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PCR-enriched Differential Display of Gene Variation

Jason X. Feng[1*] • Bruce W. Stallsmith[2] • Maria R. Davis[2†] • Gopi K. Podila[2†]**

[1] AT Bioscience, LLC, Shelby Center, Huntsville, 301 Sparkman Drive, AL. USA 35899
[2] Department of Biological Science, University of Alabama in Huntsville, Shelby Center, 301 Sparkman Drive, Huntsville, AL. USA 35899
[†]Both of these authors are deceased after an on-campus shooting

Corresponding authors: * **rjxfeng@yahoo.com** ** **stallsb@email.uah.edu**

ABSTRACT

Discovery of genetic variation-based biomarkers is time-consuming and cost-prohibitive. We have developed an integrated high-throughput procedure to comprehensively screen and identify differentially expressed genetic variation-based biomarkers and biomarker-related protein-coding genes. These identified biomarkers and marker-genes will be further utilized to analyze their corresponding chromosome variation using reverse genetic method from published data. These biomarkers, the marker-related genes and their genomic variations will play critical roles in clinical diagnosis, drug discovery and pathogenesis research of diseases. In this study, differentially expressed genes between two mRNA samples are discriminately amplified and displayed. Newly discovered special genetic sequence information is analyzed and further used to develop diagnostic biomarkers. This approach can be used to screen biomarkers from cancer genomes, genetic diseases, and for microorganism characterization.

Keywords: biomarker gene, discriminative polymerase chain reaction, genetic biomarkers, mutation, reverse bioinformatic analysis
Abbreviations: **DNA**, deoxyribonucleic acid; **RNA**, ribonucleic acids; **PCR**, polymerase chain reaction; **PDDGV**, PCR-enriched differential display of gene variation

INTRODUCTION

In the last decade, genetic biomarkers have been shown to play critical roles in all biological fields (Sidransky 2002). Genetic biomarkers represent variant sequences on genomic deoxyribonucleic acid (DNA) or ribonucleic acids (RNA) from normal or diseased organisms. They not only characterize unique functions in individual organisms or potentially alter cell function to induce cell pathogenesis, but are indicators of different disease developmental stages, and are effectively used in biomedical research and early detection of cancer and identification of pathogenic microorganisms (Sidransky 2002).

To meet the high demand for biomarkers in various research and diagnostic fields, a number of biotechnologies have been developed. The restriction fragment length polymorphism (RFLP) method was the first screening technique to detect DNA variation (Botstein *et al.* 1980). However, it is costly, laborious and a large amount of genetic variation in a fragment remains undetected. Liang *et al.* (1993) developed an mRNA differential display technique, which detects a few of the nucleotide differences on the untranscribed region (UTR) of a transcript to identify differentially expressed mRNA molecules. Subtractive complementary DNA (cDNA) hybridization has been a useful tool to identify and isolate differentially expressed genes (Duguid *et al.* 1990), but it is less efficient in the analysis of low abundance messenger RNA (mRNA) molecules. To solve this issue, a PCR-based suppressive subtractive hybridization method was developed (Diatchenko *et al.* 1996; Lukyanov *et al.* 1996) to detect genetic variation in individual organisms in the fields of cancer, genetic diseases and microbiology. Amplified fragment length polymorphism PCR (AFLP-PCR or AFLP) (Vos *et al*. 1995; Caballero *et al.* 2008) is a DNA fingerprinting technique for the genotyping of biodiversity on the genomic DNA level and is visualized on a denaturing polyacrylamide gel. Constant denaturant gel electrophoresis (Borresent *et al.* 1991) and single-strand conformation polymorphism (SSCP) (Orita *et al.* 1989; Hayashi *et al*. 1993) are used for genomic DNA analysis or for the detection of variation in a known single gene by utilizing mobility differences of electrophoresis between mutant DNA and wild-type DNA. Restriction-mediated differential display (Fisher 2001) identifies differentially expressed genes using selective 3′-elongated oligonucleotide primers to generate subpools of restricted cDNA fragments and display them on agarose or polyacrylamide gels. DNA microarrays have enabled researchers to conduct large scale quantitative experiments in the genetic characterization of individual organisms or particular tissues or cell types as well as new gene identification and disease research. However, this technology depends on known DNA probes for its screening (Stoughton 2005), which limits its application and also results in time consuming experimental work.

