- SPIA™ uses proprietary DNA/RNA random primers for the cDNA synthesis and subsequent cDNA amplification by NuGEN's linear single primer isothermal amplification. WT-SPIA™ generates about 10ug of cDNA from 10ng total bacterial RNA in about 4 hours. The method is suitable for gene expression analysis with Affymetrix GeneChip® arrays or pre-amplification for quantitative PCR assays. Results are presented for global gene expression profiling using GeneChip® arrays and real-time PCR data from two biological paradigms in two independent species, Pseudomonas aeruginosa and E. coli. The linearity, accuracy and reproducibility of WT-SPIA™ are demonstrated by statistically significant correlation of differential gene expression determination in amplified and non-amplified cDNA using TaqMan (R^2=0.96) and the highly sensitive and reproducible data for differential gene expression obtained with GeneChip® arrays.

INTRODUCTION

Microarrays provide a powerful tool for parallel expression analysis of large numbers of genes. However, the challenge is to have enough total RNA to generate meaningful gene expression data. This is particularly challenging for prokaryotes, samples for clinical diagnosis, obtained at different pathological stages or during disease progression and therapeutic intervention, where small amounts of RNA are expected. Moreover, many pathogenic microorganisms are difficult to culture, or suitable culture conditions are unknown. Sample mRNA amplification is one solution to this problem. NuGEN™ Inc has developed a novel technology, Ribo-SPIA™, for global amplification of mRNA. Ribo-SPIA™ is a rapid, isothermal linear amplification method that generates single stranded cDNA products (complementary to the original mRNA). This novel technology is the basis for NuGEN's Ovation™ family of products for highly efficient amplification and labeling of mRNA from eukaryotes, requiring very small amount of samples (as little as 5 ng total RNA), with very high fidelity and accuracy. The amplification products are directly suitable for labeling and fragmentation for gene expression analysis on various array platforms including Affymetrix GeneChip®, Agilent oligonucleotide, CodeLink® arrays, and others.

We further advanced the technology for the amplification of prokaryotic RNA by using specially designed DNA/RNA chimeric primers for random initiation of the first strand cDNA synthesis, and subsequent amplification of the whole transcript, using the novel Ribo-SPIA™ procedure and reagents. The feasibility of this new approach is demonstrated herein for global gene expression analysis in Pseudomonas aeruginosa and E. Coli. total RNA.

Figure 1

WT-SPIA™ RNA Amplification and Labeling Process

First Strand cDNA Synthesis:

Single strand cDNA is prepared from total RNA using a uniquely designed random DNA/RNA chimeric primer and reverse transcriptase. The DNA portion of the primer randomly hybridizes to the mRNA to initiate the first strand cDNA synthesis. The resulting cDNA/mRNA complex has a unique RNA target sequence at the 5´ end of the cDNA.

Second Strand cDNA Synthesis:

A heating step followed the first strand cDNA synthesis results fragmentation of the mRNA in the cDNA/mRNA complex, DNA polymerase then generates second strand cDNA. It also generates DNA copy that is complementary to the 5´ portion of the primer, therefore a unique sequence is incorporated into the second strand cDNA. A double stranded cDNA with an RNA/DNA heteroduplex of unique sequence at one end is formed at the end of the 2nd strand cDNA synthesis.

Single Primer Isothermal Linear Amplification (SPIA™):

The isothermal linear amplification step uses a second DNA/RNA chimeric primer, DNA polymerase and RNase H. RNase H removes the unique RNA sequence in the double stranded cDNA revealing the sequence for the amplification DNA/RNA chimeric primer. DNA polymerase synthesizes cDNA starting at the 3´ end of the DNA/RNA amplification primer and displacing the existing forward strand. RNA at the 5´ end of the newly synthesized strand is again being removed by RNase H, therefore exposing the priming site for the DNA/RNA amplification primer to initiate next round of DNA synthesis. The process of DNA/RNA primer binding, DNA synthesis and strand displacement and RNA digestion work simultaneously with multiple DNA polymerase molecules amplifying same template, leading to rapid linear isothermal amplification.

Amplified Product Fragmentation and Labeling:

A proprietary chemical and enzymatic process developed by NuGEN was employed to accomplish cDNA fragmentation and labeling at one step.

