

RESEARCH ARTICLE

Novel reference gene microseminoprotein-beta based prostate cancer antigen 3 urine test: Enhanced diagnostic performance for prostate cancer and feasibility of non-digital rectal examination urine sampling

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Abstract

Background: The diagnosis of prostate cancer (PCa) is limited by low specificity and invasiveness. This study aims to establish a highly accurate, noninvasive reverse transcription-quantitative polymerase chain reaction (RT-qPCR)-based urinary prostate cancer antigen 3 (*PCA3*) assay system and explore the feasibility of using first-morning urine instead of post-digital rectal examination (DRE) samples.

Methods: Three prospective multicenter cohort studies were conducted on 643 Chinese men. Urine *PCA3* expression was quantified by RT-qPCR, using either the novel reference gene microseminoprotein-beta (*MSMB*) or conventional kallikrein-related peptidase 3 (*KLK3*, encoding prostate-specific antigen, PSA). Diagnostic performance was evaluated through receiver operating characteristic (ROC) analysis in Cohort I ($n = 176$), with validation in Cohort II ($n = 350$). In Cohort III ($n = 117$), the diagnostic accuracy of *PCA3/MSMB* test was compared between DRE and non-DRE urine samples.

Results: The *PCA3/MSMB* RT-qPCR system showed high precision and stability. In Cohort I, the *PCA3/MSMB* test demonstrated superior diagnostic performance compared with the *PCA3/KLK3* test, with a significantly higher area under the receiver operating characteristic curve (AUC) (0.869 vs. 0.830, $p = 0.0468$), higher sensitivity (82.1% vs. 79.0%), and higher specificity (77.8% vs. 74.1%). Cohort II confirmed these results, yielding a sensitivity of 82.63% and a specificity of 77.50%. In Cohort III, no significant difference in diagnostic performance was observed between non-DRE and DRE urine samples (AUC 0.859 vs. 0.850, $p = 0.6262$), suggesting the potential for simplified sample collection.

Conclusion: The *PCA3/MSMB* urine test is a promising noninvasive diagnostic tool for PCa; it can reduce unnecessary biopsies and improve detection accuracy using non-DRE urine samples.

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KEYWORDSMSMB, non-DRE, noninvasive diagnosis, *PCA3*, prostate cancer, RT-qPCR, urine**1 | INTRODUCTION**

Prostate cancer (PCa) is the second most prevalent cancer in men worldwide, with 1 467 854 new cases and 397 430 deaths reported in 2022^[1]. The annual percentage change in age-standard incidence rates for PCa worldwide from 2014 to 2019 was 0.32%^[2]. Early-stage localized PCa can be treated radically with radiation or surgery^[3,4]. However, once the disease has metastasized, radical treatment is no longer available, resulting in a poorer prognosis^[5,6]. Therefore, early diagnosis is crucial for improving patient survival.

The gold standard for PCa diagnosis is puncture biopsy, however, it is an invasive procedure associated postoperative complications, including bleeding and infection^[7-11]. Prostate-specific antigen (PSA) is currently the most commonly used biomarker for PCa screening and diagnosis, but it has low specificity, especially in the so-called gray zone (4.0–10.0 ng/mL), where the specificity is only 25%–40%, resulting in a large number of unnecessary biopsies and reduced utility in clinical practice^[12-14].

Several biomarkers, such as the prostate health index (PHI), the *TMPRSS2-ERG* fusion gene, 4K score, and prostate cancer antigen 3 (*PCA3*), have been shown to improve the specificity and sensitivity of PCa diagnosis, reducing unnecessary biopsies in patients^[15-19]. Among these biomarkers, *PCA3* is the most PCa-specific gene identified to date. *PCA3* expression is restricted to prostate tissue and is absent in other normal human tissues^[18]. Located on chromosome 9q21-22, *PCA3* encodes a long non-coding RNA that is highly overexpressed in PCa tissue compared to benign tissue^[18]. However, the area under the receiver operating characteristic curve (AUC) of *PCA3* for the clinical PCa detection is approximately 0.75, which remains sub-optimal, and its diagnostic utility is further limited by the requirement for urine collection after digital rectal examination (DRE).

In this study, we developed a *PCA3*-based reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assay for the noninvasive diagnosis of PCa. We chose the microseminoprotein-beta (*MSMB*) gene as a reference gene instead of the traditional kallikrein-related peptidase 3 (*KLK3*). *MSMB*, also known as prostate secretory protein, is a nearly prostate-specific protein secreted by the luminal epithelial cells in the prostate. *MSMB* is one of the three most abundant proteins secreted by the prostate^[20,21]. The *MSMB* gene, located on chromosome 10q11.2, is abundantly and specifically expressed in prostate tissue. These characteristics make it an attractive reference gene, expected

to provide more accurate and reliable normalization of *PCA3* expression levels^[22].

The study population included men with suspected PCa. To investigate the feasibility of utilizing non-DRE urine as an alternative to DRE urine for PCa diagnosis, morning urine and post-DRE urine samples were collected, and *PCA3* expression in urinary sediment was measured using RT-qPCR. Combining the *PCA3* levels with clinicopathologic findings, we evaluated the diagnostic performance of the assay for PCa detection.

