Methods

Novel multiple 5'-amino-modified primer for DNA microarrays

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Abstract

For DNA microarray analysis, total RNA is reverse-transcribed, labeled by incorporating fluorescent dye into the cDNA, and used to hybridize microarray. This protocol requires a minimum of 20 \( \mu \)g of total RNA. To overcome the sample limitation, an RNA amplification technique has been developed. Although it needs less RNA, this amplification technique is relatively expensive, time consuming, and, unfortunately, has been found to introduce bias. In this study, we designed a novel 5'-amino-modified primer and used it for priming cDNA synthesis. The novel primer has a special structure that contains four Uni-Link molecules with two nucleotide (thymine) residues inserted between them as spacers. This novel primer is used in the reverse-transcription reaction for cDNA synthesis. Using the novel 5'-modified primer combined with indirect labeling method, cDNA probes can be prepared with much less total RNA (5 \( \mu \)g or less) without amplification producing optimal results after hybridization of arrays. This primer can also be used to label nucleotides for other purposes.

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Keywords: Microarray; Uni-Link molecules; Novel multiple 5'-amino-modified primer; Signal intensity

Contents

Introduction ............................................................. 0
Results and discussion................................................... 0
Methods ............................................................... 0
Materials and preparations .............................................. 0
Microarray fabrication ................................................ 0
RNA extraction ......................................................... 0
Probe preparation and purification .................................... 0
Dye coupling and purification ........................................ 0
Hybridization and washing ............................................ 0
Scanning and data analysis ............................................ 0
Acknowledgments .................................................... 0
Appendix A. Supplementary data......................................... 0
References .......................................................... 0

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Expression profiling using DNA microarrays is rapidly becoming a popular technique in biomedical research. A successful microarray experiment must be both sensitive and accurate for the detection of gene expression profiles. To analyze gene expression patterns in cells or tissues, the mRNAs are reverse-transcribed and cDNAs are labeled by incorporating fluorescent dye into them [1, 2]. First-strand cDNA fluorescence labeling protocols have been optimized to achieve higher sensitivity and lower variability [3]. Currently, an aminoallyl indirect labeling method using fluorescence Cy dyes (Cy3 and Cy5) has been widely used in research laboratories. This method requires at least 15–20 μg of total RNA [3]. For small tissue samples, RNA amplification is the preferred technique for labeling the probes [4–6]. Although RNA amplification can use much less total RNA (1–5 μg) for the reaction, it is relatively expensive, time consuming, and labor intensive compared to current aminoallyl labeling methods. Furthermore, the amplification process has been shown to introduce bias and amplify inappropriately a selective set of genes [7, 8]. To improve the sensitivity of the assay, new labeling materials and primers have been developed and tested [9–11].

In this study, we describe a new primer for cDNA synthesis that requires only 5 μg of total RNA or less for the preparation of fluorescently labeled probes. In the newly designed 5'-amino-modified primer, Uni-Link molecules [12] are conjugated to the 5' end of an oligo(dT) sequence without modification of structure of thymidines. Following the current indirect labeling protocol using newly designed multiple 5'-amino-modified primer, the probes generated gave much stronger signal compared with probes using a regular oligo(dT) primer and several other 5'-modified primers. Probe preparation with fluorescent dye labeling using our novel 5'-modified primer is relatively simple and inexpensive compared with other methods.

