

# Noninvasive multianalyte diagnostic assay for monitoring bladder cancer recurrence

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**Background:** The purpose of this study was to establish the clinical performance of a urine-based assay, called a multianalyte diagnostic readout, in monitoring for bladder cancer recurrence.

**Methods:** This was a prospective, multicenter, single assessment observational study. The multianalyte diagnostic readout uses a combination of one protein and three DNA biomarkers. Urine samples from 733 patients undergoing monitoring for bladder cancer recurrence were analyzed for matrix metalloproteinase-2 levels, the presence of mutant *FGFR3* DNA, and hypermethylation of the *NID2* and *VIM* genes. The probability of a patient having (positive predictive value) or not having (negative predictive value) recurrent bladder cancer was determined by *FGFR3* alone or all four biomarkers combined, respectively.

**Results:** Cystoscopy/biopsy diagnosed 63 patients with bladder cancer recurrence at the time of study assessment. The four-biomarker assay identified 237 patients as having a low probability of disease recurrence, 231 of whom were determined by cystoscopy as not having recurrent cancer, resulting in a negative predictive value of 97.5% at 90.5% sensitivity. The *FGFR3* assay identified 49 patients with *FGFR3* mutations, 19 of whom were confirmed by biopsy as having cancer, resulting in a positive predictive value of 38.8%, with 95.5% specificity.

**Conclusion:** The urine-based multianalyte diagnostic readout assay was able to delineate the patient population into those highly likely to have bladder cancer recurrence, those unlikely to have recurrent disease, and those with an average risk for bladder cancer recurrence.

**Keywords:** *FGFR3*, matrix metalloproteinase-2, *NID2*, *VIM*, recurrent bladder cancer

## Introduction

Bladder cancer is the seventh leading cause of death in the US, and the disease was estimated in 2008 to cause about 150,000 deaths worldwide.<sup>1</sup> The majority of bladder cancer patients present with disease that does not invade muscle, with an estimated recurrence rate in this group of patients being >50%.<sup>2</sup> About 10%–30% of this patient population will have recurrent disease which progresses to muscle invasion within 5 years.<sup>3,4</sup>

Due to the risk of recurrence and progression, established guidelines recommend that patients with nonmuscle-invasive bladder cancer should be monitored after initial diagnosis and treatment.<sup>5,6</sup> The American Urological Association and National Comprehensive Cancer Network recommend surveillance every 3–6 months for 3 years and at least yearly thereafter.<sup>7</sup> Surveillance for patients who have been treated for nonmuscle-invasive bladder cancer may include urine cytology, imaging, cystoscopy, and biopsy.<sup>6</sup>

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Currently, two major assays for detecting bladder cancer are based on cystoscopy and biopsies. Cystoscopy has been considered the “gold standard” surveillance diagnostic modality for nonmuscle-invasive bladder cancer, and requires a degree of invasiveness, as well as both clinician-related and patient-related time commitment and expense.<sup>8</sup> Moreover, iatrogenic injury to the urethra and bladder as well as risk of infection may ensue.<sup>8</sup> Currently approved biomarkers for detecting bladder cancer rely on a single quantitative cutoff, above which a patient is defined as having cancer and below which a patient is defined as cancer-free. Single cutoff assays attempt to maximize sensitivity and specificity simultaneously; neither value is optimal and may result in many false-positive or false-negative results,<sup>9</sup> thereby falling short of providing clinically relevant information.<sup>10</sup> For example, for a biomarker assay with 80% sensitivity and 80% specificity, 20% of the positive samples will not have cancer and likewise 20% of negative samples will have cancer, creating ambiguity in the results.

A urine-based multianalyte diagnostic readout assay has been developed that combines the performance of one protein (matrix metalloproteinase-2, MMP-2) and three DNA (Nidogen 2 [*NID2*], Vimentin [*VIM*], and fibroblast growth factor receptor 3 [*FGFR3*] gene) markers for identifying patients with a high likelihood (positive predictive value, PPV) or low likelihood (negative predictive value, NPV) of having recurrent bladder cancer. The multianalyte diagnostic readout assay uses two cutoffs, one to achieve high NPV and another to achieve high PPV. The combined biomarkers were used to achieve a high NPV, and *FGFR3* alone was used to obtain a high PPV.

