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Rational primer design greatly improves differential display-PCR (DD-PCR)

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ABSTRACT

Since its conception in 1992, differential display PCR (DD-PCR) has attracted widespread interest. Theoretically an attractive cloning approach, it combines the comparative analysis of several samples with the sensitivity of PCR. Although a large number of studies embracing this technology have been initiated, few novel genes of interest have been identified, suggesting that the method has not realised its potential. The present report shows that by modifying primer design, sampling of differentially expressed genes can be greatly enhanced and relevant genes can be isolated. Using our modified conditions DD-PCR efficiently screens a wide range of gene expression levels, in which differences are represented on a linear scale.

We studied gene induction in mouse thymocytes, using exposure to antibodies against the T-cell receptor (TCR) or phorbol-12-myristate-13-acetate (PMA) and Ca^{++}-ionophore A23187 as model systems. Thymocyte suspensions were stimulated in the presence of cycloheximide (CHX, 10 mg/ml) for 4 h with plate-bound antibody to TCR (H57, 10 µg/ml adsorbed overnight) or 250 ng/ml A23187 in combination with 20 ng/ml PMA. Control cells were cultured in medium alone or in the presence of CHX. RNA was prepared by spinning the cell lysate through a CsCl cushion. cDNA was prepared with SuperscriptII (Gibco-BRL) and oligo-dT primer according to manufacturers instructions. PCR was performed with the primers indicated using [^{33}P]dATP and hot start. The reaction mixtures were overlayered with melted paraffin, SuperTaq (HT Biotechnology, Cambridge, UK) was added and the tubes were put into a preheated thermocycler (Omnigene, Hybaid). Hot start was found to improve reproducibility. The annealing temperature for the first five cycles was 36°C, and 38°C for the subsequent 35 cycles. The PCR products were separated on 5% denaturing long ranger gels (FMC BioProduct, USA). Differential bands were cut from dried gels, reamplified and cloned into a T/A vector (1). Recombinant clones were screened by PCR, plasmid DNA was prepared and sequenced. All PCR reactions were run in duplicate.

Primers and cycling conditions in DD-PCR protocols were originally designed such that 100–150 different primer combinations encompass most of the estimated 15 000 expressed genes (2,3). Mitogen stimulation of mature T cells up-regulates more than 100 genes, among them CD69 and Nur77 (4,5). Assuming that mitogen stimulation is similarly effective in thymocytes one would predict on average one difference per primer combination. However, using primers and conditions as described (2,6) we encountered two types of problems. First, we observed far fewer differences than anticipated and second we isolated predominantly 10mer/10mer fragments (Table 1). 10mer/10mer products are in general not desirable since anchored oligo-dT primers are intended to bias the amplification to 3′-untranslated regions (3′-UTR) of genes, reducing the number of potentially amplified bands and minimising artefacts arising from variations in cDNA quality (cDNA length) or contamination with genomic DNA. Both problems can be explained by the following notions. First, the predominant amplification of 10mer/10mer products is the result of the different characteristics of random 10mer and oligo-dT primer. Because G/C interactions are stronger than those between A and T, 10mer primers with a G/C content of 50–60% are predicted to bind more strongly than the A/T-rich oligo-dT primer. As DD-PCR is a competitive reaction where primers and cDNAs are in equilibrium (see below) the amplification of 10mer/10mer products is favoured. Second, sequences in 3′-UTR often are A/T rich, making binding of random 10mer primers less likely and oligo-dT/10mer products will occur less frequently than expected.

Table 1. Retrospective analysis of oligomer useage among differentially expressed bands from conventional and modified (rational) DD-PCR

Upstream primer	Fragments isolated	10mer/10mer products	Percent 10/10mer	Ref.
Random 10mer	14	12	86	
	15	14	93	7
Rational primer	7	0	0	

We reasoned that by designing primers with an A/T content of 60–80%, corresponding more closely to the A/T content typically seen in 3′-UTR, both problems might be resolved. Taking this idea further, some primers were not only A/T rich but also resembled sequence motifs found in the 3′-UTR of immediate early genes (Jun/Fos family members). To compensate for the lower affinity these new primers are 12–14 bp in length (UTR-10: ^{c}/gTT^{c}/gTTT-^{c}/gTTT^{c}/g; UTR-11: gTA^{c}/g^{c}/gT^{c}/g^{c}/gATTTA; UTR-12: CATTGT-TATATTA). The oligo-dT primer and the new random primer had to be used in a 1:1 or 1:2 ratio. A 5-fold excess of dT12VG yielded many oligo-dT/oligo-dT products (bands amplified independently of the random primer, Fig. 1A, upwards pointing arrow) which could be suppressed by decreasing the oligo-dT concentration. Concomitantly, the amplification of molecules involving both primers could be improved (Fig. 1A, downwards pointing arrow). The ratio between the primers determines the outcome of the reaction implying that DD-PCR is a competitive equilibrium PCR; if cDNA and primer are in equilibrium, sequences with favourable

