A novel ultra-sensitive method for the detection of *FGFR3* mutations in urine of bladder cancer patients – Design of the Urodiag® PCR Kit for surveillance of patients with non-muscle-invasive bladder cancer (NMIBC)

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Technical advance

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Abstract

Background We have recently developed a highly accurate urine-based test, named Urodiag®, associating *FGFR3* mutation and DNA methylation assays for recurrence surveillance in patients with low-, intermediate-, and high-risk NMIBC. Previously, the detection of four *FGFR3* mutations (G372C, R248C, S249C and Y375C) required amplification steps and PCR products were analyzed by capillary electrophoresis (Allele Specific-PCR, AS-PCR), which was expensive and time-consuming. Here, we present the development a novel ultra-sensitive multiplex PCR assay as called “Mutated Allele Specific Oligonucleotide-PCR (MASO-PCR)”, generating a cost-effective, simple, fast and clinically applicable assay for the detection of *FGFR3* mutations in voided urine.

Methods Comparative clinical performances of MASO-PCR and AS-PCR technologies were performed from 263 urine DNA samples (87 *FGFR3* mutated and 176 *FGFR3* wild-type). In the development of Urodiag® PCR Kit, we studied the stability and reproducibility of each all-in-one PCR master mix (single reaction mixture including all the necessary PCR components) for MASO-PCR and QM-MSPCR (Quantitative Multiplex Methylation-Specific PCR to co-amplify *SEPTIN9*, *HS3ST2* and *SLIT2* methylated genes) assays.

Results Complete concordance (100%) was observed between the MASO-PCR and AS-PCR results. Each PCR master mix displayed excellent reproducibility and stability after 12 months of storage at -20°C, with intra-assay standard deviations lower than 0.3 Ct and coefficient of variations (CV) lower than 1%. The limit of detection (LoD) of MASO-PCR was 5% mutant detection in a 95% of wild-type background. The limit of quantification (LoQ) of QM-MSPCR was 10 pg of bisulfite-converted DNA.

Conclusions We developed and clinically validated the MASO-PCR assay, generating cost-effective, simple, fast and clinically applicable assay for the detection of *FGFR3* mutations in urine. We also designed the Urodiag® PCR Kit, which includes the MASO-PCR and QM-MSPCR assays. Adapted to routine clinical laboratory (simplicity, accuracy), the kit will be a great help to urologists for recurrence surveillance in patients at low-, intermediate- and high-risk NMIBC. Reducing the number of unnecessary cystoscopies, it will have extremely beneficial effects for patients (painless) and for the healthcare systems (low cost).

Background

Bladder cancer (BCa) is the fifth most common malignancy in the industrialized countries, with 549,393 new cases diagnosed and 199,922 deaths in 2018 [1]. During diagnosis, around 75% to 85% of patients present non-muscle invasive bladder cancer (NMIBC) tumors and more of them are stage Ta (70%), followed by T1 (20%) and Tis (10%) [2]. The remaining cases (20-25%) are detected with muscle invasive bladder cancer (MIBC) tumors (stages T2 to T4). The primary treatment of patients with NMIBC is transurethral resection of bladder tumor (TURBT). However, more than 70% of these patients will develop tumor recurrence within 2 years, and almost 90% will have a recurrence of their disease within 15 years

[2]. The high rate of intravesical recurrences and disease progression require follow-up of treated NMIBC patients with cystoscopy and urine cytology at regular intervals (every 3–6 months during the next several years), making it the most expensive of all cancers [3]. Cystoscopy is an uncomfortable and costly invasive procedure. Cytology is known to have a low sensitivity for detecting low-risk (low-grade) disease [4]. In this context, there is a clear clinical need to find reliable markers to monitor the recurrence in NMIBC. Genetic and epigenetic alterations contribute to the development of human cancer and then can be used as biomarkers for cancer detection, including BCa [5]. Among these candidates, the fibroblast growth factor receptor 3 gene (*FGFR3*) appears to be the most frequently mutated in BCa. Ten different *FGFR3* mutations have been described in this disease [6, 7], but four of them (G372C, R248C, S249C, and Y375C) account for > 95% cases [8]. *FGFR3* has been proposed as a urine prognostic marker for the early diagnosis and detection of recurrences in patients with low-risk NMIBC [9-11]. Aberrant DNA methylation has been shown to play an important role in cancer [12, 13]. DNA hypermethylation of the CpG sites located in the promoter regions and/or exon 1 of tumor-suppressor genes are observed in many human cancers as bladder tumors [14-16]. Prior works have shown that the detection of *FGFR3* mutations added with methylation analysis could be a promising method for the diagnosis and surveillance of NMIBC patients [17]. In a previous work, we have shown that an integrated genetic/epigenetic analysis could lead to the development of highly accurate urine-based test for the surveillance of patients with NMIBC [18]. Here, we developed and clinically validated the MASO-PCR assay, generating a cost-effective, simple, fast and clinically applicable assay for the detection of *FGFR3* mutations in urine. We designed Urodiag® PCR Kit, a new urine-based lab test, for recurrence surveillance in patients at low-, intermediate and high-risk NMIBC.

Methods

Urine collection and capture of exfoliated bladder cells with a membrane filter

Urine samples (100 ml) were collected (n= 26) from the first miction in the morning into a clean sterile container (120 ml, Thermo Scientific Samco). Collection was carried out from healthy volunteers with no known urological diseases. Urine samples were pooled and stored at 4 ° C for up to 72 hours prior analysis. Each donor gave consent before study participation.