Here, we describe a demonstrated procedure, PCR-enriched Differential Display of Gene Variation (PDDGV). PDDGV is a high-throughput procedure to comprehensively analyze gene expression profiling and identify altered cDNA molecules in their sequences. In this procedure, most of the common sequences of cDNA fragments are eliminated using the subtraction hybridization method (Diat-

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chenko *et al.* 1996). Differentially expressed cDNAs from the rest of the cDNA mixture are amplified using discriminative PCR and displayed on a non-denatured polyacrylamide gel for comparison analysis. After comparison with reference cDNA (driver), differently mobilized unique DNA fragments are cut, cloned and analyzed. These identified cDNA variants are useful sources for the development of genetic biomarkers, and can be used for reverse bioinformatic analysis to analyze variations in the genomic DNA and for isolating biomarkers-related protein coding genes. Identified biomarkers and biomarker-related genes are also powerful tools for the development of other tools in the fields of clinical practice, drug discovery and biomedical research.

MATERIALS AND METHODS

General preparation

In this study, the targeted tester mRNA is from lung squamous cell carcinoma and referenced driver mRNA is from the adjacent normal tissue of the lung cancer for comparison analysis.

The cancer tissues and adjacent normal tissues were purchased from Invitrogen Co. (CA, USA). mRNAs from each tissue were prepared using PureYield™ RNA Midiprep and PolyATtract® mRNA Isolation kits (Promega, WI, USA). Two cDNA libraries, lung squamous cell carcinoma cDNA as a tester and lung normal tissue cDNA as a driver were constructed using a cDNA construction kit from Invitrogen following the manufacturer's instructions. The two cDNA libraries were stored at -80°C pending further processing.

Other supplies: All restriction enzymes, T4 DNA ligase, calf intestinal alkaline phosphatase (CIP), T4 polynucleotide kinase and DNA size ladders were purchased from New England Biolabs (MA, USA). Advantage® DNA polymerase was purchased from Clontech (CA, USA). Gel extraction and PCR purification kits were purchased from Qiagen (CA, USA). Silver-staining kit and pGEM®-T cloning vector were purchased from Promega. Adapter linkers and oligonucleotides for primary PCR and selective PCR were synthesized by Bioneer Company (CA, USA). All other chemicals used for making solutions were purchased from Sigma-Aldrich USA (MO, USA). All solutions were prepared from the protocols of Sambrook *et al*. (2001). Sterile distilled deionized (SDD) water was purified with a Milli-Q system (MA, USA) in our laboratory. An ABI prism 7000® thermocycler (ABI, CA, USA) was used for PCR throughout the experiment.

This PDDGV technique utilizes differential genetic expression patterns between disease-specific tissue or individual cell type and normal tissue or wild-cell type for the detection of altered and paneled pathogenic or cell-specific protein-coding gene (mRNA) sequences. The success of this procedure relies on discriminative PCR and comparative screening all of the differentially expressed genes on a silver-stained polyacrylamide gel from one form of cancer / genetic disease or cell type to obtain a complete set of the variant mRNA molecules. Second, sequence-determined genetic biomarkers are used in a BLAST search of data on the web sites of the National Center for Biotechnology Information (NCBI) and European Bioinformatics Institute (EBI) to determine mutated genetic sequences and mutation rate in each biomarker-gene. PCR-amplified biomarkers are used as probes to recover the disease or cell-associated genes.

Stage I: cDNA subtraction and primary PCR

The stage I protocol for PCR-select cDNA subtraction is similar to the method described by Diatchenko *et al.* (1996) with some modifications to fit into the differential display process in stage II. Tester cDNA from lung squamous carcinoma and driver cDNA from the adjacent normal lung tissue cDNA were used in this study.

cDNA subtraction: Six µg of each cDNA library (tester and driver) was separately digested with 30 U of each enzyme *Rsa*I and *Alu*I in a 100 µl reaction volume at 37°C for 20 hrs. Five µl of each digested reaction was loaded and run on a 1% agarose tris-boric acid-EDTA (TBE) gel to confirm that digestion was complete. Digested samples showed smeared cDNA ranging from 100 to 1000 base pairs with a few distinctive bands on the gel (**Fig. 1**). The rest of the digested tester cDNA was dephosphated using 3 µl of calf intestinal alkaline phosphate (CIP) at 37°C for 1 hr and cleaned with the PCR purification kit. Tester and driver cDNAs were separately concentrated into 20 µl of SDD water using 3 M NaOAC (pH 5.2) and the ethanol precipitation method. Ten µl (10 µM or 0.3 µg) of each adapter linker (1 and 2, **Table 1**) was phosphorylated using 1 µl of T4 polynucleotide kinase in a 20 µl volume at 37°C for 15 min before ligation.