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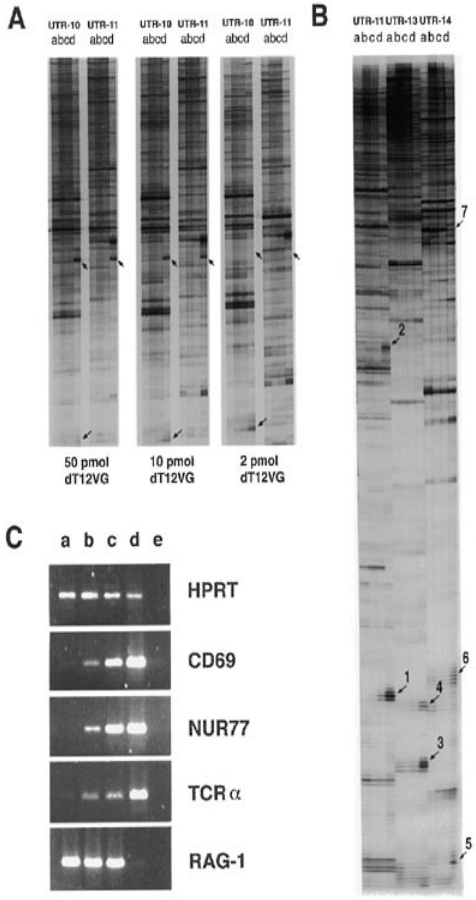

Figure 1. Characteristics and performance of rational primer. DD-PCR or normal PCR with cDNA from thymocytes freshly isolated *ex vivo* (lane a), thymocytes cultured for 4 h in CHX (lanes b–d) plus anti-TCR (lane c) or PMA + A23187 (lane d). Lane e contains no cDNA. (**A**) DD-PCR with various concentrations of dT12VG primer (50, 10, 2 pmol) and 10 pmol UTR-10 or UTR-11. (**B**) Efficiency of rationally designed primers. Bands 1–7 were reproducible differences. (**C**) PCR performed on cDNA adjusted for equal HPRT levels. Nur77, TCRα and RAG-1 are comparable to DD-PCR (B) (also compare text).

primer on/off rates will compete out the amplification of other bands.

DD-PCR reactions were performed with three of the newly designed rational primers in combination with a dT12VG primer (Fig. 1B). The numbers correspond to fragments which were differentially amplified reproducibly. On cloning, all fragments were bona fide rational primer/oligo-dT products, a significant improvement on results with the original protocol (Table 1). Of the seven fragments, bands 1, 2 and 7 represented Nur77, the TCRα chain and RAG-1, respectively, all known to be regulated during thymocyte activation. None of the rational primers were designed to match the 3′-UTR of those particular genes. The differential expression of Nur77, TCRα and RAG-1 was confirmed by RT-PCR (Fig. 1C).

Our results emphasize that DD-PCR must be viewed as a competitive equilibrium PCR. As a consequence of this, differences in expression should be represented in a linear fashion reflecting adequately the quantitative differences in the starting material. To test this, cDNA from PMA + A23187 stimulated thymocytes was serially diluted with cDNA from unstimulated thymocytes and

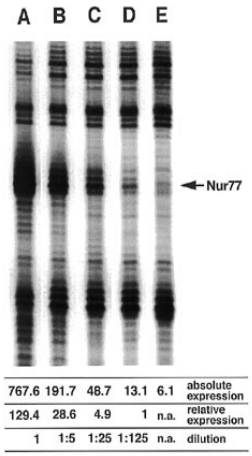

Figure 2. Linear representation of expression levels by DD-PCR. cDNA from thymocytes stimulated with PMA + A23187 was serially diluted with cDNA from unstimulated thymocytes: undiluted (**A**), diluted 1:5 (**B**), 1:25 (**C**) or 1:125 (**D**). (**E**) DD-PCR products using cDNA from unstimulated thymocytes. The arrow indicates the position of the Nur77 fragment. A 125-fold dilution of stimulated cDNA (D) still gave a detectable difference when compared to *ex vivo* thymocytes (E). Expression levels were determined on a phosphorimager and are given as arbitrary units (absolute expression) or were background-corrected ratios determined as follows [(X) – (E)]/[(D) – (E)], where (X) stands for lanes (A) to (D).

subjected to DD-PCR analysis using the primers dT12VA and UTR-11. As shown in Figure 2 the relationship between cDNA input and Nur77 band intensity is indeed linear.

Since DD-PCR was described the enthusiasm of many groups to apply it has turned increasingly into frustration when failing to identify large numbers of novel and relevant genes. A reason for this might be that the competitive nature of the underlying PCR was not fully appreciated. Using conventional primers many gene fragments compete for amplification such that only a small fraction of potential products are sufficiently amplified and displayed. With the rational to predominantly amplify 3′-UTR novel, 3′-UTR biased primers were designed. Although our primer might miss genes with non-A/T rich 3′-UTR our pilot studies show that they allow the rapid isolation of many gene fragments, some from genes with known relevance. To date we have tested 60 primer combinations and recovered ~100 differentially expressed bands leading to a considerable collection of differentially expressed genes. Due to the competitive nature of DD-PCR, differences depicted follow a linear scale. With these modifications it should be possible to identify 2–3-fold differences in expression or differential expression in a fraction of the cell population, a prerequisite for the study of more complex biological questions.

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