Journal of Biochemistry and Molecular Biology, Vol. 37, No. 4, July 2004, pp. 439-444

JOURNAL OF Biochemistry and Molecular Biology

Comparison of Hybridization Behavior between Double and Single Strand of Targets and the Application of Asymmetric PCR Targets in cDNA Microarray

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Received 24 September 2003, Accepted 6 November 2003

Double stranded targets on the cDNA microarray contain representatives of both the coding and noncoding strands, which will introduce hybridization competition with probes. Here, the effect of double and single strands of targets on the signal intensity and the ratios of Cy5/Cy3 within the same slide were compared. The results show that single stranded targets can increase the hybridization efficiency without changing the Cy5/Cy3 ratio. Based on these results, a new strategy was established by generating cDNA targets with asymmetric PCR, instead of conventional PCR, to increase the sensitivity of the cDNA microarray. Furthermore, the feasibility of this approach was validated. The results indicate that the cDNA microarray system based on asymmetric PCR is more sensitive, with no decrease in the reliability and reproducibility as compared with that based on conventional symmetric PCR.

Keywords: Asymmetric PCR, cDNA microarray, Hybridization, Sensitivity, Single-stranded DNA

Introduction

cDNA microarray analysis (Schena *et al*., 1995; DeRisi *et al*., 1996; Schena *et al*., 1996; Schena *et al*., 1998; Stears *et al*., 2003) has become the most widely used technique for the study of gene expression patterns on a genomic scale. This technology is the latest in a line of techniques to exploit a potent feature of the DNA duplex-the sequence complementarities of the two strands. The first strand of a cDNA probe (non-coding strand) in the liquid phase will hybridize against the solid phase coding strand of the target. The introduction of solid supports greatly increases the range of applications of the method, and sets the trail to array-based methods (Southern *et al*., 1999). However, this introduces complexity to the hybridization system due to uncertain factors, such as the nature of modified solid surface, steric effects and immobility of the solid phase targets. Particularly, when double-stranded PCR products (dsDNA) are immobilized on solid slide targets, nucleic acid analysis has been promoted to an unprecedented level of complexity because of the hybridized competition in the microarray system. This competition not only increases the complexity of hybridization, but also influences the hybridization efficiency, which is closely associated with the sensitivity of the microarray.

The sensitivity has been one of the key points in the utility of current microarray formats. Many genes with important biological function have low transcript level, and rare mRNAs make up 80-90% of total mRNA (Nallur *et al*., 2001; Salin *et al*., 2002). Large amounts of mRNA or total RNA is usually required because of relatively low sensitivity. To keep more valuable information, it is important to increase detection sensitivity (Kooperberg *et al*., 2002).

It is known that a single stranded molecule can hybridize with complementary DNA more efficiently without competitive pressure. However, large-scale preparation of single stranded targets is relatively difficult and unpractical. Therefore, one possible strategy to increase the sensitivity of a cDNA microarray is to replace the dsDNA targets from conventional PCR with ds-ssDNA mixture targets produced by asymmetric PCR. Asymmetrical PCR, which uses a large

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excess of one primer to that of the other, has been used to produce a partial ssDNA target (Mao *et al*., 1999). To date, there have been no reports on the application of asymmetrical PCR targets on a microarray, or the systematical comparison of hybridization behavior between ssDNA and dsDNA arrays. Accordingly, at least two questions need to be answered before applying asymmetric PCR in the cDNA microarray fabrication; how much the single-stranded DNA could improve the fluorescent signals, and whether ssDNA would introduce significant ratio bias.

In this report, the hybridization behaviors of ssDNA and dsDNA targets were systematically validated. Moreover, the optimal asymmetric PCR parameters were described for the manufacture of effective ds-ssDNA arrays with highly reproducible, accurate and reliable performance.

Materials and methods

Preparation of targets

Double strands of cDNA targets All the cDNA clones with the same orientation insert in the pGEM-T Vector plasmids were provided by United Gene Holding, Ltd (Shanghai, China). The cDNA inserts were amplified with standard PCR procedure using M13 primers (M13F: CGCCAGGGTTTTCCCAGTCACGA and M13R: AGCGGATAACAATTTCACACAGG). The PCR reaction system contained 0.2 mM dNTP, 1.5 mM $MgCl_2$, 0.1-1 ng template, 0.2 μM forward and 0.2 μM reverse primers, and 5U rTaq DNA polymerase (Takara Biotechnology Co., Ltd). The mixture was incubated for 30 s at 94°C and 40 cycles of PCR were performed at 94°C for 30 s, 60°C for 30 s and 72°C for 2 min. Then an incubation for 6 min at 72°C, followed by reduction to and maintenance at 4. PCR products were purified as described previously (Sambrook *et al*., 1989).

ds-ss DNA mixture In asymmetrical PCR, the reaction conditions and purification were the same as with conventional PCR, except for the use of 1 μM forward and 0.02 μM reverse primers.