**Results and discussion**

The Uni-Link aminomodifiers were used to generate the 5'-amino-modified primers. The Uni-Link aminomodifier contains a primary amine group in its structure and allows reaction with any amino-reactive molecule. By repetitive coupling cycles, multiple Uni-Link aminomodifiers with the same amount of amino groups can be added to the 5' end of the primer synthesized [12]. Unlike other 5'-end-labeling molecules, the Uni-Link molecule is conjugated only to the 5' end of the nucleotide without any requirement for modification of the nucleotide structure. In this study, several 5'-amino-modified primers were designed and tested. The structures of the 5'-end-modified primers are shown in Fig. 1. Basically, different numbers of Uni-Link aminomodifiers were conjugated onto the 5' end of an 18-mer oligo(dT) sequence. Fig. 1A depicts the structure of the regular oligo(dT) (18-mer) primer and Figs. 1B, 1C, and 1D are three different 5'-end-modified oligo(dT) primers. The first modified primer has only one amino group at the 5' end of the oligo(dT) sequence (Fig. 1B). The second modified primer has multiple amino groups at the 5' end of the oligo(dT) sequence. A total of five Uni-Link molecules with the same number of amino groups are consecutively conjugated at the 5' end of the oligo(dT) sequence in Fig. 1C. The third modified primer has four Uni-Link molecules with 2 nucleotide (thymine) spacers between them at the 5' end of the oligo(dT) sequence (Fig. 1D). All four primers have identical 18-mer oligo(dT) sequence without any structure modification. The only difference between these primers is the 5'-end modification of the oligo(dT), either with or without Uni-Link molecules. The cDNA probes were synthesized using the same indirect labeling protocol with the incorporated aminoallyl–dUTP. The resulting cDNA probes contain not only the internal amine-binding sites (from incorporated aminoallyl–dUTP), but also various extra amine-binding sites at the 5’ terminus. Unlike direct labeling approaches, the indirect labeling method used in our study did not stipulate incorporation of dye during the reverse-transcription (RT) reaction. In fact, RT reaction and dye-coupling reaction were two separate reactions for probe preparation.

cDNA with aminoallyl–dUTP was labeled with Cy dyes and used to perform self-on-self hybridization experiments using 5 μg human universal total RNA labeled with Cy5, comparing with the same amount of RNA labeled with Cy3 dye. We compared the results between regular oligo(dT) as a primer (Fig. 2A), the single 5'-amino-modified oligo(dT) as a primer (Fig. 2B), the multiple 5'-amino-modified primer without the spacer (Fig. 2C), and the novel multiple 5'-amino-modified primer with spacers (Fig. 2D). Triplicate hybridizations were performed...
with each primer. When regular oligo(dT) was used as a primer, the signal intensities of most genes were low (<10^3) (Fig. 2A and Table 1). A higher level of variation in the signals with the low intensity of hybridization was observed. The scatter plot using single 5'-amino-modified primers showed a better linearity of the signal intensity distribution and gave better results compared with regular oligo(dT) primers. The overall mean signal intensity increased by two- to fourfold in comparison to the oligo(dT) primer method (Fig. 2B and Table 1). The multiple 5'-amino-modified primer without the spacer did not show a better result compared with the single 5'-amino-modified primer (Fig. 2C and Table 1), even though this primer contains more free amine-binding sites at its 5' end. In contrast, when multiple 5'-amino-modified primers with spacers (the novel 5'-amino-modified primer) were used, the mean signal intensity dramatically increased by eight- to ninefold compared with oligo(dT) primer and by two- to threefold compared with the single 5'-amino-modified primer and multiple 5'-amino-modified primer without the spacer (Table 1). The scatter plot of the novel 5'-amino-modified primer showed a great linearity along the diagonal line, indicating the strong signal and less variation (Fig. 2D).

To determine the sensitivity and the resolution using the novel 5'-amino-modified primer for reverse-transcription reaction and fluorescent dye labeling, two parameters, median signal-to-background ratio (SBR) and median signal-to-noise ratio (SNR), were carefully examined (Table 1). These parameters not only served to assess the array quality, but also were used as indicators for the sensitivity and the resolution. Median SBR is a direct measure of raw data between the signal intensity of the feature and the background in the surrounding area [13]. Higher ratio indicates stronger signal and lower background. The commonly accepted low limit for SBR is 2 [13]. As shown in Table 1, the priming method with the novel 5'-amino-modified primer gave the highest SBR values in both Cy3 and Cy5 channels (7.0 and 6.2, respectively), indicating that this method gave the best signal sensitivity compared with other methods. Median SNR is another important parameter for the array quality control. A higher SNR indicates higher
signal over background noise. A signal-to-noise ratio of 3 is commonly considered the low limit for detection [13]. This parameter can be used as an indicator of the array resolution; the higher the ratio, the better the resolution. As shown in Table 1, the novel 5'-amino-modified probes gave the highest SNR values in both channels, compared with other methods (12.6 for Cy3 and 11.5 for Cy5). Compared with current labeling methods using regular oligo(dT) primers, novel 5'-amino-modified primers not only greatly improved the signal sensitivity and gave a better resolution, but also showed lower background noise.