Four µl of each phosphorylated adapter 1 and 2 were ligated to 300 ng of digested and dephosphorylated tester cDNA fragments A/B groups and driver cDNA fragments A/B groups, each in 20 µl reaction volume with 1X T4 ligase buffer and 1 µl of T4 DNA ligase (400 U/µl) at 16°C overnight. The reaction was inactivated by heating to 65°C for 10 min and stored at -20°C.

To subtract common sequences from expressed genetically varied tester cDNA fragments, 5 µl of the unligated driver cDNA fragments (1250 ng) was added into a PCR reaction containing 2 µl (30 ng) of each adapter 1-ligated tester cDNA A and adapter 2-

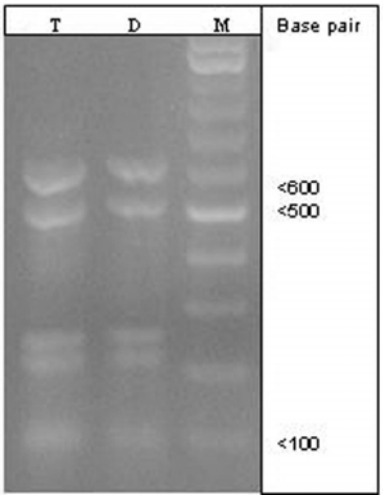

Fig. 1 Digestion of tester and driver cDNA using *Rsa*I and *Alu*I restriction enzymes. Legend: M: 100 base pair DNA marker. T: digested tester cDNA fragments. D: digested drive cDNA fragments.

Table 1 DNA sequences of adapters and oligonucleotide primers.

Adaptor 1	5′ ATTAACCCTCACTAAAGGGAGGGGCGACGACTCCTGGAGCCCG 3′
	3′ CCTCGGGC 5′
Adaptor 2	5′ ATTAACCCTCACTAAAGGGATTGACACCAGACCAACTGGTAATG 3′
	3′ ACCATTAC 5′
PCR primer 1	5′ GAGGGGCGACGACTCCTGGAGCCCG 3′
P3G	5′ GCGACGACTCCTGGAGCCCGG 3′
P3A	5′ GCGACGACTCCTGGAGCCCGA 3′
P3T	5′ GCGACGACTCCTGGAGCCCGT 3′
P3C	5′ GCGACGACTCCTGGAGCCCGC 3′
P4G	5′ CACCAGACCAACTGGTAATGG 3′
P4A	5′ CACCAGACCAACTGGTAATGA 3′
P4T	5′ CACCAGACCAACTGGTAATGT 3′
P4C	5′ CACCAGACCAACTGGTAATGC 3′

ligated tester cDNA B, respectively, 1 µl of SDD water and 2 µl of 5X hybridization buffer (2.5 M NaCl/250 mM Hepes, pH 8.3). The first hybridization in each tube was performed at 98°C for 1.5 min, and then annealed for 10 hrs at 68°C in the thermocycler. After the first hybridization, the two samples were combined and 2 µl of newly heat-denatured unligated driver cDNA (800 ng) in 1X hybridization buffer (0.5 M NaCl/50 mM Hepes, pH 8.3) was added. The second hybridization for this mixed sample was performed at 68°C for 10 hrs. This second hybridization sample was then diluted in 100 µl of dilution buffer (20 mM Hepes, pH 8.3/50 mM NaCl/0.2 mM EDTA). The diluted sample was heated at 72°C for 7 min and stored at -20°C.

Primary PCR: Ligated and unsubtracted driver cDNA and subtracted tester cDNA were separately amplified in a PCR reaction of 50 µl, containing 31 µl of SDD water, 5 µl of 10X PCR buffer, 0.5 µl of Advantage® *Taq* DNA polymerase, 10 µl of 10X deoxyribonucleotide triphosphate (dNTP, 2 mM), 1 µl of diluted DNA sample, 2 µl of DNA primer 1 (10 µM, **Table 1**), which have common 5′ sequences of adapter 1 and adaptor 2. Two PCR mixtures were incubated at 72°C for 5 min, and then 27 cycles of 94°C for 30 sec, 66°C for 30 sec and 72°C for 1.5 min. Following this PCR, 10 µl of each reaction was run on a 2% agarose gel in 1X tris-acetate-EDTA (TAE) buffer to determine amplification efficacy and size range. Three µl of each reaction was diluted into 27 µl (1:9) of SDD water and stored at -20°C for subsequent processing.