2 | MATERIALS AND METHODS**2.1 | Study population**

Three prospective multicenter studies were conducted at three medical centers (West China Hospital of Sichuan University, the Second Affiliated Hospital Zhejiang University School of Medicine, and the First Affiliated Hospital of Wenzhou Medical University) during February to May 2024 (Cohort I), May to December 2024 (Cohort II), and January to March 2025 (Cohort III). A total of 674 subjects were initially enrolled; 31 were excluded due to their urine RNA concentrations was below 5 ng/ μ L or A260/A280 ratios fell outside the 1.8–2.1 range. Thus, 643 subjects were included in the final analysis. Urine samples from Cohort I ($n = 176$) and Cohort II ($n = 350$) were collected after DRE, while paired DRE and non-DRE samples from the same patients were collected for Cohort III ($n = 117$). Participants included men scheduled for prostate biopsy who meet at least one of the following criteria: (1) suspicious prostate nodule on DRE; (2) suspicious lesion identified by transrectal prostate ultrasound (TRUS) or magnetic resonance imaging (MRI); (3) serum PSA > 10 ng/mL; (4) serum PSA 4–10 ng/mL, with abnormal f/t PSA (free PSA/total PSA) or prostate-specific antigen density (PSAD). Patients who had been treated with anti-androgen drugs within 1 year or had a previous history of PCa or surgery for PCa were excluded. Pathological examination of biopsies was performed by urologic pathologists at each medical center. The study was performed in accordance with the Declaration of Helsinki. The study protocols were approved by the Ethics Committee of West China Hospital of Sichuan University (approval number: HX-IRB-AF-I2-V4.0), the Second Affiliated Hospital Zhejiang University School of Medicine (approval number: 2024-MD-365), and the First Affiliated Hospital of Wenzhou Medical University (approval number: FAHWMU-2024-PMD-140), and written informed consent was obtained from each participant.

2.2 | Sample collection and processing

A 20 mL of DRE urine was collected following a standardized DRE^[23]. A 40 mL of non-DRE urine was collected from the first morning urine. Urine samples were collected using a disposable sampler (Hangzhou York Biotech, Zhejiang Hangzhou Instrument Record No. 20190694), a collection tube containing 10 mL of urine preservation solution. Samples were shipped to the central laboratory at room temperature and immediately stored at a 4°C.

Nucleic acid extraction was performed within 5 days of collection using a nucleic acid extraction kit (Hangzhou York Biotech, Zhejiang Hangzhou Instrument Record No. 20190691) following the manufacturer's instructions. RNA concentration and purity were measured using NanoDrop (Thermo Fisher, NanoDrop One, USA). Subjects with sample RNA concentrations below 5 ng/μL or A260/A280 ratios outside 1.8–2.1 were excluded.

2.3 | RT-qPCR assay

PCA3 transcript levels were quantified in the urine sediment using the time-resolved fluorescence-based quantitative RT-qPCR protocol. *KLK3* or *MSMB* served as the reference gene for relative quantification.

For reverse transcription, each reaction mix contained 4 μL reverse transcription premix (ABclonal, Cat. No. RK20403, China), and 16 μL of RNA samples to make a final volume of 20 μL. Reverse transcription was carried out at 37°C for 25 min followed by heat inactivation at 85°C for 10 s.

For PCR amplifications, each reaction mix contained 15 μL PCR premix, (HuanKai Microbial, Cat. No. HKE091), 1.25 μL of 10 μmol/L forward primer, 1.25 μL of 10 μmol/L reverse primer, 2.5 μL of probe, 10 μL of cDNA template to make a final volume of 30 μL. The *PCA3*-specific primers were as follows: forward, 5'-CAC AAAAGGAAGCACAGAGAT-3', and reverse, 5'-GTCTGT TTCCTCTCATTGGT-3'. The *MSMB*-specific primers were as follows: forward, 5'-CCAGGAGATTCAACCAGG AA-3', and reverse, 5'-CAGTCTGCCACTCCGAGTTT-3'. The *KLK3*-specific primers were as follows: forward, 5'-GCATTGAACCAGAGGAGTTCT-3', and reverse, 5'-CAC AGCATGAACCTTGGTCA-3'. Reactions were performed with the qPCR system in the following conditions: 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 10 s and annealing/extension at 60°C for 30 s.

2.4 | Evaluation of precision, linearity, and anti-interference capability of the RT-qPCR system for *PCA3/MSMB* test

To assess the precision of RT-qPCR system for *PCA3/MSMB* test, twenty RT-qPCR analyses were performed on

strongly positive (mixture of 15 000 copies/μL for both *PCA3* RNA and *MSMB* RNA) and weakly positive samples (mixture of 200 copies/μL for both *PCA3* RNA and *MSMB* RNA). Cycle threshold (Ct) values and the coefficients of variation were calculated. The *PCA3* RNA and *MSMB* RNA reference standards were synthesized by Huzhou Hippo Biotechnology Co., Ltd. The copy number concentrations of *PCA3* and *MSMB* RNA were converted by the following formula: Concentration of copy number (copies/μL) = $\frac{N(\text{ng} / \mu\text{L})}{M(\text{g} / \text{mol})} \times 6.02 \times 10^{14}$ where *N* is the RNA concentration detected by NanoDrop (Thermo Fisher, NanoDrop One, USA) and *M* is the molecular mass of the RNA fragments.