We found that the novel multiple 5'-amino-modified primer with spacers gave stronger signals and optimal sensitivity. Since 5'-end-modified primers allow the extra dye molecules to be added onto the 5' terminus of the synthesized cDNA sequences, this approach substantially generated more fluorescence compared with the regular oligo(dT) primer. When the modified primer is used for cDNA synthesis, two potential problems, dye quenching and primer hybridization stability, need to be carefully considered. In Randolph and Waggoner’s multiply labeled fluorescent DNA probes study [14], 27-mer oligo(dT) primers were synthesized with various modified bases, which allowed fluorescent dyes to be attached. Hybridization stability, specificity, and fluorescence intensities of the primers were carefully tested. Their study indicated that modification of the thymidine structure in the oligo(dT) sequence significantly affected hybridization to target DNA. More modified thymidines in the oligo(dT) primers lowered the hybridization stability [14]. When 6 modified thymidines were incorporated into the 27-mer oligo(dT) sequence (each modified thymidine was separated by 4 thymidines), the melting temperature decreased to 37.6 from 49.7°C. These observations indicate that the hybridization stability was decreased and therefore it is not surprising that the labeled fluorescence intensity was decreased even though more Cy dye binding sites were introduced into the sequence. Additionally, when 5 modified thymidines were incorporated into the 27-mer oligo(dT) sequence (each modified thymidine was separated by 5 thymidines), the labeled fluorescence intensity reached the highest level. Again, a dynamic balance between hybridization stability and dye labeling efficiency is highlighted here. Waggoner’s paper concluded that, to stabilize the primer hybridization and prevent dye–dye quenching, the dye molecules should be separated by at least 5 nucleotides [14].

In contrast, in our approach, we conjugated Uni-Link molecules at the 5' end of oligo(dT) sequence and consequently they did not modify the thymidine structure and therefore did not interfere with the hybridization affinity of the primer. Since no structure was modified, the mere introduction of two thymidines (or it can be any nucleotide) provided the space to avoid the dye quenching and improved the signal intensity.

Our results further indicate that because of the quenching between dye–dye interactions, conjugated Uni-Link molecules at the 5' end would not give a stronger signal than the single conjugated molecule, even though there are more primary amine group binding sites at the 5' end. Two nucleotides as a spacer play a critical role in enhancing the labeling signals. The stronger signals obtained by using novel 5'-amino-modified primers suggest that the spacer between the Uni-Link molecules not only allows cyanine dyes to couple to the primary amine sites easily, but also can effectively prevent the quenching of the fluorescent signals between dye–dye interactions.

We next determined the amount of total RNA required for microarray probe labeling with our novel 5'-amino-modified primer and compared gene expression profiles using regular oligo(dT) primer. For this, total RNA isolated from human U251 glioblastoma and CCD27 fibroblast cell lines was labeled and arrays were hybridized. For oligo(dT) primer, 20 μg of total RNA from the U251 cell line was labeled with Cy5 and RNA from a fibroblast cell line with Cy3. For novel multiple 5'-amino-modified primer, 5 μg of total RNA samples from U251 and CCD27 cell lines was labeled with Cy5 and Cy3, respectively. Three 17,000 human oligonucleotide arrays were hybridized for each group. Genes that were highly significant between the U251 and the CCD27 cell lines at a ratio of either greater than or less than threefold were selected for data comparison. The correlation coefficient of results between hybridizations using oligo(dT) primer with 20 μg of total RNA and novel 5'-amino-modified primer with 5 μg of total RNA was 0.91 (0.87–0.96), indicating a
high similarity of gene expression profiles between priming methods. The gene expression of selected genes is shown in Table 2 (a full list is provided in Supplementary Table 1S). These data provided a comparison between two priming methods and overall support to our conclusions that gene expression results using the novel 5′-amino-modified primers and a low amount of total RNA starting material are similar to those generated using regular oligo(dT) primers and much larger amounts of total RNA, regardless of biological significance of differentially expressed genes. When 1 μg of total RNA was used in the experiment with the novel 5′-amino-modified primer, the correlation coefficient between the oligo(dT) and the novel 5′-amino-modified primer was 0.82 (0.73–0.86). Although the correlation coefficient, 0.82, deceased from 0.91, it still indicated similar gene expression profiles (data shown in Supplementary Table 1S). These comparative results suggest that by using novel multiple 5′-amino-modified primer the amount of total RNA required for analysis can be reduced to as little as 5 μg without reduction of the quality of the experiment, and gene expression profiles are comparable to those using oligo(dT) primers that require at least 20 μg of total RNA for good signals.