The important premise, and associated clinical implication, is designing an assay with high NPV and high sensitivity, which would identify patients with a low probability of having the disease, as well as an assay with a high PPV and high specificity which would identify patients with a high probability of having the disease and who would thus require additional intervention. The multianalyte biomarker readout assay is used to identify patients unlikely to have bladder cancer at the given monitoring visit; a patient is considered not to have the disease if all biomarkers are negative. In prior studies, we have shown that *FGFR3* has high specificity and PPV, and may be used to identify patients with a high probability of having bladder cancer; patients are categorized as having the disease if their urine contains mutant *FGFR3* DNA.<sup>11,12</sup> The remaining patients are at average risk for recurrence and would continue to receive the standard of care, which

includes routine surveillance by cystoscopy<sup>5,13</sup> and possibly cytology and other biomarkers.

## Materials and methods

This was a prospective, observational, multicenter, single assessment, diagnostic study evaluation designed to assess the performance characteristics of the multianalyte biomarker readout assay relative to routine surveillance cystoscopy to detect recurrent bladder cancer. Patients were recruited from 29 independent urology or oncology practices performing follow-up cystoscopy for bladder cancer. The study was conducted according to the principles established by the Declaration of Helsinki. Appropriate institutional review boards approved the protocol and all patients gave their written informed consent.

## Study patients

Eligible patients were  $\geq 18$  years of age, had a history of nonmuscle-invasive bladder cancer, and were undergoing routine bladder cancer recurrence screening by cystoscopy for  $\geq 3$  months and  $\leq 3$  years post initial urothelial bladder cancer treatment and/or not  $>3$  years from the most recent diagnosis of bladder cancer recurrence.

One group of samples from the entire study population was chosen at random and used to determine cutoffs (derivation population,  $n = 271$ ). The second group, consisting of the remaining patient samples, was used to test assay efficacy (validation population,  $n = 733$ ). Random selection ensured that the patient populations were comprised of samples obtained from the various clinical sites and were representative of the entire study population having similar demographics and clinical characteristics.

## Study design

At the study visit, urine samples and baseline values, including age, gender, and smoking status, were collected and cystoscopy was performed. A patient was considered negative for bladder cancer if cystoscopy showed no evidence of disease or if a biopsy was performed and the biopsy was negative for cancer. All patients were considered positive for recurrence if the finding of cancer was confirmed by histopathology. Patients with indeterminate cystoscopy findings were sent for biopsy and then categorized as positive if the biopsy was positive or no evidence of disease if the biopsy was negative. In cases where a biopsy result was missing, the patient was excluded from further analysis.

The multianalyte biomarker readout assay was performed and evaluated with regard to the clinical cystoscopy and biopsy diagnosis in a blinded manner. Urine samples were collected using a standard clean catch protocol prior to cystoscopy and stored at  $-80^{\circ}\text{C}$ . Samples for DNA analysis were stabilized with 25 mM ethylenediamine tetra-acetic acid. Genomic DNA was isolated from the urine samples (4 mL) using the QIAamp MinElute virus vacuum kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, and stored at  $-20^{\circ}\text{C}$ .

## MMP-2 protein quantitation

MMP-2 urine protein levels were quantified by enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN) using 50  $\mu\text{L}$  of neat urine, as per the manufacturer's instructions. Samples were assayed in duplicate.

## FGFR3 mutation assay

Genomic DNA isolated from 4 mL of urine was amplified using oligonucleotide primers specific for human *FGFR3* to amplify DNA from exons 7, 10, and 15 (Supplemental Table 1). Polymerase chain reaction (PCR) amplification was performed using a Bio-Rad C1000 thermal cycler under the following conditions: 10 seconds at  $95^{\circ}\text{C}$ , 30 seconds at  $65^{\circ}\text{C}$ , and 10 seconds at  $72^{\circ}\text{C}$  for 50 cycles. DNA amplification was confirmed by agarose gel analysis of primary PCR products.