To assess the linearity, 10-fold serial dilutions (1×10^2 to 1×10^6 copies/μL) of *PCA3* and *MSMB* RNA mixtures were tested in triplicate. The correlation coefficients between the Ct values and the logarithmic values of the corresponding RNA concentrations of *PCA3* and *MSMB* were calculated.

To assess the anti-interference capability, 20 potential interfering substances were added to urine samples from both healthy men and PCa patients. The twenty interfering substances were: 2 g/L hemoglobin, 60 g/L albumin, 0.35 mmol/L bilirubin, 1.5 mmol/L uric acid, 60 mmol/L glucose, 40 mmol/L cholesterol, 0.4 g/L vitamin C, 1.5 mmol/L acetaminophen, 5% whole blood, 50 μmol/L levofloxacin, 12 μmol/L penicillin, 1.2 μmol/L doxazosin, 25 mg/L atorvastatin, 0.3 mmol/L metformin, 30 mg/L al-fuzosin, 0.3 mmol/L allopurinol, 0.25 μmol/L amlodipine, 5×10^6 CFU/mL *Escherichia coli*, 5×10^6 CFU/mL *Staphylococcus aureus*, and 5×10^6 CFU/mL *Candida albicans*. Urine samples from healthy men and PCa patients without added interfering substances were used as controls. The *PCA3/MSMB* test was performed on the interference and control samples. Anti-interference was evaluated by comparing Ct values and diagnostic accuracy between interference and control samples. Compared with the control samples, the Ct values and ΔCt values of the samples containing interfering substances showed no significant differences. Moreover, in urine containing interfering substances, the diagnostic accuracy of the *PCA3/MSMB* test remained 100% (with the cut-off value set at 5.32), indicating that these substances did not interfere with the RT-qPCR assay.

2.5 | Statistical analysis

Statistical analyses were performed using GraphPad Prism (version 8.0.2) and MedCalc (version 23.0.1). For comparison between two groups of continuous variables, the Welch *t* test or the Mann-Whitney *U* test was used as a nonparametric alternative when the data failed the Kolmogorov-Smirnov test for normality. For comparisons among three groups of continuous variables, one-way analysis of variance (ANOVA) or the Kruskal-Wallis test was applied accordingly. Qualitative variables were

compared using the chi-square test. Pearson's correlation coefficient was calculated for correlations analyses. The sensitivity and specificity of diagnostic performance were evaluated using receiver operating characteristic (ROC) curves, with the AUC serving as a measure of the marker's discriminative efficacy. The 95% confidence intervals (CIs) and comparisons of AUCs were performed using MedCalc software. Paired samples were compared using DeLong's test, while independent samples were compared using the Hanley and McNeil method. Youden index was used to determine the optimal cut-off value for model scores. Statistically significant differences were set at $p < 0.05$. ****: $p < 0.0001$.

3 | RESULTS

3.1 | Patient characteristics

A total of 643 urine samples were collected in three independent prospective clinical trials (Cohort I: $n = 176$; Cohort II: $n = 350$; Cohort III: $n = 117$). Table 1 summarizes the patient characteristics. In Cohort I, 95 of 176 men (53.98%) had positive biopsy results, compared with 190 of 350 men (54.29%) in the Cohort II and 55 of 117 men (47.01%) in Cohort III. No statistically significant differences in baseline characteristics were observed among the cohorts, except for fPSA levels.

3.2 | Precision, linearity, and anti-interference capability of the RT-qPCR system for *PCA3/MSMB* test

As shown in Figure 1A,B, the coefficients of variation (CVs) of Ct values in strongly positive samples were

0.18% for *PCA3* and 0.23% for *MSMB*. For weakly positive samples, the CVs were 0.46% and 0.27%, respectively. These results demonstrate the high precision of the RT-qPCR assay. In terms of linearity, the correlation coefficients between Ct values and the logarithmic values of *PCA3* and *MSMB* RNA concentrations were -0.9999 and -1.0000 , respectively, indicating the near-perfect linearity of the RT-qPCR assay (Figure 1C,D). In samples containing interfering substances, the diagnostic accuracy of the *PCA3/MSMB* test remained 100% (cut-off value: 5.32), and the differences in Ct and Δ Ct values relative to control samples were less than 1 and not statistically significant (Figure 1E,F and Table S1). These results demonstrate that the *PCA3/MSMB* RT-qPCR assay exhibits potent anti-interference capability and performs reliably in complex urine matrices.

3.3 | Diagnostic performance

A total of 176 urine samples with corresponding clinical diagnostic data were collected from 95 PCa patients and 81 benign patients in Cohort I, serving as the training cohort. *PCA3* expression levels were detected by RT-qPCR using *MSMB* as the reference gene (*PCA3/MSMB* test). For comparison, *PCA3* expression was also normalized to the commonly used reference gene *KLK3* (*PCA3/KLK3* test).

The diagnostic performance of these biomarkers is summarized in Table 2. The AUC of the *PCA3/MSMB* test was 0.869 (95% CI: 0.810–0.915; $p < 0.0001$), with an optimal cut-off value of 5.32, a sensitivity of 82.1%, and a specificity of 77.8%. The AUC of the *PCA3/KLK3* test was 0.830 (95% CI: 0.767–0.883; $p < 0.0001$), with an optimal cut-off value of 3.72, a sensitivity of 79.0%, and a specificity of 74.1% (Figure 2A). AUC of the *PCA3/MSMB* test

TABLE 1 Patient characteristics.

