

# Prostate Cancer Marker Panel With Single Cell Sensitivity in Urine

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**Background.** Over one million men undergo prostate biopsies annually in the United States, a majority of whom due to elevated serum PSA. More than half of the biopsies turn out to be negative for prostate cancer (CaP). The limitations of both the PSA test and the biopsy procedure have led to the development for more precise CaP detection assays in urine (e.g., PCA3, TMPRSS2-ERG) or blood (e.g., PHI, 4K). Here, we describe the development and evaluation of the Urine CaP Marker Panel (UCMP) assay for sensitive and reproducible detection of CaP cells in post-digital rectal examination (post-DRE) urine.

**Methods.** The cellular content of the post-DRE urine was captured on a translucent filter membrane, which is placed on Cytoclear slides for direct evaluation by microscopy and immuno-cytochemistry (ICC). Cells captured on the membrane were assayed for PSA and Prostein expression to identify prostate epithelial cells, and for ERG and AMACR to identify prostate tumor cells. Immunostained cells were analyzed for quantitative and qualitative features and correlated with biopsy positive and negative status for malignancy.

**Results.** The assay was optimized for single cell capture sensitivity and downstream evaluations by spiking a known number of cells from established CaP cell lines, LNCaP and VCaP, into pre-cleared control urine. The cells captured from the post-DRE urine of subjects, obtained prior to biopsy procedure, were co-stained for ERG, AMACR (CaP specific), and Prostein or PSA (prostate epithelium specific) rendering a whole cell based analysis and characterization. A feasibility cohort of 63 post-DRE urine specimens was assessed. Comparison of the UCMP results with blinded biopsy results showed an assay sensitivity of 64% (16 of 25) and a specificity of 68.8% (22 of 32) for CaP detection by biopsy.

**Conclusions.** This pilot study assessing a minimally invasive CaP detection assay with single cell sensitivity cell-capture and characterization from the post-DRE urine holds promise for further development of this novel assay platform. *Prostate* 75:969–975, 2015.

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**KEY WORDS:** prostate cancer; urine; biomarker panel; cell capture

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Conflict of interest: Henry M. Jackson Foundation for the Advancement of Military Medicine (HJF) has filed a patent application on isolation and detection of cancer cells described in this manuscript, on which S.S., G.P., S.T., and K.P.N. are co-inventors. A patent on the mouse monoclonal anti-ERG antibody, 9FY, has been issued to the HJF on which S.T., A.D., and S.S. are co-inventors. The antibody has been licensed to the Biocare Medical, where T.S. and D.T. are employees. Other than technical assistance, Biocare Medical has not played a role in the design and conduct of the study.

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## INTRODUCTION

Early and minimally-invasive diagnosis of cancer requires the development of sensitive and specific methodologies, such as fine needle aspiration from tumor lesions, the detection of circulating tumor cells (CTCs) from blood, or cancer cells present in urine [1–4]. Prostate cancer (CaP) is the most prevalent non-skin cancer and the second leading cause of cancer death in men in the United States. The widely used prostate specific antigen (PSA) test has greatly benefited early detection of CaP for the past 20 years. However, its limitations have been increasingly recognized [5].

Over one million men undergo prostate biopsies annually in the United States, a majority of them due to elevated serum PSA [6]. The biopsy procedure, which is the gold standard of CaP diagnosis, is invasive and painful, the side effects are significant, sometimes serious. More than half of the biopsies are negative for CaP partially because serum PSA can be elevated for reasons other than CaP (true negative biopsy), or because biopsy needles often miss tumor foci in the prostate (false negative biopsy). Recent efforts to develop non-invasive alternatives are focusing on urine-based molecular assays (e.g., *PCA3*, *TMPRSS2-ERG* [1]) and blood-based molecular assays (e.g., Prostate Health Index [PHI], four kallikreins score [4K] and CTC assays) [7–10]. As the prostate secretions exit through the urethra it was realized that cells from the prostate, including CaP cells, can be shed into the urine, especially after digital rectal examination (DRE), which can be used for CaP detection by specific immunocytochemistry (ICC) staining [11]. Cytology procedures for isolating cells from urine include cytopsin preparation, ThinPrep filtration and centrifugation [1,11,12], which have been shown to be beneficial in the detection of bladder cancer. However, these methods lack the sensitivity in the context of CaP, even with post-DRE urine.

