During the first study, we observed (Table 1, study I) that the EDTA and heparinized plasma were both stable refrigerated for 48 h after thawing. By day 6, Hcy had decreased in both plasma types. We did not expect to observe the significantly increased values for Hcy in the frozen heparinized plasma compared with the EDTA plasma. We speculated that the gel barrier and/or freezing of the plasma on the gel was the cause of the observed difference.

In the second study (Table 1, study II), the significant difference observed in the first study between heparinized and EDTA plasma was not observed when heparinized plasma was stored at 2–8 °C on the gel rather than frozen on the gel. Thus, freezing the plasma on the gel was the cause of the observed difference.

In conclusion, these studies indicate that Hcy cannot be stored frozen on the PST separator gel. However, Hcy is stable for 72 h in heparinized plasma when stored on the gel at 2–8 °C. This stability and ease of handling has considerable practical application for assuring accurate patient results for Hcy assays when there is a time delay between blood collection and testing.

**Table 1. Comparison of EDTA and heparinized plasma at different times in studies I and II.**

<table>
<thead>
<tr>
<th></th>
<th>EDTA plasma</th>
<th>Heparinized plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Study I (n = 21)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 1</td>
<td>9.8 (2.6)</td>
<td>11.3 (2.8)</td>
</tr>
<tr>
<td>Day 3</td>
<td>9.9 (2.6)</td>
<td>11.4 (2.7)</td>
</tr>
<tr>
<td>Day 6</td>
<td>8.6 (2.5)</td>
<td>9.6 (2.6)</td>
</tr>
<tr>
<td>Day 6 vs day 1, % difference</td>
<td>−12.20</td>
<td>−15.00</td>
</tr>
<tr>
<td><strong>Study II (n = 15)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 h</td>
<td>9.4 (2.4)</td>
<td>9.8 (2.6)</td>
</tr>
<tr>
<td>48 h</td>
<td>9.5 (2.6)</td>
<td>9.9 (2.6)</td>
</tr>
<tr>
<td>72 h</td>
<td>8.5 (2.4)</td>
<td>9.7 (2.5)</td>
</tr>
</tbody>
</table>

Somatic mutations were only slightly higher and considered to be statistically different (P = 0.0005), the observed differences were only slightly higher and considered to be negligible.

Repeated-measures ANOVA was used to compare Hcy stability for the 2 plasma types. EDTA plasma Hcy was stable for 48 h, whereas Hcy in heparinized plasma (PST) was stable on the gel for 72 h at 2–8 °C (P = 0.466). Although PST results compared with EDTA results were statistically different (P = 0.0005), the observed differences were negligible.

In conclusion, these studies indicate that Hcy cannot be stored frozen on the PST separator gel. However, Hcy is stable for 72 h in heparinized plasma when stored on the gel at 2–8 °C. This stability and ease of handling has considerable practical application for assuring accurate patient results for Hcy assays when there is a time delay between blood collection and testing.

### References


DOI: 10.1373/clinchem.2005.054122

Clinical Chemistry 51, No. 8, 2005 1555
The general principle underlying the AS-PCR technique is to design a mutation-specific primer that produces the preferential amplification of a specific mutant allele (4). We compared the results obtained with this assay with those obtained by direct sequencing in a series of 95 DNA samples extracted from UCC tumors. We also analyzed matching tumors and voided urine from 20 patients. Our results demonstrate the sensitivity, specificity, and reliability of this technique for detecting FGFR3 mutations in both tumors and urine from patients with UCC.

Ninety-five primary tumor samples were collected from patients admitted to the Department of Urology of Hospitals Henri Mondor (Creteil) and CHRU (Besancon) and who had received no previous treatment. Patients gave written informed consent, and we collected matched tumors and blood samples. Tumors from each patient were snap-frozen and stored at −80 °C. DNA from tumor samples was extracted as described previously (5). DNA was extracted from the blood with use of the QIAamp system (Qiagen S.A.).

An additional 20 formalin-fixed, paraffin-embedded tumors were studied. In some of these tumors, the tumor tissue was manually microdissected under a microscope to remove regions of healthy mucosa. DNA was extracted from paraffin-embedded tissues by use of the DNeasy® Tissue reagent set (Qiagen).

Tumors were graded according to the WHO classification of 1973 (6), and stage was determined according to the 1997 TNM classification guidelines (7).

In these 20 cases, samples of voided urine (25–100 mL) collected at the time of diagnosis were tested in parallel with their matching tumor samples. Freshly voided urine samples were stored at 4 °C. The urine samples were centrifuged within 12 h after being voided, and cell pellets were stored at −20 °C until the DNA was extracted (usually within 1 week). DNA was extracted from urinary cells by use of the Qiagen DNA extraction reagent set. The DNA concentration was measured fluorometrically with Picogreen quantification reagents (Biopros; Interchim).

PCR primers were carried out in an Eppendorf thermocycler (Mastercycle; VWR). PCR was performed in a final volume of 25 μL containing 5 ng of genomic DNA, 1× PCR buffer (Perkin-Elmer Taq polymerase buffer), 200 μM each of the deoxynucleoside triphosphate, 2.5 mM MgCl2 for PCR1 or 2 mM MgCl2 for PCR 2, 2.5 μL of dimethyl sulfoxide, 2.5 U of gold Taq polymerase (Perkin-Elmer), and primers at the concentrations indicated in Table 1 of the Data Supplement that accompanies the online version of this Technical Brief at http://www.clinchem.org/content/vol51/issue8/. Cycling conditions were as follows: 95 °C for 5 min followed by 35 cycles of 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 15 s.