100 ml of each pooled urine sample were poured using a 50 ml syringe and passed through a single-use filter cartridge (Filter), presenting a nylon disc filter of 11 µm porosity and 25mm diameter. The Filter was rinsed with 5 ml of 1X PBS (pH 7.4) before being disconnected from the syringe. To avoid saturation, urine sample was passed through the filter under gentle positive pressure.

Urine DNA extraction

Urine DNA isolation has been carried out directly from bladder cells captured on Filter using the QIAamp DNA Blood Mini Kit (Qiagen). If the Filter has been stored at -20°C, it has to be left 5-10 min at room temperature before DNA extraction. 442 µl of lysis buffer (220 µl 1X PBS, 22 µl Proteinase K and 220 µl

AL buffer) were added on the filter. Bladder cells have been homogenized by passing 3 times through a syringe equipped with a 21 gauge needle (Terumo) to shear genomic DNA. The lysate was collected into a 2 ml tube and incubated at 56°C for 15 minutes and then centrifuge briefly to reduce foam. Subsequent processing was done according to the DNA purification protocol. All centrifugation steps were carried out in a benchtop microcentrifuge (14,000 RPM) at room temperature. DNA was eluted from the column into a clean 1.5 ml tube by adding 50 ml of AE buffer to the column, then incubated at room temperature for 5 minutes, and centrifuged for 1 minute. DNA concentration was determined with Qubit 4 fluorometer (Invitrogen) using the highly sensitive Qubit quantification assay. All genomic DNA samples were diluted or concentrated to obtain a final concentration of 1.25 ng/µl. We determined the optimal conditions for storage and shipping of the filters before DNA extraction. All samples were examined for DNA (5 ng) integrity via PCR amplification of the *GLOBIN* gene.

Reproducibility and stability study

We studied the reproducibility of the urine filtration (17 urine samples belonging to Pool 1 to 4). We also studied the stability of filters, recovered after filtration (9 filters belonging to Pool 5 to 7), according to temperature and storage time. This study requires the following steps: DNA extraction from filter, DNA quantification and PCR amplification of the *GLOBIN* gene.

Bisulfite DNA modification

30 ng of universal methylated human DNA standard (Zymo Research) were modified by sodium bisulfite using the EZ DNA Methylation kit (Zymo Research) according to the manufacturer's instructions. The PCR tubes (0.2 ml) were placed in a thermal cycler and we performed the followings steps: 37°C for 15 min followed by 50°C for 15h30 (overnight). For DNA purification, all centrifugation steps were carried out in a benchtop microcentrifuge (14,000 RPM) at room temperature. Bisulfite modified DNA was eluted from the column into a clean 1.5 ml tube by adding 10 ml buffer M-Elution buffer to the column, then incubated at room temperature for 5 minutes, and centrifuged for 1 minute.

Multiplex Real-time PCR

All PCR reactions were performed on the real-time PCR instrument StepOnePlus™ (Thermo Fischer Scientific) with a final volume of 20 µl. The PCR cycling parameters were: initial denaturation at 95°C for 5 min followed by 40 cycles of 15s at 95°C, 45 s at 60°C. The fluorescence data was acquired at the end of each cycle.

FGFR3 mutation analysis using Mutated Allele Specific Oligonucleotide-PCR (MASO-PCR)

The MASO-PCR technology is presented in Figure 2a. Our technology was performed to simultaneously detect four mutations of the *FGFR3* gene (*FGFR3*mut) with 6Fam-S249C and Vic-Y375C (MASO-PCR1) and 6Fam-R248C and Vic-G372C (MASO-PCR2). PCR was conducted using 5 ng of DNA template (1.25 ng/µl), 1X Quantifast Multiplex PCR (Qiagen), 500 nM of primers (Eurogentec) and 200 nM of probe

(Thermo Fischer Scientific) in a final volume of 20 µl (16 µl reaction mix and 4 µl of DNA). The DNA integrity has been checked by amplification of the Ned-*GLOBIN* gene included as an internal control. The primers and probe sequences are listed in Table 1a.

Methylation analysis using Quantitative Multiplex Methylation Specific-PCR (QM-MSPCR)

We used the QM-MSPCR, a highly sensitive and specific PCR developed previously by our team [18]. The QM-MSP technology is presented in Figure 2b. We performed two QM-MSPCR for co-amplification of 6Fam-*SEPTIN9* with Vic-*ALBUMIN* (QM-MSPCR1) and 6Fam-*HS3ST2* with Vic-*SLIT2* (QM-MSPCR2), respectively. QM-MSPCR reactions were performed with 4 µl of bisulfite-converted positive control DNA (100% methylated) and 16 µl of PCR mix containing 1x KAPA PROBE FAST qPCR Master Mix ABI Prism (KAPA Biosystems), 400 nM primers (Eurogentec) and 250 nM TaqMan-MGB probes (Thermo Fischer Scientific). *ALBUMIN* sequence has been designed without CpG site and used for normalizing the DNA amounts. All primers are presented in Table 1b.