Acknowledgment

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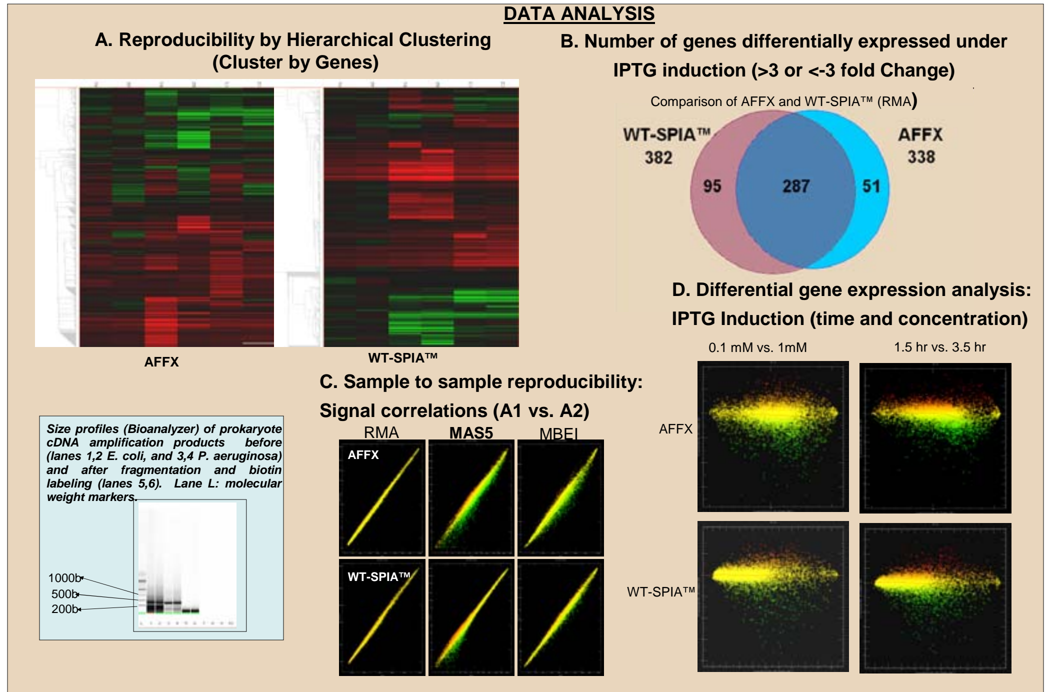
DATA ANALYSIS



VALIDATION OF WT-SPIA™ AMPLIFICATION PROCESS

WT-SPIA™ reproducibility: qPCR (SYBR® Green) CT values for non amplified cDNA and for WT-SPIA™ amplification products

Pseudomonas aeruginosa

Sample	PhzR Ave. Ct			oprD Ave. Ct			rpoA Ave. Ct			rpsB Ave. Ct		
	cDNA	WT-SPIA™	Amp fold	cDNA	WT-SPIA™	Amp fold	cDNA	WT-SPIA™	Amp fold	cDNA	WT-SPIA™	Amp fold
12M	N/D	31.0	N/D	27.7	21.4	552	27.8	21.2	827	32.1	27.0	258
22M	N/D	32.1	N/D	28.3	21.1	1298	27.8	20.4	1590	30.7	24.5	575
32M	N/D	33.9	N/D	30.7	20.3	8179	31.6	21.8	7699	32.5	24.1	2369
14M	34.0	26.4	3392	27.3	20.6	731	29.5	22.3	1271	33.1	27.2	429
24M	35.8	27.8	4495	28.3	20.5	1508	30.2	22.4	1927	33.6	26.3	1358
34M	33.0	26.5	1617	27.1	20.2	776	28.6	21.8	915	32.1	25.9	568
Ave.			3168			2174			2371			926