Single strand of cDNA and RNA targets A cDNA cone of GDNF (Hs.248114) of 750 bp was chosen to make a simulant DNA microarray. Single-stranded DNA was isolated from an asymmetric PCR product by cutting gels. RNA was made from T7 *in vitro* transcription using a dsDNA template (Wang *et al*., 2000; Yue *et al*., 2001). Purified DNA or RNA was quantified with UV260/280. The purified targets were dissolved in 3 × SSC (standard saline citrate, 0.45 M NaCl and 0.045 M sodium citrate) buffer pH 7.0 before arraying.

DNA arraying process The targets were spotted onto silylated slides (Shanghai Biostar Genechip, Inc.) using a Cartesian PixSys 7500 motion control robot (Cartesian Technologies, Irvine, USA) fitted with ChipMaker Micro-Spotting Technology (TeleChem International, Sunnyvale, USA). Glass slides with spotted molecules were then hydrated for 2 h in 70% humidity, dried for 0.5 h at room temperature and UV cross-linked (65 mJ/cm). They were further processed at room temperature by soaking in 0.2% sodium dodecyl sulfate (SDS) for 10 min, distilled H_2O for 10 min and 0.2% sodium borohydride ($NaBH_4$) for 10 min. Lastly, the slides were dried again prior to use.

In the simulant array, there types of GDNF target (dsDNA, ssDNA and ss-RNA) were spotted fivefold, in a series of 2-fold concentration dilutions. In the cDNA microarray, 96 human dsDNA and corresponding ds-ss DNA mixtures were spotted onto the same slides.

Preparation of probes The fluorescent cDNA probes of GDNF were made from reverse transcription of the *in vitro* transcription RNA with Cy3-dCTP or Cy5-dCTP (Amersham Bioscience, Uppsala, Sweden), and then purified according to the methods of Schena *et al* (Schena *et al*., 1995).

Tissue samples were kindly provided by Changzheng hospital in Shanghai. The RNA samples from normal human fetal and cancerous livers were prepared as previously described (Li *et al*., 2002) and then separately labeled with Cy3-dCTP and Cy5-dCTP, as above.

Hybridization and washing Microarrays were pre-hybridized with Hybridization Solution (5 × SSC, 0.2% SDS and 50% formamide) containing 0.5 mg/ml denatured salmon sperm DNA at 42°C for 6 h. The labeled probe mixture was lyophilized with a vacuum pump, dissolved in 20 μl hybridization solution and then fluorescent mixtures were denatured for 5 min at 95°C. The denatured probe mixtures were applied to the pre-hybridized microarray that had been boiled for 5min, and placed in a humid 42°C chamber for 15-17 h. After hybridization, the microarray slides were washed once at 60°C with washing solution, 2 × SSC and 0.2% SDS solution, and again with 0.1 × SSC and 0.2% SDS solution, followed by 0.1 × SSC solution, for 10 min each, then dried at room temperature.

Signal detection and data analysis The arrays were scanned with a ScanArray 4000 (GSI Lumonics, Bellerica, USA). The acquired images were analyzed by GenePix pro 3.0.5 software (Axon Instruments, Inc). All the spot signals had background-subtracted intensities. The signals in two channels were normalized within each array using a total intensity approach.

In order to extend the linear range of the scanner, every array was scanned 2 or 3 times at 95% laser power, with photomultiplier tube gain (PMT) settings ranging from 60-95%. The range of PMT settings were determined by finding the lowest power required to bring the brightest spots into linear range and highest power with reasonable background intensity. The average ratios (AR) of the signals between two scans were calculated using the spots with signals that were both within a good linear range (2,000-50,000). The data from the highest PMT were chosen for further analysis, where the saturated signal was rectified with the product of the corresponding signal within the linear range of the lower laser power and AR of both scans (Aimee *et al*., 2002).

To minimize artifacts arising from low expression values, only genes with raw intensities greater than the mean background value plus two standard deviations of all spots in at least one channel were chosen for further analysis (Epstein *et al*., 2001).