Detection of FGFR3 mutations using MASO-PCR in patients with NMIBC

We developed a sensitive Mutated Allele Specific Oligonucleotide–PCR (MASO-PCR) assay to detect *FGFR3* mutations using multiplex real-time PCR. We selected 263 urine DNA samples, including 176 *FGFR3* wild-type (wt) and 87 *FGFR3*-mutated (mut) previously validated by AS-PCR, from NMIBC patients (AUVES cohort, project reference RECF0998-PHRC 2003) [11]. For initial diagnosis the distribution of patients (n= 57) among low/intermediate and, high-risk NMIBC was 51%/23% and 26%. For follow-up the distribution of patients (n= 30) among low/intermediate and, high-risk NMIBC was 56%/17% and 27%.

The distribution of *FGFR3* mutations was:

For initial diagnosis (n= 107): *FGFR3*mut (n= 57): S249C (n=31), Y375C (n=14), R248C (n=7), G372C (n=3) and R248C/S249C (n=2), and *FGFR3*wt (n=50).

For follow-up (n= 156): *FGFR3*mut (n= 30): S249C (n=20), Y375C (n=4), R248C (n=3), G372C (n=2) and R248C/S249C (n=1), and *FGFR3*wt (n= 126).

MASO-PCR: *FGFR3* positive control, primer specificity, and determination of limit of detection (LoD)

Construction of the control plasmids containing *FGFR3* mutations

Positive control plasmids were designed to incorporate the region of the *FGFR3* mutation. The synthetic mutated *FGFR3* sequences (FGFR3mut) were synthesized and inserted into pMA-T vector using GeneArt (ThermoFisher Scientific). Each positive control plasmid was confirmed by sequencing before use.

- *FGFR3*mut plasmid n°1 (2571 bp): Plasmid pMAT (2374 bp) + *FGFR3* sequence with S249C and Y375C mutations (197 bp) (Additional file 1: Figure S1a)

- *FGFR3*mut plasmid n°2 (2560 bp): Plasmid pMA-T (2374 bp) + *FGFR3* Sequence with R248C and G372C mutations (186 bp) (Additional file 1: Figure S1b)

Primer pair specificity

We used the same *FGFR3* primer pairs (Table 1a) to amplify *FGFR3* mutations with the Fast SYBR Green PCR master mix (SG-PCR, ThermoFisher Scientific). PCR reactions were performed in duplicate onto two separated runs. In each 20 µL reaction, G372C, R248C, S249C, and Y375C were amplified with a 1X SG (10 µl), 200 nM of primers and *FGFR3*mut plasmid (4µl) corresponding in a final volume of 20 µl. The thermal cycling conditions included an initial denaturation at 95°C for 3 min followed by 40 cycles: 95°C for 3 s and 60°C for 20 sec. At the end of PCR reactions, a melting curve analysis was carried out to check the specificity of the primers. Each melting curve was determined by heating the PCR product from 70°C to 95°C and monitoring the fluorescence at a transition rate of +0.3°C. The melting temperature (Tm) was calculated using the StepOnePlus software (Life Technologies) and also estimated by Howley's formula: [67.5 + (0.41* %G-C) - (395/length of amplicon)].

LoD for analysis of *FGFR3* mutations

The diploid human genome comprises about 6.10^9 base pairs (bp). Plasmids (2.50 ng/µl) were diluted at 2.10^6 (6.10^9 divided by $2.6.10^3$) in the standard human DNA (*FGFR3*wt, 2.50 ng/µl), leading to a 1:1 ratio (*FGFR3*mut/*FGFR3*wt) and dilutions were used as *FGFR3* positive controls. To determine the LoD, a serial dilution series of the each *FGFR3* positive control (see above) was produced at 50%, 10%, 5%, and 1% with *FGFR*3wt (1.25 ng/µl). 4 µl of each dilution (5 ng) were amplified by MASO-PCR and Cts were analysed by applying a predefined threshold (DRn) at 0.15 for *GLOBIN*, S249C, Y375C, G372C and 0.24 for R248C, with Cts values above the threshold as positive and below the threshold as negative. All dilutions were amplified and then analyzed in duplicate on the same plate to PCR in two independent runs.

QM-MSP: Positive control and determination of the limit of quantification (LoQ)

The LoQ of each gene was determined on each duplex QM-MSP1 and QM-MSP2 by performing a dilution range with 10, 1, 0.1 and 0.01 ng of bisulfite-converted positive control DNA (100% methylated). The Cts were analysed using a threshold value (DRn) of 0.10 for determining the limit of DNA quantity and amplification efficiency (E). Each dilution was done in duplicate on the same PCR plate in two independent runs.

Stability and reproducibility study of all-in-one PCR master mixes

The all-in-one PCR master mixes were prepared in a single reaction mixture including all the necessary components (PCR, primers and TaqMan-mgb probes) for mutation and methylation assays using MASO-PCR and QM-MSPCR technology, respectively. We studied the stability of each all-in-one PCR master mix

(MASO-PCR1,2 and QM-MSPCR1,2) from aliquots that were run in triplicate and stored at -20°C for 0, 1, 2, 3, 4, 6, 9 and 12 months.

Results

Reproducible and efficient DNA extraction from bladder cells captured on a membrane filter

To assess if filtration allows capturing the fraction of bladder cells in the sample, DNA was isolated and amplified using real-time PCR. Urine filtration was reproducibly obtained for 17 pooled urine samples belonging to Pool 1 to 4. In Figure 1, we have reported the amounts of DNA (mean±standard deviation) recovered from each filter (F) with 132±17 ng (Pool 1, n = 3), 128±13 ng (Pool 2, n= 3), 187±15 ng (Pool 3, n= 7) and 185±21 ng (Pool 4, n= 4). The integrity of each extracted urinary DNA (10 ng) was confirmed by amplification of the *GLOBIN* gene.

