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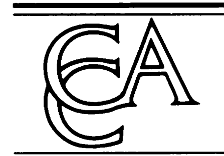

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Short communication

Use of longer extension phases to improve yield of high molecular weight products in differential display PCR

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1. Introduction

Since its first description by Liang and Pardee in 1992 [1], PCR-based differential display (DD–PCR) has become a widely-used routine method for the discovery of differentially regulated genes in such diverse experimental settings as the study of diseases such as cancer [2] and diabetes mellitus [3], and developmental biology [4]. We have been using this technique for several years to study gene regulation during macrophage foam cell formation [5]. Despite numerous improvements and technical modifications, DD–PCR remains a technically demanding method, a fact which has prompted the coining of the term 'differential dismay' to describe the frustration experienced by many who have attempted to use the procedure [6]. Good results demand a strategy for

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avoidance of false positives such as that previously described by our group [7], and a PCR protocol and electrophoresis conditions which produce clear, reproducible band patterns. Modifications which have been proposed to improve DDPCR include use of primers tailored to amplify members of a particular gene family [8], techniques for optimization of annealing temperature [9], and optimization of primer design [10].

The performance of the DNA polymerase from *Thermus aquaticus* (Taq polymerase) falls during PCR as a result of repeated heating to 94°C. This leads to reduced representation of high molecular weight templates and PCR products with an average size of no more than 350 bp, representing 3′ untranslated transcripts. We reasoned that successive prolongation of the extension time during DD–PCR would overcome this drop in performance, thus allowing templates of high and low molecular weights to be amplified to a similar degree. The extension time was increased in stepwise fashion because of the risk of non-specificity which may occur with excessive extension times early in PCR. We also designed protocols to examine the effects of increasing annealing temperature and reducing the number of cycles, modifications which have been reported to increase specificity and improve the detection of small differences in gene expression, respectively.

2. Materials and methods

Monocytes were isolated from healthy volunteers by monocytapheresis and countercurrent elutriation as previously described [11]. The cells were cultivated in RPMI 1640 supplemented with 20% of pooled heterologous human serum (PAA Laboratories, Linz, Austria) for 16 d to allow development of macrophages. This procedure for isolating monocytes was approved by the local Ethics Committee. Total RNA was isolated using guanidinium isothiocyanate and phenol/chloroform extraction as described [12]. Genomic DNA was removed by DNase treatment with a MessageClean kit (GenHunter Corporation, Nashville, TN). cDNA synthesis was performed using MMuLV reverse transcriptase (Display Systems Biotech, Los Angeles, CA), 5′-T_{11}AG-3′ or 5′-T_{11}AC-3′ primer and the manufacturer's protocol. DD–PCR was performed with a commercially available kit (Display Systems Biotech) according to the manufacturer's instructions using displayTAQ FL (Display Systems Biotech), a combination of the downstream primer corresponding to the cDNA synthesis and a decamer upstream primer (the following primer combinations were used in different PCRs: 5′-T_{11}AG-3′ with 5′-GATCAAGTCC-3′, 5′-T_{11}AC-3′ with 5′-TGGTAAAGGG-3′ and 5′-T_{11}AC-3′ with 5′-GGTACTAAGG-3′) and α-[^{35}S]dATP (ICN Biomedicals, Eschwege, Germany). To identify what we have termed 'spurious true positives' (i.e. differences in band intensity which are due

to interindividual variation and not to the experimental manipulation under study), all DD–PCRs were performed twice in parallel from duplicate cell preparations [7]. All reactions were prepared on ice using the same cDNA and the same reagents. A thermocycler with a heatable lid (GeneAmp PCR System 9600, Perkin-Elmer, Norwalk, CT) was used in all cases. The standard PCR protocol [A] that we used was as follows: pre-denaturing at 94°C for 5 mm, followed by 40 cycles of denaturing at 94°C for 30 s, annealing at 40°C for 90 s, and extension at 72°C for 60 s, followed by a final extension time at 72°C for 7 mm, as recommended by Display Systems Biotech. Five modified PCR protocols were tested as follows. Prolonged extension PCR protocols [B]: Pre-denaturing at 94°C for 5 mm, followed by 40 cycles of denaturing at 94°C for 30 s, annealing at 40°C for 90 s, and extension at 72°C for $[60+(n-1)\times m]$ s where n is the cycle number and m the prolongation time ($m=10$, 20, 30 or 40 s), followed by a final extension at 72°C for 7 mm. This modification approximately doubled the time required for a PCR reaction from 3.5 to 7.9 h. Prolonged extension protocols using higher annealing temperature protocols [C]: same as prolonged extension PCR conditions (protocol B) except for an increase in the annealing temperature to 42°C. Prolonged extension PCR protocol using reduced number of PCR cycles [D]: same as prolonged extension PCR conditions (protocol B) except for a decrease in the number of PCR cycles to 35. On completion of the PCR reaction, 10 μl of denaturing dye was added to the 20 μl of each sample and the samples were applied after denaturation at 94°C for 5 mm to a 6.0% denaturing polyacrylamide gel (Genomyx, Foster City, CA) and separated using a GenomyxLR™ sequencer (Genomyx). Separation was performed at 100 W for $2\frac{1}{2}$ h. After drying and washing, gels were exposed to BioMax MR-I films (Eastman Kodak Company, Stuttgart, Germany) for 1 day at 4°C.

