IVYSPRING INTERNATIONAL PUBLISHER

Theranostics

2016; 6(9): 1415-1424. doi: 10.7150/thno.15250

Research Paper

Colorimetric *TMPRSS2-ERG* Gene Fusion Detection in Prostate Cancer Urinary Samples via Recombinase Polymerase Amplification

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Received: 2016.02.12; Accepted: 2016.04.27; Published: 2016.06.15

Abstract

TMPRSS2 (Exon 1)-*ERG* (Exon 4) is the most frequent gene fusion event in prostate cancer (PC), and is highly PC-specific unlike the current serum prostate specific antigen (PSA) biomarker. However, *TMPRSS2-ERG* levels are currently measured with quantitative reverse-transcription PCR (RT-qPCR) which is time-consuming and requires costly equipment, thus limiting its use in clinical diagnostics. Herein, we report a novel rapid, cost-efficient and minimal-equipment assay named "FusBLU" for detecting *TMPRSS2-ERG* gene fusions from urine. *TMPRSS2-ERG* mRNA was amplified by isothermal reverse transcription-recombinase polymerase amplification (RT-RPA), magnetically-isolated, and detected through horseradish peroxidase (HRP)-catalyzed colorimetric reaction. FusBLU was specific for *TMPRSS2-ERG* mRNA with a low visual detection limit of 10^5 copies. We also demonstrated assay readout versatility on 3 potentially useful platforms. The colorimetric readout was detectable by naked eye for a quick yes/no evaluation of gene fusion presence. On the other hand, a more quantitative *TMPRSS2-ERG* detection was achievable by absorbance/electrochemical measurements. FusBLU was successfully applied to 12 urinary samples and results were validated by gold-standard RT-qPCR. We also showed that sediment RNA was likely the main source of *TMPRSS2-ERG* mRNA in urinary samples. We believe that our assay is a potential clinical screening tool for PC and could also have wide applications for other disease-related fusion genes.

Key words: *TMPRSS2-ERG*, FusBLU

Introduction

Prostate cancer (PC) is the most common form of cancer and the second leading cause of cancer death in males of developed countries [1]. The customary screening approach for prostate biopsies is mainly based on high serum prostate specific antigen (PSA) levels and/or abnormal digital rectal examination (DRE). PSA is the primary PC biomarker used by clinical practitioners and has led to a significant increase in PC diagnosis since its introduction [2]. However, due to its low PC-specificity, the majority of PSA-diagnosed cases are actually benign and do not require medical intervention. This has in turn led to redundant medical expenses as well as negative effects on patients' overall well-being [3]. Thus, there is a pressing need for better PC diagnostic biomarkers, particularly one with high PC-specificity for discriminating benign cases from aggressive PC cases.

In 2005, Tomlins and co-workers identified recurrent PC fusion genes between the promoter of transmembrane protease, serine 2 (*TMPRSS2*) and the coding sequence of erythroblastosis virus E26 (*ETS*)

family members [4]. These chromosomal rearrangements-induced fusions resulted in androgen-dependent overexpression of oncogenic *ETS* transcription factors [5]. The fusion of *TMPRSS2* exon 1 to *ERG* exon 4 (*TMPRSS2-ERG*) is the most frequent subtype of these gene fusions, appearing in about 50% of PC patients and 90% of all PC gene fusions [6]. More importantly, *TMPRSS2-ERG* is highly PC-specific and absent in non-PC samples. Furthermore, *TMPRSS2-ERG* may correlate with PC aggression and metastatic potential [7-9]; potentially making them more attractive as diagnostic and prognostic biomarkers than serum PSA.

TMPRSS2-ERG mRNA is commonly detected from the cell sediment fraction of urine [10, 11]. Recently, Nilsson and co-workers had reported *TMPRSS2-ERG* detection in urinary exosomes [12]. Additionally, it is also highly plausible that *TMPRSS2-ERG* mRNA is freely circulating within the urine sample. Given that *TMPRSS2-ERG* is detectable from multiple sources within urine [13], it could be worth investigating the primary source (circulating free mRNA in whole urine, cellular RNA or exosomal RNA) of the fusion mRNA. This could potentially lead to a more in-depth understanding of the cancer biology and aid in future assay development.

Currently, quantitative reverse transcription PCR (RT-qPCR) is mainly used to measure *TMPRSS2-ERG* mRNA levels in urinary samples [4, 10, 14]. However, this approach is too time-consuming and laborious for use in a clinical setting. Therefore, the development of newer, faster and convenient assays for quantifying *TMPRSS2-ERG* mRNA in clinical samples may aid in improved PC screening and treatment monitoring.

Colorimetric assays have been developed for a wide range of target analytes and offer benefits such as low cost, short assay time, visual readout, and quantitative detection via absorbance measurements [15]. In particular, the reaction between tetramethyl benzidine (TMB) and horseradish peroxidase (HRP) in presence of hydrogen peroxide (H_2O_2) to produce a blue-colored product [16] has been employed in many colorimetric assays, with ELISA as a well-known example. Additionally, it has been demonstrated by previous studies [16-18] that TMB could serve as an electrochemical substrate. Given the excellent simplicity, sensitivity and speed of electrochemical biosensors [19-21], it may be ideal to adapt TMB-based colorimetric assays for alternative electrochemical readouts to exploit such advantages. Therefore, we hypothesized that a TMB-based assay which displays a quantitative colorimetric/ electrochemical signal change in *TMPRSS2-ERG* presence could be potentially useful for diagnostic purposes.