Stage II: Discriminative PCR and differential display, subcloning and DNA sequencing

Discriminative PCR amplifications were performed using sixteen pairs of combinatorial DNA primers (**Tables 1** and **2**), P3 primer from adapter 1 with G, A, T, C on the end and P4 primer from adapter 2 with G, A, T, C on the end. Each of the paired PCR samples (ligated subtracted tester cDNA vs unsubtracted driver cDNA) contained 1 µl of each combinational P3 and P4 primer (5 µM) and 18 µl of reaction solution each from the tester master mixture (8 µl of the tester cDNA primary PCR dilution, 36 µl of 10 X PCR buffer, 36 µl of 10X dNTP (10 uM), 8 µl of Advantage® DNA polymerase and 236 µl of SDD water) or from the driver master solution. Alternatively, the driver cDNA primary PCR dilution was used, but not tester cDNA. Sixteen pairs of PCR reactions were performed at 14 cycles of 94°C for 2 min, 72°C for 2 min and one cycle of 72°C for 7 min. After PCR, 5 µl of each sample was analyzed on a 2% agarose gel in 1X TAE buffer to pre-screen amplification efficacy for each set of PCR amplification.

Table 2 Combinatorial pattern of oligonucleotide primers for discriminated PCR.

	P3G	P3A	P3T	P3C		P3G	P3A	P3T	P3C
P4G	X	X	X	X	P4A	X	X	X	X
P4T	X	X	X	X	P4C	X	X	X	X

Differential display of PAGE

Each amplified pair of PCR samples with 1X DNA loading buffer was loaded on a 4% non-denatured polyacrylamide gel with 10% glycerol in 0.5X TBE running buffer (gel size: 25 cm × 40 cm × 0.4 mm). In addition, three µl of each PCR sample with 1X DNA gel loading buffer from the driver sample and tester sample of discriminative PCR was mixed, then denatured and renatured to form hybrid-duplex sample, and loaded on the third lane of each group.

The gel was run at 350 V for 1 hr and 175 V for 16 hrs in 0.5X TBE buffer. After running, the gel was silver-stained using a kit from Promega, following the manufacturer's instructions.

By comparing to adjacent driver cDNA PCR bands, each unique band on the tester cDNA lane was cut and dissolved into 10 µl of tris-EDTA (TE) buffer. After the gel solution was boiled for 3 min and cooled to room temperature, each DNA gel solution was re-amplified in 50 µl of reaction, each containing 1 µl of the same combination primer pair, 5 µl of 10X Advantage® PCR buffer, 5 µl of 10X dNTP (2 mM), 10 µl of the DNA gel solution, 1 µl of Advantage® DNA polymerase and 27 µl of SDD water. The PCR reaction was carried out in the thermocycler programmed for 30 cycles of 94°C for 30 sec, 72°C for 2 min and final 72°C for 7 min. Each PCR band isolated was purified from 1% agarose gel using the gel extraction kit and cloned into a pGEM®-T easy DNA vector following manufacturer's instructions. Each insert sequence in pGEM®-T easy vector was analyzed twice by using an ABI PRISM® 310 DNA sequencer (ABI, CA, USA), to confirm the correct sequence information.

RESULTS AND DISCUSSION

As a limited amount of RNA was available for our PDDGV procedure, we used tester and driver cDNA libraries in the experiment to guarantee enough of the cDNAs needed for continuous steps and recovery of biomarker-genes from the same library. To obtain the optimal size range of digested cDNA molecules to be physically displayed on a polyacrylamide gel, we tested a number of restriction enzymes, singly and in combination, for their ability to digest the cDNA libraries correctly. After trial digestions, we chose the combination of *Rsa*I and *Alu*I from tested enzymes, *Hind*III, *Eco*RI, *Hae*III, *M*seI, *Mbo*I, *Alu*I, *Hinf*I, *Dde*I (**Fig. 1**).

