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Systematic Analysis of Intrinsic Factors Affecting Differential Display

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ABSTRACT

Differential display (DD) is a widely used method for identifying differentially expressed genes. To improve further the efficiency and reproducibility of the method, this report systematically examines four critical parameters of standard DD-PCR. Specifically, the study determined the optimal annealing temperature, elongation time, dNTP concentration, and arbitrary primer concentration. By using a thermal cycler that was capable of displaying a temperature gradient across a PCR plate, it was possible to determine (in a single experiment) the effect of different annealing temperatures. The optimal annealing temperature for a 13-mer arbitrary primer fell within a broad range of 40°C–50°C. Elongation times over a range of 30–120 s worked best. The optimal concentration for dNTPs was within a very broad range of 2–50 μ M, with higher amounts allowing for greater pipetting accuracy. The most favorable concentration for the arbitrary primer was also within a broad range of 0.1–2.0 μ M. A primer concentration below this range greatly reduced the efficiency of the amplification process. In conclusion, the experimental findings delineated the best possible DD conditions for a more reliable assessment of differential gene expression.

INTRODUCTION

Temporal and spatial expression of the 30 000–50 000 different genes encoded by the human genome is a highly regulated process that determines both the normal and pathological growth and developmental of an organism. Therefore, understanding the mechanisms of these normal and pathological processes requires identification, isolation, and characterization of differentially expressed genes.

Differential display (DD) is one such tool that has already helped thou-

sands of researchers in the world to interpret genomic information (2,7). DD compares gene expression in cells by first converting mRNAs to cDNAs using one of three individual anchored oligo-dT primers that differ from each other at the terminal 3' non-T base. The use of anchored primers enables the homogeneous initiation of cDNA synthesis at the beginning of the poly(A) tail for any given mRNA. The resulting three subpopulations of cDNAs are further amplified and labeled with either isotopes or fluorescent dyes by PCR in the presence of a second set of short, random primers. The length of a random primer is designed such that, by probability, each will recognize 50–100 mRNAs under a given PCR condition (9). As a result, 3' mRNA termini, defined by any given pair of anchored-primer and random primer, are amplified and displayed by denaturing PAGE. Side-by-side comparisons of such cDNA patterns between or among relevant mRNA samples would reveal differences in gene expression. Differentially expressed cDNA bands can be retrieved, cloned, and sequenced for further molecular characterization.

Although technically rather simple, the method can be error-prone because of both intrinsic and extrinsic factors of DD (1,3). The extrinsic factors, such as pipetting errors, quality of reagents, primers, enzymes, and the types of PCR tubes and thermal cyclers, have been well documented (8). Knowledge of these factors has greatly improved the successful rate of DD applications, which has been validated by 300–400 DD publications in each of the past five years. Although the intrinsic factors had primarily focused on primer design (10,12), little effort was made to evaluate systematically other critical factors such as the annealing temperature, elongation time, and concentrations of both dNTPs and primers. In terms of primer design, the one-base anchored primers, in combination with random 13-mers (10), have clearly proven to be the most popular and reliable, based on the number of successfully cloned genes published so far. However, this study is intended to identify "rate-limiting" factors that could contribute to the incidents of false-positive results. In an effort to identify the intrinsic factors

critical for DD-PCR, we have systematically examined the effects of the annealing temperature, elongation time, and the concentrations of both dNTPs and random primers on the overall efficiency and reproducibility of DD-PCR. The critical parameters defined by this study should be helpful in further improving the sensitivity and reproducibility of DD.

MATERIALS AND METHODS

Cell Lines and Culture

Rat embryo fibroblast cell line Rat1 and its derivative transformed by H-ras oncogene T101-4 (5) were routinely grown in DMEM (Invitrogen, Carlsbad, CA, USA) with 10% FCS (HyClone, Logan, UT, USA) and 1% penicillin-streptomycin (Invitrogen) at 37°C with 10% CO₂.

Total RNA Isolation

Total RNA was isolated using the RNeasy[®] reagent (GenHunter, Nashville, TN, USA) according to the manufacturer's instructions. To remove all contaminating chromosomal DNA, isolated total RNA was treated with DNase I using a MessageClean[®] Kit (GenHunter). The cleaned total RNA was resuspended in DEPC-treated water before use for DD.

Differential Display

Differential display was performed using the RNAimage Kit (GenHunter), following standard DD conditions (6). For the evaluation of different parameters of DD-PCR, standard DD conditions were followed (10), except for the individual parameters being examined. *Taq* DNA polymerase (5 U/ μ L) was from Qiagen (Valencia, CA, USA), and α -[³³P]-dATP (2000 Ci/mmol) was from New England Nuclear (Boston, MA, USA). PCR amplification was then performed in an Eppendorf[®] Mastercycler[®] Gradient thermal cycler (Brinkmann Instruments, Westbury, NY, USA), according to the following schedule: 94°C for 30 s, 40°C for 2 min, and 72°C for 1 min for 40 cycles, followed by 72°C for 5 min. The ampli-

fied cDNAs were then separated on a 6% denaturing polyacrylamide gel as described previously (4).

RESULTS AND DISCUSSION

To systematically determined the optimal DD-PCR conditions, we focused on four major parameters: annealing temperature, elongation time, and concentrations of both dNTPs and random primers. All four parameters were systematically examined by varying each parameter one at a time while standardizing the rest of the PCR conditions (10). The DD-PCR was designed to be able to display a ras oncogene specific target, Pai 2 (2), which serves as a positive control for the accuracy of DD-PCR. Core RT and PCR mixtures were used whenever necessary to compare, in

duplicate, the mRNA expression between control and ras oncogene-transformed rat embryo fibroblasts.