Effects of filter storage conditions

Concentration and recovery rate of the genomic DNA (Pools 5 to 7) according to filter storage conditions were summarized in Table 2. Filters of groups B (5 days at room temperature, RT) and C (5 days at -20°C) were compared to filters belonging to group A (0 day of storage). No significant differences were found among A and C, but there is a significant difference between A and B. Indeed, the DNA yields of groups A, B and C were 100%, 54±13% (mean±standard deviation) and 112±23%, respectively. We have successfully verified the integrity of each isolated DNA by amplifying a segment of the *GLOBIN* gene. The amount of DNA obtained under all these conditions was greater than 40 ng, corresponding to the amount required to perform the test. In this study, we showed that the filter could be stored for 5 days at RT and, if a long-term storage is required until DNA extraction, optimal conditions are obtained at -20°C.

Validation of primer pairs for the detection of *FGFR3* mutations by MASO-PCR

The melting curves for G372C, R248C, S249C, and Y375C mutations by SG-PCR are represented in additional file (Figure S2). The temperature of melting (Tm) for each amplicon was 83.81±0.01°C for G372C, 86.80±0.02°C for R248C, 87.54±0.01°C for S249C and 84.85±0.02°C for Y375C. By applying, the Howley's formula, we obtained equivalent Tm as compared with those given by the melting curves, with 83.87°C for G372C (63.7% G-C, 72 bases), 86.85°C for R248C (71.8 % G-C, 78 bases), 87.57°C for S249C (73.2% G-C, 82 bases) and 84.77°C for Y375C (65.5% G-C, 79 bases), respectively. These two methods allowed us to validate the specificity of each primer pair.

Sensitive detection of *FGFR3* mutations by MASO-PCR

The limit of detection (LoD) of the Mutation assay with 5 ng was set at 5% of mutant sequences in a background of 95% normal DNA (Figure 3a-b). This means that in the presence of a DNA sample containing less than 5% mutant (~15 copies), the MASO-PCR would be unable to detect the 4 mutations of the *FGFR3* gene (G372C, R248C, S249C, and Y375C). The positive reactions (amplification curves)

were carried out in duplicate onto two separate runs with a very good reproducibility. Cts were obtained with cut-off values (DRn) of 0.15 for *GLOBIN*, G372C, S249C, Y375C and 0.24 for R248C (Figure 3c-d).

MASO-PCR Accurately predicts recurrence of patients with NMIBC

By applying these threshold values, we clinically validated the MASO-PCR technology from 263 urine DNA samples (87 *FGFR3* mutated and 176 *FGFR3* wild-type). A complete concordance (100%) was observed between the MASO-PCR as compared with AS-PCR results. Sensitivity was defined as the ability of the MASO-PCR assay to detect *FGFR3* mutations (+) and specificity as the ability of assay to identify the absence of *FGFR3* mutations (-) in primary NMIBC tumor (diagnosis) as well as recurrence (follow-up). We successfully demonstrated the capacity of the MASO-PCR assay for detecting at least 15 copies of *FGFR3* mutant alleles in 5 ng of wild type DNA with a sensitivity and specificity of 100% in urine of patients with low-, intermediate- and high-risk. All data are shown in Table 3.

Sensitive quantification of DNA methylation by QM-MSPCR

QM-MSPCR was used to amplify *ALBUMIN*/ *SEPTIN9* (QM-MSPCR1) and *HS3ST2*/ *SLIT2* (QM-MSPCR2) duplexes with titration of bisulfite-converted positive control DNA (100% methylated) at various concentrations (10, 1, 0.1, 0.01 ng/well). At each dilution, the cycle threshold (Ct) was determined with bisulfite-converted positive control DNA (100% methylated). The Cts were analysed by using threshold value (DRn) of 0.10. Both calibration curves gave a slope of about -3.32, which corresponds to PCR efficiency (E) close to 100%. More precisely, the slope values were -3.31, -3.34, -3.29, and -3.30 for *ALBUMIN* (E= 100.5%), *SEPTIN9* (E= 99.2%), *HS3ST2* (E= 101.4%). and *SLIT2* (E= 100.8%), respectively. These results reflect very high amplification efficiency. We determined that the limit of quantification (LoQ) of each target gene could be detected with 10 pg of DNA. In addition, the high value of the correlation coefficient (greater than 0.99) indicates that an almost perfect linearity is obtained over the entire range. Data are represented in Figure 4a for QM-MSPCR1 and Figure 4b for QM-MSPCR2.

High stability and reproducibility of "all-in-One" PCR master mixes

The all-in-one PCR master mixes allow using solutions containing all the necessary reagents for PCR amplification of DNA. In table 3, Ct values of each target gene are indicated in function of storage time of the MASO-PCR and QM-MSPCR solutions. We have successfully verified the reproducibility and stability of each all-in-one solution after 12 months of storage at -20°C, showing intra-assay standard deviations lower than 0.3 Ct and coefficient of variations (CV) lower than 1% (Table 4).

