The principle of the assay is as follows: Theophylline-specific antibody binds theophylline-MPA in the absence of theophylline and thus prevents the inhibition of IMPDH by theophylline-MPA. The enzyme activity is greatest when theophylline is absent. Theophylline, when present, binds to its antibody, thus freeing up theophylline-MPA. Free theophylline-MPA binds to catalytically active IMPDH and inhibits the enzyme by preventing the release of XMP. The rate of formation of NADH is measured at 340 nm and is correlated to theophylline concentration. The rates observed with 0, 5, 10, 20, and 40 mg/L theophylline calibrators were, respectively: 105, 98, 93, 85, and 78 milliabsorbance units/min at 340 nm on the Hitachi 917 analyzer.

We used Passing–Bablok regression statistics to compare the theophylline-MPA method on the Hitachi 917 with the fluorescence polarization method on the Integra 700. Patient plasma samples were used in the method comparison. Regression statistics were as follows: $y = 0.962x + 0.077$; median distance (95) = 1.991; $n = 51$; $R = 0.982$; median, 11.3 ($x$), 10.8 ($y$); minimum, 8.3 ($x$), 8.8 ($y$); maximum, 29.5 ($x$), 31.0 ($y$).

We conclude that these results indicate the potential use of IMPDH as a homogeneous enzyme immunoassay technology as shown for theophylline.

The R1 reagent formulation used was 100 mmol/L Tris, 100 mmol/L KCl, 80 mmol/L IMP, 4 mmol/L TCEP, 6 mmol/L EDTA, 1.47 mmol/L theophylline-MPA, 4 mmol/L Sutacide A, 0.1 g/L (theophylline) monoclonal antibody, IMPDH-II (adjusted to rate), final pH 8.0. The R2 reagent formulation used was 1 mmol/L NAD, 4 mmol/L Sutacide A, 1.75 mL/L Nonidet P-40 (0.175%), final pH 6.0. The (theophylline) monoclonal antibody was a purified Roche Diagnostics monoclonal.

The $N$-acetyltransferase 2 (NAT2) gene is autosomal, dominant, and intronless with an open reading frame of 870 bp. It is located on chromosome 8p22. NAT2 enzyme detoxifies and inactivates drugs and xenobiotics in the liver. NAT2 polymorphisms confer phenotypes categorized as slow, intermediate, or rapid acetylators with broad interethnic variation. There are 26 known alleles, and each allele variant is a combination of one, two, three, or four nucleotide substitutions. Within the coding
region there are seven missense mutations (G191A, T341C, A434C, G590A, A803G, A845C, and G857A) and four silent mutations (T111C, C282T, C481T, and C759T) (1, 2). The wild-type NAT2*4 allele is associated with the rapid acetylator phenotype and does not have any nucleotide substitutions. The phenotype can be predicted with 95% accuracy by genotyping. (3–5).

We present a method for performing multiple-polymorphism genotyping of the NAT2 gene. A single amplification of NAT2 is performed with sequential probing for multiple polymorphisms by active electronic arrays. The method permits application of multiple DNA samples in singleton for high-throughput genotyping. Rapid high-throughput determination of genotypes may aid in clinical epidemiology studies and in routine clinical practice.

Published primer sequences were used to perform PCR to generate a 1212-bp amplicon (6). The primers were 5′-AAT TAG TCA CAC GAG GA-3′ (forward) and 5′-biotin-TCT AGC ATG AAT CAC TCT G-3′ (reverse). The biotinylated primers permit capturing of the amplicons to the microarray surface where they remain embedded through interaction with streptavidin in the permeation layer. The agarose permeation layer containing streptavidin interacts with streptavidin in the permeation layer. Known heterozygotes were used to normalize hybridization efficiency between Cy5 and Cy3 dye-labeled reporters. Heterozygotes were verified by dye-terminator sequencing on ABI 377 DNA sequencers in both forward and reverse directions. A biallelic fluorescence intensity ratio 1:3 was defined as heterozygous and a ratio ≥1:5 was defined as homozygous. Genotypes were assigned using the manufacturer’s recommended biallelic fluorescence intensity ratios. No genotype designations were made for fluorescence intensity ratios between 1:3 and 1:5. Homozygous wild-type alleles hybridized with Cy5-labeled reporter probe, whereas homozygous polymorphic alleles hybridized only with Cy3-labeled reporter probes. Each heterozygous complex hybridized with both labeled probes for each allele pair tested.

Sequential probing with reporter probe pairs was used to analyze SNP in the amplicon. The A803G polymorphism was detected first because of the relatively lower T_m for its reporters. At the end of the first reporting, the array was subjected to a reporter dehybridization protocol in the fluorescence reader. Using this protocol, we stripped the probes from the array by ramping the temperature to 40 °C followed by a l-histidine buffer wash. We confirmed complete probe dehybridization by verifying the absence of any fluorescence signal. We then sequentially reprobed with the reporters for G191A followed by T341C.