Herein, we describe the FusBLU assay, a novel approach to detect and quantify *TMPRSS2-ERG* fusion mRNA. By the innovative merger of isothermal reverse-transcription recombinase polymerase reaction (RT-RPA) and HRP-catalyzed colorimetric readout, *TMPRSS2-ERG* mRNA in urine could be specifically amplified and detected by naked-eye or by quantitative absorbance measurements. The versatility of the assay readout was further shown by conveniently performing FusBLU on a portable potentiostat for electrochemical detection. The practical application of our assay was demonstrated using PC cell lines and patient urinary samples. We also applied our assay to detect *TMPRSS2-ERG* mRNA in whole urine, urinary sediments and exosomes of PC urinary specimens in order to investigate the primary source of the mRNA.

Materials and Methods

RNA extraction from cell lines

DuCap and LnCap cells were kindly donated by Matthias Nees (VTT, Finland); Gregor Tevz (APCRC, Australia); and Michelle Hill (UQDI, Australia). DuCap and LnCap cells were cultured in RPMI-1640 growth media (Life Technologies, Australia) supplemented with 10% fetal bovine serum (Life Technologies, Australia) in a humidified incubator (Sanyo MCO-19AIC, Japan) containing 5% CO_2 at 37 ºC. Total RNA was extracted by lysing cells with Trizol® reagent (Life Technologies, Australia) before adding chloroform to separate RNA into a clear upper aqueous layer. Then, RNA was precipitated from the aqueous layer and resuspended in 50 µL of RNase-free water. RNA purity and integrity were checked using a NanoDrop Spectrophotometer (Thermo Fisher Scientific, Australia)

RNA extraction from clinical urinary samples

Ethics approval was obtained from The University of Queensland Institutional Human Research Ethics Committee (Approval No. 2004000047). Informed consent was obtained from all subjects prior to sample collection and methods pertaining to clinical samples were carried out in accordance with approved guidelines.

For whole urine RNA extraction, 1 mL of fresh urine sample was briefly mixed with 500 µL of lysis buffer [22] (150 mM Tris-HCI pH 8.0, 4.5 M guanidium-HCI, 3% v/v Triton-X and 1.5 mM EDTA). Total RNA was purified from 25 µL of cell lysate using the Agencourt AMPure XP kit (Beckman Coulter, Australia). Briefly, two volumes of Solid Phase Reversible Immobilization (SPRI) reagent was incubated with the cell lysate for 10 minutes. The

RNA-bound magnetic beads were then separated from the lysate using a magnet and washed twice with 80% ethanol. Finally, total RNA was eluted in 25 µL of RNase-free water.

For urinary cell sediment RNA extraction, urinary samples (30–50 mL) were centrifuged (Beckman Coulter Allegra™ X-22R, Australia) at 700 *g*, 4ºC for 10 min and urinary sediments (supernatant was kept for exosomal RNA extraction) were washed with ice-cold 10 mM PBS buffer before being centrifuged again at the same conditions. Then, 25 µL of lysis buffer was added to a 50 µL fraction of PBS-suspended urinary sediments with vigorous mixing to release total RNA. Next, total RNA was purified from 25 µL of cell lysate using the procedure as described for whole urine RNA extraction.

For urinary exosomal RNA extraction, microvesicles in the supernatant (after urinary sediment centrifugation) was firstly pelleted by ultracentrifugation (Beckman Coulter Optima™ XL-100 K, Australia) at 100 000 g, 4ºC for 90 min and resuspended in 200 µL of 10 mM PBS buffer. Then, the Exo-spin™ kit (Cell Guidance Systems, USA) was used accordingly to manufacturer's instructions to isolate exosomes from the pellet. To ensure that extracted RNA was of exosomal origin, the isolated urinary exosomes were treated with RNase A (New England Biolabs, Australia) to degrade all non-exosomal RNA before RNA extraction. Exosomal total RNA was extracted by Trizol® reagent (Life Technologies, Australia) using the procedure as described for RNA extraction from cells.

Colorimetric FusBLU assay

Synthetic oligonucleotide and primer sequences used in our experiments were obtained commercially (IDT, Singapore). RT-RPA primer sequences were designed based on the fusion junction of the most common *TMPRSS2* (Exon 1)*:ERG* (Exon 4) mRNA isoform reported in literature [4]. The RT-qPCR primers were also designed to amplify the same target *TMPRSS2:ERG* isoform.

For cell line experiments, the TwistAmp Basic RPA kit (Twist-DX, UK) was used with slight modifications to manufacturer's instructions. Briefly, 1 µL of extracted RNA, 50 units of MMuLV reverse transcriptase (New England Biolabs, Australia), 375 nM of each primer (Table 1) and 20 nM of biotinylated dUTPs (Thermo Fisher Scientific, Australia) were added to make a 12.5 µL reaction volume prior to incubation at 43°C for 20 min. For all other experiments, the TwistAmp Basic RT-RPA kit (Twist-DX, UK) with pre-included reverse transcriptase was used with the same modifications to manufacturer's instructions. The 43ºC amplification temperature, which is above the manufacturer's recommended optimum temperature, was required for higher amplification specificity.

