

Efficient reamplification of differential display products by transient ligation and thermal asymmetric PCR

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ABSTRACT

A new method for specific reamplification of DDRT-PCR products is presented. After transient ligation of the primary DDRT-PCR fragments into a T-vector, the cDNAs of interest were reamplified by hemi-nested PCR and thermally asymmetric cycles. In contrast to the originally described protocol, this method of reamplification is specific, sensitive, reproducibly gives a high yield of DNA and allows direct sequencing of the reamplified product without purification or cloning.

We used the differential display technique to investigate the effect of a blood meal on the expression of *Anopheles gambiae* midgut-specific genes and thereby identify genes potentially involved in the sporogonic cycle of the human malaria parasite *Plasmodium falciparum*. The differential display technique (DDRT-PCR) (1–3) is a method that can be used for analysis of changes in gene expression by detecting differential production of mRNA and which requires a small amount of RNA. This method has been used successfully to identify a variety of differentially expressed genes (2,4–6). However, it has some drawbacks such as the large number of false positives generated, and different modifications of the original protocol have been proposed to limit the production of false positives or to facilitate the identification of putative positives (2,7–10). Another disadvantage is the difficulty in selectively re-amplifying the target cDNA, which is a necessary step in verifying the expression pattern of the DDRT-PCR band (8,11). The original procedure for reamplification resulted, in our hands, in poor yields even after two rounds of re-amplification which in most cases generated non-specific PCR products. Furthermore, such direct reamplification often led to the production of fragments which contained the same arbitrary upstream primer at both ends (8,9 and unpublished results). These products have no T-tail and may correspond either to mRNA or to contaminating DNA. To circumvent these problems, we developed a procedure for re-amplification at high annealing temperature using a transient ligation step (11) and a modified oligo-dT anchored primer (DDT3). To increase the specificity and the yield of the reaction, three hemi-nested reactions are performed with thermal asymmetric cycles (12).

Total RNA was isolated from mosquito midguts dissected prior to and after the blood meal by using TRI REAGENT™ (13) and DNase I. 300 ng of total RNA from each pools were reverse transcribed with four downstream anchored primers, (T₁₁AA, T₁₁AC, T₁₁AG and T₁₁CA) and amplification was performed with 24 upstream 10mer primers (Display Systems Tandil S.A., Paris)

essentially as described (1). As exemplified in Figure 1A, numerous PCR products were found differentially displayed, at least 10 fragments for each combination of primers tested. Selected c-DNAs fragments were efficiently reamplified as follows. Fragments were eluted from the gel and resuspended in 10 µl DEPC-treated water (11). An aliquot of 3 µl of eluted cDNA was incubated at 72°C for 30 min in 10 µl with 1 U of Hitaq DNA polymerase (Bioprobe), 0.4 mM of dATP, 1× PCR buffer and added to the ligation reaction in a final volume of 20 µl, containing 20 ng of PCR-II vector (Original TA cloning kit™, Invitrogen), 400 U of T4 DNA ligase (Biolabs) and 1× ligase buffer. After overnight incubation at 16°C, the ligation product was precipitated with 5 M NaCl and isopropanol at room temperature. The pellet was rinsed twice with 200 µl of 70% ethanol and resuspended in 10 µl DEPC-treated water. The ligation mixture was then used as template for a series of three hemi-nested PCR using DDT3 in conjunction with three nested primers annealing to vector sequences in the vicinity of the cloning site (T7-1; T7-2; T7-3) as follows. The primary PCR amplification mixture (20 µl) contained 10 µl of ligation product, 0.2 µM T7-1 primer, 5 µM DDT3 primer, 200 µM of each dNTP, 2 U of Hitaq DNA polymerase and 1× PCR buffer. The secondary (20 µl) and tertiary (100 µl) PCR amplifications were performed using 25 µM of each dNTP, 2.5 µM DDT3 primer and 0.2 µM specific primers T7-2 and T7-3. Two µl of 100-fold dilutions of the primary PCR products were used in the secondary PCR; 10 µl of 100-fold dilutions of the secondary PCR products were used in the tertiary PCR. Each PCR was performed in a Perkin-Elmer Cetus DNA Thermal Cycler 9600. Primer sequences and T_m , and thermal setting are described in Table 1. The primary and secondary PCRs use thermal asymmetric cycles for hybridization of primers with different T_m s. During the first five cycles at high stringency a linear amplification primed by the vector internal primer is favored, thereby increasing the amount of specific template for the following amplification cycles at reduced stringency. In addition, in the primary PCR one low stringency cycle was carried out to facilitate the initial annealing of the DDT3 through its T-track. As described by Liu and Whittier (12), the non-specific products corresponding to fragments primed by only one primer are eliminated by carrying out three PCR successively with nested specific primers.