To address these limitations, we describe in this report the development and evaluation of the Urine CaP Marker Panel (UCMP) assay for the detection of CaP cells from post-DRE urine with single cell sensitivity.

## MATERIALS AND METHODS

### Cell Lines

VCaP, LNCaP, and NCI-H660 prostate cancer cells, T24 bladder cancer cells, McCoy's 5A Modified Medium, and fetal bovine serum (FBS) were purchased from ATCC (Manassas, VA). DMEM and RPMI-1640 cell culture media and glutamine were purchased from Life technologies (Carlsband, CA). VCaP cells were maintained in DMEM supplemented

with 10% FBS. LNCaP cells were maintained in RPMI-1640 supplemented with 10% FBS, 2.8 mM L-glutamine. NCI-H660 cells were maintained in RPMI-1640 medium supplemented with 5% FBS, 4 mM L-glutamine, 0.005 mg/ml insulin, 0.01 mg/ml transferrin, 30 nM sodium selenite, 10 nM  $\beta$ -estradiol, and 10 nM hydrocortisone. T24 bladder cancer cells were maintained in McCoy's 5A Modified Medium, supplemented with 5% FBS. All cells were cultured in a 5% CO<sub>2</sub> humidified incubator at 37°C.

### Cell Spiking and Recovery

In controlled spiking experiments, urine samples from healthy volunteers stored at  $-80^{\circ}\text{C}$  were thawed and pre-cleared by centrifugation at 3,000g for 15 min. Cultured CaP or bladder cancer cells were collected, counted and titrated into the urine sample to achieve the approximate number of cells required (10 cells or 100). Saccomanno's fixative (BBC Biochemical, Mount Vernon, WA) was added immediately in a 1:1 ratio and the samples were incubated at room temperature for a minimum of 2 hr. The cell and urine mix was then filtered, and stained with 4',6-diamidino-2-phenylindole (DAPI) (Life Technologies) for 10 min. After excess liquid was removed by aspiration, the filter was mounted with Prolong Gold anti-fade mounting medium (Life Technologies) under a round cover glass. Cell number was assessed visually by counting intact nuclei on the filter using a Leica DMIRE2 inverted microscope (Leica Microsystems, Bannockburn, IL).

### Optimization of Cell Fixation Buffer and Filter Pore Size

Due to the variability of the pH, protein and cellular debris in the urine [13] the specimen collection procedure was optimized to maintain cellular structure and allow filtration of urine through the filter (Supplementary Table S1). The content of urine debris was assessed on several freshly collected control urine specimens from healthy volunteers. To assess these variables, 10 ml of urine was spiked with known number of cells from a CaP cell line, VCaP, and incubated with equal volumes of the PreservCyt or Saccomanno's fixative for up to 4 hr at room temperature. Following the incubation, the urine was filtered through a filter membrane of 2, 5, or 8  $\mu\text{m}$  pore size. The captured cells were stained directly on the membrane with DAPI, and visually assessed to ascertain that cells were properly fixed and that their cellular and nuclear structure remained intact. Both PreservCyt and Saccomanno's fixative offered sufficient fixation preservation of cell structure (Supple-

mentary Table S1). The flow-rate of urine through the filters is dependent not only on debris content but also fixative used. Low debris urine fixed with either fixative passed through the 2, 5, and 8  $\mu\text{m}$  filters easily. Urine containing medium debris filtered through 8  $\mu\text{m}$  filters with ease following incubation with either buffer but has decreased flow rate through the 5  $\mu\text{m}$  pores when stabilized with PreservCyt. In the high debris urine samples only Saccomanno's fixative allowed smooth filtration through both the 5 and 8  $\mu\text{m}$  pore filters, in contrast to the PreservCyt solution, which clogged both filters (Supplementary Table S1).