Table 1. Oligonucleotide sequences used in experiments.

Oligos	5'-Sequence-3'
RT-RPA TMPRSS2-ERG Fwd Primer Sequence	CCTGGAGCGCGGCAGGAAGCCTTATCAGTTG
RT-RPA TMPRSS2-ERG Rev Primer Sequence	GCTAGGGTTACATTCCATTTTGATGGTGAC
RT-RPA Housekeeping Fwd Primer Sequence	GCTATGCCGATCGGGTGTCCGCACTAAGTT
RT-RPA Housekeeping Rev Primer Sequence	GACGGGGTCTCGCTATGTTGCCCAGGCTGG
RT-qPCR Fwd Primer Sequence	CCTGGAGCGCGGCAGGAAGCCTTATCAGTTG
RT-qPCR Rev Primer Sequence	TCCTGCTGAGGGACGCGTGGGCTCATCTTG

For colorimetric detection of RT-RPA products, 2 µL of RT-RPA products was incubated with 1 µL of 1:1000 diluted streptavidin (SA)-HRP (BD Biosciences, Australia), 1 µL of SA-magnetic beads (New England Biolabs, Australia) and 10 µL of wash buffer (10 mM PBS buffer, 0.5% triton-X) for 5 min. Then, a magnet was used to separate the beads and beads were washed three times with wash buffer (10 mM PBS buffer, 0.1% Tween20). Lastly, 25 µL of 1-Step™ TMB substrate solution (Thermo Fisher Scientific, Australia) was added to the beads and the mixture was observed for color change after 5 min. For each sample, two separate RT-RPA assays were performed for independent detection of *TMPRSS2-ERG* and housekeeping (RN7SL1) RNA. For quantitative measurements of color change, absorbance readings were recorded at 650 nm with a NanoDrop Spectrophotometer (Thermo Fisher Scientific, Australia). For each sample, relative *TMPRSS2-ERG* level was calculated by normalizing the absorbance value of *TMPRSS2-ERG* to the respective housekeeping RN7SL1 as follows:

$$\text{Relative } \textit{TMPRSS2-ERG} \text{ Level} = X_{Tmprss2\text{-}ERG} \,/\, X_{\mathrm{RN7SL1}}$$

where $X_{Tmprss2:ERG}$ and X_{RN7SL1} are the average absorbance readings at 650 nm for *TMPRSS2-ERG* and housekeeping RN7SL1 RNA respectively.

Alternative electrochemical readout

For electrochemical detection of RT-RPA products, total RNA was isolated from patient urinary sediments and after which, the same steps as described previously for colorimetric FusBLU assay were performed. At 5 min after TMB-H_2O_2 addition, 500 mM H_2SO_4 was added to stop the reaction and activate TMB for electrochemical detection. The electrodes used in our experiments were

screen-printed electrodes (DRP110, Dropsens, Spain) with carbon as working and counter electrodes, and silver as reference electrode. Briefly, 45 µL of the resulting mixture after H_2SO_4 addition was pipetted onto the electrode surface, and ensured that the solution was in contact with all three electrode types (i.e. working, counter, and reference). Amperometry measurements were carried out using a portable µSTAT 400 bipotentiostat/galvanostat (Dropsens, Spain) at 150 mV, 30 s. Each sample's electrochemical measurement was scored for relative *TMPRSS2-ERG* level in the same manner as described previously for absorbance readings. All measurements were performed at room temperature.

RT-qPCR

The KAPA SYBR® FAST One-Step qRT-PCR kit (KAPA Biosystems, Australia) was used to set up a single reaction volume of 10 µl for each sample. Each reaction volume consist of 1X KAPA SYBR® FAST qPCR Master Mix, 200 nM of each forward and reverse primer (Table 1), 1X KAPA RT Mix, 50 nM ROX dye and 30 ng of cell line total RNA template. RT-qPCR was performed using the Applied Biosystems® 7500 Real-Time PCR System (Thermo Fisher Scientific, Australia). The cycling protocol was: 42°C for 10 min to synthesize cDNA, followed by 95°C for 5 min to deactivate RT before cycling 35 times (95°C for 30 s, 50°C for 30 s and 72°C for 1 min) and finished with 72°C for 10 min.

Results

FusBLU assay for *TMPRSS2-ERG* detection

As illustrated in Fig. 1, our colorimetric assay utilized a combination of isothermal RT-RPA and TMB-based colorimetric readout for detecting *TMPRSS2-ERG* mRNA. Total RNA was firstly isolated from various sources used in our study and specific primers were used to amplify the *TMPRSS2-ERG* mRNA region at a constant temperature of 43ºC. During the isothermal RT-RPA, biotinylated dUTPs were randomly incorporated into the newly-synthesized strands. Then, SA-magnetic beads and SA-HRP were added to select for and label biotinylated RT-RPA products through biotin-streptavidin interactions. Lastly, TMB-H_2O_2 was added for color change reaction to test for the presence of HRP which in turn signifies the presence of RT-RPA products (i.e. *TMPRSS2-ERG* positive).