Enrichment and discriminative PCR

Subtractive PCR done in the first stage of this procedure greatly enriched mutated cDNA molecules in subtracted tester cDNA fragments, similar to the technique described in the published subtractive hybridization method (Diatchenko *et al.* 1996). The second stage procedure allowed highly efficient PCR selection of altered genetic sequences and also decreased false-positive results. Highly selective PCR using 16 pairs of the primers as shown in **Tables 1** and **2** not only divided the whole cDNA pool into 16 subpools clearly seen on a PAGE gel, but most importantly, only amplified matched nucleotide sequence between 3′-end of the primer pairs and tester/driver cDNA fragments. In this experiment, combinational groups 11, 12 and 16 of DNA primers were not amplified after the PCR (data not shown), which indicate that the 3′-end of the primer pairs does not have a sequence match with the digested cDNA molecules. Through this discriminative PCR, sequence-altered tester cDNA molecules matched with DNA primer sequences were differentially amplified, and displayed different mobility on a non-denaturing polyacrylamide gel compared to driver cDNA molecules. The glycerol in the gel solution allowed for better resolution of DNA bands (Pennings *et al.* 1992) and easy isolation and subcloning of DNA fragments from the gel. **Fig. 2** shows differentially displayed cDNA fragments between driver and tester cDNA molecules. To observe the potential mismatched hybrids of single-stranded tester and driver cDNA pools, we loaded the hybrids in the third lane in each group. However, resolution of single-stranded DNA was not enough to discriminate mismatched hybrids in each lane from the whole cDNAs of its subset.

Genetic analysis of the differentially displayed DNA bands: All unique DNA bands were cut and subcloned into pGEM®-T easy vector and subsequently sequenced. All of the cDNA insert sequence information from the clones was determined after deleting vector and adapter sequences using the align program of the NCBI. Forty-five cDNA inserts with altered genetic sequences and their protein homologues were screened and further analyzed using a BLAST search from the data banks of NCBI and the EBI to: 1) Align to normal gene and genomic sequences to check mutated rate and sequences as potential genetic markers; and 2) Define the potential marker-associated gene functions and their roles in the carcinogenesis of cells. Identified biomarkers and their DNA sequence comparison will be published separately.

The PDDGV technique described here is a convenient, efficient and fast procedure for genetic biomarker discovery. When compared to cDNA subtraction and other technologies, our procedure, after subtractive enrichment of tester-