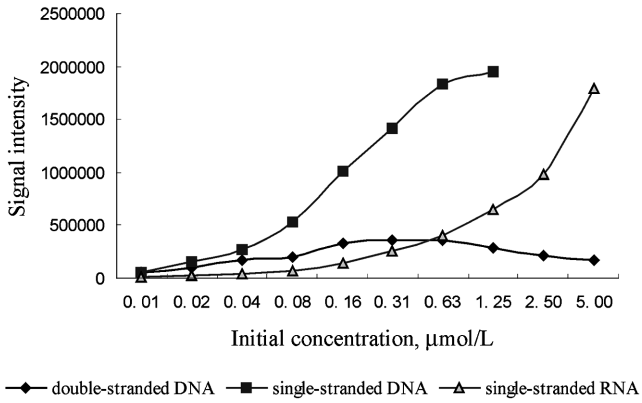


Fig. 1. Comparison of signal intensity of different targets. Arrays with 2-fold dilution serials of dsDNA, ssDNA and RNA were incubated with a 3 : 1 ratio of Cy5/Cy3 labeled DNA. The saturated signal was rectified with the product of corresponding signal within a linear range (2,000-50,000) with lower laser power, and average ratios (AR) of both scans. Signal intensity of each spot was the sum of the calibrated Cy5 and Cy3 signals.

Results

Comparison of hybridization behavior among ssDNA, ssRNA and dsDNA target using the simulant array In order to understand how double- and single-stranded targets virtually affected hybridization efficiency, the simulant array was made with 2-fold dilution series of double-stranded DNA, single-stranded DNA and RNA target. The array was incubated with a Cy5 and Cy3 labeled probe mixture, at a ratio of 3 : 1.

As the results show in Fig. 1, the sum of the calibrated Cy5 and Cy3 signal intensities increased along with increases of the ssDNA and RNA concentration, with no decline trend found in the ssDNA and RNA even in the higher concentration spots. In contrast, the double-stranded target gave the highest signal intensity at a concentration of 0.63 μmol/l, and then the signal declined. The relatively low signals of RNA at concentrations from 0.01 to 0.63 μmol/l were possibly due to RNA degradation. The highest signal of ssDNA in our experiment was 1.94×10^6 at 1.25 μmol/l and that of RNA was 1.79×10^6 at 5 μmol/l, 5.4 and 3.7 times higher, respectively, than the highest signal of dsDNA.

Although the signals were significantly different between the various targets, the average ratios of dsDNA, ssDNA and RNA were 3.21, 2.94 and 3.22, respectively (seen in Table 1). The ratios remained relatively stable, around 3.0, regardless of the type or concentration of the targets. The same results were observed when the input probe ratios for labeling with Cy3 versus Cy5 were 1 : 9 and 1 : 27 (data not shown). Our result substantiates the Winzelers (Winzeler *et al*., 1999) assumption that the amount of target DNA deposited on the glass slide would have little or no impact on any observed differentially expressed ratios. However, that is not in agreement with Yues (Yue *et al*., 2001) results, which indicated target DNA arrayed at input concentrations <1.0 μg/10 μl results in an underestimate or compression of the observed differential expression, with more compression occurring at lower DNA concentrations. It was assumed that the underestimate of the ratio in Yues research may have been caused by the weak signals that were outside the linear detection range at such concentrations.

These results suggest that single-stranded targets can greatly increase hybridization efficiency without altering the signal ratios of the two channels. However, large-scale preparation of ssDNA or RNA targets is relatively difficult and unpractical. Furthermore, RNA is unstable during the manipulation process due to RNase contamination. An alternative way of target preparations is to use the ds-ssDNA mixture generated by asymmetric PCR.

Validation of asymmetric PCR cDNA arrays

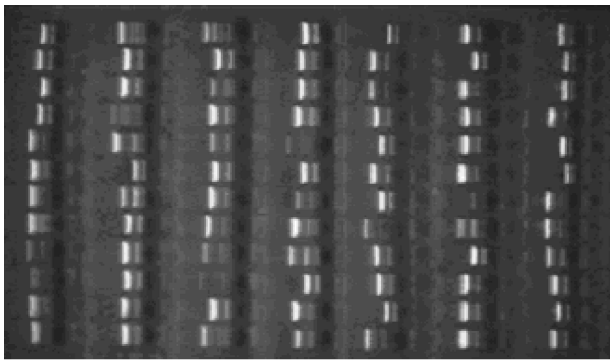
Asymmetric PCR can improve the signal intensity of the cDNA microarray compared with conventional PCR In order to acquire stable asymmetric PCR products of various gene templates, preliminary experiments were performed to optimize the asymmetric PCR. The result of the optimal ratio of forward to reverse primers to produce higher amounts of ssDNA was 50 : 1 (1000 nM : 20 nM) (data not shown). In addition, the yields of the single-strand products did not increase with initial templates increment. The results show that 0.1-1 ng of template in an 80 μl PCR reaction solution is an appropriate concentration for asymmetric PCR, which agrees with the previous report (Kinjo *et al*., 1998).