Characteristics	Cohort I	Cohort II	Cohort III	<i>p</i> -value
Patients, <i>n</i>	176	350	117	/
Age, year, mean (median; IQR)	67.81 (68.00, 63.00–73.00)	68.01 (68.00, 63.00–73.00)	68.46 (68.00, 63.00–74.00)	0.8820
tPSA, ng/mL, mean (median; IQR)	18.79 (10.10, 6.570–15.10)	19.27 (10.09, 6.728–16.80)	19.50 (11.81, 6.963–17.62)	0.2816
fPSA, ng/mL, mean (median; IQR)	2.757 (1.454, 0.9423–2.961)	2.477 (1.475, 0.9710–2.457)	3.099 (2.036, 1.183–3.235)	0.0311
f/tPSA, mean (median; IQR)	0.1777 (0.1600, 0.1100–0.2200)	0.1701 (0.1500, 0.1000–0.2200)	0.1844 (0.1600, 0.1075–0.2325)	0.6217
PCa diagnosis, <i>n</i> (%)	95 (53.98)	190 (54.29)	55 (47.01)	0.3713
ISUP, mean	2.653	2.758	3.026	0.6580
GS 6, <i>n</i> (%)	24 (25.26)	42 (22.11)	7 (18.42)	0.7217
GS 7, <i>n</i> (%)	45 (47.37)	89 (46.84)	16 (42.11)	
GS 8–10, <i>n</i> (%)	26 (27.37)	59 (31.05)	15 (39.47)	

Note: PSA, ISUP, or GS information was not available for some patients, and only the information obtained was included in the statistics.

Abbreviations: f/tPSA, the ratio of free to total PSA; fPSA, free PSA; GS, Gleason score; IQR, interquartile range; ISUP, International Society of Urological Pathology grading of PCa; PCa, prostate cancer; tPSA, total prostate-specific antigen.

