Preincubation Superior to Strict Competition with Mismatched Oligonucleotides for Detecting α1-Antitrypsin Deficiency

To the Editor:

We (1) and others (2) have shown that mismatched oligonucleotides improve the specificity of detection of point mutations by allele-specific oligonucleotide (ASO) methods applied to polymerase chain reaction (PCR) products. Two variations of competition have been described, one with simultaneous hybridization with excess unlabeled mismatched oligonucleotide and a specific radiolabeled probe (strict competition) (2), the other with preincubation with excess unlabeled mismatched oligonucleotide, followed by separate hybridization with a specific radiolabeled probe (preincubation) (1). We performed direct comparison of these methods, using increasing amounts of competing oligonucleotide (2, 10-, 20-, and 50-fold molar excess) to obtain quantitative data on the specificity and sensitivity of these procedures.

The α1-antitrypsin (AAT) gene was used as a model system. The sequence of the PCR products of the AAT gene, obtained from homozygous M and Z patients' samples, and heterozygous MZ samples, were verified by cycle-sequencing (Gibco BRL, Glasgow, UK). The sample concentrations were adjusted so that equivalent amounts of each product were applied. The AAT control (200 mg/L) was diluted 10-fold in sterile water and 2 μL (40 ng) of this was spotted onto nylon membrane. Duplicate dots of homozygous M, heterozygous MZ, homozygous Z, and pA1T were spotted on the membrane before hybridization performed as described previously (1).

The M and Z oligonucleotides were diluted to 5 μmol/L for use as unlabeled competing oligonucleotides. We also used 2 μL (10 pmol) of this dilution for end-labeling with γ32P as described previously (1). To minimize the effect of variations in specific activity of the oligonucleotides, we labeled the probes freshly each day and conducted the experiments on consecutive days.

Nonspecifically bound labeled probe was removed as before (1).

The filters were exposed to a preflashed Fuji RX blue-sensitive film at −80°C for 18 h with CaWO4 intensifying screens. We have previously demonstrated (1) that a linear signal is obtained for up to 24 h under these conditions. The autoradiograms were scanned with a Beckman Appraise automatic densitometer (Beckman Instruments, Brea, CA).

As expected, conventional use of ASOs under the conditions described did not discriminate between M and Z (Fig. 1). The use of a twofold excess of unlabeled competing oligonucleotide in the prehybridization solution resulted in good differentiation with the Z probe but the M probe still showed some residual binding (−15%) to the Z target sequence. We have previously shown that complete differentiation can be obtained by using a higher temperature for the posthybridization wash (1). Use of 10-fold excess unlabeled competing oligonucleotide in the prehybridization solution gave perfect differentiation for both M and Z probes under the conditions used, and the strong signal obtained indicated good specificity and sensitivity (Fig. 1).

We have successfully used identical preincubation conditions to detect multiple single-point mutations in the tumor suppressor gene, p53 (3). These results suggest that similar conditions maybe useful for detecting many single-point mutations, thus obviating the need to determine each one empirically.

These studies highlight how subtle variations in the conditions used in competition assays can significantly influence the specificity of single-point mutation detection and demonstrate the superiority of a preincubation step over strict competition.

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References


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Negative Interference with a Thyrotropin IRMA from an Unidentified Serum Constituent

To the Editor:

We have found that the CoTube™ TSH IRMA kit (Bio-Rad Diagnostics Group, Hercules, CA) underestimates thyrotropin (TSH) in some sera, and that the underestimation can be overcome by dilution of the sample with the manufacturer's 0 mIU/L calibrator.

As shown in Table 1, increasing values on dilution can be seen at TSH concentrations as low as 36 mIU/L; however, not all samples between 30 and 100 mIU/L exhibit this behavior. Upon progressive dilution, most samples reach a plateau value. A plateau was not reached with sample 4 in Table 1 before all of the sample was consumed. The manufacturer's literature...
Table 1. Increase in TSH (mIU/L) in Bio-Rad IRMA of eight samples upon dilution.

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<thead>
<tr>
<th>Dilution</th>
<th>1:5</th>
<th>1:10</th>
<th>1:100</th>
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<tr>
<td>None</td>
<td>40.9</td>
<td>69.9</td>
<td>74</td>
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<td>40.9</td>
<td>69.6</td>
<td>103</td>
<td>108</td>
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<td>44.7</td>
<td>69.5</td>
<td>79.5</td>
<td>84</td>
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<td>76.6</td>
<td>153.3</td>
<td>433</td>
<td>636</td>
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<tr>
<td>90.8</td>
<td>146.8</td>
<td>142</td>
<td>134</td>
</tr>
<tr>
<td>49.4</td>
<td>67.8</td>
<td>72</td>
<td>80</td>
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<tr>
<td>98.9</td>
<td>284</td>
<td>279</td>
<td>273</td>
</tr>
<tr>
<td>36.4</td>
<td>288.7</td>
<td>310</td>
<td>330</td>
</tr>
</tbody>
</table>

states that this IRMA procedure does not experience the high-dose "hook effect" at TSH concentrations below <250 mIU/L (1992 instruction manual, CoTube TSH IRMA, p. 13). From that information and the appearance of our data, we do not believe that these aberrant results are due to a high-dose hook effect; rather, they appear to be the result of some endogenous interfering substance.

Regression data from a correlation study, with assay of unaffected samples by the Bio-Rad IRMA TSH procedure (B), Immunon1™ (Miles Diagnostics Division, Tarrytown, NY; M), and Access™ (Sanofi Diagnostics Pasteur, Chaska, MN; S) systems, yielded: AS = 0.971B + 0.1 (r = 0.991, n = 102, SE = 4.1 mIU/L), and M = 0.980B + 0.1 (r = 0.994, n = 107, SE = 9.4 mIU/L). Thus, with samples that do not demonstrate this phenomenon, the correlations between the isotopic and the two nonisotopic procedures are quite good. Furthermore, we found that for those samples that required dilution with the Bio-Rad procedure, the concentrations found on the diluted specimens correlated well with those by the nonisotopic procedures (within what was considered to be acceptable errors, given the very large dilutions that had to be made). However, the initial IRMA-measured concentrations for these specimens ranged from 20 to 97 mIU/L (all <100 mIU/L, the concentration of the IRMA's high calibrator), so an operator would not ordinarily have reassayed these samples after dilution. On dilution, their concentrations ranged from 31 to 285 mIU/L.

We now routinely re-assay after dilution all samples that yield an initial TSH result ≥10 mIU/L. At present, this means that we repeat about two samples per day (~2% of our TSH requests), and about 25% of these exhibit this apparent interference. All of the data presented above are for specimens with high concentrations of TSH, because for all practical purposes it is only these that can be diluted. However, our correlation study produced two outliers with relatively low TSH, that might be explained by this same phenomenon—e.g., TSH concentration of 0.87 mIU/L with the Bio-Rad procedure (confirmed by repeat analysis) and 2.2 and 1.8 mIU/L with the Immunon and Access systems, respectively. Thus, there may also be cause for concern at much lower concentrations of TSH, with risk of misclassification of patients.

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