Assay specificity

To demonstrate the specificity of FusBLU in detecting *TMPRSS2-ERG*, we applied our assay to total RNA from two different PC cell lines. DuCap and LnCap are well-studied cell lines with the presence and absence of *TMPRSS2-ERG* respectively [4]. As shown in Fig. 2A, our assay was able to specifically amplify *TMPRSS2-ERG* mRNA from DuCap RNA and subsequently generated a blue color change. On the other hand, when the assay was

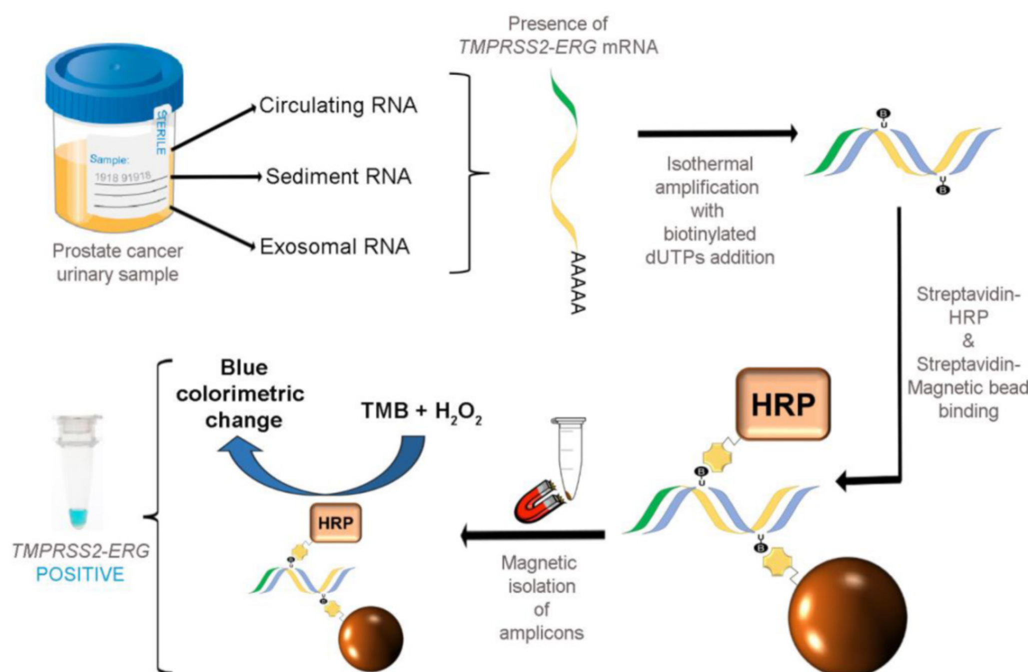


Figure 1. FusBLU assay for rapid *TMPRSS2-ERG* detection. Total RNA is isolated from urine and *TMPRSS2-ERG* mRNA is specifically amplified by RT-RPA isothermally. During strand polymerization, biotinylated dUTP bases are randomly incorporated and subsequently, SA-magnetic beads and SA-HRP are added to RT-RPA products for coupling through biotin-SA interactions. After magnetic isolation of RT-RPA products, TMB is added and a blue color change indicates the presence of *TMPRSS2-ERG*.

performed on LnCap RNA, RT-RPA products were not generated at a detectable level colorimetrically (Fig. 2A). The specific colorimetric detection of *TMPRSS2-ERG* could be followed by naked-eye observation of color change or more quantitatively by absorbance measurements (Fig. 2B). Furthermore, gel electrophoresis was also used to verify that the RT-RPA primers used in our experiments were specific and generated 216 bp products only in *TMPRSS2-ERG* positive DuCap samples. RN7SL1 housekeeping RNA was used as a loading control to validate successful RNA isolation from the cell lines (Fig. 2A). In addition, control experiments without the reverse transcriptase did not generate any RPA products, thus demonstrating that amplification was dependent on *TMPRSS2-ERG* mRNA, not DNA targets (Fig. 2).