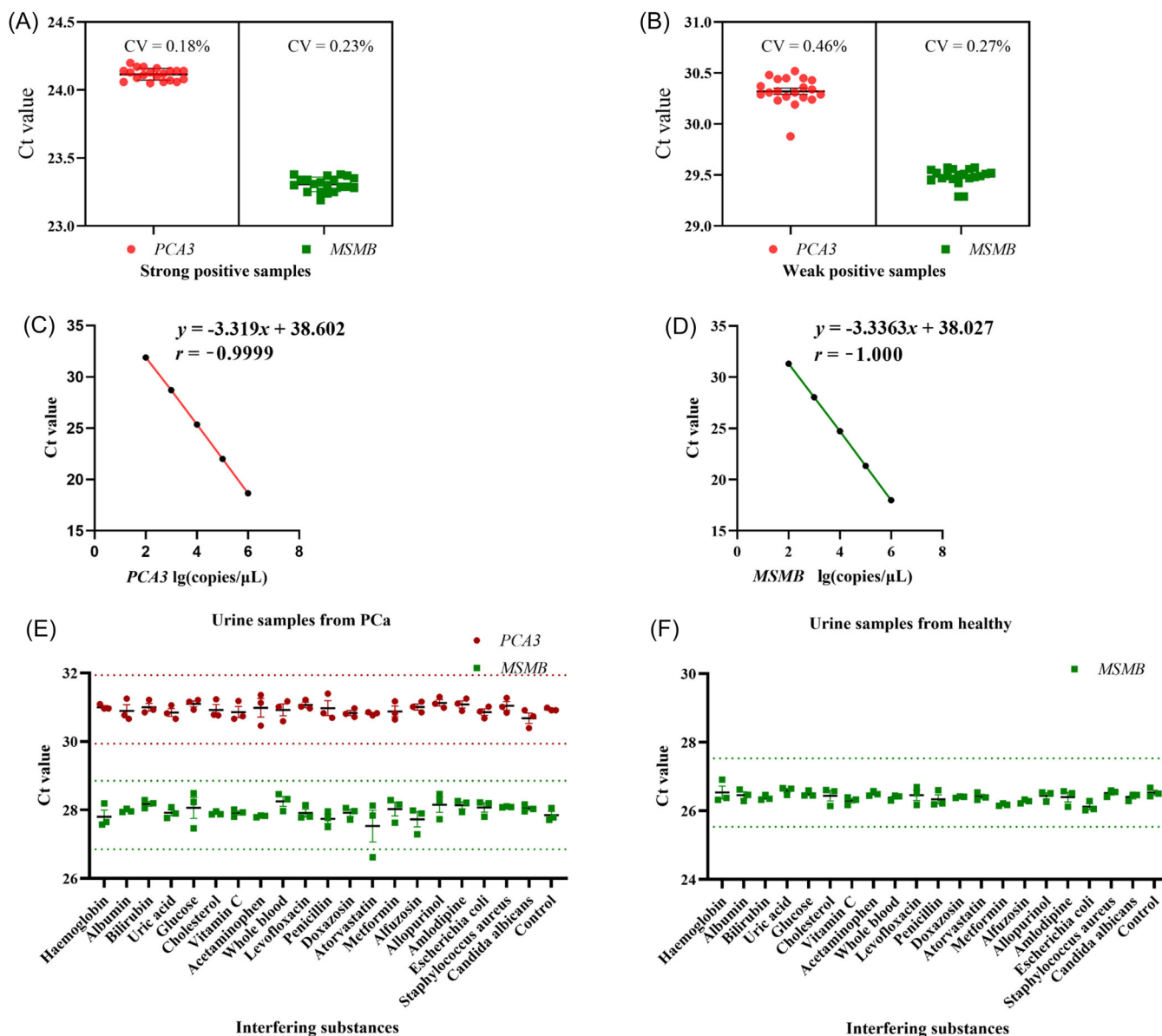


FIGURE 1 Precision, linearity, and anti-interference capability of reverse transcription-quantitative polymerase chain reaction (RT-qPCR). (A, B) Twenty RT-qPCR analyses were performed on strongly positive samples and weakly positive samples. (C, D) The coefficients of variation (CVs) for Ct values of both *PCA3* and *MSMB* were less than 0.5%, which indicates that the RT-qPCR analyses have high precision. The correlation coefficients between Ct values and the logarithmic values of *PCA3* and *MSMB* RNA concentrations were -0.9999 and -1.0000 , respectively, demonstrating near-perfect linearity of the RT-qPCR assay. (E, F) The difference in Ct values between the interference and control samples was less than 1, indicating that the *PCA3/MSMB* test has a potent anti-interference capability. Dashed line: ± 1 range of the mean *PCA3* (red) or *MSMB* (green) Ct values of control samples.

was significantly higher than that of the *PCA3/CLK3* test ($p = 0.0468$), confirming that *MSMB* is a more effective reference gene than *CLK3*. Using *PCA3* alone yielded an AUC of 0.764, and adding *MSMB* significantly improved performance (AUC 0.869 vs. 0.764; $p = 0.0055$). In contrast, adding *CLK3* did not produce a statistically significant improvement (AUC 0.830 vs. 0.763; $p = 0.0856$).

In the “gray zone” subgroup (4.0–10.0 ng/mL), *MSMB* also outperformed *CLK3*. As shown in Table 3, within this subgroup (Cohort I), the *PCA3/MSMB* test

achieved an 80.6% diagnostic accuracy (95% CI: 69.2%–88.6%), with an 87.9% sensitivity (95% CI: 70.9%–96.0%) and 74.4% specificity (95% CI: 57.6%–86.4%). In comparison, the *PCA3/CLK3* test showed 76.4% accuracy (95% CI: 64.7%–85.3%), 81.8% sensitivity, and 71.8% specificity. Clinically, this translates to fewer unnecessary biopsies (25.6% false-positive rate for *PCA3/MSMB* vs. 28.9% for *PCA3/CLK3*) and reduced missed diagnoses (12.1% false-negative rate vs. 17.6%) within this clinically challenging subgroup. As shown in Figure 2B, the Ct value for *MSMB* were significantly lower than

TABLE 2 Diagnostic performance of *PCA3*, reference genes and *PCA3* combined reference genes.

	AUC	Cut-off value	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
<i>KLK3</i> Ct	0.536	29.71	78.9	33.3	58.14	57.45
<i>MSMB</i> Ct	0.485	26.53	51.6	60.5	60.49	51.58
<i>PCA3</i> (<i>KLK3</i> batch) Ct	0.763	32.92	84.2	61.7	72.07	76.92
<i>PCA3</i> (<i>MSMB</i> batch) Ct	0.764	32.94	86.3	63.0	73.21	79.69
<i>PCA3/KLK3</i> Δ Ct	0.830	3.72	79.0	74.1	78.13	75.0
<i>PCA3/MSMB</i> Δ Ct	0.869	5.32	82.1	77.8	81.25	78.75

Abbreviations: AUC, area under receiver operating characteristic curve; *KLK3*, kallikrein-related peptidase 3; *MSMB*, microseminoprotein-beta; NPV, negative predictive value; PPV, positive predictive value; *PCA3*, prostate cancer antigen 3; *PCA3/KLK3* Δ Ct, Ct [*PCA3*]-Ct [*KLK3*]; *PCA3/MSMB* Δ Ct, Ct [*PCA3*]-Ct [*MSMB*].