### Cell Capture Efficiency

To test whether our method showed enhanced cell recovery over reported methods [11] low numbers of cells from established CaP cell lines (10 or 100 cells) were spiked into pre-cleared urine samples, and the number of cells recaptured on the filter that stained positive with DAPI were counted. Approximately, 76 and 79% of VCaP cells were recovered from urine spiked with 10 and 100 cells, respectively. About 82 and 72% of LNCaP cells were recovered from urine spiked with 10 and 100 cells, respectively. About 71 and 85% of NCI-H660 cells were recovered from urine spiked with 10 and 100 cells, respectively (Fig. 1b).

### Patient Specimens

This study was approved by the Institutional Review Boards at the Walter Reed National Military Medical Center (Bethesda, MD). Urine samples were collected following a physician orchestrated DRE. The DRE was performed by multiple providers (urologists) following a strict standard protocol. Briefly, firm pressure was applied on the prostate (to slightly depress the prostate surface) from the base to the apex and from the lateral to the median line for each lobe. Exactly three strokes per lobe were performed (a total of six strokes). The urine specimens were quickly stabilized and any cells in the urine specimen were fixed by the addition of Saccomanno's fixative at a 1:1 ratio. Samples were transported and stored at room temperature, and filtered as described below at the Center for Prostate Disease Research Laboratory (Rockville, MD). An initial feasibility cohort of 10 patients was assessed, followed by an assessment of 53 post-DRE urine specimens. Among the 63 patients, specimens from 57 patients were evaluable. Specimens from six patients were considered as non-evaluable due to the detection of three or fewer cells on the filter (Supplementary Table S2).

### Filtration and ICC

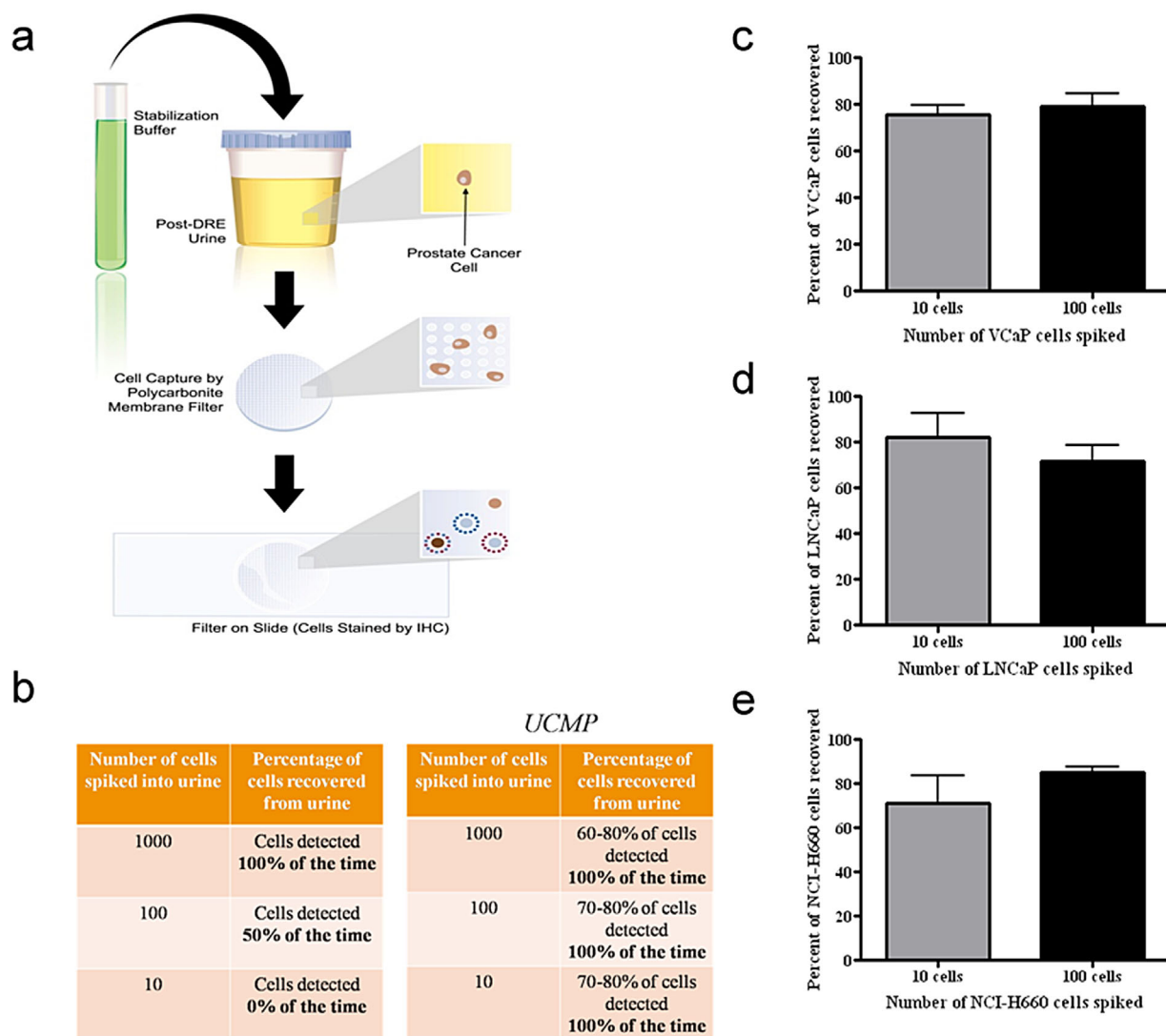
Urine samples were filtered by using the Swinney filtration apparatus (Sterilitech Corporation, Kent, WA), which was assembled from a 20 ml two-part disposable syringe, a 13 mm polypropylene in-line holder, and a 5  $\mu\text{m}$ /13 mm polycarbonate hydrophilic membrane filter. The membrane filter was first pre-wet by passing approximately 5 ml of TBS (Biocare Medical, LLC, Concord, CA) before fixed urine samples were filtered, followed by a flush with 10 ml of TBS. The membrane was then removed from the holder, placed on a Cytoclear glass slide (Sterilitech) and outlined with an Imm-Edge pen (Vector Laboratories, Burlingame, CA). Each experimental step was performed at room temperature, and TBS was used for washings between each step.