We used DNA samples from tumors harboring 4 known FGFR3 mutations (R248C, S249C, G372C, and Y375C) to set up the PCR conditions so that only mutated DNA and not wild-type DNA was amplified in 2 different PCRs: PCR1 for detection of the R248C and G372C mutations, and PCR2 for S249C and Y375C. In both PCRs, a pair of primers amplifying a fragment of the β-globin gene was included as an internal control. For each primer pair, a fluorescent primer was used to label the PCR product. All primers were obtained from Applied Biosystems.

We explored the effect of various conditions on AS-PCR amplification: for each AS-PCR, 3 mutation-specific oligonucleotides differing from each other by the position and type of mismatch were tested at the same concentration in a solution containing 2.5 mM Mg2+. The allele-specific primers that we tested all matched the mutated sequence at their 3′ extremity (i.e., mismatched the wild-type sequence), and contained 1 or 2 additional mismatches vs both the wild-type and mutated sequences. For each mutation, the oligonucleotide that yielded the greatest specificity compatible with efficient PCR was selected. The final PCR conditions to be used were chosen from 3 concentrations of AS oligonucleotide and 3 concentrations of Mg2+ with a fixed annealing temperature. The conditions giving the best amplification of mutated DNA with complete specificity are shown in Table 1 of the online Data Supplement.

PCR products were analyzed on an ABI PRISM 310 capillary DNA sequencer with the Genscan software.

Mutated DNA was diluted in wild-type DNA to determine the sensitivity of each mutation-specific PCR: in every case, 0.2 ng of mutated DNA mixed with 4.8 ng of wild-type DNA (1:25 dilution) yielded a detectable signal (Fig. 1). The ratio of each mutation-specific signal to that corresponding to β-globin was calculated and used as a threshold to determine whether an unknown DNA sample was to be classified as positive or negative (Fig. 1 of the online Data Supplement).

We checked the validity of our assay by use of a series of 95 DNA samples extracted from UCC tumors that had been studied by directly sequencing exons 7, 10, and 15 of the FGFR3 gene. The following mutations were found by sequencing: S249C (23 samples), Y375C (9 samples), R248C (3 samples), K652E (2 samples), K652M (1 sample), G372C (3 sample), S373C (1 sample), and G382R (1 sample). When we used sequencing as the comparison method, AS-PCR had a specificity of 90% and a sensitivity of 88% (Table 1). Five mutations found by sequencing were not looked for by AS-PCR. In 5 cases in which no mutation had been found by sequencing, AS-PCR did detect mutations (1 sample with the R248C mutation, 3 with the S249C mutation, and 1 with the Y375C mutation). It is likely that in these latter cases AS-PCR was able to detect a mutation present only in a small fraction of the cells and that could not therefore be easily be detected by sequencing. This hypothesis is supported by the dilution experiments mentioned above.

Using the same technique (AS-PCR), we tested 20 paired samples from patients with UCC for whom both a tumor sample and DNA extracted from urinary cells at the time of diagnosis were available. Ten pairs of samples had an FGFR3 mutation (1 with R248C, 6 with S249C, 2 with S373C, and 1 with Y375C), and the other 10 pairs had no detectable mutation. Matched tumors and urine from
the same patient gave exactly the same results. Mutations found according to stage and grade of tumors are shown in Table 2 of the online Data Supplement. For the S249C mutation, tumor DNA (1.25 and 0.2 ng, as indicated) was tested for the S249C mutation, tumor DNA (1.25 and 0.2 ng, as indicated) was tested in the presence of wild-type DNA so that the final amount of DNA was 5 ng in each PCR. The arrows indicate the positions of peaks corresponding to the β-globin gene fragment. * indicate the positions of peaks corresponding to the mutated FGFR3 gene fragment. Additional small peaks correspond to size markers.

Fig. 1. Genscan profiles of PCR fragments obtained from a tumor sample harboring the S249C mutation and a wild-type DNA control.

This research was funded by INSERM, Université Paris 12 and Paris 7, Association de la Recherche Contre le Cancer (ARC), Délégation à la recherche Clinique, Assistance Publique Hôpitaux de Paris (AP-HP), Program Hospitalier de Recherche Clinique (AOA 94015 and AOM 03115), and Ligue Nationale Contre Le Cancer. We acknowledge the skillful technical work of Aurélie Brice.

### Table 1. FGFR3 mutations detected by sequencing and AS-PCR.

<table>
<thead>
<tr>
<th>Mutation-positive by sequencing, n</th>
<th>Mutation-negative by sequencing, n</th>
</tr>
</thead>
<tbody>
<tr>
<td>AS-PCR+</td>
<td>38</td>
</tr>
<tr>
<td>AS-PCR−</td>
<td>48</td>
</tr>
</tbody>
</table>

* These mutations correspond to K652E (2 cases), S373C (1 case), G382R (1 case), and K652M (1 case). In 1 patient, 2 mutations (G382R and S249C) were found by sequencing, whereas only the S249C was looked for and found by AS-PCR.

#### References


DOI: 10.1373/clinchem.2005.049619