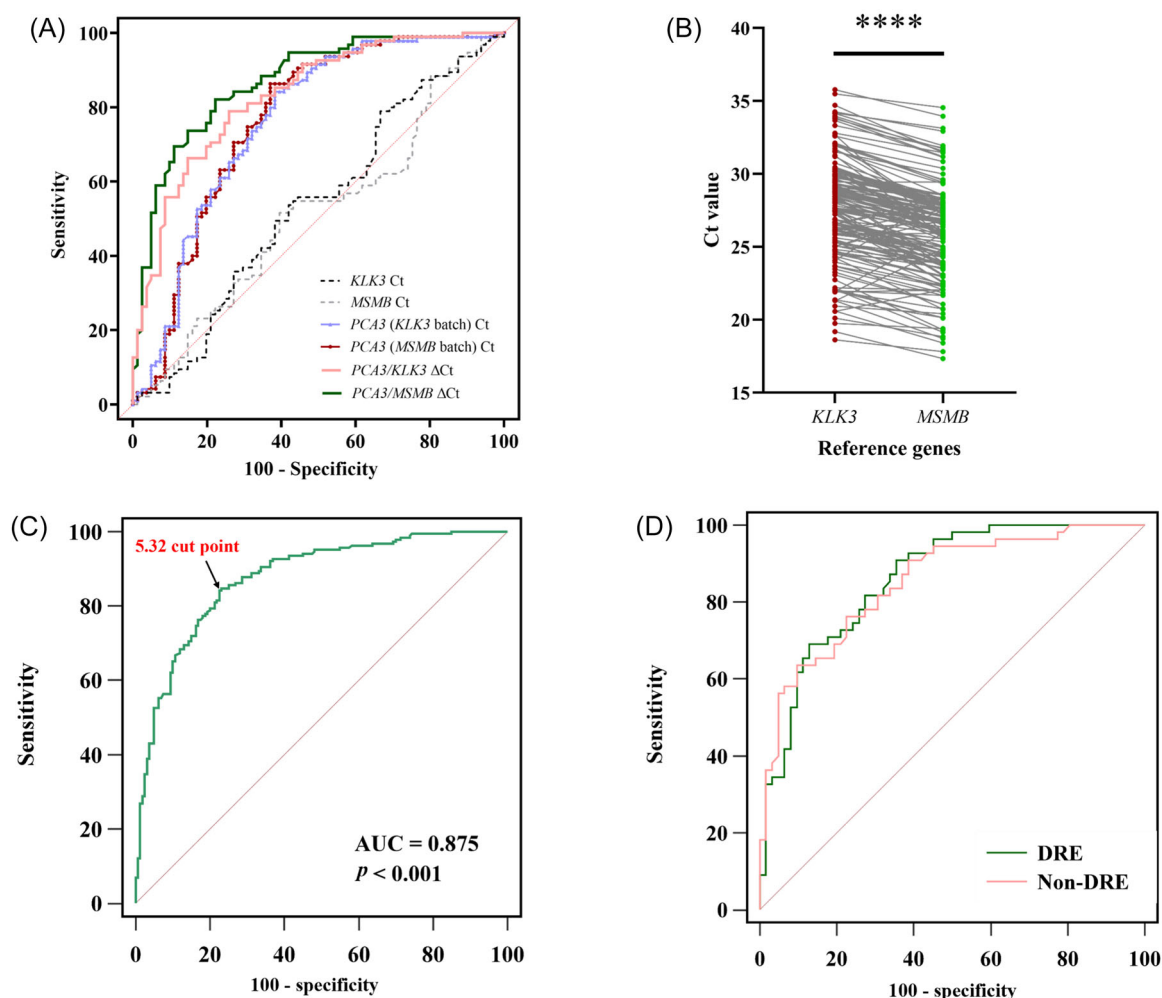


FIGURE 2 Diagnostic performance of predicting prostate cancer (PCa) by prostate cancer antigen 3 (*PCA3*)/microseminoprotein-beta (*MSMB*) test. (A) Receiver operating characteristic (ROC) curves of *PCA3*, reference genes and *PCA3* combined reference genes. (B) The Ct values for the *MSMB* and kallikrein-related peptidase 3 (*KLK3*) genes in identical samples in Cohort I. (C) ROC curve of predicting PCa by *PCA3/MSMB* test in Cohort II. (D) ROC curves for digital rectal examination (DRE) versus non-DRE urine in Cohort III. *PCA3/KLK3* Δ Ct, Ct [*PCA3*] - Ct [*KLK3*]; *PCA3/MSMB* Δ Ct, Ct [*PCA3*] - Ct [*MSMB*]; **** $p < 0.0001$.

TABLE 3 Diagnostic performance of *PCA3/MSMB* test or *PCA3/KLK3* test in patients with PSA 4-10 ng/mL in the Cohort I.

	<i>PCA3/MSMB</i> test		<i>PCA3/KLK3</i> test	
	Positive	Negative	Positive	Negative
Positive biopsy results	29	4	27	6
Negative biopsy results	10	29	11	28
Sensitivity (95% CI)	87.9% (70.9%–96.0%)		81.8% (63.9%–92.4%)	
Specificity (95% CI)	74.4% (57.6%–86.4%)		71.8% (54.9%–84.5%)	
PPV (95% CI)	74.4% (57.6%–86.4%)		71.1% (53.9%–84.0%)	
NPV (95% CI)	87.9% (70.9%–96.0%)		82.4% (64.8%–92.6%)	
False-positive (95% CI)	25.6% (13.6%–42.4%)		28.9% (7.4%–35.2%)	
False-negative (95% CI)	12.1% (4.0%–29.1%)		17.6% (16.0%–46.1%)	
Diagnostic accuracy (95% CI)	80.6% (69.2%–88.6%)		76.4% (64.7%–85.3%)	

Abbreviations: 95% CI, 95% confidence interval; NPV, negative predictive value; PPV, positive predictive value.

those for *KLK3* in identical samples, indicating a higher detectability of the *MSMB* and enabling reliable measurement from smaller sample volumes. In samples with low *KLK3* expression, the AUC value obtained using *MSMB* (AUC = 0.863) was higher than that using *KLK3* (AUC = 0.815); although this difference was not statistically significant (DeLong test $p = 0.2194$) (Figure S1).

3.4 | Diagnostic performance validation

In Cohort II ($n = 350$), using a cut-off value of 5.32, the *PCA3/MSMB* test showed a sensitivity of 82.63%, specificity of 77.50%, positive predictive value of 81.35%, and negative predictive value of 78.98% (Figure 2C and Table 4). These results closely matched those of Cohort I, confirming the robustness and reproducibility of the *PCA3/MSMB* assay.