### Single Antibody ICC

Prostate cancer cells on the filter membrane were washed twice with TBS. Slides containing the filters were placed into 1 $\times$  Reveal Decloaker retrieval solution (Biocare Medical) in a Coplin jar and a 30 min antigen retrieval step was performed using Biocare's Decloaking Chamber at 80°C. Filters were blocked for 10 min using Background Punisher blocking reagent (Biocare Medical). Filters were then incubated with the following primary antibodies: mouse ERG MAb (9FY, Biocare Medical), rabbit polyclonal AMACR (Biocare Medical), mouse MAb PSA (Biocare Medical) or mouse MAb Prostein (DAKO, Carpinteria, CA). Filters were then washed with TBS and incubated in a secondary antibody for 30 min. Specific chromogens (Biocare Medical) were used to develop the antibody specific color. ERG was developed with Betazoid 3, 3' Diaminobenzidine (DAB) for 10 min; AMACR, with Warp Red for 8 min; and, PSA and Prostein, with Ferangi Blue for 5 min. DNA was stained with DAPI diluted 1:2,000 in TBS for 5 min. Finally, filters were washed twice with TBS and mounted with Prolong Gold anti-fade solution under a cover glass.

### Multiple Antibody ICC

Filter membranes containing CaP cells were washed with TBS, treated for antigen retrieval and blocked as described for single antibody ICC. Samples were incubated with the ERG and AMACR primary antibody cocktail for 30 min, followed by 30 min incubation with MACH 2 Double Stain 2 secondary antibody (Biocare). To develop the antibody specific color, filters were incubated with Warp Red chromogen for 8 min, followed by Betazoid DAB chromogen for 10 min. After removing and washing the filter with TBS, filters were incubated in Denaturing solution (Biocare) at 1:4 ratio for 3 min followed by three washes in TBS. Filters



**Fig. 1.** **a:** Schematic representation of the assay work flow. **b:** Sensitivity of cell recovery from urine in the UCMP assay compared to literature data [11]. Recovery of (c) VCaP, (d) LNCaP, and (e) NCI-H660 cells from urine after spiking in approximately 100 or 10 cells.

were then incubated with PSA or Prostein antibody for 30 min, then rinsed and incubated with MACH 2 mouse-AP secondary antibody for 30 min. To develop the color for PSA or Prostein, filters were incubated with Ferangi Blue chromogen for 5 min. Filters were then washed once with TBS and stained with DAPI for 5 min. Filters were washed twice with TBS and mounted with Prolong Gold anti-fade solution under a cover glass. The slides were allowed to set before examination under the microscope.