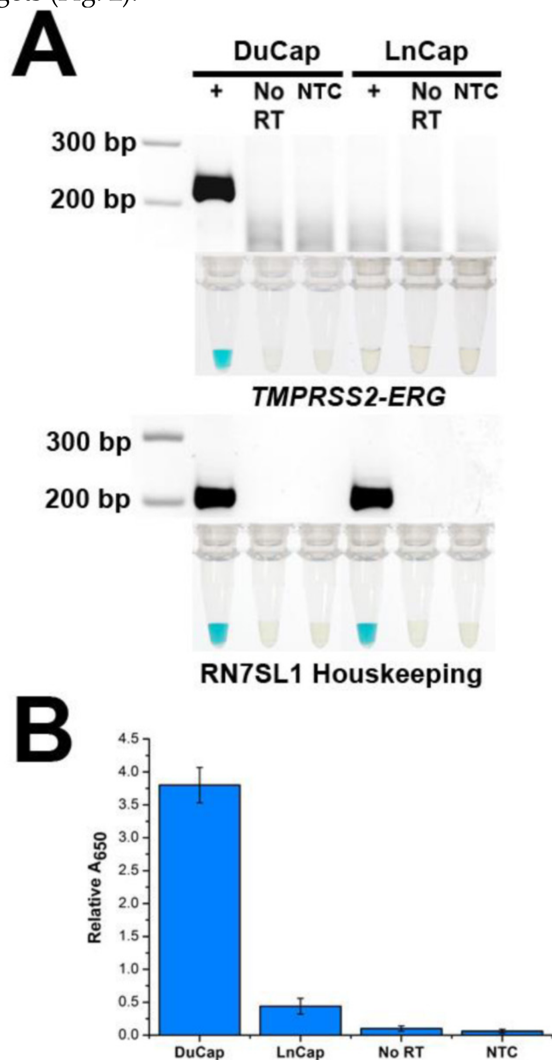


Figure 2. Specificity of FusBLU assay. (A) HRP-catalyzed colorimetric results for DuCap (fusion positive) and LnCap (fusion negative) cell lines with corresponding gel electrophoresis images after RT-RPA. Top panel: *TMPRSS2-ERG*. Bottom panel: RN7SL1. No reverse transcriptase (No RT) and no-template control (NTC) were included. (B) *TMPRSS2-ERG* levels normalized to RN7SL1. Error bars represent standard deviation of three independent experiments.

Assay sensitivity

To evaluate assay sensitivity, we prepared a titration of synthetic *TMPRSS2-ERG* mRNA (10^2-10^8 copies) into a background of LnCap total RNA to test the limit of detection (LOD). As expected, the color change intensity increased with increasing amounts of input targets (Fig. 3A). By visual detection, the assay LOD was 10^5 copies of *TMPRSS2-ERG* mRNA at which a blue color change in solution was still observable. The determination of assay sensitivity could also be followed quantitatively by absorbance measurements and assay LOD was further improved by 10-fold to 10^4 *TMPRSS2-ERG* mRNA copies with a linear range of 10^4-10^8 copies (Fig. 3B). However, it is worthy to note that the LOD of 10^5 copies by visual detection is approximately equivalent to single cell level of detection [23] and thus may potentially be adequate for *TMPRSS2-ERG* screening purpose in a clinical setting. FusBLU also showed good assay reproducibility with intra- and inter-assay variability of 12.5% and 10.5% respectively (n = 3).

Colorimetric assay performance on clinical specimens

After establishing assay specificity and sensitivity, we challenged FusBLU with urine specimens to demonstrate potential clinical utility. To this end, we isolated sediment RNA from the urinary samples of 12 men and applied FusBLU for *TMPRSS2-ERG* detection. The magnetic SPRI method provided a minimal yield of 15 ng total RNA; an amount which was sufficient for our analysis using FusBLU. Of the 12 clinical urinary samples used in our experiments: 5 (P1-P5) were metastatic hormone-refractory PC patients undergoing treatment; 5 (P6-P10) were diagnosed with PC after biopsies; and the remaining 2 (H1 and H2) were healthy controls. As shown on Fig. 4A, we could visually detect *TMPRSS2-ERG* in 8 of the patient samples (P1, P3, P4, P5, P7, P8, P9, and P10), while the remaining samples (P2, P6, H1, and H2) showed no observable color change as similar to the no-target control (NTC). For quantitative detection, the relative absorbance measurements (Eqn. 1) provided additional information of *TMPRSS2-ERG* levels in different patients (Fig. 4B). In addition, we also performed RT-qPCR, the current gold standard gene fusion detection approach on the same 12 urinary samples as a benchmark comparison. The RT-qPCR threshold cycle (Ct) values (Fig. 4D) showed that higher Ct values (i.e. lower amount of targets in samples) were generated for samples with low FusBLU-detected *TMPRSS2-ERG* levels. We found that our FusBLU results were in excellent agreement

with the RT-qPCR results, thus validating the *TMPRSS2-ERG* status of the patient samples.