Two sets of cDNAs were made by conventional and asymmetric PCR from the same 96 targets under similar optimal conditions, except for the primers proportions. The products were qualified by agarose gel electrophoresis, as shown in Fig. 2. Statistical data indicate that approximately 95 percent of clones can be amplified efficiently to generate

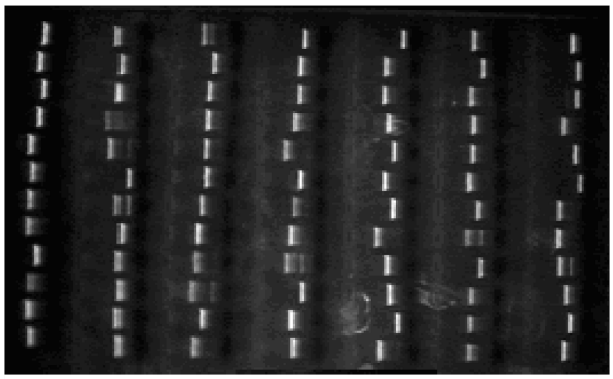
Table 1. The average ratios (Cy5 : Cy3) at serial concentrations and different types of targets

spotting concentration (μM)	0.01	0.02	0.04	0.08	0.16	0.31	0.63	1.25	2.5	5	average ratios	SD	CV
dsDNA*	2.91	3.07	3.07	3.30	3.48	3.61	3.60	3.18	2.94	2.96	3.21	0.27	0.08
ssDNA*	2.57	2.64	2.61	2.99	3.20	3.52	3.21	2.71			2.94	0.37	0.13
ssRNA*	2.42	2.90	3.26	2.74	3.00	3.95	3.21	3.27	3.61	3.84	3.22	0.48	0.15

*Average ratios of quintuplicate spots.



(A)



(B)

Fig. 2. Comparison between asymmetric PCR and conventional PCR by agarose gel electrophoresis. (A) Asymmetric PCR was performed with primer ratios of 50 : 1 (1000 nM : 20 nM) (forward to reverse primers) and 0.1-1 ng of template in an 80 μl PCR reaction solution. (B) Conventional PCR was performed under standard conditions, as described above.

sufficient products with visible ssDNA bands. Although the proportion of ssDNA and dsDNA among different genes could not be calculated exactly, and slightly varied, the overall yields of the two approaches were of the same level.

To make the cDNA microarray, both sets of purified PCR products from the asymmetric and conventional PCRs were dissolved with the spotting solution, and then spotted onto the same slide. The signal intensities and the ratios between two channels were analyzed after hybridization with the probes. 10 microarrays were performed, including 8 pairs of different tumor/normal tissues, and 2 pairs of normal/normal probes (self-comparison experiment). In a self-comparison experiment, equal amounts of the same total RNA was independently labeled with Cy3 and Cy5 and co-hybridized to the microarray. The average signal intensities of overall ds-ssDNA and dsDNA elements on all arrays were calculated. This showed that the average signal intensity of ds-ssDNA was 1.8 times higher than that of dsDNA.

Because of the detection sensitivity limitation in a microarray, many spots with low signal intensities are vulnerable to background and noise biases. Steady ratio values depend on the signal intensities, which most commonly

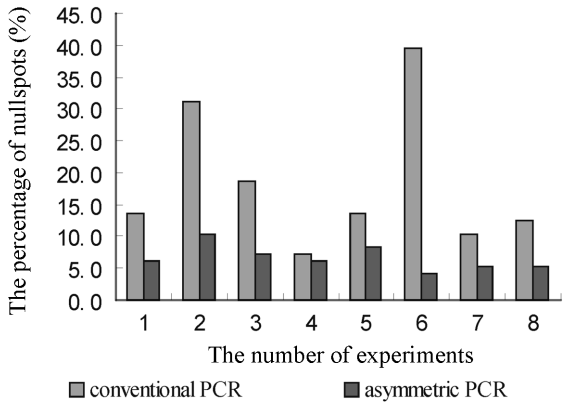


Fig. 3. Comparison of the percentage of null spots between ds-ss DNA mixture and dsDNA spots. The signal intensities from dsDNA and ds-ss DNA mixture targets were analyzed after hybridization with the probes. 8 tumor/normal tissue microarrays were performed. The spots with the raw intensities for both Cy3 and Cy5 below the threshold, which was the mean background values plus two standard deviations of the spots, filtered as null spots. The percentages of null spots of the ds-ssDNA and dsDNA targets from each slide were calculated.

deviate from 1 for low intensity spots (Quackenbush *et al*., 2002). To minimize artifacts arising from low expression values, the spots with raw signal intensity for both Cy3 and Cy5 below the threshold, including some of the rare transcript genes, were filtered as null spots. In order to see whether asymmetric PCR targets can improve the sensitivity, the percentages of null spots on each of 8 tumor/normal tissue microarrays were calculated with two different approaches (Fig. 3). The result obviously suggests asymmetric PCR can maintain more valid information than conventional PCR.