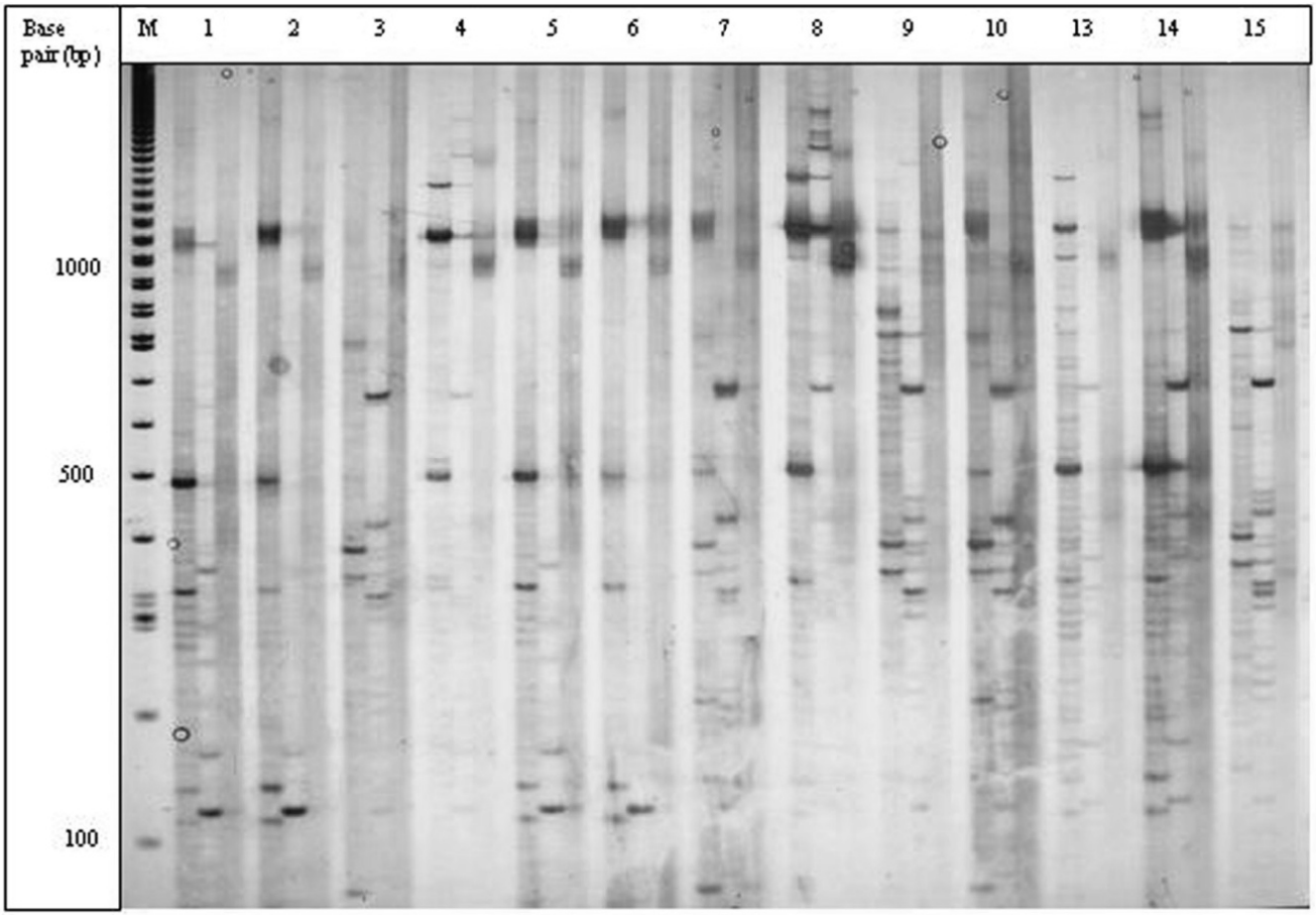

Fig. 2 Differential display of discriminative PCR between driver cDNA vs tester cDNA on non-denature polyacrylamide gel electrophoresis. Legend: M is 100 base pair DNA ladder; primer combination groups 11, 12 and 16 were not amplified by special primer pairs in pre-screening. Lanes 1–15 are discriminately PCR amplified using special primer combination. Lane numbers are the same as primer group numbers. In each group, in left to right order are amplified driver cDNA, amplified tester cDNA and hybrid of single-stranded driver and tester cDNA. Tester cDNAs show unique DNA bands in comparison to driver cDNA.

dominant cDNA molecules, can directly amplify, display and clone variant molecules of the tester cDNA, so that there is no need to work on time-consuming microarray screening using known sequence DNA probes. In addition, as the technique uses combinational digestion of two four-base cut restriction enzymes, *Rsa*I and *Alu*I, and displays discrepancy mobility of digested cDNA mocleules on PAGE gel, the PDDGV procedure could detect not only difference of UTR of mRNA like differential display RT-PCR, but also altered protein-coding sequences of expressed genes. Moreover, sub-grouped mRNA molecule pools are made more easily visible on the gel and more available for subcloning for differential expressed genes.

CONCLUSION

Due to the high demand for biomarkers in the pharmaceutical industry, healthcare provision and biomedical research in the last decade, a wealth of tools has been developed in the search for predictive and specific biomarkers (Scaros *et al*. 2005). The biomarkers can offer significant benefits throughout clinical field, biomedical research, drug discovery, the identification of biological differences among different biological species and the understanding of fundamental biological processes and relationships (Fitz *et al.* 2008).

Identification of biomarkers is a time-consuming and costly process. The PDDGV we have developed is a highly sensitive procedure and its utility has been confirmed through our experimental results. Compared to other techniques currently used, the PDDGY is faster, more convenient and costs much less without the need for prior knowledge of genetic information as is necessary in microarray procedures. This bio-procedure also overcomes issues previous methods have in time-consuming screening and losing information in low abundance mRNA molecules. This validated procedure allows a research project to be finished within six months, including the screening of differentially displayed genes and the analyses and identification of potential biomarkers and biomarkers-related genes. All of these discovered genetic biomarkers, biomarker-genes and their expressed proteins and the proteins-induced antibodies could play significant roles in biomedical research, clinical practice and drug discovery.

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