

Rapid non-radioactive TMACl hybridization protocol employing enzymatically labeled oligonucleotides

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Submitted December 12, 1990

The availability of a rapid hybridization protocol which allows the confirmation of the specificity of PCR products has become of increasing importance. Here, a novel non-radioactive protocol is described, using enzymatically 3′ biotinylated (1, 2) oligonucleotides in combination with a TMACl (tetramethylammonium chloride) hybridization procedure. TMACl rendering the dissociation temperature of oligonucleotides independent of their GC-content (3, 4) provides the advantage that the critical washes for all oligonucleotides of the same length can be performed simultaneously at the same temperature. As the dissociation temperature of 17 to 24 mers lies within a range of 55–66°C, linking an enzyme directly to the oligonucleotide is not possible. Therefore, the oligonucleotide is biotinylated first. Besides making it possible to employ oligonucleotides already on hand, this procedure circumvents a possible inhibitory action of TMACl on the SA-HRP (streptovidine conjugated horseradish peroxidase), which is coupled to the biotinylated oligonucleotide after the TMACl washes.

Performing PCR: cDNA was generated from a RNA preparation of human granulocytes following the protocol described by Chang *et al.* (5) using a primer complementary to CD45 mRNA. PCR was carried out using two primers amplifying a 294 bp fragment of CD45 cDNA.

Oligonucleotide hybridization: 3′ labeling of 60 pmol of oligonucleotide (24 mer) was performed in a final volume of 20 µl containing 1×TdT buffer (200 mM potassium cacodylate, 25 mM Tris/HCl pH 6.6, 0.25 mg/ml BSA), 6.25 mM $CoCl_2$, 30 µM Biotin-14-dATP (Gibco/BRL) and 25 U TdT(terminal transferase) (Boehringer) for 30 min at 37°C. The membranes (50 cm^2) were prehybridized for 15 min at 42°C in 10 ml of hybridization buffer (5×SSPE/5×Denhardt's/0.5% Triton X-100). Labeled probe was added (30 pmol/50 cm^2) and hybridization continued for 3 h. The membrane was washed twice for 5 min in buffer A (5×SSPE/0.1% Triton X-100) at 42°C. Stringency washes were performed twice for 15 min with 3 M TMACl/0.1% Triton X-100 at 55°C or 66°C. Membranes were then rinsed twice in buffer B (1 M urea/1% dextrane sulfate/5% Triton X-100 in PBS) to remove TMACl and incubated for 3 h in buffer B containing 1 µl/ml SA-HRP (Gibco/BRL) followed by two 5 min washes in buffer B. Subsequently, the membrane was rinsed once in buffer C (O.1 M sodium citrate, pH 5). Detection of enzyme activity was performed by preincubation (10 min) in buffer C containing 0.5mg/ml 3,3′, 5,5′-tetramethylbenzidine (TMB, Sigma) and subsequent incubation in buffer C containing 0.5 mg/ml TMB and 0.5 µl/ml H_2O_2 (30%).

Figure 1 shows the results of a PCR for the 294 bp fragment after gel electrophoresis (A) and hybridization (B, C, D, E). Increasing amounts of the PCR product were loaded onto the gel (2, 6, 10, 14 µl, lanes 1, 2, 3, 4, respectively). Hybridization has been performed with an oligonucleotide not complementary to the amplified sequence (C, E) and an oligonucleotide complementary to this sequence (B, D). The two different hybridization temperatures were chosen to further demonstrate specificity: 66°C (critical temperature for a 24 mer; B, C) and 55°C (below the critical temperature; D, E). It is clearly demonstrated, that the stringency of hybridization is temperature dependent. Faint bands can only be observed for the non-specific oligonucleotide when the critical washes are performed below the critical temperature.

This novel protocol also allows the detection of single base pair missmatches (results not shown) which is of particular importance for example in HLA class II-oligotyping (for unrelated donor selection), where extensive genetic polymorphism makes it neccessary to simultaneously hybridize a large number of membranes with many different oligonucleotides (manuscript in preparation). In conclusion, we would like to stress the usefullness of the novel combination of an oligonucleotide hybridization protocol comprising enzymatic labeling and a TMACl washing procedure with a rapid non-radioactive detection protocol rendering high specificity and easy handling.

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