Asymmetric PCR will not decrease the reliability and reproducibility of the microarray compared with conventional PCR In two independent self-comparison experiments, the Pearson correlation coefficients (R) of signal intensities between channels in ds-ssDNA mixture and dsDNA spots were 0.97 and 0.98, respectively. This suggests that an asymmetric PCR approach will not introduce bias for two dyes, and will not increase hybridization artifacts.

Eight different independent liver cancer samples versus normal fetal liver were hybridized to the cDNA microarray. Pearson correlation coefficients of Cy5/Cy3 ratios between dsDNA and ds-ssDNA on the same slide were calculated. Miki (Miki *et al*., 2001) suggested that if the coefficients of two replicated experiments were equal to or greater than 0.7, the data were considered to be reasonable, and could be used in subsequent analyses. In our result, all coefficients of the eight experiments were in accord with Mikis criterion, with an average R value of 0.926, which was close to the 0.901 obtained from our previous work on conventional PCR products (data not shown). This also suggests that the reproducibility and reliability of the asymmetric PCR method

are similar to the conventional method.

Discussion

cDNA microarray analysis can be used to monitor the expression levels of thousands of genes in a single assay, and is the most widely used technique for the study of gene expression pattern on a genomic scale. However, due to the complexity of hybridization between a solid and liquid, particularly when the target molecules spotted on the array are double-stranded, many genes with rare transcripts cannot be detected efficiently. Therefore, it is very important to establish a more sensitive microarray system that is also reproductive and reliable. To relieve the competitive hybridization pressure between the probe and anti-sense strand of the dsDNA target, the ssDNA target was prepared and systematically compared the hybridization behavior of the ssDNA and dsDNA targets. The results show that the capability of ssDNA and RNA in capturing probes was far greater than that of the dsDNA, and neither would introduce signal ratio bias to the system. Nonetheless, neither ssDNA nor RNA is practical for use in producing targets due to the high cost and complicated preparation procedure. Alternatively, the economical and simply-manipulated asymmetric PCR technique was chosen to produce the ds-ssDNA targets and validate its feasibility. This markedly decreased the average percentage of null spots of the ds-ssDNA targets compared to those of the dsDNA targets. This suggests that asymmetric PCR targets can obviously improve sensitivity and maintain more useful information than conventional PCR, which is helpful in measuring rare transcript gene expression changes.

Meanwhile, an interesting phenomenon is found in ssDNA and dsDNA hybridization. The signal intensity declines along with increase in the amount of dsDNA when the concentration is higher than 0.63 μmol/l, which has not been reported before. It is hard to identify the specific reasons for this phenomenon that involves several possible factors. First, steric crowding of the surface may exist at high-density targets (Shchepinov *et al*., 1997; Peterson *et al*., 2001). For this reason, more targets within a certain area on a solid surface may possibly reduce the target hybridization efficiency and capture fewer probes. Second, the double stranded targets at high density may exhibit a higher self-annealing rate, which can increase competitive pressure for hybridization, and then decrease the hybridization efficiency. Third, it is possible that high-density packing of targets within the confines of a small spot interferes with the fluorescence emission of the small spots (Wang *et al*., 2003). It is likely that the amount of target immobilized on a slide declines at high concentrations as this exceeds the DNA binding capacity of the slide surface (Farbrother *et al*., 2002). Obviously, this is an area for future investigation.

In summary, the present study provides evidence that single-stranded molecules may increase the hybridization sensitivity without altering the ratios of the two probes. In addition, by analyzing the sensitivity, reproducibility and reliability, it is concluded that the ds-ssDNA mixture generated by asymmetric PCR is a feasible and efficient method for preparing spotting targets.

Acknowledgments We thank Shanghai Biostar Genechip Inc. for manufacturing the microarrays, preparing the probes and performing the hybridizations used in this report. This work was supported by grant 2002AA2Z2002 from the National High Technology Research and Development Program of China (863 Program).

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