Effect of Annealing Temperature

The concept of DD is based on the selective recognition of cDNA sequences from mRNA by short, random primers, 10–13 bases in length. Speculation that these primers may be too short to give reproducible cDNA amplification has led to many variations of DD, such as using long PCR primers (12) or performing restriction digests followed by primer ligation (11). However, these modifications have led to little if any improvement in the accuracy of DD. Based on theoretical prediction of the melting temperature of PCR primers, 13-mers of 50%–60% GC content would perform poorly above an

annealing temperature of 30°C. To examine accurately the optimal annealing temperature of 13-mers in DD-PCR, an Eppendorf Mastercycler Gradient thermal cycler, capable of displaying a temperature gradient across the heating/cooling surface, was employed. This thermal cycler allows the same DD-PCR samples to be exposed to identical PCR conditions, except for different annealing temperatures ranging from 30°C to 60°C (Figure 1). The arrow indicated the detection of the positive control, Pai 2. It was quite surprising to realize that little change in the overall pattern of display mRNAs were observed over the 30°C–50°C range. Clearly, DD-PCR started to fail when the annealing temperature exceeded 50°C, especially with regard to detecting the positive control. It should be noted that the standard DD anneal-

Figure 1. Effect of annealing temperature on DD. DD-PCR was performed under standard conditions, except for the variable parameter under investigation. Duplicate RT reactions and PCR were carried out for RNA samples from normal (left two lanes) and H-ras oncogene transformed rat embryo fibroblasts (right two lanes) using core RT and PCR mixtures. Pai 2 was selected as a positive control known to be only expressed in H-ras-transformed cells (indicated arrow). The G-anchored oligo-dT primer (H-T₁₁G) and H-AP 29 13-mer primers were used for DD-PCR. Eleven different annealing temperatures ranging from 30°C to 60°C were examined.

Figure 2. Effect of elongation time on DD. DD-PCR was carried out as described in Figure 1. Six different elongation times from 15 s to 2 min 30 s were examined.

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ing temperature was originally set at 40°C–42°C (7,10), which is well within the optimal annealing temperature range identified.

Effect of Elongation Time

DD was originally designed to amplify the 3' mRNA termini (500–600 bases). However, it was argued that longer cDNA products would allow more mRNA sequences to be obtained. The most obvious way to obtain longer cDNA PCR products is to increase the extension time of PCR. To evaluate the effect of elongation time on DD, six different elongation times between 15 and 2 min 30 s were compared (Figure 2). There was a clear correlation between increased elongation time and increased length of DD-PCR products. However, an elongation time longer than 2 min led to over-amplification of

high molecular weight cDNAs, which produced smeared PCR products too complex to allow any individual cDNA band to be discerned. Also under this condition, the positive control band was barely visible. These results indicated that a broad elongation time for DD can be employed that has little effect on its reproducibility. However, elongation time longer than 2 min may not be beneficial for DD, given the over-amplification of high molecular weight PCR products. It should be noted that the standard 1-min elongation time that we described previously (10) appeared to be within an optimal range for cDNA amplification in terms of the band intensity, sensitivity, and reproducibility.

Effect of dNTP Concentration

Under standard DD-PCR conditions, cold dNTP concentrations in the

DD-PCR were intentionally kept low (2–4 μM) to allow efficient incorporation of radioactively labeled dATP (7). However, there has been no attempt to systematically look at the effect of a broader range of cold dNTP concentrations on DD. To this end, dNTP concentrations from 0.2 to 200 μM were compared in otherwise identical reactions (Figure 3). Clearly, dNTP concentrations lower than 2 μM gave very poor cDNA amplification and reproducibility in DD. Otherwise a surprisingly broad range of cold dNTP concentrations could be used with little negative impact on overall cDNA pattern and reproducibility. However, as predicted, dNTP concentrations higher than 50 μM appeared to cause some inhibition of cDNA product labeling as cDNA bands became fainter. Maintaining a dNTP concentration of 10–50 μM could potentially increase the repro-

Figure 3. Effect of dNTP concentration on DD. DD-PCR was performed as described in Figure 1. Eight different dNTP concentrations from 0.2 to 200 μM were compared.

Figure 4. Effect of arbitrary primer concentration on DD. DD-PCR was carried out as described in Figure 1. Seven different arbitrary primer concentrations from 0.02 to 20 μM were tested.

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ducibility of DD, since a slight variation in dNTP concentration caused by pipetting error within this range (as opposed to the standard 2–4 μM range) will less likely cause false positives in the amplified cDNA pattern.

Effect of Random Primer Concentration

To examine the effect of random primer concentration on DD, the H-AP29 primer concentration was varied 1000-fold, from 0.02 μM up to 20 μM in DD-PCR. The reproducibility of the DD cDNA pattern increases with increasing concentrations of primers from 0.02 to 1 μM (Figure 4). Primer concentration lower than 0.1 μM not only gave poor reproducibility but also a weaker signal of amplified cDNAs (Figure 4). The standard 0.2 μM final primer concentration appears to be at the lower end of the optimal random primer concentration range that gives a highly reproducible DD pattern.

In summary, we have determined that DD-PCR can tolerate a rather broad range of annealing temperatures and elongation times. However, the major factors that appear to influence negatively the reproducibility of DD are low concentrations of dNTP and random primers. As expected, the suboptimal concentration of these components will make DD-PCR amplification susceptible to pipetting errors in aliquoting these reagents. Therefore, our findings suggest that increasing the final concentrations of dNTPs greater than 2 μM and arbitrary primers greater than 0.2 μM is likely to improve the reproducibility of DD. For example, the optimal DD-PCR that we described previously (10) may benefit by increasing the dNTP and arbitrary primer concentrations to 4 μM and 0.4 μM , respectively. Such adjustments should help to allow for slight pipetting errors of these critical components.

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