To provide a simple value for informing patients, we convert the Ct value to the relative *PCA3* expression level using the following formula, referred to as *PCA3* index:

$$PCA3 \text{ Index} = 2^{-\Delta Ct} \times 1000,$$

where ΔCt is the Ct value of *PCA3* minus the Ct value of *MSMB*, that is, $\Delta Ct = Ct [PCA3] - Ct [MSMB]$. For cut-off value of 5.32, the *PCA3* index equals 25. A *PCA3* index higher than 25 suggests a high risk of *PCa*.

3.5 | Diagnostic performance of DRE versus non-DRE urine samples

To investigate whether non-DRE urine samples provide the same diagnostic performance as DRE urine samples, 117 paired DRE and non-DRE urine samples were collected from the same patients in Cohort III. *PCA3/*

TABLE 4 Diagnostic performance of *PCA3/MSMB* test in Cohort II with a cut-off value of 5.32.

	Biopsy result		Total
	Positive	Negative	
<i>PCA3/MSMB</i> $\Delta Ct \leq 5.32$	157	36	193
<i>PCA3/MSMB</i> $\Delta Ct > 5.32$	33	124	157
Total	190	160	350
Sensitivity		82.63%	
Specificity		77.50%	
PPV		81.35%	
NPV		78.98%	

Abbreviations: *MSMB*, microseminoprotein-beta; NPV, negative predictive value; *PCA3*, prostate cancer antigen 3; PPV, positive predictive value.

MSMB test was performed on all samples (Figure 2D). The AUC values of DRE and non-DRE urine samples were 0.859 (95% CI: 0.783–0.917; $p < 0.0001$) and 0.850 (95% CI: 0.772–0.909; $p < 0.0001$), respectively, demonstrating comparable diagnostic accuracy ($p = 0.6262$ difference of AUCs). These findings indicate that non-DRE urine samples have the potential to replace DRE urine samples, thereby simplifying specimen collection.

4 | DISCUSSION

This study employed the novel reference gene *MSMB* to establish the *PCA3/MSMB* test, which demonstrates superior diagnostic performance. It demonstrated that the test holds potential to replace DRE urine samples with non-DRE urine samples, thereby simplifying sample collection. Multiple studies and meta-analyses have evaluated the diagnostic accuracy of *PCA3*, almost all of which have used post-DRE urine and *KLK3* as the

reference gene^[18,24–27]. A comprehensive meta-analysis of 46 clinical trials involving 12 295 subjects reported that the pooled sensitivity of 0.65, specificity of 0.73, and AUC of 0.75, indicating acceptable diagnostic performance^[28]. The present study developed a highly sensitive and specific diagnostic using a novel reference gene *MSMB* and demonstrated improved diagnostic accuracy in both DRE and non-DRE urine samples. This improvement has the potential to reduce reliance on invasive biopsies.

In this study, the RT-qPCR system for the *PCA3/MSMB* test exhibited strong anti-interference capability, high precision, and excellent linearity, confirming its reliability in complex clinical settings. These analytical validation results support its suitability as a stable and robust diagnostic assay.

The diagnostic performance of the *PCA3/MSMB* test was validated in Cohort I, with a sensitivity of 82.1% and a specificity of 77.8%, which are comparable to or exceed those reported in previous *PCA3*-based studies^[18,29,30]. Furthermore, the *PCA3/MSMB* test demonstrated higher diagnostic accuracy than the *PCA3/KLK3* test, especially in the PSA gray zone to reduce over-biopsies and underdiagnosis. These results suggest that *MSMB* is a more reliable reference gene for *PCA3* testing than the traditional *KLK3* in PCa diagnosis.

The Cohort II further confirmed the diagnostic performance of the *PCA3/MSMB* test, with similar sensitivity and specificity to the Cohort I. The similar sensitivity and specificity observed across independent cohorts enhance confidence in the robustness and reproducibility of this assay. This is particularly important given the heterogeneity of PCa and the need for accurate and consistent biomarkers to guide clinical decision-making^[31,32].

Current *PCA3* and other molecular assays generally require post-DRE urine collection, adding complexity to clinical workflows. This study investigated whether non-DRE urine samples could achieve similar diagnostic performance to DRE urine samples. The results showed that non-DRE urine had comparable diagnostic performance. This finding offers substantial clinical advantages: non-DRE sampling supports home-based collection, simplifies primary care workflows, facilitates repeat sampling, and improves overall patient experience by eliminating DRE-related discomfort. This is particularly useful for patients on active surveillance, in whom periodic noninvasive monitoring is essential. The specific mechanism by which the *PCA3/MSMB* test maintains stability without DRE remains incompletely elucidated. This may be attributed to the superior detectability of *MSMB*, thereby sustaining detection performance with a limited number of exfoliated cells present in morning urine. Further studies are warranted to elucidate these potential mechanisms.

