

# Multi-Analyte Diagnostic Readout (MADR): Combining Protein and DNA Markers to Maximize Clinical Performance

Cecilia A. Fernández<sup>1</sup>, John Millholland<sup>1</sup>, Ian C. Summerhayes<sup>2</sup>, John A. Libertino<sup>2</sup>, R. Jeffrey Karnes<sup>3</sup> and Anthony P. Shuber<sup>1</sup>  
<sup>1</sup>Predictive Biosciences Inc., Lexington, MA 02421, <sup>2</sup>Lahey Clinic Medical Center, Burlington, MA 01850, <sup>3</sup>Mayo Clinic, Rochester, MN 55905

## Abstract

### Background

We have recently reported the development of a non-invasive diagnostic assay using urinary Matrix Metalloproteinases (MMPs) as monitors of disease-free status and bladder cancer in high-risk populations. Using a novel approach called Clinical Intervention Determining Diagnostic (CIDD), we identified with high confidence those patients without bladder cancer, who could then be excluded from additional intervention. In order to refine this assay and maximize Negative Predictive Value (NPV) driven by sensitivity, we have now added additional protein markers and developed a Real Time PCR assay to detect FGFR3 mutations in urine. FGFR3 mutations have been identified in 30-50% of bladder cancer patients, and are associated with low-stage non-invasive tumors where sensitivity reaches ~70%. FGFR3 mutations have been detected in the urine of bladder cancer patients, making this an attractive non-invasive DNA marker.

### Methods

We measured and compared MMP-2 and MMP-9 levels by ELISA and ADAM12 by western in a cohort of 181 patients undergoing monitoring for bladder cancer recurrence, 25 of which had a confirmed recurrence. We are currently analyzing this cohort for the presence of eight FGFR3 mutations, and have modeled results to ultimately combine both the protein and DNA analyses into one assay.

### Results

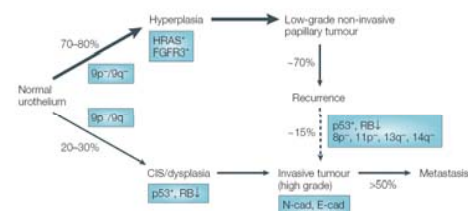
In this recurrence monitoring population, using MMP-9 alone we identify with high confidence (91% NPV) those patients who do not have cancer (49%). Although above 90%, this NPV was not driven by high sensitivity (68%). We therefore increased the number of markers in order to reduce the number of cancers missed (increase sensitivity) as well as to maximize NPV. Using the prevalence of FGFR3 mutations in the urine of bladder cancer patients from other studies, we simulated FGFR3 detection in these samples. We then tested for MMP-2 and ADAM12 protein levels. Using these 3 additional markers sensitivity increased to 96% resulting in the identification of 27% of patients who do not have cancer at 98% NPV.

### Conclusions

The novel Multi-Analyte Diagnostic Readout (MADR) concept described here combines the best performance characteristics of protein biomarkers and DNA biomarkers into one assay for optimal clinical performance. Using this approach, the detection of an FGFR3 mutation in the urine of patients undergoing recurrence monitoring would effectively increase the sensitivity and NPV at the established protein cutoffs, resulting in the identification of patients who could be excluded from receiving invasive procedures. All remaining patients would continue to receive the existing standard of care procedure.