Unlike conventional approaches, the indirect labeling method used in our study does not stipulate incorporation of dye during the reverse transcription (RT) reaction. RT reaction and dye-coupling reactions are two separate reactions for probe preparation. The cDNA is labeled with fluorescent dyes after it is synthesized and aminoallyl–dUTP replacing dTTP is incorporated. The addition of dye at the aminoallyl–dUTP is a standard procedure [15]. The enhanced labeling observed in our study is based on the creation of unique extra amine sites in addition to internal amine binding sites within the cDNA. Since these amine sites were labeled with dyes, it generated amplification of signals on a per-molecule basis.

Previously, Xiang et al. reported the use of a random hexamer with 5′-end amino-modification as a primer for reverse-transcription to enhance signal intensity [11]. In this study, the authors modified a thymidine residue at a fixed position, 5′, with an 8- to 9-carbon chain ending in a primary amine. Dye molecules were then added to amino groups in both cDNAs and the incorporated primers during the labeling reaction. This technique allowed amplification of fluorescence signals generated by microarray hybridization. In contrast to this technique, we introduced multiple aminoallyl–dUTP at many sites, creating amplified amino-modified labeling sites. We did not modify any thymidines and thus the structure was unchanged at the 5′ end.

This novel 5′-amino-modified primer can also be used for any nucleotide labeling methods. For example, (a) generating sensitive PCR primers for labeled PCR products and (b) generating sensitive probes for in situ hybridization. In addition, the combination of PCR and microarray technology will provide a powerful tool to detect and discriminate the subtypes of pathogens [16–18]. Our novel 5′-amino-modified method will not only allow detection of pathogens, but also quantify the amount of each subtype. Since biotin–N-hydroxysuccinimide ester can be conjugated to the primary amine group of the novel primer, the signal can be amplified even more if biotin–streptavidin conjugates are applied with this structure. Finally, our new approach is relatively simple, time saving, and inexpensive compared with other methods, especially compared with the current amplification method. Thus, this technique will be a good choice for laboratories that have limited RNA supply from precious tissues.

### Methods

#### Materials and preparations

5′-Amino-modified oligo(dT) (18-mer) primers were synthesized in the CBER core facility; however, these 5′-amino-modified primers can also be synthesized elsewhere. The following materials were used: Uni-Link aminomodifier (Cat. No. 635703; BD Biosciences), Cy3 monofunctional reactive dye (Cat. No. PA23001; Amersham Pharmacia) and Cy5 monofunctional reactive dye (Cat.
No. PA25001; Amersham Pharmacia), Stratascript RT (Cat. No. 600085; Stratagene), 100 mM dNTP set (Cat. No. 10297018; Invitrogen), aminoallyl–dUTP (Cat. No. A0410; Sigma), human Cot-1 DNA (Cat. No. 15279-011; Invitrogen), poly(dA) (Cat. No. 27-7988-01; Amersham Pharmacia), universal human reference RNA (Cat. No. 740000; Stratagene), DMSO (Cat. No. D8418; Sigma), MinElute PCR purification kit (Cat. No. 28006; Qiagen).

Microarray fabrication

The human microarrays containing approximately 17,000 oligonucleotides were fabricated on poly-L-lysine-coated glass slides using an OmniGrid microarrayer (GeneMachine, San Carlos, CA, USA). The human genome oligonucleotides were purchased from Operon/Qiagen. Each oligo is a 70-mer that was selected from 750 nucleotides of the 3' end of the ORF. Detailed information regarding coating glass slides with poly-L-lysine, printing oligonucleotides on the slides, and postprocessing the printed slides is presented at the Web site of the Advanced Technology Center/National Cancer Institute/NIH (http://nciarray.nci.nih.gov/reference).

RNA extraction

Total RNA from U251 human glioblastoma and CCD27 fibroblast cell lines was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol.

Probe preparation and purification

For preparation of cDNA probes with oligo(dT) primer, 20 µg of total RNA in 19 µl dH2O was used for reverse transcription with 1 µl oligo(dT) primer (18-mer; 1 µg/µl). The mixture was incubated at 70°C for 5 min and quickly chilled in ice for 5 min. The total 10 µl reaction mixture contained 10 × first-strand buffer, 3 µl; 20 × aa–dTTP/dNTP (10 mM dATP, 10 mM dGTP, 10 mM dCTP, 6 mM dTTP, and 4 mM aminoallyl–dUTP), 2 µl; 0.1 M DTT, 3 µl; Stratascript RT (Stratagene), 2 µl and was added to the RNA–primer mix and incubated at 42°C for 60 min.