## RESULTS

### Direct Detection of Captured Cells From Urine

The procedure flowchart for the detection of CaP cells from post-DRE urine by the Urine CaP Marker

Panel (UCMP) assay is shown in Figure 1a. This method enables whole cell detection using highly specific prostate tissue or CaP specific protein biomarkers. The post-DRE urine was stabilized immediately after collection by the addition of Saccomanno's fixative at a 1:1 ratio. Following incubation for 2–48 hr at room temperature, urine specimens were filtered through five micron polycarbonate hydrophilic filters (Sterlitech, Kent, WA). After washing, filters were placed onto Cytoclear microscope slides (Sterlitech). The cellular content captured on the filter membrane was directly analyzed for protein biomarkers using ICC as these filter membranes are translucent. A key advantage of this approach was the elimination of the extra step of cell transfer from the membrane to glass slides resulting in loss of captured cells.

Due to the high variability of pH, protein and cellular debris in the urine [13], we optimized the specimen collection procedure for cellular stability, fixation and for urine flow through filter membranes (Supplementary Table S1). To determine membrane pore size and optimal buffer for cell fixation, urine samples with various degrees of debris were assessed for the ability of the urine to pass through, while at the same time preventing the loss of cellular content and morphology.

### Sensitivity Improved to Single Cell Detection

To test whether our method indeed enhanced cell recovery over reported methods describing a limit of reliable detection of a thousand cells [1,11], we spiked 10 or 100 cells from established CaP cell lines into pre-cleared urine samples and counted the number of cells recovered on the filter using DAPI nuclear staining. (Fig. 1b). We were able to recover the cells 100% of the time, even when spiking in as few as 10 cells (Fig. 1c–e). The sensitivity of detecting a single cell with this assay was tested by spiking one green fluorescent protein (GFP) labeled LNCaP cell into 10 ml urine. The spiked cell was recovered and microscopically detected eight out of eight times, demonstrating the high sensitivity of this cell-capture approach.

### Antibody Panel Assures Prostate and CaP Cell Specificity

In addition to sensitive capture of cells the specificity of the method relied on the performance of antibodies selected for the ICC staining. We utilized a highly specific anti-ERG monoclonal antibody (9FY) developed in our laboratory [14]. ERG protein is frequently expressed in tumor cell specific manner in CaP as a result of prevalent *TMPRSS2-ERG* gene fusions [1,14,15]. Moreover, studies have shown diagnostic utility of *ERG* or *TMPRSS2-ERG* transcript measurements in post-DRE urine [16–18]. Our marker panel also included a widely used CaP diagnostic marker,  $\alpha$ -methylacyl-CoA racemase (AMACR/P504S) [2,19]. Two prostate epithelial cell specific markers, PSA [20] and Prostein (P501S) [21,22], were used for enumeration of cells of prostatic origin. We first established the detection of ERG (nuclear-brown), AMACR (cytoplasmic-red), Prostein or PSA (cytoplasmic-blue) expression individually by ICC in VCaP and LNCaP CaP cell lines (Fig. 2a). T24 bladder cancer cells were used as the negative control for each antibody stain. In order to enhance the clinical applicability and work flow of the assay, we developed a multiplexed staining protocol for simultaneous

detection of the biomarker panel. As expected, ERG, AMACR, and Prostein or PSA expression was apparent in VCaP cells, AMACR and PSA in LNCaP cells (Fig. 2b). No expression of any of these markers was detected in the T24 negative control cells. We developed a scoring system for the assay where samples with a score of two or higher (positive for at least one prostate and one CaP marker, or two CaP markers) were called positive for the presence of CaP cells (Fig. 2c).