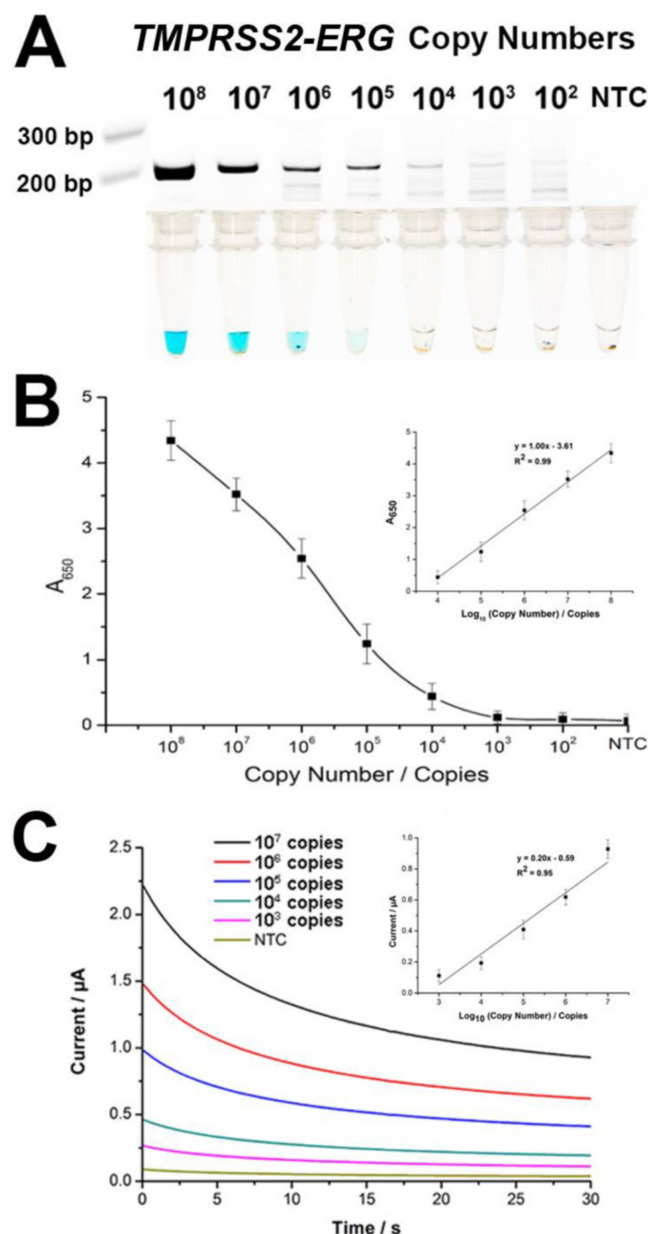


Figure 3. Sensitivity of FusBLU assay. (A) HRP-catalyzed colorimetric change generated from 10^2 - 10^8 copies of synthetic *TMPRSS2-ERG* mRNA, and corresponding gel electrophoresis images after RT-RPA. (B) Calibration plot of the average absorbance measurements at different initial RT-RPA input amounts. Inset shows the analogous linear calibration plot. (C) HRP-catalyzed current change generated from 10^3 - 10^7 copies of synthetic *TMPRSS2-ERG* mRNA. Inset shows the analogous linear calibration plot. Error bars represent standard deviation of three independent experiments.

Alternative electrochemical readout

Since TMB is electrochemically active and considering the multiple advantages of an electrochemical biosensor [20, 24, 25], we integrated the use of a portable potentiostat as an alternative FusBLU readout strategy to further demonstrate the potential for clinical screening applications. We firstly established the LOD of the electrochemical readout by a titration assay, and observed that 10^3 *TMPRSS2-ERG* copies were detectable with a linear range of 10^3-10^7 copies (Fig. 3C).

We next used the same collection of 12 urinary samples to demonstrate the electrochemical readout. Fig. 4C showed the *TMPRSS2-ERG* levels detected electrochemically were similar to that of spectrophotometry (Fig. 4B) and RT-qPCR (Fig. 4D). Taken together, these results indicated that FusBLU was a robust method for detecting *TMPRSS2-ERG* levels in urine and could be readily adapted for naked-eye, spectrophotometrical or electrochemical readout platforms.