*FGFR3* mutations were detected utilizing a PCR-clamping methodology. Wild-type blocking oligonucleotides containing locked nucleic acid bases surrounding known mutation sites were included, along with real-time PCR primers and dual-labeled Taqman probes (Supplemental Table 1). The locked nucleic acid primers, which have a higher annealing affinity with wild-type than mutant DNA, inhibit wild-type DNA elongation, resulting in preferential amplification of mutant DNA. PCR was performed in the absence of primers containing locked nucleic acid to control for levels of DNA in each reaction. Real-time PCR amplification was performed using a Roche Light Cycler

real-time thermal cycler (10 seconds at  $95^{\circ}\text{C}$ , 30 seconds at  $60^{\circ}\text{C}$ , and 10 seconds at  $72^{\circ}\text{C}$  for 50 cycles). Samples were excluded from analysis if there was not sufficient input DNA as determined during the initial PCR reaction. The ratio of amplification cycles of positive controls (plasmids containing known *FGFR3* mutations) to negative controls (wild-type DNA) were used to assess if a sample did or did not contain a mutation. All PCR reactions were performed in duplicate.

## NID2 and VIM methylation analysis

Methylation of *NID2* DNA was evaluated using conventional methylation-specific PCR. A maximum of 2  $\mu\text{g}$  of genomic DNA purified from 8 mL of urine was bisulfite-converted using the Epiect bisulfite kit (Qiagen) following the manufacturer's instructions. The resulting converted DNA was eluted into 30  $\mu\text{L}$  of molecular grade  $\text{H}_2\text{O}$  and stored at  $-20^{\circ}\text{C}$ . Conventional methylation-specific PCR was performed using methylation-specific primers to the *NID2* promoter (Supplemental Table 1) in the Bio-Rad C1000 thermal cycler (10 seconds at  $95^{\circ}\text{C}$ , 10 seconds at  $60^{\circ}\text{C}$ , and 10 seconds at  $72^{\circ}\text{C}$  for 50 cycles) and the methylation threshold determined by densitometry.

Methylation of *VIM* was evaluated using quantitative real-time methylation-specific PCR from DNA prepared as described above. Quantitative real-time methylation-specific PCR was performed using methylation-specific primers and probes (Supplemental Table 1) to the *VIM* promoter region in the Roche Light Cycler 480 (Roche, Basel, Switzerland) under the following conditions: 60 seconds at  $95^{\circ}\text{C}$ , 60 seconds at  $65^{\circ}\text{C}$ , and 60 seconds at  $72^{\circ}\text{C}$  for 50 cycles. A standard curve was included in all *VIM* assays to determine the amount of methylated *VIM* DNA present in each sample.

To ensure that a negative result for either gene was accurate, a minimum input of 20 ng of DNA, as determined by quantitative real-time methylation-specific PCR amplification of unmethylated *ACTIN-B*, was required. Samples negative for hypermethylation that did not meet the minimum DNA inputs were excluded from further analysis.

## Statistical analysis

The primary endpoint was to test the ability of the multianalyte biomarker readout assay to evaluate a patient population undergoing monitoring for bladder cancer recurrence using the two cutoff approach, where one cutoff included all markers and the other cutoff included only *FGFR3*. Performance of the combined biomarker assay focused on sensitivity and NPV for the purpose of identifying patients who were unlikely to have recurrent disease, and performance of *FGFR3* alone focused

**Table 1** Summary of derivation population performance characteristics<sup>a</sup>

	Sensitivity	NPV	Specificity
<i>NID2</i> , <i>FGFR3</i> , <i>VIM</i> , and MMP-2, n (%)	85 (41/48)	96 (153/160)	67 (153/229)
95% CI	72–94	91–98	60–73

**Notes:** <sup>a</sup>Cutoffs for the different biomarkers were: MMP-2 < 1.100 ng/mL, *NID2* < 600 K, and *Vimentin* < 0.19 ng. For *FGFR3*, a patient is either positive or negative for mutant DNA.

**Abbreviations:** CI, confidence interval; NPV, negative predictive value.

on specificity and PPV for the purpose of identifying patients with recurrent disease. Statistics of categorical variables included number and percentage of subjects. Subgroup analysis included cancer stage and grade.