Design of Urodiag® PCR Kit

The Urodiag® PCR Kit is an in vitro diagnostic test intended for the qualitative detection of *FGFR3* somatic mutations (G372C, R248C, S249C, Y375C) and the quantification of three DNA methylation markers (*HS3ST2*, *SEPTIN9*, *SLIT2*) by stable multiplex PCR in urine of NMIBC patients. The PCR kit is composed of 8 tubes (4 for the Mutation assay, 3 for the Methylation assay and 1 tube with sterile water)

(Table 5). Each tube contains all the components (PCR mastermix, primers and probes) necessary to carry out Mutation and Methylation assays.

Discussion

Due to the high recurrence rate of bladder cancer, NMIBC tumors require regular surveillance: cystoscopy combined with urine cytology remains the reference examination, making it the most expensive of all cancers [19]. Cystoscopy depends on operator, is costly, uncomfortable, and carries a risk of infection and urethral strictures. Cytology has a good sensitivity for detecting high-risk NMIBC, but for detection of low-risk NMIBC, its sensitivity falls between 4 and 31% [20]. As the bladder is the only storage organ for urine, urine provides a convenient source for detection of exfoliated bladder tumor cells. Previous works showed that the exfoliation of tumor cells into the urine depends on tumor characteristics such as size, stage, grade, and that the filtration of urine samples, as compared to the centrifugation step, increased the diagnostic accuracy of BCa [21, 22]. In hematuria, this observation could be due to the removal of contaminating leukocytes that are smaller size (5-10 µm) than bladder cells (>15µm), and this size difference could be exploited to enrich samples for tumor cells. In order to optimize the accuracy of our test, the filtration of urine samples was carried out by a disposable syringe filter device. A variety of commercially available urinary molecular markers have been introduced for initial diagnosis and detection of tumor recurrence for the follow-up of NMIBC patients. These non-invasive tests include the measurement of markers such as MCM5 protein (ADXBLADDER test, sensitivity/specificity of 76%/69%, $52 per test), 15 DNA methylation patterns (Bladder Epicheck test, overall sensitivity of 67% with 40%/89% in low-/high-risk and specificity 88%, not yet marketed), bladder tumor associated antigen (BTA, sensitivity/specificity of 58%–71%/73%, $40 per test), proteins detected on fixed urothelial cells (ImmunoCyt, sensitivity/specificity of 67%–86%/75%–79%, $200 per test), nuclear matrix protein 22 (NMP22, sensitivity/specificity of 71%–73%/73%–80%, $25 per test), chromosomal aberrations detected by fluorescence in situ hybridization (UroVysion, sensitivity/ specificity of 72%/83%, $800 per test), gene expression levels of five targets (*ABL1*, *CRH*, *IGF2*, *UPK1B*, *ANXA10*) by RT-PCR (Xpert BC Monitor, overall sensitivity 46% with 40%/86% in low-/high-risk and specificity 90%, $165 per test) [23-30]. However, due to their limited specificities or sensitivities, the markers proposed to date have not been widely adopted in daily clinical practice. *FGFR3* is a receptor tyrosine kinase that is somatically altered in a number of malignancies including BCa. Prior studies have reported that activating mutations of *FGFR3* are particularly common in NMIBC (~80%) versus MIBC (~20%) [31,32]. In NMIBC, the four most common mutations are found in exons 7 and 10 with S249C (60%) and R248C (10%) in exon 7, and Y375C (20%) and G372C (5%) in exon 10 [33,34]. Zuiverloon and colleagues described that these four mutations can be detected in urine and used to develop a non-invasive test for the diagnosis and monitoring of patients with NMIBC [34]. In addition, it has been shown that high-risk tumors have generally more hypermethylated genes than low-risk groups [35]. Consistently with all these observations, we have identified and clinically validated a combination of genetic and epigenetic urinary markers (mutations of *FGFR3* added to hypermethylation of *HS3ST2* (heparin sulfate sulfotransferase), *SEPTIN9* and *SLIT2*) which, due to their strong complementarity, afforded the best diagnostic accuracy for the surveillance in

NMIBC patients presenting low-, intermediate- and high-risk of recurrence. In comparison with the tests above mentioned, our combined test gives the best clinical performances: sensitivity/specificity/NPV respectively equal to or greater than 95%/76%/99% [18]. During the present study, we designed the Urodiag® Kit so that it contains all the necessary components to use our test in clinical routine. To increase the diagnostic accuracy of BCa, we showed the feasibility of enriching the exfoliated bladder cells with a unique syringe filter to replace traditional centrifugation. We showed that this device was able to isolate DNA with reproducibility, high purity and sufficient quantity for subsequent MASO-PCR and QM-MSPCR amplification. We have developed and clinically validated the MASO-PCR to detect *FGFR3* gene mutations with outstanding accuracy with 100% sensitivity/specificity, equivalent to the results that can be obtained using capillary electrophoresis for DNA analysis (AS-PCR). Consequently, the mutation and methylation assays can be carried out on the same real time quantitative PCR machine, facilitating the implementation of the Urodiag® Kit in laboratories. To simplify the PCR workflow, we prepared the all in one master mixes, solutions containing all the necessary reagents for MASO-PCR and QM-MSPCR PCR, with two main advantages: reduction of pipetting errors and time saving.

Conclusions

We showed that the Mutation assay (MASO-PCR) and Methylation assay (QM-MSPCR) could be simultaneously performed on the same real time quantitative PCR machine, facilitating the implementation of the Urodiag® PCR Kit in laboratories. It has been designed as a urine-based laboratory test that provides a simple, fast, reliable and low-cost (~ $100 per test) method for diagnosis and individualized surveillance for patients with low-, intermediate- and high-risk NMIBC. Leading to a significantly reduction of repetitive cystoscopies, it presents major benefits for the quality of life of the patients during their follow-up, the work of the urologists and in terms of cost reduction for health care systems.