For preparation of cDNA probes using different 5’-amino-modified primers, 5 µg of total RNA in 12 µl dH2O and 1 µl primer (18-mer; 1 µg/µl) was incubated at 70°C for 5 min and quickly chilled on ice for 5 min. The total 7-µl reaction mixture (10 × first-strand buffer, 2 µl; 20 × aa–dTTP/dNTP, 1.5 µl; 0.1 M DTT, 2 µl; Stratascript RT (Stratagene), 1.5 µl) was added to the RNA–primer mix and incubated at 42°C for 60 min.

After incubation, MinElute PCR purification kits were used to purify the cDNA; dH2O was added to make up to a total volume of 60 µl. Then, 300 µl of PB binding buffer (Qiagen MinElute PCR Purification Kit) was added to the mixture, loaded on a MinElute column, and spun for 1 min at maximum speed. Flowthrough was discharged and the column was washed twice with PE washing buffer followed by spinning for 1 min at maximum speed. cDNA was eluted by adding 15 µl elution buffer to the center of the column and incubation for 2 min, followed by centrifugation at maximum speed for 1 min. The eluate was concentrated by spin-drying.

Dye coupling and purification

For dye coupling, the cDNA pellet was resuspended into 5 µl 2× coupling buffer (0.2 M NaHCO3, pH 9.0). Five-microliter aliquots of Cy3 or Cy5 dye were mixed well by pipetting (final concentration of coupling reaction 0.1 M NaHCO3, pH 9.0; total reaction 10 µl) and then placed in a dark box at room temperature for 60 min. After coupling, 50 µl dH2O was added to the mixture, and then 300 µl PB buffer was added. Labeled cDNA was purified on MinElute column as described above except the cDNA was eluted in 10 µl of elution buffer. After elution, Cy3- and Cy5-labeled cDNA probes were mixed together and their volumes adjusted to 25 µl with dH2O.

Hybridization and washing

A total of 25 µl of purified cDNA was mixed with 1 µl (1 µg) poly(dA) (Amersham Pharmacia Biotech), 1 µl (1 µg) Cot-1 human DNA (Invitrogen), 1 µl (4 µg) yeast tRNA (Invitrogen), 1 µl 10% SDS, and 6 µl 20 × SSC to a total volume of 35 µl. The probe was denatured for 2 min at 100°C and then added onto arrays, which were hybridized at 65°C for 16–18 h. All hybridizations were performed in triplicate for good quality technical repeats. After hybridization, array slides were washed with 2 × SSC with 0.1% SDS for 2 min, 1 × SSC for 3 min, and finally 0.2 × SSC for 2 min at room temperature. Slides were then quickly spin-dried before scanning.

Scanning and data analysis

Microarray slides were scanned using a GenePix 4000B scanner (Axon Instruments, Inc., Foster City, CA, USA) with a 10-µm resolution. Scanned microarray images were exported as TIFF files to GenePix Pro 5.0 software for image analysis. The raw images were collected at 16-bit/pixel resolutions that displayed all pixels in a 0 to 65,535 count dynamic range. To determine the sensitivity and resolution of each method, each array was scanned with identical PMT voltage setting (750 for Cy5 and 700 for Cy3).

The mean and median signal intensity, median SNR, and median SBR parameters generated by the GenePix 4000B scanner were used for comparisons between different 5’-end-labeled primers and the quality control analyses. For advanced data analysis, data files were imported into mAdb
(microarray database) and analyzed by software tools provided by the Center for Information Technology, NIH.

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The 5′-amino-modified oligo(dT) primers were synthesized and purified by the core facility of the CBER/FDA. The oligonucleotide microarray slides used in this study were printed at CBER. We thank Dr. Amy X. Yang also for printing oligonucleotide microarray slides and Dr. Robert Aksamit and Dr. Vladimir Chizhikov of CBER/FDA for critically reading the manuscript. We also thank John Powell and his colleagues at the Center of Information Technology, NIH, for array database support.

Appendix A. Supplementary data

Supplementary data for this article may be found on ScienceDirect.

References