## Negative predictive value

Assessment of NPV used a composite of cutoffs for all the biomarkers. Cutoff values were selected to achieve  $\geq 95\%$  NPV and approximately 90% sensitivity when in combination and as previously described by us.<sup>14</sup> A patient was classified as negative if all biomarkers were negative. A patient was classified as positive/intermediate if one or more of the biomarkers did not meet the cutoff criteria. NPV was calculated as follows:

$$\text{NPV} = \frac{\text{True cystoscopy negative}}{\text{All multianalyte biomarker readout assay negatives}}$$

## Positive predictive value

Evaluation of PPV utilized *FGFR3* mutational status. A patient was considered positive if their urine contained mutant *FGFR3* DNA. A patient was considered indeterminate/negative if the urine contained wild-type *FGFR3* DNA. PPV was calculated as follows:

$$\text{PPV} = \frac{\text{True cystoscopy-biopsy positive}}{\text{All } FGFR3 \text{ assay positives}}$$

## Results

Of the 1153 patients enrolled in the study, 271 were used as the derivation population to establish the cutoff values for the multianalyte biomarker readout assay and 733 were

analyzed as the validation population (Figure 1). A total of 149 samples were not evaluable due to incorrect consent forms, samples not received or damaged during shipping, or samples where at least one of the marker results were missing (Figure 1).

## Establishment of biomarker cutoffs: derivation population

Four biomarkers were evaluated to determine what combination would give the highest sensitivity and NPV for the study endpoint. We have previously reported on a limited population trial evaluating the use of a DNA/protein combination assay for patients undergoing recurrence monitoring.<sup>11</sup> As with previous studies, the marker combination used here includes hypermethylation markers. Hypermethylation of *VIM* can also be detected in the urine of bladder cancer patients.<sup>15</sup> Preliminary studies in our laboratory have shown that samples positive for one hypermethylation marker often overlap with samples that are positive for other hypermethylation markers tested (data not shown). In this study, the markers included the DNA biomarkers *NID2*, *FGFR3*, and *VIM*, and the protein marker, MMP-2. For the derivation population, 271 patients from this study were randomly chosen to establish cutoffs for the four biomarkers. Cutoffs for quantitative markers were established to achieve  $\sim 90\%$  sensitivity while maximizing specificity when in combination. To obtain maximum NPV at high sensitivity, we first applied binary markers, and then established cutoffs for the other markers one by one, as previously described.<sup>11</sup> Of the 271 patients, 42 had

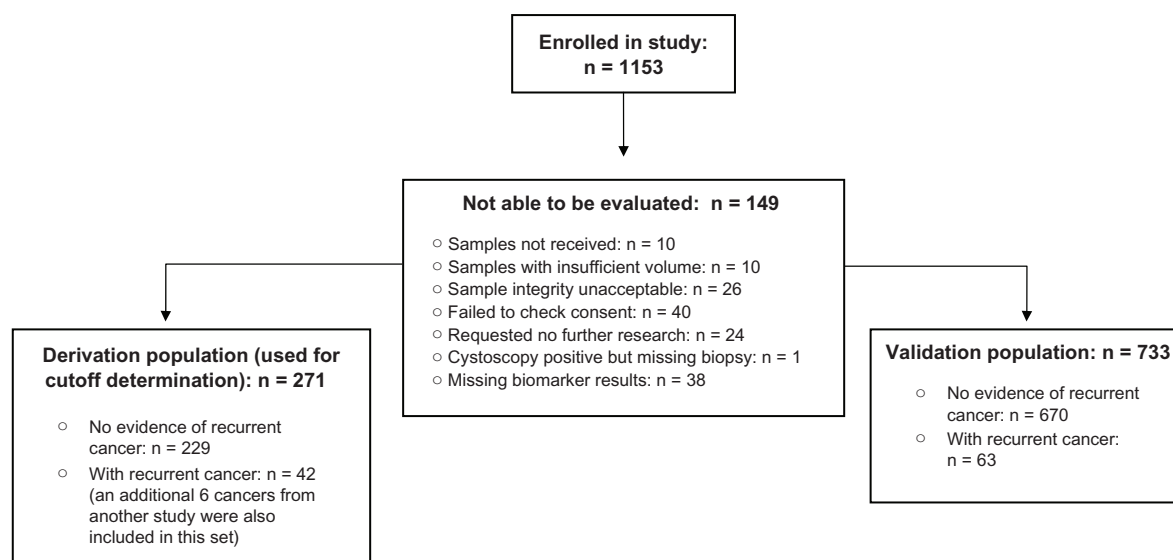


Figure 1 CONSORT diagram.



recurrent cancer and the remaining 229 had no evidence of disease based on cystoscopy or biopsy. Six additional patients with bladder cancer from an independent study with demographics similar to those of the current study were included in the derivation population to increase the number of cancers.