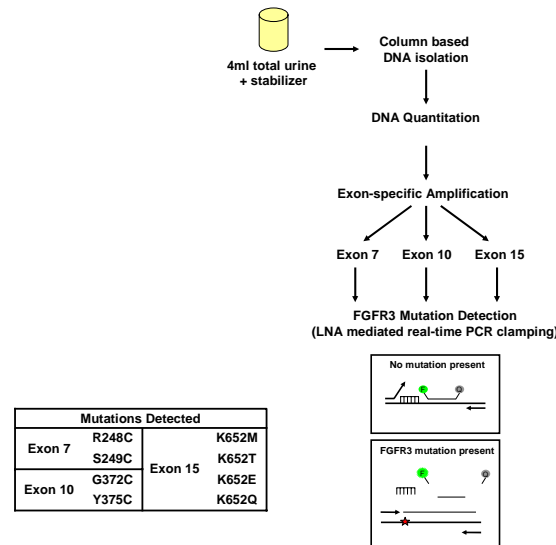
## Introduction

To complement our existing MMP assay, we have developed a non-invasive assay for the detection of FGFR3 mutations in urine. As shown below, FGFR3 mutations occur primarily in low grade, non-invasive tumors, while MMPs are associated with higher grade, invasive tumors. Our FGFR3 assay utilizes a novel sample prep method and a two-step PCR amplification process. As with our previous studies, urinary MMP levels were determined by ELISA and cutoffs were determined to maximize Negative Predictive Value in a population of patients undergoing monitoring for bladder cancer recurrence. Using these cutoffs and our proprietary Clinical Intervention Determining Diagnostic concept, patients with levels below the cutoffs could be excluded from further intervention. To further maximize NPV, we have also added the protein marker ADAM-12, which has also been shown to be associated with bladder cancer status. Our new assay would combine MMPs, ADAM-12 and FGFR3 mutation detection in the same non-invasive test. Here, we demonstrate detection of FGFR3 mutations in bladder cancer tissue, as well as matched tissue and urine samples. In addition, we model the performance of a combined MMP-9/MMP-2/ADAM-12/FGFR3 assay using estimated detection rates for FGFR3 from current literature to determine the impact of combining DNA and protein markers into one assay.



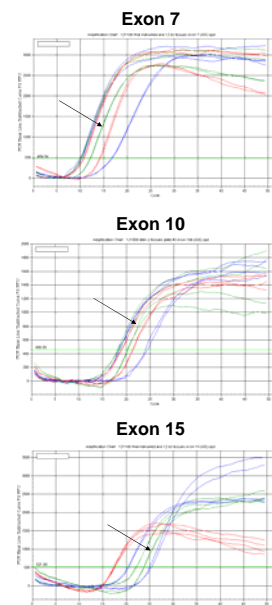
Taken from Xue-Ru. *Nature Reviews/Cancer*, vol. 5: 713-725, Sept. 2005.

## FGFR3 Mutation Detection Assay



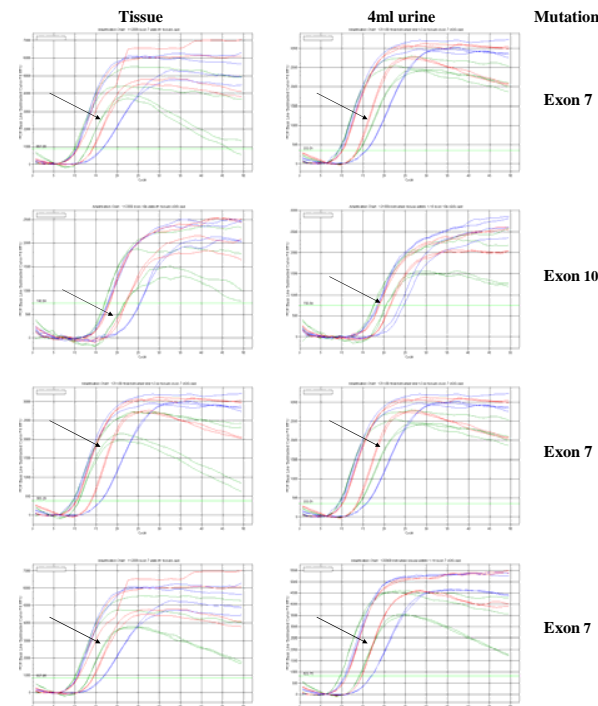
**Figure 1. Assay Design.** DNA is extracted from 4ml of urine using a modified Qiagen Virus Vacuum kit. The extracted DNA is then quantitated by real-time PCR, and subjected to 50 cycles of PCR amplification for three FGFR3 exons (7, 10, and 15). These PCR products are then used as template for our real-time mutation detection assay. The real-time assay utilizes blocking oligonucleotides containing locked-nucleic acid which suppress the amplification of wild-type DNA.

## Detection of FGFR3 Mutations in Bladder Cancer Tissue



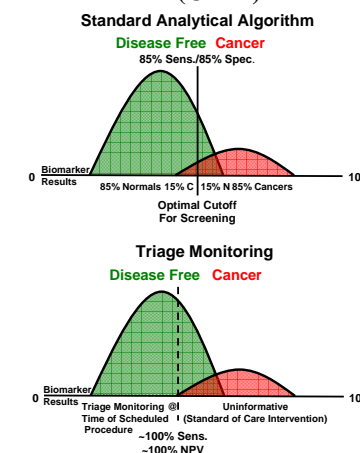
**Figure 2.** DNA extracts from bladder cancer tissue were processed for FGFR3 mutation detection. Shown above are tumors positive for each FGFR3 exon. Samples were initially quantitated by real-time PCR, and then subjected to an initial round of PCR amplification for exons 7, 10, and 15. Real-time mutation detection amplifications were carried out using these primary PCR products. Each sample was amplified in the presence or absence of LNA blocking oligonucleotide (green lines). When a mutation is present, amplification curves are shifted to the left (arrows). In addition, control DNA (blue lines), and control DNA with 1% mutant plasmid (red lines) were also amplified with and without LNA blocking oligonucleotide.