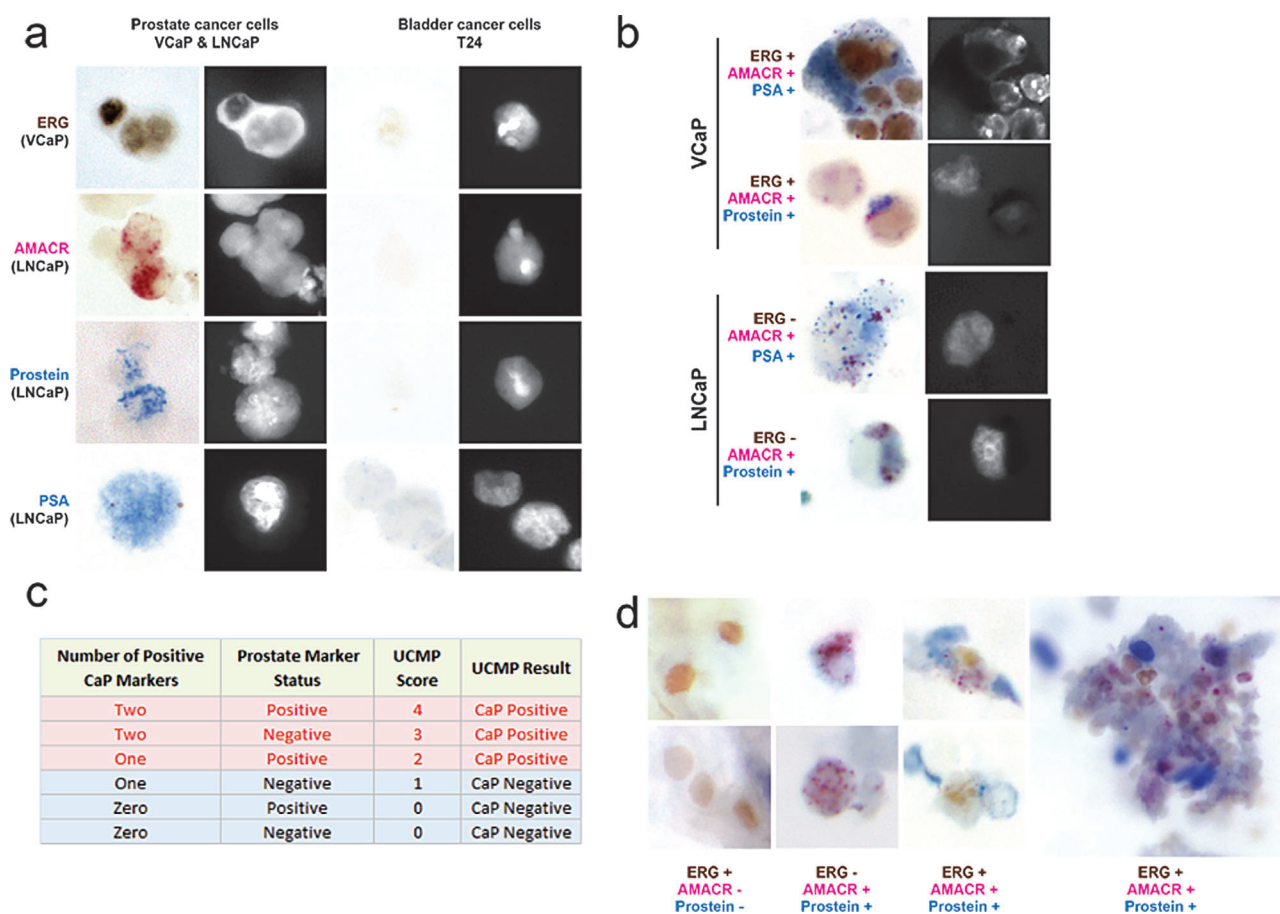
### Feasibility Demonstrated in Patients Undergoing Diagnostic Prostate Biopsy