The assay assessed the performance of the four biomarkers in combination to maximize sensitivity and NPV (Table 1). The optimal cutoffs for the individual markers were: MMP-2 < 1.100 ng/mL; *VIM* < 0.190 ng; *NID2* < 600 K; and *FGFR3* = negative. *FGFR3* was used to assess PPV, given that it has high specificity and is known to be associated with bladder cancer.<sup>16–20</sup>

## Validation population

The validation population consisted of 733 patients; 670 (91.4%) of the patients did not have recurrent bladder cancer and 63 (8.6%) had documented recurrence as evidenced with cystoscopy/biopsy (Figure 1). Baseline demographics and disease characteristics were similar between the two patient groups (Table 2). The four-biomarker combination identified 237 patients as unlikely to have recurrent cancer, 231 of whom were confirmed not to have cancer by cystoscopy resulting in a NPV of 97.5% (Table 3) and a sensitivity of 90.5%. In general, the results of the assay were not influenced by the grade and stage of the tumor (Table 4).

Cystoscopy/biopsy identified 63 patients with bladder cancer recurrence. Mutant *FGFR3* DNA was present in the urine of 49 patients, 19 of whom were identified by cystoscopy/biopsy to have recurrent bladder cancer. These findings indicate that *FGFR3* had a specificity of 95.5% with 38.8% PPV.

**Table 2** Baseline demographics and disease characteristics

Characteristic	Biopsy positive (n = 63)	Biopsy/cystoscopy negative (n = 670)
Mean age, years ± standard deviation	71 ± 11.4	69 ± 10.9
Male, n (%)	49 (77.8)	513 (76.6)
Determination of disease diagnosis, n (%)		
Biopsy	63 (100.0)	100 (14.9)
Cystoscopy	0	570 (85.1)
Smoking status, n (%)		
Yes	43 (68.3)	520 (77.6)
No	20 (31.7)	137 (20.4)
Unknown	0	13 (1.9)

## Discussion

This prospective, multicenter, single assessment, observational study evaluated the performance characteristics of the multi-analyte diagnostic readout assay compared with cystoscopy in identifying recurrent bladder cancer. The assay used a combination of four biomarkers, ie, *NID2*, *VIM*, and *FGFR3* DNA biomarkers, and the MMP-2 protein biomarker, to identify patients with a low probability of having recurrent bladder cancer. The *FGFR3* assay has a high specificity for detecting mutant *FGFR3* DNA in urine, and was used to detect patients with a high likelihood of having recurrent bladder cancer. The four-biomarker combination had an NPV of 97.5% for identifying patients unlikely to have recurrent disease. Importantly, the sensitivity and NPV of the assay was high across all stages and grades of tumors.

The combination of the three DNA-based biomarkers with the MMP-2 protein marker resulted in an assay with a sensitivity comparable with that of cystoscopy in detecting patients without bladder cancer, suggesting that this assay may identify patients who might be excluded from invasive diagnostic procedures at a given follow-up visit. Typically, either protein or DNA markers are multiplexed as independent marker sets. In contrast, in the multianalyte diagnostic readout assay, the biomarkers are combined into a protein-DNA multiplex format. Protein markers usually have high sensitivity due to the fact that the methods for measuring their presence are highly quantitative; however, these same methods are often limited in specificity. In contrast, DNA markers typically have high specificity (for example, a mutation is either present or not) but low sensitivity. This study found that combining protein and DNA markers into a multiplex format resulted in an assay that accurately identified patients unlikely to have recurrent bladder cancer. These findings are consistent with a prior study that evaluated the ability of a similar protein-DNA multiplexed assay to detect recurrent bladder cancer.<sup>11</sup>

*FGFR3* mutations are prevalent in noninvasive low-grade tumors, and due to the high specificity of this biomarker, the presence of mutant DNA in a patient's urine is likely indicative of bladder cancer.<sup>16–20</sup> In this study, *FGFR3* mutations were detected in the urine of 19 of 63 patients who had cancer with high specificity (95.5%) and with 38.8% PPV, higher than other markers when adjusted for prevalence.<sup>21</sup> Although *FGFR3* was also positive in the urine of 30 patients who were negative for cancer by cystoscopy, it is possible that in the bladder cancer survivor patient population, the likelihood of an *FGFR3*-positive/cystoscopy-negative finding is higher than in other symptomatic populations.<sup>12</sup> Given that bladder