Investigating *TMPRSS2-ERG* source in urine

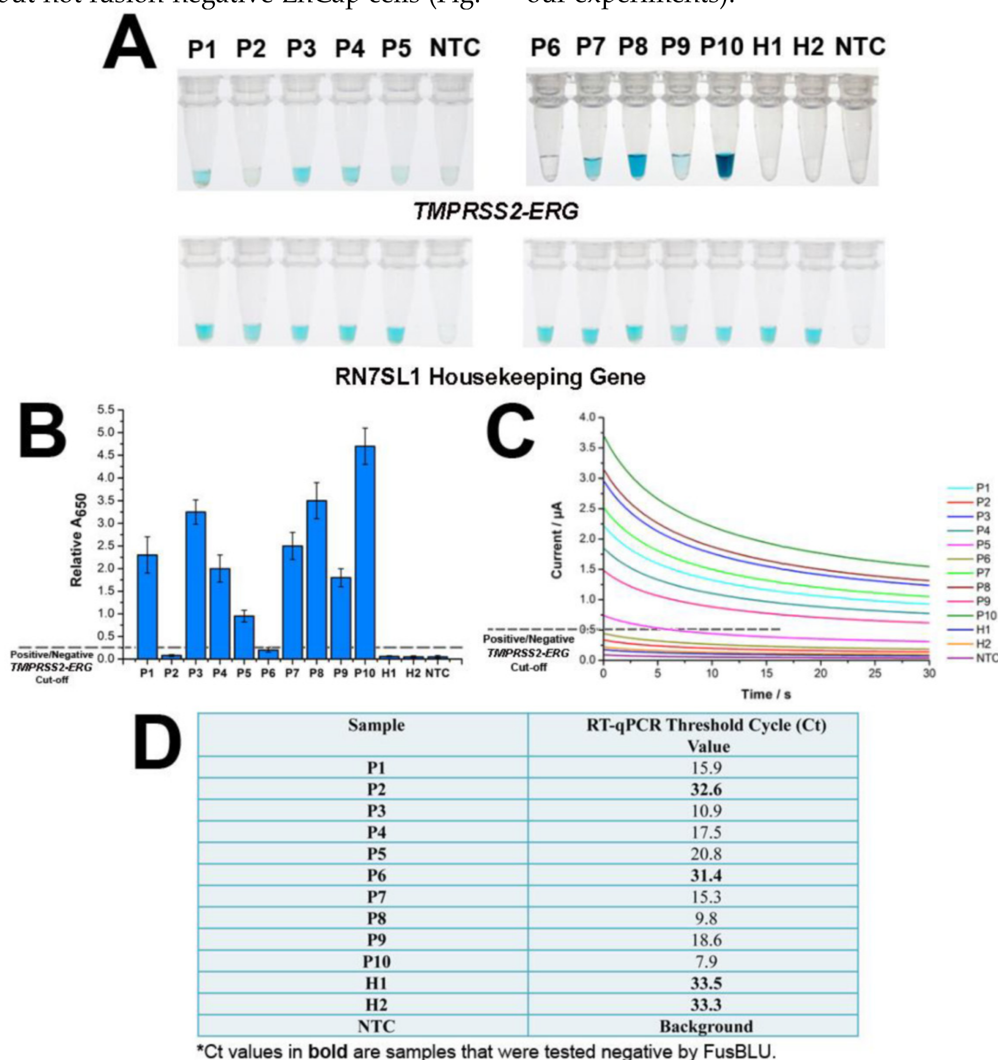
After demonstrating FusBLU's potential for clinical applications, we used our assay to investigate the primary source of *TMPRSS2-ERG* mRNA in urine. The potential sources included cell-free circulating RNA, cell sediment RNA and exosomal RNA. To this end, 3 *TMPRSS2-ERG*-positive PC patients (P1, P3, and P4) were first selected (Fig. 4) and total RNA was then isolated from the three candidate sources and assayed with FusBLU. As shown on Fig. 5A, the sediment fraction gave the strongest color intensity change (i.e. highest amount of *TMPRSS2-ERG* mRNA), followed by circulating free fraction, while the exosomal fraction contain the least amount of *TMPRSS2-ERG* mRNA. In addition, quantitative absorbance measurements showed that *TMPRSS2-ERG* mRNA in the sediment fraction was 2-fold and 12-fold higher than in the circulating free and exosomal fractions respectively (Fig. 5B). Absorbance results were also validated by gel electrophoresis following RT-RPA of the isolated total RNA from the various candidate sources (Fig. 5A). Our results therefore suggested that the sediment fraction is likely the main source of *TMPRSS2-ERG* mRNA in urine.

Discussion

Recurrent gene fusions between *TMPRSS2* exon 1 and *ERG* exon 4 is one of the emerging PC biomarker alternative to serum PSA. *TMPRSS2-ERG* offers higher PC specificity, potential prognostic value and is detectable in urine as a non-invasive biomarker [10, 11]. However, the conventional RT-qPCR approach for measuring *TMPRSS2-ERG* levels is not ideal for routine clinical screening due to long assay time and the need for specialized instrument [4, 14]. Therefore, we have developed FusBLU, an assay (Fig. 1) which combined isothermal RT-RPA and HRP-based colorimetric readout for quick and

quantitative *TMPRSS2-ERG* detection in 75 min without requiring expensive readout instruments. With FusBLU, we demonstrated (i) potential *TMPRSS2-ERG* detection in clinical urinary samples with a quick and convenient visual readout; (ii) quantitative *TMPRSS2-ERG* detection by alternative absorbance/electrochemical readout platforms; (iii) cell sediment RNA as potentially the main source of *TMPRSS2-ERG* mRNA in patient urinary samples.

In order for the assay to be used for *TMPRSS2-ERG* detection in real biological samples such as urine specimens, assay specificity and sensitivity are of paramount importance. High assay specificity is required to avoid false-positive/negative results due to detection of non-target mRNA and high assay sensitivity is required to detect trace amounts of *TMPRSS2-ERG* mRNA from a complex background of other biological molecules. FusBLU was highly specific for *TMPRSS2-ERG* mRNA in fusion-positive DuCap cells but not fusion-negative LnCap cells (Fig. 2). This high assay specificity was largely enabled by designing RT-RPA forward primer across the fusion junction to selectively amplify only gene fusion events. In future, this flexibility of primer design may also allow specific detection of other *TMPRSS2-ERG* isoforms for multiplexed FusBLU gene fusion detection. Our assay visual detection limit of 10^5 copies of *TMPRSS2-ERG* mRNA (Fig. 3A); which was approximately single-cell level [26], may be especially useful for non-invasively detecting the low levels of *TMPRSS2-ERG* in urine. Furthermore, this visual LOD could be further improved up to 100-fold with easily-adaptable absorbance/electrochemical readouts. In comparison to other standard urinary *TMPRSS2-ERG* detection methodologies such as RT-qPCR, FusBLU required lower sample input (10 ng total RNA vs. 50 ng total RNA for RT-qPCR in our experiments) and was able to produce results within a shorter timeframe (75 min vs. 150 min for RT-qPCR in our experiments).



