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

	Mean (SD), μmol/L	
	EDTA plasma	Heparinized plasma
Study I (n = 21)		
Day 1	9.8 (2.6)	11.3 (2.8)
Day 3	9.9 (2.6)	11.4 2.7()
Day 6	8.6 (2.5)	9.6 (2.6)
Day 6 vs day 1, % difference	−12.20	−15.00
Study II (n = 15)		
0 h	9.4 (2.4)	9.8 (2.6)
48 h	9.5 (2.6)	9.9 (2.6)
72 h	8.5 (2.4)	9.7 (2.5)

matic cycling method. The assay was performed on a Hitachi 917 chemistry analyzer.

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 appears to be the contributing factor to the difference and is not recommended.

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 only slightly higher and considered to be 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.

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Sensitive Allele-Specific PCR Assay Able to Detect *FGFR3* Mutations in Tumors and Urine from Patients with Urothelial Cell Carcinoma of the Bladder, *Ashraf A. Bakkar,[1] Viviane Quach,[2] Anaïg Le Borgne,[2] Marianne Toublanc,[3] Dominique Henin,[3] Hervé Wallerand,[5] François Radvanyi,[4] Hugues Bittard,[5] Vincent Ravery,[6] Laurent Boccon Gibod,[6] Sixtina Gil Diez de Medina,[1] Dominique K. Chopin,[1] and Bernard Grandchamp[2*]* ([1] EMI INSERM 03-37 and Service d'Urologie, Université Paris 12, AP-HP, Hôpital Henri Mondor, Créteil, France; [2] Service de Biochimie et Génétique, [3] Service d'Anatomie et Cytologie Pathologiques, and [6] Service d'Urologie, Université Paris 7, AP-HP, IFR02, Hôpital Bichat-Claude Bernard, Paris, France; [4] UMR 144, CNRS Institut Curie, Paris, France; [5] Service d'Urologie CHRU Besançon, France; * address correspondence to this author at: Service de Biochimie et Génétique, Hôpital Bichat, 46 rue Henri Huchard 75018 Paris, France)

Activating somatic point mutations in exons 7, 10, and 15 of the *FGFR3* gene are frequently observed in urothelial cell carcinoma (UCC) *(1, 2)*. These mutations have been found primarily in superficial papillary pTa tumors and were absent in carcinoma in situ *(3)*.

Given the high frequency of *FGFR3* mutations and the possible implication of this receptor in the development of UCC, it was important to develop a simple, fast, and reliable method to identify these mutations in greater detail as a potential tool for the diagnosis and follow-up of UCC patients.

Ten different *FGFR3* mutations have been described in UCC, but 4 of them (R248C, S249C, G372C, and Y375C) account for >95% of cases *(2)*. These mutations therefore represent an excellent target for assays, such as allele-specific PCR (AS-PCR), that depend on the specific detection of point mutations.

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 tumoral 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 (Bioprobes; Interchim).

PCRs 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 $MgCl_2$ for PCR1 or 2 mM $MgCl_2$ 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, 61 °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 Mg^{2+}. 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 Mg^{2+} 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

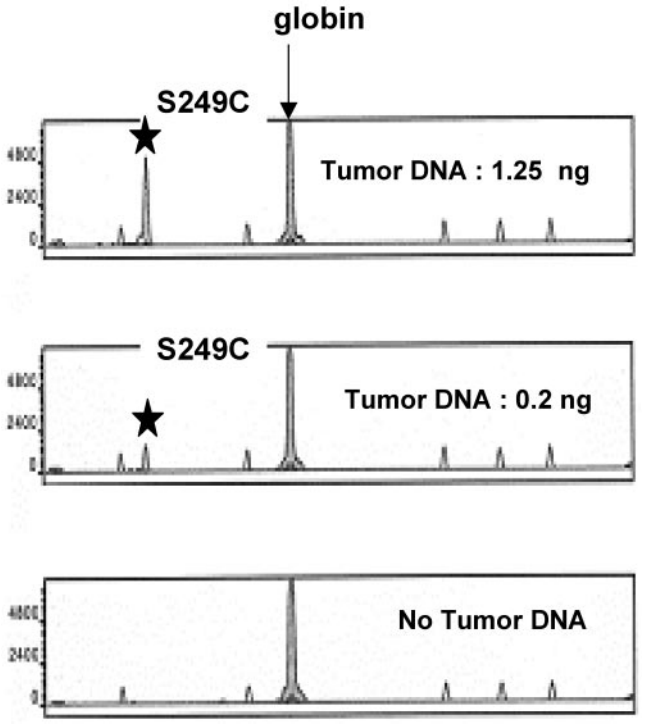

Fig. 1. Genscan profiles of PCR fragments obtained from a tumor sample harboring the S249C mutation and a wild-type DNA control.

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.

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.

The PCR assay we developed permits the accurate detection of the 4 most common (≈95% of mutated cases) *FGFR3* mutations found in UCC. This assay is based on the specific amplification of mutated DNA. In a recent report, van Rhijn et al. *(8)* detected *FGFR3* mutations in only 11 of 21 urine samples from patients whose tumor contained an *FGFR3* mutation. The lower sensitivity of detection reported in that study by single-strand conformation polymorphism analysis of PCR products was thought to result from a dilution effect caused by the presence of healthy cells in the urine. Although our present data need to be confirmed in larger series, these preliminary findings support the idea that *FGFR3* mutations can be detected with high sensitivity in urinary cells when a mutation is present in the matching tumor. Our test seems to be suitable for future routine clinical practice. In particular, the use of a sensitive PCR test instead of repeated cystoscopy is likely to have important potential clinical implications for the follow-up of patients with a *FGFR3* mutation in the initial tumor. However, because our study was carried out on primary tumors rather on recurrent tumors, which are usually smaller, a prospective study of the diagnostic value of this test for the detection of recurrences should be undertaken.

Because *FGFR3* mutations occur at a high frequency in low-grade tumors, our test complements other available tests to provide early, noninvasive detection of bladder cancer. Indeed, these tests, such as morphologic cytology or microsatellite analysis of the urine, are more sensitive for high-grade than for low-grade tumors *(8–10)*.

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

	Mutation-positive by sequencing, n	Mutation-negative by sequencing, n
AS-PCR+	38	5
AS-PCR−	5[a]	48

[a] 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.

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