**Table 3** Assay performance characteristics

	Sensitivity	Specificity	NPV	PPV
Combined four biomarkers, % (n)	90.5 (57/63)	34.5 (231/670)	97.5 (231/237)	–
95% CI	80.4–96.4	30.9–38.2	94.6–99.1	–
<i>FGFR3</i> , % (n)	30.2 (19/63)	95.5 (640/670)	–	38.8 (19/49)
95% CI	19.2–43.0	93.7–97.0	–	25.2–53.8

**Abbreviations:** CI, confidence interval; NPV, negative predictive value; PPV, positive predictive value.

cancer survivors are frequently monitored by cystoscopy, it is possible that the *FGFR3* assay may detect the presence of a cancer before it is visible by cystoscopy.<sup>5–7,13</sup> In support of this idea, a recent study found that patients positive for an *FGFR3* mutation in their urine but who were considered cancer-free by cystoscopy often had bladder cancer recurrence shortly thereafter.<sup>20</sup> These findings suggest that patients who have *FGFR3* mutant DNA in their urine may benefit from additional surveillance. In addition, given that the *FGFR3* assay can identify patients with low-grade tumors, there may be advantages to using this assay in conjunction with other assays that are typically more sensitive for high grade and stage tumors, such as cytology.<sup>22</sup> In fact, a recent study found that the combination of *FGFR3* and cytology had a specificity of 99.5% and a PPV of 87.5% for diagnosing bladder cancer in patients with microhematuria or macrohematuria.<sup>12</sup>

Cystoscopic follow-up for nonmuscle-invasive bladder cancer is the main form of surveillance within the urologic community.<sup>13</sup> Due to the high frequency of local recurrence and

the potential for stage progression, the American Urological Association guidelines recommend lifelong follow-up for patients with nonmuscle-invasive bladder cancer.<sup>23</sup> The most commonly utilized strategy involves patient assessment every 3 months for the first 2 years following the initial diagnosis, followed by every 6 months for an additional 2–3 years, and then annually thereafter.<sup>5,13</sup> Reducing the number of cystoscopies administered to screen for recurrent bladder cancer may reduce patient burden and increase compliance.<sup>24</sup> The high NPV of the four biomarker assay (97.5%) may assist in the successful identification of patients with a very low probability of recurrent disease.

## Conclusion

In this study, the noninvasive, urine-based multianalyte diagnostic readout assay uses a combination of four biomarkers to achieve high NPV for identifying a subgroup of patients who are unlikely to have bladder cancer recurrence and *FGFR3* to identify another subgroup with a high likelihood of recurrence. This approach provides a noninvasive, urine-based assay to triage a population of patients undergoing routine monitoring for recurrence and correctly identify those patients who should be further evaluated with additional diagnostic procedures.

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**Table 4** Summary of multianalyte diagnostic readout assay performance characteristics by tumor TNM stage and grade

Grade, n (%)	Sensitivity by grade/stage	Specificity for all cancer-negative subjects	NPV by grade/stage versus all cancer-negative subjects
I	31/37 (83.8)	231/670 (34.5)	231/237 (97.5)
95% CI	68.0–93.8	30.9–38.2	94.6–99.1
II	8/8 (100.0)	231/670 (34.5)	231/231 (100.0)
95% CI	63.1–100.0	30.9–38.2	98.4–100.0
III	16/16 (100.0)	231/670 (34.5)	231/231 (100.0)
95% CI	79.4–100.0	30.9–38.2	98.4–100.0
Unknown	2/2 (100.0)	231/670 (34.5)	231/231 (100.0)
95% CI	15.8–100.0	30.9–38.2	98.4–100.0
<b>Stage</b>			
Ta	43/49 (87.8)	231/670 (34.5)	231/237 (97.5)
95% CI	75.2–95.4	30.9–38.2	94.6–99.1
T1	8/8 (100.0)	231/670 (34.5)	231/231 (100.0)
95% CI	63.1–100.0	30.9–38.2	95.4–100.0
Tis	5/5 (100.0)	231/670 (34.5)	231/231 (100.0)
95% CI	47.8–100.0	30.9–38.2	95.4–100.0
Unknown	1/1 (100.0)	231/670 (34.5)	231/231 (100.0)
95% CI	2.5–100.0	30.9–38.2	95.4–100.0

**Note:** <sup>a</sup>Biomarker combination included *NID2*, *FGFR3*, *VIM*, and *MMP-2*.

**Abbreviations:** CI, confidence interval; NPV, negative predictive value.

## Disclosure

CAF and APS are employees of Predictive Biosciences. NDS has no conflict of interest to disclose.