3. Results and discussion

Successive prolongation of the extension phase of the PCR reaction in steps of 10, 20, 30, and 40 s per cycle substantially increased the intensity of the bands obtained. Prolongation in steps of 20 s per cycle gave slightly better results than a prolongation in steps of 10 s, but a further increase in the size of the steps to 30 or 40 s showed no additional benefit (data not shown). Fig. 1 shows that the intensity of cDNA bands obtained by DD–PCR increased significantly if protocols were used with a stepwise prolongation of the extension phase in steps of 20 s per cycle (protocols B, C, and D; lanes B, C and D) compared to the standard protocol (protocol A, lane A). Stepwise lengthening of the extension phase using protocol B increased band intensity without

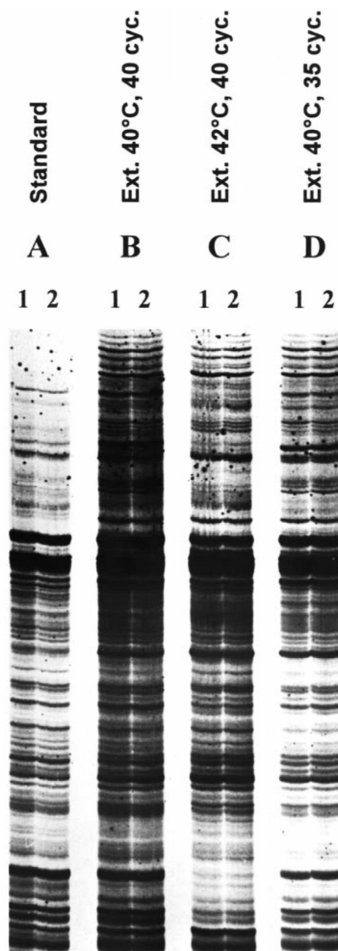

Fig. 1. Use of longer extension phases to improve yield of high molecular weight products in differential display PCR. **DD–PCR using a variety of protocols.** The same reactions performed in duplicate are shown in lanes 1 and 2 using primer combination 5′-T_{11}AG-3′ and 5′-GATCAAGTCC-3′. All reactions were prepared with total RNA from 16-day-old human macrophages. [A] **Standard PCR protocol;** pre-denaturing 94°C, 5 min; 40 cycles of 94°C, 30 s; 40°C, 90 s; 72°C, 60 s; final extension 72°C, 10 min. [B] **Prolonged extension PCR protocol;** pre-denaturing 94°C, 5 min; 40 cycles of 94°C, 30 s; 40°C, 90 s; 72°C, $[60 + (n - 1) \times 20]$ s; final extension 72°C, 10 min. [C] **Prolonged extension with higher annealing temperature PCR protocols;** same as prolonged extension PCR conditions (protocol B) except for an increase in the annealing temperature to 42°C. [D] **Prolonged extension PCR protocol using reduced number of PCR cycles;** same as prolonged extension PCR conditions (protocol B) except for a decrease in the number of PCR cycles to 35. The intensity of products obtained by DD–PCR with a stepwise prolonged extension phase (lane B) is increased compared to the standard protocol (lane A), with a striking increase in the number of high molecular weight bands. Amplification of high molecular weight products is markedly improved even when our prolonged extension protocol is used with an increased annealing temperature of 42°C (lane C) or a reduced number (35) of PCR cycles (lane D).

generation of new or spurious bands. Apart from a general increase in band intensity, the major difference between the results of protocol B and the standard protocol was the striking increase in the number and intensity of high molecular weight PCR products. Even when the annealing temperature was increased to 42°C (a manoeuvre which is known to increase the stringency of primer annealing leading to more specific amplification [9]), stepwise lengthening of the extension phase resulted in markedly increased intensity and number of high molecular weight bands compared to the standard protocol (lane C). It has been reported that reduction in the number of PCR cycles improves detection of differentially expressed bands [13]. We therefore, employed a protocol in which prolonged extension times were combined with a reduction in the number of cycles 35 (protocol D). This resulted in a decrease in band intensity compared to the prolonged extension protocol using 40°C annealing temperature (lane D versus lane B). However protocol D also increased the number of high molecular weight products compared to the standard protocol (lane D versus lane A). Similar results were obtained with a variety of combinations of downstream and upstream primers (not shown), indicating that this is a general effect. We amplified, cloned and sequenced several differentially regulated high molecular weight bands. These were found to correspond to expressed sequence tags with molecular weights of more than 600 bp (data not shown).

Successive prolongation of the extension times significantly improves the yield of high molecular weight DD–PCR products with no increase in spurious bands or PCR artifacts. Such longer PCR products are more likely to include part of the 3′ coding region. They are, therefore, more likely to be homologous to cDNAs in existing databases, are easier to clone, and are more suitable for production of probes for hybridization experiments such as Northern blotting or library screening. Protocols for production of longer PCR products either by means of longer PCR primers [14] or by increasing the concentration of dNTPs or prolonging the extension time to 90 s [15] have been published. However, these methods have the respective disadvantages of limiting the number of PCR products or of reduced specificity, limitations which are avoided by our protocol.

Each of our modified protocols has its own particular usefulness. Protocol B (40 cycles, 40°C annealing temperature, successive prolongation of extension times in 20 s steps) produced a general increase in band intensity, particularly those of higher molecular weight, and is a good general-purpose protocol. It is conceivable that protocol B might produce non-specific bands under certain circumstances. In such cases, increasing the annealing temperature to 42°C (protocol C) increases stringency while still showing good amplification of high molecular weight products. The high cycle number in protocol B might obscure smaller differences in gene expression. This can be dealt with by reducing the cycle number to 35 (protocol D) without loss of high molecular weight bands.

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