E. coli

Sample	dnaJ (3') Ave. Ct			dnaJ (5') Ave. Ct			dnaK Ave. Ct			clpA-2 Ave. Ct		
	cDNA	WT-SPIA™	Amp fold	cDNA	WT-SPIA™	Amp fold	cDNA	WT-SPIA™	Amp fold	cDNA	WT-SPIA™	Amp fold
104	31.2	24.1	2821	30.5	21.8	3444	27.3	18.8	3059	29.7	23.2	1070
204	33.1	24.9	6177	31.7	22.5	5363	28.4	19.7	3590	30.9	23.4	2224
105	34.0	26.4	3820	34.2	24.4	8291	31.4	22.7	3932	30.6	24.0	1204
205	34.1	26.5	3958	34.7	24.4	3987	30.7	22.5	2577	30.4	23.7	1792
106	32.5	24.8	3993	31.7	22.2	6223	29.0	20.1	4132	31.2	24.9	1003
206	31.2	23.9	3228	31.1	21.5	6791	28.5	19.5	4923	30.5	23.6	1467
Ave.			4000			5683			3702			1460

qPCR (TaqMan®): Genes Implicated in IPTG Induction Induced with 0.1mM IPTG

Gene of Interest (GOI)	Growth State	GOI Average C_T	Normalizer Average C_T	GOI Relative Expression
ydfa; B1571	3.5 hrs	25.98 +/- 0.44	26.80 +/- 0.16	4.1
cDNA	1.5 hrs	27.12 +/- 0.18	25.75 +/- 0.28	1
Ydfa; B1571	3.5 hrs	23.23 +/- 0.43	24.91 +/- 0.17	12.3
WTA	1.5 hrs	25.01 +/- 0.32	23.12 +/- 0.41	1
nac; B1988	3.5 hrs	33.50 +/- 0.23	26.80 +/- 0.16	-5.18
cDNA	1.5 hrs	31.20 +/- 0.22	25.75 +/- 0.28	1
nac; B1988	3.5 hrs	26.54 +/- 0.19	24.91 +/- 0.17	-7.68
WTA	1.5 hrs	23.87 +/- 0.32	23.12 +/- 0.41	1
glcB; b2976	3.5 hrs	33.12 +/- 0.18	26.80 +/- 0.16	-9.18
cDNA	1.5 hrs	30.50 +/- 0.27	25.75 +/- 0.28	1
glcB; b2976	3.5 hrs	24.03 +/- 0.22	24.91 +/- 0.17	-15.18
WTA	1.5 hrs	21.12 +/- 53	23.12 +/- 0.41	1

qPCR (TaqMan®): Pseudomonas Genes Regulated by Quorum Sensing

Gene of Interest (GOI)	Growth State	GOI Average C_T	Normalizer Average C_T	GOI Relative to Uninduced
PA0105	Induced	31.22+/- 0.44	30.22 +/- 0.32	5.7
Non-Amplified cDNA	Uninduced	33.01 +/- 0.16	31.05 +/- 0.11	1
PA0105	Induced	25.54 +/- .51	27.26 +/- 0.17	7.6
WTA	Uninduced	27.89 +/- 0.02	28.32 +/- 0.21	1
PA3334	Induced	27.50 +/- 0.67	30.22 +/- 0.32	23.1
Non-Amplified cDNA	Uninduced	32.02 +/- .32	31.05 +/- 0.11	1
PA3334	Induced	22.82 +/- 0.56	27.26 +/- 0.17	32.4
WTA	Uninduced	28.12 +/- 0.81	28.32 +/- 0.21	1
PA3877	Induced	31.56 +/- 0.91	30.22 +/- 0.32	12.9
Non-Amplified cDNA	Uninduced	35.23 +/- 0.97	31.05 +/- 0.11	1
PA3877	Induced	22.83 +/- 0.89	27.26 +/- 0.17	15.7
WTA	Uninduced	27.81 +/- 0.12	28.32 +/- 0.21	1

Pseudomonas aeruginosa Samples:
12M, 22M, 32M: Biological triplicates, non-induced
14M, 24M, 34M: Biological triplicates, Induced

E. coli samples:
104, 204: Biological duplicates; 0.1mM IPTG; 1.5 hrs (A1, A2)
105, 205: Biological duplicates; 0.1mM IPTG; 3.5 hrs (B1, B2)
106, 206: Biological duplicates; 1.0mM IPTG; 1.5 hrs (C1, C2)

MATERIALS AND METHODS

Bacterial strains and total RNAs: E. coli samples were derived from E. coli w3110 strain. Total RNAs were extracted from induced with different concentration of IPTG (0.1mM or 1.0mM) for different times (1.5 and 3.5 hrs). 100 series and 200 series were from E. coli W3110 transformed with two plasmids, pklr1, and pKQV4, and are biological replicates for the study. Plasmid pklr1 contained a gene of interest.