We acknowledge several limitations of this study. First, all participants were Chinese men; therefore,

generalizability to other ethnic groups requires further validation. Second, pathological evaluation was performed by local pathologists at each center without centralized review, so the impact of inter-observer variability in Gleason (ISUP) grading was not assessed. Third, the study focused on diagnostic accuracy at the time of biopsy and did not include long-term outcomes; thus, the prognostic value of the *PCA3/MSMB* test remains unknown. Fourth, although PSAD was an inclusion criterion, prostate volume data were not collected, limiting further analysis of the relationship between PSAD and *PCA3/MSMB* performance. Finally, head-to-head comparisons with other commercially available biomarker panels (e.g., PHI, 4K score) were not performed, and future studies should evaluate the comparative clinical and economic value of *PCA3/MSMB* testing.

Clinically, the *PCA3/MSMB* test should be used as an adjunctive tool in patients with equivocal or borderline diagnostic findings. In the PSA gray zone (4.0–10.0 ng/mL), a positive *PCA3/MSMB* result favors proceeding with biopsy, whereas a negative result may support deferring biopsy in favor of multiparametric MRI (mpMRI) and close follow-up. However, a negative *PCA3/MSMB* test does not eliminate the need for biopsy in patients with high-risk features such as suspicious DRE findings or PI-RADS ≥ 4 lesions on mpMRI. Conversely, in patients with low PSA (< 4.0 ng/mL) but concerning clinical findings, a positive *PCA3/MSMB* result may justify closer surveillance or biopsy.

We acknowledge that several authors are employees of Hangzhou York Biotech Co., Ltd., the manufacturer of the assay kits used in this study. To minimize potential bias, the study design oversight, clinical recruitment, sample collection, and pathological diagnosis were independently conducted by three academic medical centers. All biopsy results were assessed by urologic pathologists blinded to assay results. Data analyses were performed jointly by company and academic investigators, and independent verification was carried out before manuscript submission. All authors had unrestricted access to the raw data and participated in data interpretation and manuscript approval. The entire research process was supervised by the ethics committees of all participating institutions, and academic independence was maintained throughout study design, result interpretation, and manuscript preparation. These procedures were implemented to ensure the objectivity and scientific integrity of the study.

5 | CONCLUSION

The *PCA3/MSMB* urine test is a noninvasive and accurate tool for PCa diagnosis, outperforming *PCA3/KLK3* assay. Its compatibility with non-DRE samples simplifies clinical implementation, and this promising noninvasive

diagnostic method has the potential to reduce unnecessary biopsies and improve the overall accuracy of PCa detection.

AUTHOR CONTRIBUTIONS

Jianglei Xu contributed to conceptualization, data curation, formal analysis, validation, investigation, methodology, writing—original draft, and writing—review and editing. Lu Yang and Cong Shi were involved in data curation, formal analysis, visualization, resources, investigation, and writing—review. Yiwei Jiang and Jing Chen were involved in visualization, resources, investigation, and writing—review. Xiaoxiao Sun, Xian Wang, and Jianzhong Jiang contributed to data curation, visualization, methodology, and writing—review. Xuejuan Gao contributed to data curation, formal analysis, visualization, supervision, and writing—review. Xiuzhi Duan, Xuchu Wang, and Yiyi Xie were involved in resources, data curation, visualization, and writing—review. Zhixian Yu and Zhihua Tao contributed to resources, supervision, data curation, methodology, project administration, and writing—review and editing. Xiaobing Dou contributed to resources, supervision, methodology, project administration, and writing—review and editing. Chunyu Jin contributed to conceptualization, formal analysis, methodology, supervision, funding acquisition, project administration, and writing—review and editing.

CONFLICT OF INTEREST STATEMENT

Hangzhou York Biotech Co., Ltd. is the manufacturer of the disposable sampler, nucleic acid extraction kits, and RT-qPCR reagent kits used in this study. Authors Jianglei Xu, Cong Shi, Xiaoxiao Sun, Xuejuan Gao, Xian Wang, Jianzhong Jiang, and Chunyu Jin are employees of Hangzhou York Biotech Co., Ltd. These employee-authors were involved in assay development, optimization, and validation, centralized laboratory testing, data curation and initial statistical analyses, and participated in drafting and revising the manuscript. Study design oversight, clinical recruitment, sample collection, and pathological diagnosis were performed by investigators at the independent academic medical centers (West China Hospital of Sichuan University, the Second Affiliated Hospital Zhejiang University School of Medicine, and the First Affiliated Hospital of Wenzhou Medical University), and pathology was reported by local urologic pathologists blinded to assay results. Data analysis and interpretation were conducted collaboratively between company employees and independent academic co-authors; all authors had full access to the complete dataset and took final responsibility for the integrity of the data and the accuracy of the data analysis and interpretation. None of the company-employed authors hold equity or stock in Hangzhou York Biotech. A patent related to the disposable sampler ("Urine sample RNA stabilizing solution and preparation

method," application number: CN201911102059.0) has been filed. No patents related specifically to *MSMB* as a reference gene have been submitted. This study was funded by the Zhejiang Provincial Leading Project for Leading Geese Plan (2023C03059). The remaining authors no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. This prospective study was approved by the Ethics Committee of West China Hospital of Sichuan University (approval number: HX-IRB-AF-I2-V4.0), the Second Affiliated Hospital Zhejiang University School of Medicine (approval number: 2024-MD-365), and the First Affiliated Hospital of Wenzhou Medical University (approval number: FAHWMU-2024-PMD-140). Informed consent was obtained from all individual participants included in the study.

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SUPPORTING INFORMATION

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