Abbreviations

BCa: Bladder cancer; Ct: Cycle threshold, CV: Coefficient of variation, MASO-PCR: Mutated Allele Specific Oligonucleotide-PCR; LoD: Limit of detection; LoQ: Limit of quantification; NMIBC: Non-muscle invasive bladder cancer; NPV: negative predictive value; PBS: Phosphate buffered saline; QM-MSPCR: Quantitative Multiplex-Methylation Specific PCR; RT: Room temperature; TURBT: Transurethral resection of bladder tumor

Declarations

Ethics approval and consent to participate

Free and written consent was obtained from all individuals participant of this study. Approval was obtained by the Paris Bichat-Claude Bernard hospital ethics committee (approval number: 2004/15).

Consent for publication

Not applicable.

Availability of data and materials

The authors declare that [the/all other] data supporting the findings of this study are available within the article [and its supplementary information files].

Competing interests

JPR and CH are founding members of OncoDiag SAS. JPR is the R&D head and CH is the CEO.

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Authors' contributions

JPR performed the study design and the development of methodology. JPR collected data. CH and JPR *were responsible* for data interpretation and *manuscript* preparation. CH and JPR were responsible for the revision of the manuscript. CH and JPR reviewed, read and approved the final version of the manuscript.

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Additional File Information

Additional file 1: Figure S1. Construction of mutated *FGFR3* plasmids

Additional file 2: Figure S2. Melting curve PCR analysis for the *FGFR3* mutations

Tables

Please see the supplementary files section to view the tables.

Table 1 Primers sequences for MASO-PCR and QM-MSPCR

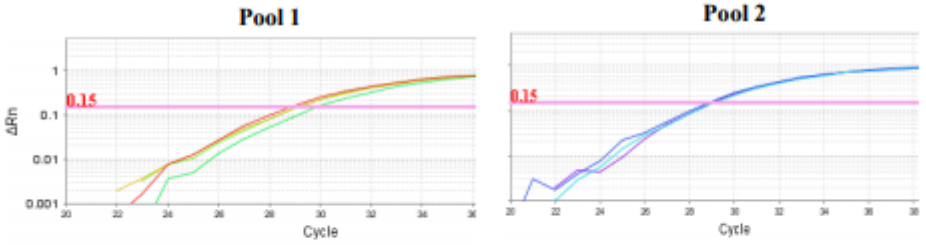
Table 2 Relationship between filter storage conditions, concentration and amount of urine DNA

Table 3 Ultra-sensitive MASO-PCR method for surveillance of NMIBC patients

Table 4 High performance of PCR Master mix all-in-one

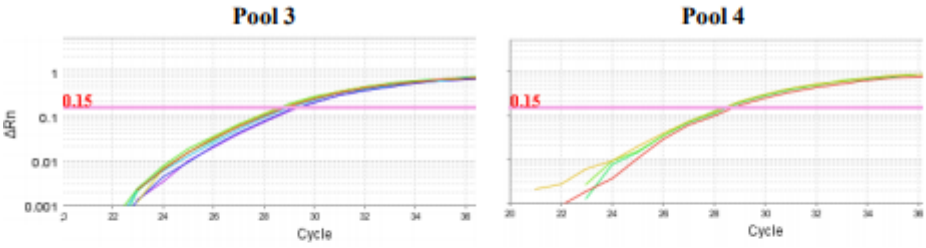
Table 5 Components of the Urodiag® PCR Kit

Figures



Filter (F) (n= 3)	Conc., ng/µl	Amount, ng
F1	3.30	152
F2	2.66	122
F3	2.68	123

Filter (F) (n= 3)	Conc., ng/µl	Amount, ng
F1	2.56	118
F2	3.09	142
F3	2.63	121



Filter (F) (n= 7)	Conc., ng/µl	Amount, ng
F1	3.98	183
F2	4.39	202
F3	4.42	203
F4	4.20	193
F5	3.56	164
F6	3.89	179
F7	3.76	173

Filter (F) (n= 3)	Conc., ng/µl	Amount, ng
F1	3.42	157
F2	4.39	202
F3	4.38	201
F4	3.93	181

Figure 1

DNA integrity assessed by PCR amplification of Globin gene DNA concentrations were determined by fluorometry. The GLOBIN gene was amplified with an amount of urine DNA comprised between 10 and 18 ng (4 µl of DNA sample) from each Filter (F). Amplification curves are shown from Pool 1 to Pool 4, respectively.