Using this strategy we efficiently reamplify 23 out of 40 (57.5%) DDRT-PCR fragments obtained from different experiments, whereas only 31% (11/48) could be reamplified at very low yield using the original protocol. Figure 1B shows an example of products obtained by our method. Specific products of reamplification were visible after the subsequent secondary and tertiary reactions. In most cases (17/23), only one molecular species of cDNA was amplified. Using

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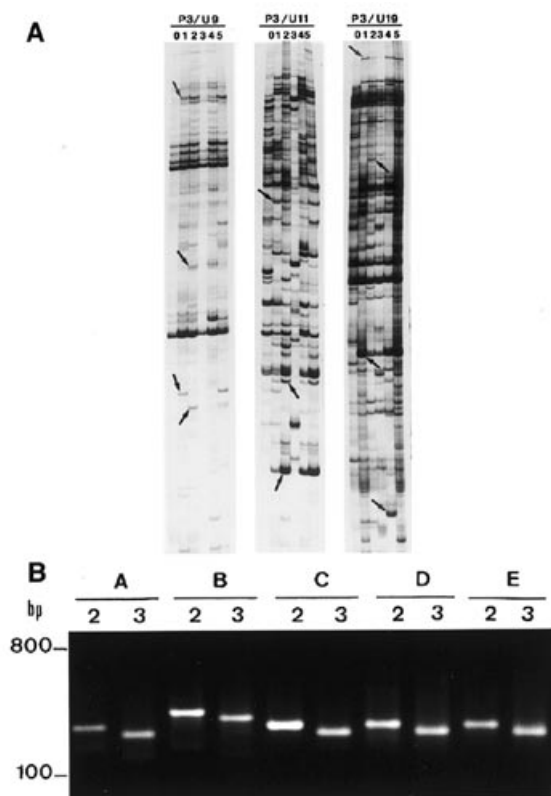


Figure 1. (A) Examples of banding patterns obtained using the DDRT-PCR technique and non-denaturing gel with midgut RNA of sugar-fed mosquitoes (lanes 0), and blood-fed mosquitoes 14–16 h (lanes 1); 17–19 h (lanes 2); 20–22 h (lanes 3); 23–25 h (lanes 4) and 26–28 h (lanes 5) after the meal. The 3' primer was (dT)₁₁AG (P3) and the 5' primers were GATTTGCGAG (U9), GAACTGACAC (U11) and TAGAACGAGG (U19). Arrows indicate selected fragments that are differentially regulated by the blood meal. (B) Agarose gel analysis of five different DDRT-PCR products reamplified by thermal asymmetric hemi-nested PCR after transient ligation. Each pair of lanes contains products from consecutive secondary (2) and tertiary (3) reactions for a given selected PCR fragment.

the transient ligation system (11) and low annealing temperature (40°C) we were unable to obtain reamplification products.

We found that precipitating the ligation product with isopropanol at room temperature was essential as reported previously (14). We also found that rinsing the DNA pellet twice with 70% ethanol was required (data not shown). Large amounts of DNA (up to 5 µg) could be produced after the third nested PCR and were directly sequenced with the Sequenase PCR product Sequencing kit and [³³P]dATP using primer T7-3. Unpurified templates yielded clear sequence profiles (not shown), reflecting the high specificity of the reamplified product. The reamplified fragments could also be used for *in vitro* production of anti-sense RNAs using the promoter for the T3 RNA polymerase incorporated into the DDT3 primer. Such RNAs can be used in RNase protection experiments.

In conclusion, the described protocol allows efficient reamplification of T-tailed DDRT-PCR products thus alleviating the difficulties encountered in using the original protocol (1). Although the differentially displayed band may contain several PCR products (15), our method will favor the amplification of a T-tailed product. The high purity of the reamplified product allows direct sequencing

and facilitates subsequent experiments such as RT-PCR, RPA or reverse northern blot (8,10) without a cloning step thereby saving much time and effort.

Table 1. Primer sequences and cycling conditions used for the three thermal asymmetric PCR

Reactions and primers	Cycle numbers	Thermal setting
Primary (T7-1/DDT3)	1	94°C 5 min
	5	94°C 30 s, 66°C 1 min, 72°C 2 min
	1	94°C 30 s, 25°C 3 min, ramping to 72°C over 3 min, 72°C 2 min
	15	94°C 30 s, 40°C 1 min, 72°C 2 min
	1	72°C 5 min
Secondary (T7-2/DDT3)	1	94°C 5 min
	5	94°C 30 s, 72°C 2 min
	20	94°C 30 s, 53°C 1 min, 72°C 2 min
1	72°C 5 min	
Tertiary (T7-3/DDT3)	1	94°C 5 min
	20	94°C 30 s, 53°C 1 min, 72°C 2 min
	1	72°C 5 min

The melting temperature (T_m) of T7-1 (5'-TGCAAGGCGATTAAGTTGGGTAAC), T7-2 (5'-AACGCCAGGGTTTCCCAGTCACACG), T7-3 (5'-GTTGTAAACGACGGCCAGTGA) are 61, 66 and 60°C respectively as calculated with the formula $69.3 + 0.41 (\%GC) - 650/L$, where L is primer length (16). The anchored primer [DDT3: 5'-AATTAACCCTCACTAAAGGG(T)₁₁VN] has an overall T_m of 61°C. Annealing temperatures for the high-stringency cycles were set 5°C higher than the calculated T_m of the specific primer (12).

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