To further confirm the functionality of the UCMP assay, we assessed a feasibility cohort of 63 post-DRE patient urine specimens (Supplementary Table S2) prospectively collected from consecutive patients undergoing diagnostic prostate biopsy. After cell-capture and ICC assays evaluable specimens (57 of 63, 90.5%) were analyzed for CaP marker (*ERG*, *AMACR*) and prostate epithelial cell marker (Prostein or *PSA*) expression (Fig. 2d). Specimens that scored positive according to the UCMP assay (Fig. 2c) were compared to blinded results of the biopsies. This assay achieved a sensitivity of 64% (16 UCMP positives of the 25 biopsy positives) and a specificity of 68.8% (22 UCMP negatives of the 32 biopsy negatives). The sensitivity and specificity in this small feasibility cohort were comparable to the *PCA3* (sensitivity 47–66%, specificity 60–76%) and *TMPRSS2-ERG* assays (sensitivity 35–40%, specificity 70–80%) developed for post-DRE urine [1,2,18,23]. Similar to the *PCA3* assay, this assay will also have utility for patients needing repeat biopsy after initial negative result.

No relationship between assay positivity and disease grade was noted. In patient subsets with biopsy Gleason grade of 3 + 3, 3 + 4, and 4 + 3 or higher, 8 of 13 (61%), 3 of 4 (75%), and 4 of 7 (57%) had positive UCMP assay, respectively, with a *P*-value of 1.00. The relationship between race and assay results was evaluated. Eleven of 30 (36.7%) CA and 13 of 22 (59.1%) AA had a positive UCMP assay; however, the apparently higher assay positivity in AA patients did not reach significance (*P* = 0.109).

## DISCUSSION

About a third (31.2%, 10 of 32) of the biopsy negative patients had a positive UCMP assay potentially indicating biopsies missing tumor area (false negative). Although no evidence is available to confirm the existence of tumor foci missed by the biopsies, the identification of patients with potentially false negative biopsies is one of the main advantages



**Fig. 2.** **a:** Individual detection of ERG (nuclear-brown), AMACR (cytoplasmic-red), Prostein or PSA (cytoplasmic-blue) expression in spiked VCaP and LNCaP cells by ICC. T24 bladder cancer cells were used as negative control. **b:** Triple staining by ERG, AMACR, and PSA, or ERG, AMACR, and Prostein, of spiked VCaP and LNCaP cells. **c:** Summary of the UCMP scoring system. Samples that score 2 or more (positive for at least one prostate and one CaP marker, or two CaP markers) are considered CaP positive. **d:** Representative examples of ICC images of cells captured from post-DRE urine specimens of four patients.

of this assay, as these patients may most likely benefit from repeat biopsies. Theoretically, all biopsy positive cases should be positive in the UCMP assay; however, the assay was negative in 9 of the 25 biopsy positive cases. As our cell-capture method can pick up a single cell, we speculate that post-DRE urine of patients with CaP may not always contain CaP cells, possibly due to the location and/or size of the tumor. This observation also highlights the need for improvement in approaches releasing CaP cells into urine.

The most important improvement of this assay over previous efforts is its sensitivity for capturing minute amounts of cells from the urine. In bladder cancer, where much more tumor cells are released into the urine (typically thousands of cells available for capture), cytological assays have been used efficiently with traditional cell capture approaches [24]. In CaP, one thousand cells were required for consistent cell capture from urine, which is often not present in post-DRE urine specimens, limiting the applicabil-

ity of the specific assay [11]. The UCMP assay takes advantage of translucent filter membranes, on which the captured cells can be directly analyzed by ICC and microscopy, without the extra step of transferring the cells from the membrane to a glass slides with a massive loss of captured cells. This simple technical innovation may be incorporated into other biofluid-based cytological assays to improve cell capture efficiency and assay sensitivity.

## CONCLUSIONS

In summary, the UCMP assay described here is optimized for the detection of small amounts of cellular material from urine specimens and the detection of cancer cells. The innovative cell-capture approach using translucent membranes ensures single cell sensitivity, a significant improvement over existing methods, as no further transfer of cells to microscope slides is required. The well-preserved cells co-

stained by CaP markers (ERG and AMACR) and prostate epithelium markers (Prostein or PSA) provide visual analysis of biomarkers in whole cells, ensuring the specificity of the assay. The simple but highly sensitive cell-capture method combined with the multiplexed ICC staining is easy to accommodate in a routine pathology laboratory setup. The UCMP assay may also be adapted for other cancers or pathologic conditions where whole cell based biomarker analyses (protein, DNA and RNA) will be of diagnostic or prognostic value.

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