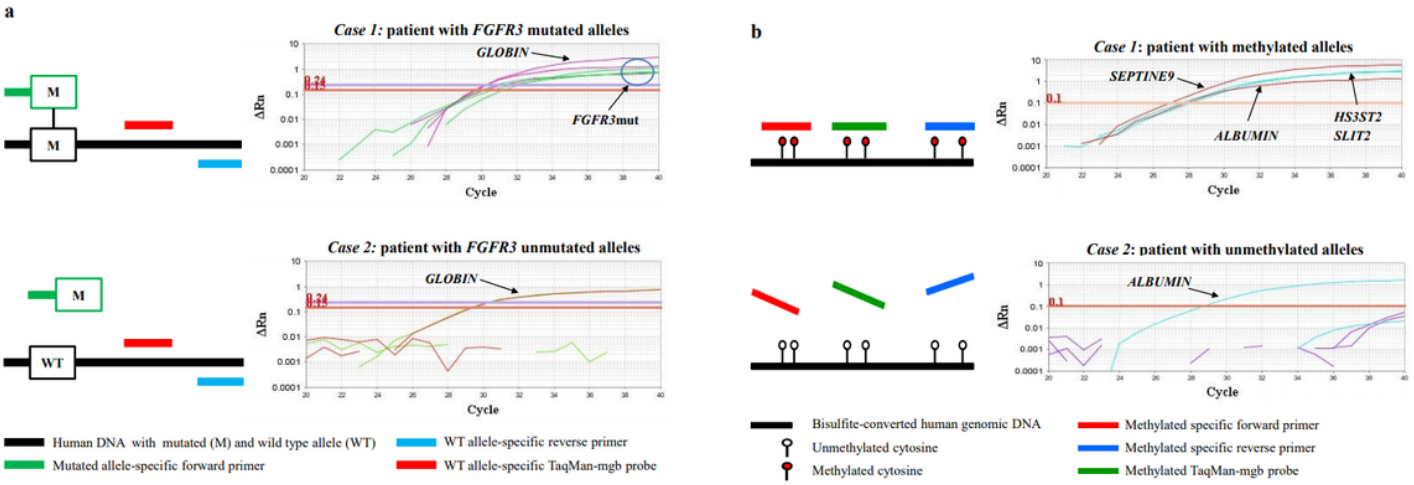
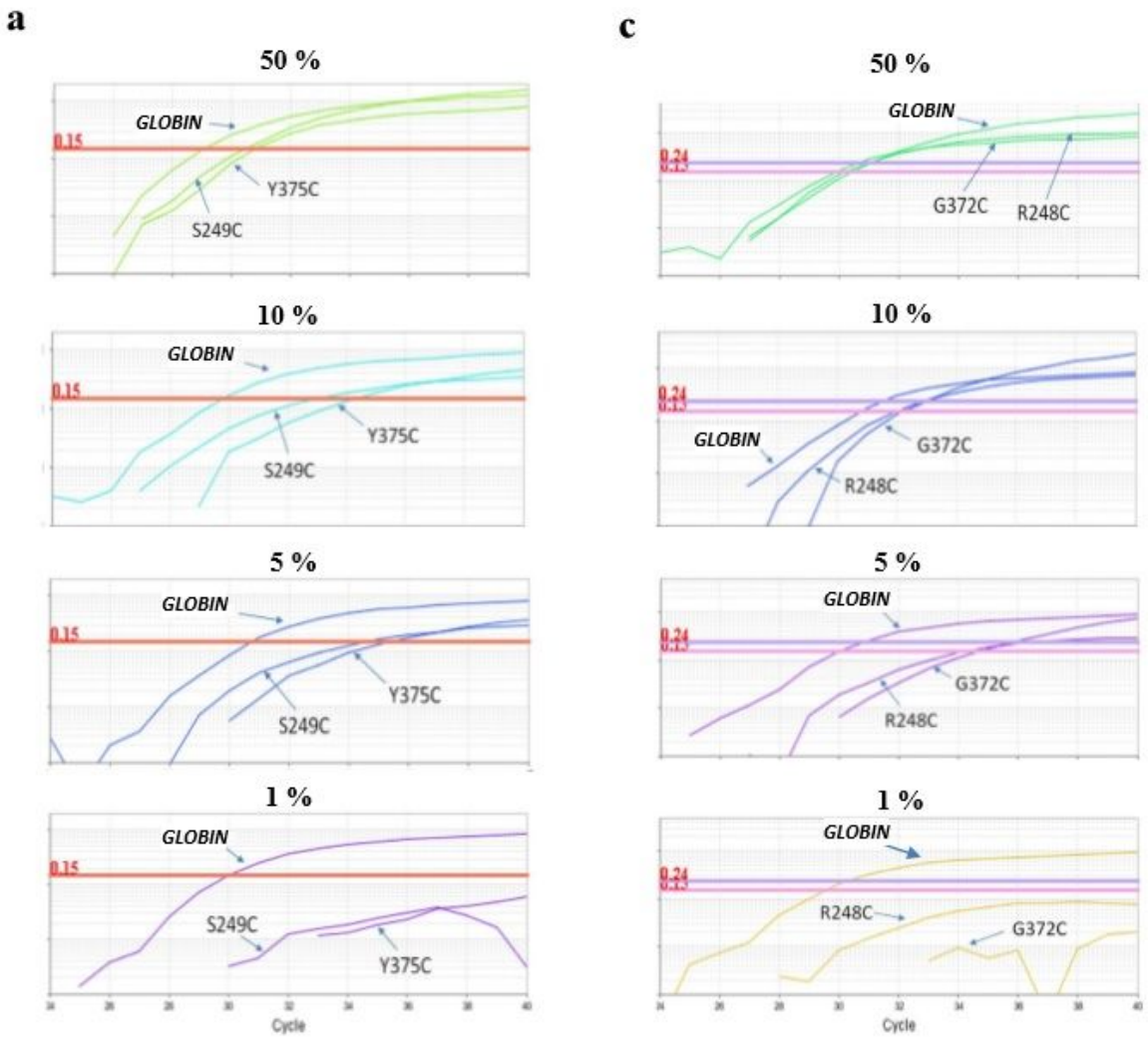