P. aeruginosa strain PAO-JP2 was grown in the presence or absence of exogenous autoinducer (C4-HSL = 2 μM). The magnitude of gene induction (Fold change) is calculated for PAO-JP2 with exogenous autoinducer compared to PAO-JP2 cultures grown aerobically in modified FAB. Values are reported for early stationary phase growth when differential expression was observed in both growth phases. RNA was extracted from biological replicates.

RNA amplification: The WT-SPIA™ for prokaryote procedure is schematically illustrated in Figure 1. All incubations were carried out in a thermocycler. 20ng total RNA samples were amplified using protocol and reagents similar to that of the Ovation™ Amplification System (NuGEN, San Carlos, CA) with the following modifications: 1). The first strand synthesis was carried out by incubation at 25ºC for 10 min., followed by 0.5 hr incubation at 42ºC. 2). The second strand cDNA synthesis was carried out at 50ºC for 0.5 hour. The procedure generates about 10ug amplified cDNA in about 4 hours. 8ug amplified cDNA were fragmented and labeled for microarray application.

Amplified cDNA products were quantified by a spectrophotometer. The sizes of amplification products before and after fragmentation-labeling were analyzed on Agilent 2100 BioAnalyzer.

GeneChip® arrays: E. coli GeneChip® arrays (Affymetrix) were used in these studies. Microarray targets were prepared either by Affymetrix recommended protocol (non amplified) or by the WT-SPIA™ and NuGEN's fragmentation-labeling protocol. GeneChip® hybridization and process for all the targets were carried out according Affymetrix' protocol for eukaryotic GeneChip®. Microarray data analysis was performed using the various commercial microarray data analysis methods including MAS5, RMA and MBEI.

Q-PCR: ABI PRISM™ 7900 Sequence Detection System (Applied Biosystems, Foster City, CA) was used to quantify cDNA. TaqMan® assays were run as recommended (10 min. at 95ºC followed by 40 cycles of 95ºC for 15 sec., 60ºC for 60 sec.) by the manufacturer. Primers were designed using Primer Express® v1. Real time PCR quantifications of genes of interest (GOI) were performed with either non-amplified cDNA generated by random priming reverse transcription (non-amplified) or following amplification using WT-SPIA™ protocol (Amplified). The normalizer for data analysis for the calculation of increased or decreased expression level of the gene of interest (GOI) was the ribosomal RNA subunit. Real Time qPCR with SYBR® Green was carried out using Opticon® (MJ Research) with 2x master mixture of QuantiTect SYBR® Green PCR Kit (Qiagen), and the following thermo-cycle program: 15 min. at 95ºC to activate the DNA polymerase, followed by 45 cycles of 30 seconds each at 95ºC, 55ºC and 72ºC.

CONCLUSIONS

The novel WT-SPIA™ procedure for prokaryote RNA amplification and fragmentation-labeling is fast, simple, sensitive and reproducible:

- **Efficient and rapid RNA amplification for expression analysis in total RNA samples from E. coli and P. aeruginosa was demonstrated.**
- **The method generates about 10µg cDNA from as little as 5 ng total RNA in about 4 hours, and >6 µg of fragmented and biotin labeled cDNA targets, for prokaryote expression analysis on GeneChip® arrays.**
- **Highly reproducible and accurate amplification, and determination of differential expression are demonstrated by quantification of specific transcripts (TaqMan® and real-time PCR with SYBR® Green), in concordance with non-amplified samples.**
- **Performance of WT-SPIA™ generated targets on E. coli GeneChip® arrays was similar to or better than that of non-amplified cDNA generated by the recommended AFFX protocol, as shown by sample to sample signal correlation, differential gene expression of IPTG induction (M vs A plots), and hierarchical clustering by signal.**
- **The method enables highly sensitive differential gene expression analysis for prokaryotes.**