Benchmarks

Reverse Slot Blot for the Verification of cDNAs Identified through Differential Display

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Differential display, first described by Liang and Pardee (4), has become a powerful screening tool for the identification of cell- or stage-specific gene expression. The technique relies on efficient reverse transcription of mRNA and subsequent amplification of this cDNA with as many random primer combinations as possible. RT-PCR products are separated on denaturing sequencing gels, visualized through autoradiography, and bands of interest are eluted from the gel and sequenced. The central focus for improvements to the technique since its inception has been to reduce the high number of false positives and improve the efficiency of re-amplification of target genes. During the differential display procedure, there are several sources of false positives. These include cross-contamination of the RNA samples being compared, identically sized fragments co-migrating on the differential display gel, inefficient PCRs, and contamination during the re-amplification process. Numerous methods have been utilized in an attempt to identify false positives. The most common of these is Northern blot analysis (4), which requires large amounts of RNA. Liang et al. (3) and Zimmermann and Schultz (7) resorted to performing duplicate or triplicate RT-PCRs from a single RNA preparation and choosing only reproducible bands for further study. Another approach uses plasmid dot blotting to screen cloned cDNA fragments, using cDNA PCR products as probes (2). Methods to separate co-migrating cDNA fragments include restriction enzyme digestion and partial DNA sequencing (6) and modified single-stranded conformational polymorphism (mSSCP) analysis (5). These methods all have various drawbacks, including lengthy time requirements or the need for large amounts of RNA.

Minimizing the number of procedures that have to be performed for confirmation of results is optimal for any technique aimed at reducing false positives. We have devised a reverse slot blot technique that is performed using re-amplified eluted fragments, thereby eliminating unnecessary sub-cloning or sequencing reactions.

We have used differential display to identify stage-specific gene expression in the asexual and gametocyte stages of the malaria parasite *Plasmodium falciparum*, with a view to blocking this transition, which is vital to the transmission of the parasite to its mosquito host.

Figure 1. Differential display analysis of *P. falciparum* asexual (A) and gametocyte (G, G') stage mRNA. Arrows indicate some of the differentially expressed cDNAs. G and G' represent mRNA preparations from different gametocyte cultures.

Differential display (Figure 1) was performed essentially as described (4). Approximately 200 ng *P. falciparum* strain 3D7 total RNA were reverse-transcribed with 2-base anchored primers to select for the 5' end of the polyA tail, using SuperScript IITM RNase H⁻ Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). The PCR was performed with 11 random 10- and 13-mers (IDT, Coralville, IA, USA) designed with AT content of 50%–70% to accommodate the high AT content of the *P. falciparum* genome. Each random primer, in combination with each of the 2-base anchored primers, was included in separate reactions to maximize the combinations available for comparison. To isolate gene fragments, the gel was carefully realigned with the autoradiograph, and a sterile scalpel was used to excise the cDNA fragments. A second exposure to X-ray film was then performed to confirm that the correct fragments had been excised. To elute the DNA from the acrylamide gel, the bands were finely sliced and placed in 30 μ L Milli-Q[®] water (Millipore, Bedford, MA, USA). The sample was centrifuged through a pipet filter tip at 10 000 $\times$ g for 10 min. Ten microliters of this were used for an initial 25- μ L PCR containing 0.2 μ M primers, 2.5 μ L 10 $\times$ Takara PCR Buffer, 0.25 μ L rTaq DNA polymerase (Takara Bio, Shiga, Japan), and dNTPs at a final concentration of 70 μ M, de-

Figure 2. Reverse slot blot to confirm stage-specific expression of differentially displayed *P. falciparum* gene fragments 1 and 2. RT-PCR was performed with primer combinations designated 55-1G (55-1 is the arbitrary 13-mer 5'-GTCCAATTAGATG-3'; G refers to the 2-base anchored primer polydT₁₂VG, where V = G, A, and C). The image shows the up-regulation of fragment 1 in the asexual stages (A) of the parasite and the unique expression of fragment 2 in gametocytes (G).

Benchmarks

noted PCR1. One microliter of PCR1 was used as a template for a second PCR, which was set up in triplicate, pooled, precipitated with ethanol, and resuspended in a final volume of 20 μ L Milli-Q water. Two microliters were assessed on a standard 1% agarose gel. Using this technique, we attained a re-amplification rate of greater than or equal to 90%.

To verify that the re-amplified products were indeed expressed in a stage-specific manner, we developed a modified reverse slot blot technique (Figure 2). In brief, 2–5 μ L re-amplified DNA, representing 70–90 ng, were added to 195 μ L denaturing solution (0.4 M NaOH, 25 mM EDTA) and denatured at 95°C for 2 min. This was then transferred onto nylon membranes (Amersham Biosciences, Little Chalfont, UK) and UV cross-linked for 4 min at 302 nm. α -³²P RT-PCR products from new asexual and gametocyte preparations were prepared as described above.

Identical concentrations of asexual stage and gametocyte RNA were used for the RT-PCRs, which were performed with the original primer combinations from the differential display gel. Blots were placed in 5-mL test tubes and probed with 800 μ L hybridization buffer (5 $\times$ SSPE, 5 $\times$ Denhardt's solution, 0.5% SDS) containing up to 25 μ L α -³²P RT-PCR products. Asexual and gametocyte probe concentrations were normalized by comparing the incorporation of α [³²P]dATP, assessed by 10% TCA precipitation and liquid scintillation counting (1). To confirm differentially expressed fragments that originated from different primer combinations, all α -³²P RT-PCR products were combined to probe a single blot. The blot was washed in 0.2 $\times$ SSC/0.1% SDS for 10 min with vigorous rotation at room temperature, followed by a similar wash at 65°C. Stringency was varied if necessary, according to standard protocols (1). The blot was exposed to X-ray film for 30 min in a cassette containing enhancing screens. Exposure times were varied according to signal intensity.

This novel, sensitive, and rapid method ascertains both the reproducibility of the differential display technique and the authenticity of differential display results. Since the probe is an RT-PCR product and re-amplified cDNA fragments are immobilized on the membrane, only small quantities of RNA are required (50–500 ng). The technique is highly sensitive, and eluted fragments can be verified as being true positives within two days of re-amplification.

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