Figure 2

Design of Mutation and Methylation PCR assays



b

	Mean Ct±SD of replicates	
	S249C	Y375C
50%	30.35 ± 0.12	30.72 ± 0.16
10%	33.05 ± 0.12	34.14 ± 0.24
5%	34.49 ± 0.11	35.08 ± 0.08
1%	ND	ND

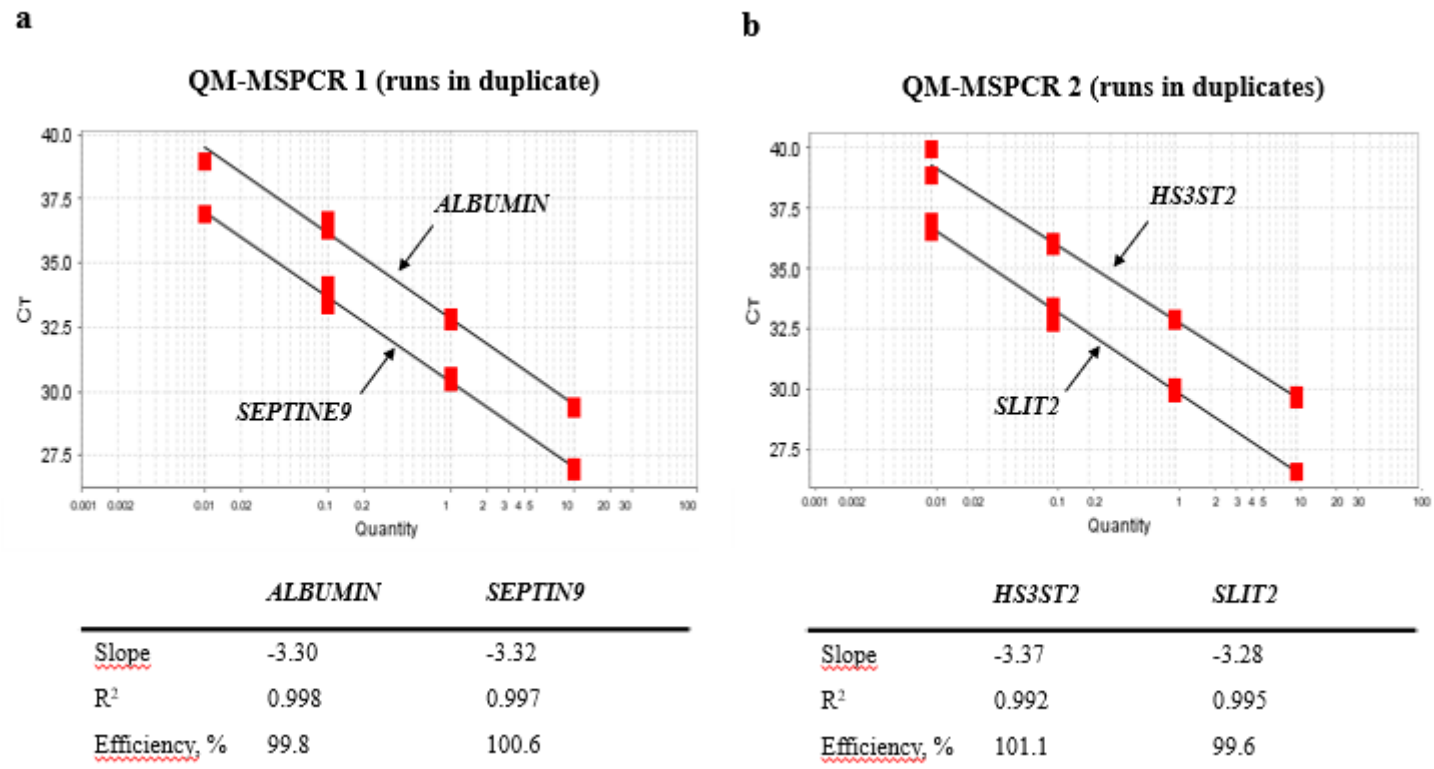
d

	Mean Ct±SD of replicates	
	G372C	R248C
50%	30.05 ± 0.09	30.79 ± 0.11
10%	32.47 ± 0.21	33.44 ± 0.25
5%	34.02 ± 0.09	35.22 ± 0.15
1%	ND	ND

Results are shown as mean Ct obtained from duplicates in two independent runs
Ct: Cycle threshold; SD: Standard deviation; ND: not detectable

Figure 3

LoD for the Mutation assay DNA from FGFR3 mutant plasmids was diluted into the wild-type DNA (standard human DNA). The proportion of mutant DNA was 50%, 10%, 5%, and 1%, respectively. The representative amplification curves (a, c) and mean Ct values (b, d) are shown in the detection of the FGFR3 S249C/Y375C (a, b) and R248C/G372C (c, d) mutations by MASO-PCR.



	ALBUMIN	*SEPTIN9*
Slope	-3.30	-3.32
R^2	0.998	0.997
Efficiency, %	99.8	100.6

	HS3ST2	*SLIT2*
Slope	-3.37	-3.28
R^2	0.992	0.995
Efficiency, %	101.1	99.6

Figure 4

LoQ for the Methylation assay

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Table3.pdf
- Table5.pdf
- Additionalfile2FigureS2.ppt
- Table4.pdf
- Additionalfile1FigureS1.ppt
- Table2.ppt
- Table1.pdf