Representative genotyping results for the NAT2*T341C polymorphism are shown in Fig. 1. The genotype designation for amplicon 1 is homozygous for the polymorphic allele. This is based on a fluorescence intensity ratio ≥1:5 between Cy3 and Cy5 dye-labeled reporters. Furthermore, the data are normalized with a known heterozygote confirmed previously by sequencing. Similarly, the genotype designation for amplicon 10 is homozygous for the wild-type allele. The genotypes for the remaining sample amplicons shown in Fig. 1 are designated as heterozygous with Cy5/Cy3 biallelic fluorescence ratios ≤1:3.

Overall, for NAT2*T341C, 79 of 83 (95.2%) amplicons
were genotyped in a single hybridization run on one microarray. Of these, 32.5% were wild type, 50.6% were heterozygous, and 12% were homozygous for the polymorphism (Table 1). For NAT2*A803G, 80 of 83 (96.4%) amplicons were genotyped in a single hybridization run on a microarray, of which 34.9% were wild type, 49.4% were heterozygous, and 12% were homozygous for the polymorphism. For NAT2*G191A, 71 of 83 (86%) amplicons were genotyped in a single run, of which 100% were wild type.

In practice, <100% of samples could be genotyped in a single run. For NAT2*T341C, there were three amplicons that could not be assigned a genotype because the biallelic ratio was between 1:3 and 1:5. There were two amplicons for NAT2*A803G and six amplicons for NAT2*G191A that were not assigned a genotype for the same reason. Of the 83 amplicons that were analyzed, there was only one DNA sample that could not be genotyped for any of the SNPs because of failed PCR. This may be attributable to the poor quality of the template DNA used for PCR or to human error in either the PCR set-up or genotyping steps. In some cases, in spite of successful PCR, the amplicon(s) had a lower DNA concentration than required. There were five NAT2*G191A amplicons for which the fluorescence signals were too low to make a designation. Furthermore, three sets of stabilizers and reporters were used for the three different SNPs that were analyzed. Although sequential probing was done on the same amplicon, the amplicon concentration thresholds required for the three SNPs were different. This is because of differences in base-pairing interactions among the stabilizers and target DNA as well as differences in base-stacking interactions between stabilizer and reporter pairs. It is therefore possible that, whereas one SNP could be assayed successfully for a test amplicon, analysis of another SNP on the same amplicon failed.

Microarray genotyping results were confirmed by direct DNA sequencing with 100% concordance. Sequential scanning for polymorphisms within an amplicon could be performed up to five times without significant degradation of the signal-to-noise ratio. This approach has been extended to four additional four SNPs (C282T, C481T, G590A, and G857A), using a second microarray, and achieved comparable results.

Sequential probing is a rapid and accurate method for genotyping multiple polymorphisms in large amplicons and intronless genes. The method can be applied to test large number of samples, using active electronic arrays. This sequential probing approach is particularly useful in epidemiologic studies because of its relative simplicity and high sample throughput, which could improve the cost-effectiveness for genotyping.

### Table 1. Genotyping results for NAT2 polymorphisms in this study.

<table>
<thead>
<tr>
<th>SNP</th>
<th>Genotyped/single run</th>
<th>Wild type*</th>
<th>Heterozygous</th>
<th>Mutant*</th>
</tr>
</thead>
<tbody>
<tr>
<td>T341C</td>
<td>79/83 (95.2%)</td>
<td>32.5%</td>
<td>50.6%</td>
<td>12%</td>
</tr>
<tr>
<td>A803G</td>
<td>80/83 (96.4%)</td>
<td>34.9%</td>
<td>49.4%</td>
<td>12%</td>
</tr>
<tr>
<td>G191A</td>
<td>71/83 (86%)</td>
<td>100%</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Homozygous.

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**Mass Spectroscopy as a Discovery Tool for Identifying Serum Markers for Prostate Cancer**, John J. Hlavaty,1 Alan W. Partin,2 Felicity Kusinitz,1 Matthew J. Shue,2 Adam Stieg,1 Kate Bennett,1 and Joseph V. Briggman1*

Prostate cancer is the second most common malignancy in men, after skin cancer, and the second most common cause of cancer death in men over age 60 years, after lung cancer. This year, ~198 100 new cases of prostate cancer will be diagnosed in the US, and an estimated 31 500 men will die of prostate cancer (1). Five-year survival is close to 100% when the disease is diagnosed and treated with definitive local therapy while it is still organ-confined, but in approximately one-third of men diagnosed with clinically localized disease, the disease has spread beyond the confines of the prostate at the time of surgery (2, 3).

The Food and Drug Administration approved a serum