## Real Time FGFR3 Mutation Detection in Matched Tumor and Urine Samples

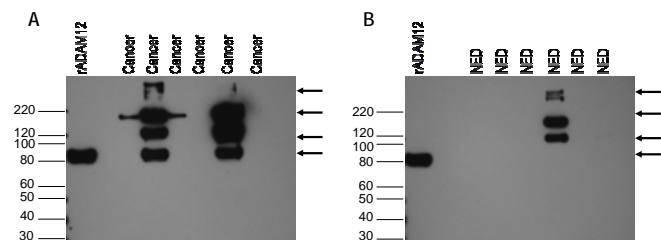


**Figure 4.** Tissue samples and paired urine sediments were analyzed for FGFR3 mutations. As can be seen in these four examples, the mutation present in tissue (left panel, arrow) can also be detected in a 4ml equivalent volume of urine (right panel, arrow). DNA was extracted from fresh frozen or formalin-fixed, paraffin embedded tumor tissue samples according to the manufacturer's protocol using the Qiagen QiaAMP DNA Mini kit or DNA FFPE Tissue kit, respectively. The paired urine sediments were resuspended to the original urine volume, and DNA was isolated using a modified Qiagen Virus Vacuum kit protocol. Extracted DNA from all sources was then quantitated by real-time PCR, and 50ng of tissue or urine derived DNA was subjected to an initial round of PCR amplification for exons 7, 10, and 15. Real-time mutation detection amplifications were carried out using these primary PCR products.

## Clinical Intervention Determining Diagnostic (CIDD)



## ADAM-12 as a Marker of Bladder Cancer Recurrence



**Figure 5.** Presence or absence of ADAM-12 in the urine of patients undergoing recurrence monitoring was determined by western using ADAM-12-specific monoclonal antibodies. Representative gels are shown above for a group samples from patients who had recurrent cancer (A) and samples from cancer survivors who at the time of testing had No Evidence of Disease (NED). In these sample set, a number of ADAM-12 reactive bands were observed: a band of ~84kD, consistent with the latent secreted form of ADAM-12, a band of ~120kD, a band of ~220kD and bands of >220kD. Samples were considered positive if any bands of these molecular weights were present.

## Clinical Performance Using Multiple Markers

Marker	Number of Simulations	n	Cutoff	NPV	Sens.	POE	Cancers Missed
MMP-9	0	25 Cancers 159 NEDs	MMP-9<0.517	91% [82-96%]	68% [47-85%]	49% [41-57%]	8
MMP-9 + FGFR3	1	25 Cancers 159 NEDs	MMP-9<0.517	95% [87-98%]	84% [64-95%]	39% [31-47%]	4
MMP-9 + FGFR3 + ADAM-12	1	25 Cancers 159 NEDs	MMP-9<0.517 ADAM-12<1	97% [88-100%]	92% [74-99%]	36% [28-44%]	2
MMP-9 + FGFR3 + ADAM-12 + MMP-2	1	25 Cancers 159 NEDs	MMP-9<0.517 ADAM-12<1 MMP-2<0.413	100% [92-100%]	100% [86-100%]	27% [20-35%]	0
MMP-9 + FGFR3 + ADAM-12 + MMP-2	1000	25 Cancers 159 NEDs	MMP-9<0.517 ADAM-12<1 MMP-2<0.413	98% [92-100%]	96% [86-100%]	27% [20-35%]	0-1

## Summary

- FGFR3 mutations are associated with non-invasive bladder cancer while MMPs and ADAM-12 are associated with invasive tumors.
- We have developed a sensitive, non-invasive assay to detect FGFR3 mutations from as little as 4ml of urine.
- Using the inherent specificity of DNA and the increased sensitivity of proteins, MADR provides one test that delivers extremely high Negative Predictive Value.