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## Supplemental table

**Table S1** Sequence of primers for multianalyte diagnostic readout assay

Assay	Reagent	Sequence
FGFR3 primary PCR	Exon 7 forward	5' GCG GTC CCA AAA GGG TCA GTA CAG TGG CGG TGG TGG TGA GGG AG 3'
	Exon 7 reverse	5' GCG GTC CCA AAA GGG TCA GTA CGC ACC GCC GTC TGG TTG G 3'
	Exon 10 forward	5' GCG GTC CCA AAA GGG TCA GTA CGG TCT GGC CCT CTA GAC TCA 3'
	Exon 10 reverse	5' GCG GTC CCA AAA GGG TCA GTA CGA AGA AGC CCA CCC CGT A 3'
	Exon 15 forward	5' GCG GTC CCA AAA GGG TCA GTA CCC TGC CCT GAG ATG CT 3'
	Exon 15 reverse	5' GCG GTC CCA AAA GGG TCA GTA CCG TCC TAC TGG CAT GAC C 3'
FGFR3 mutation detection	Exon 7 forward	5' GCG TCA TCT GCC CCC A 3'
	Exon 7 reverse	5' CAC CGC CGT CTG GTT G 3'
	Exon 7 LNA	5' AGA <u>GCG</u> <u>CTC</u> <u>CCC</u> G 3' (LNA bases are underlined)
	Exon 7 probe	5' FAM-CCC GCC TGC AGG ATG GGC CGG T-lowa Black FQ 3'
	Exon 10 forward	5' GGC CTC AAC GCC CAT GT 3'
	Exon 10A reverse	5' TAG CTG AGG ATG CCT GCA TA 3'
	Exon 10B reverse	5' CCG TAG CTG AGG ATG CCT G 3'
	Exon 10A LNA	5' ATA <u>CAC</u> <u>ACT</u> <u>GCC</u> <u>CGC</u> CT 3' (LNA bases are underlined)
	Exon 10B LNA	5' GCC TGC <u>ATA</u> <u>CAC</u> <u>ACT</u> 3' (LNA bases are underlined)
	Exon 10 probe	5' FAM-CCG AGG AGG AGC TGG TGG AGG CTG AC-lowa Black FQ 3'
	Exon 15 forward	5' CAA TGT GCT GGT GAC CGA G 3'
	Exon 15 reverse	5' CCG GGC TCA CGT TGG TC 3'
	Exon 15 LNA	5' GGT CGT <u>CTT</u> <u>CTT</u> <u>GTA</u> <u>GT</u> 3' (LNA bases are underlined)
	Exon 15 probe	5' FAM-CTG GCC CGG GAC GTG CAC AAC CTC GAC T-lowa Black FQ 3'
	Nid2	Nid forward
Nid reverse		5' CTA CGA AAT TCC CTT TAC GCT 3'
VIM	VIM forward	5' GCG GTC CCA AAA GGG TCA GTA CTT CGG GAG TTA GTT CGC GTT 3'
	VIM reverse	5' GCG GTC CCA AAA GGG TCA GTA CAC CGC CGA ACA TCC TAC GA 3'
	VIM probe	5' ACG AAA AAT AAC GAT AAC CTA AAC GAC GAC GA 3'
ACTB	ACTB forward	5' TAG GGA GTA TAT AGG TTG GGG AAG TT 3'
	ACTB reverse	5' AAC ACA CAA TAA CAA ACA CAA ATT CAC 3'
	ACTB probe	5' TGG GGT GGT GAT GGA GGA GGT TTA GTA AGT TTT TT 3'

**Abbreviations:** LNA, locked nucleic acid; PCR, polymerase chain reaction.

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