Sample	RT-qPCR Threshold Cycle (Ct) Value
P1	15.9
P2	**32.6**
P3	10.9
P4	17.5
P5	20.8
P6	**31.4**
P7	15.3
P8	9.8
P9	18.6
P10	7.9
H1	**33.5**
H2	**33.3**
NTC	**Background**

*Ct values in **bold** are samples that were tested negative by FusBLU.

Figure 4. FusBlu assay on clinical urinary samples. (A) HRP-catalyzed colorimetric results of 12 urinary samples over two different assay runs (1st run (Left): P1-P5; 2nd run (Right): P6-H2). Top panel: *TMPRSS2-ERG*. Bottom panel: RN7SL1. (B) *TMPRSS2-ERG* levels normalized to RN7SL1. (C) TMB-derived currents of 12 urinary samples compared to no template-control (NTC). (D) RT-qPCR threshold cycle (Ct) values for each sample as validation for FusBLU results. Error bars represent standard deviation of three independent experiments.

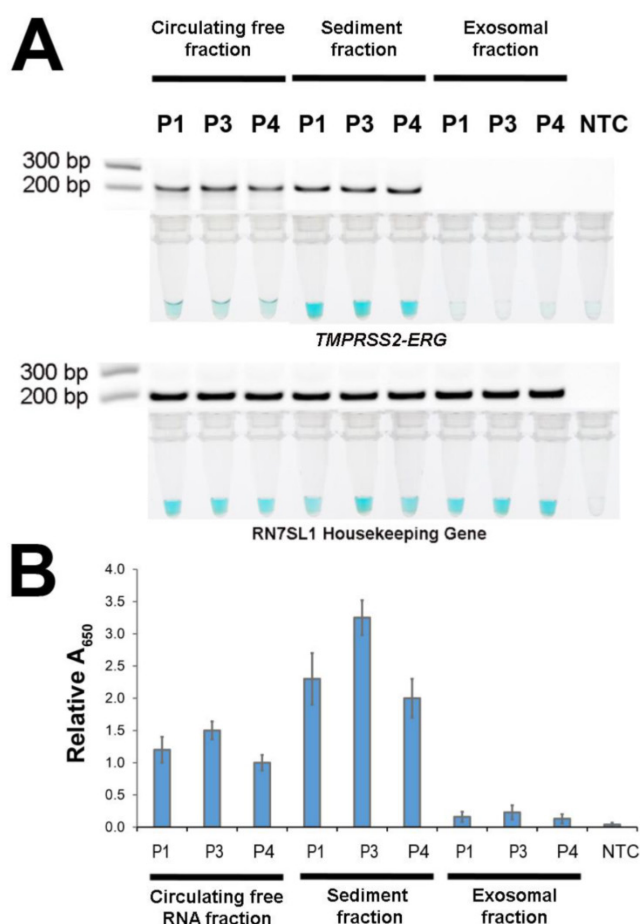


Figure 5. Source of *TMPRSS2-ERG* mRNA in urine. Total RNA isolated from circulating free, sediment, and exosomal fractions of 3 patient urine samples were studied (A) HRP-catalyzed colorimetric changes and corresponding gel electrophoresis images after RT-RPA. Top panel: for *TMPRSS2-ERG*. Bottom panel: RN7SL1. (B) *TMPRSS2-ERG* levels normalized to RN7SL1. Error bars represent standard deviation of three independent experiments.

To demonstrate the potential clinical utility of FusBLU, we applied our assay to 12 urinary samples (Fig. 4) and found excellent agreement between our assay results and current gold-standard RT-qPCR results (Fig. 4D). Importantly, the healthy and NTC samples generated negligible background signals, and varying *TMPRSS2-ERG* levels were detected for the other samples with good reproducibility over independent runs. These data therefore suggested the reliability and functional applicability of our assay for routine diagnostics. Furthermore, the presence of *TMPRSS2-ERG* in the urine samples of the late-stage metastatic, hormone refractory samples was consistent with previous reports in literature [27, 28]. Hence, our results suggested that FusBLU may be a useful screening tool for the *TMPRSS2-ERG* positive metastatic subtype of aggressive PC. Considering the good assay sensitivity as well as speed and simple naked-eye evaluation of color change detection; FusBLU may also be a rapid and convenient preliminary PC screening tool for guiding clinical decisions. In addition, quantitative absorbance measurements of the color intensity can also provide additional information that could be beneficial for tracking patient response to treatment or for relapse monitoring. Lastly, electrochemical approaches have high potential for rapid, sensitive, inexpensive and portable biosensing [29-31] and thus have attracted great interest for clinical diagnostic developments. To this end, we exploited TMB's compatibility as an electrochemical substrate and adapted FusBLU into an electrochemical assay with a portable potentiostat to highlight the versatility of the method (Fig. 4C). Despite the use of a portable potentiostat, the FusBLU electrochemical readout provided the highest detection sensitivity (10^3 copies) out of the three different readout platforms. In short, FusBLU is potentially a flexible methodology that could be tailored to specific diagnostic needs.

While it is known that *TMPRSS2-ERG* mRNA is present in urine, it is not previously clear where the fusion mRNA originated from. Hence, identifying the primary source of *TMPRSS2-ERG* mRNA in urine might be useful for future assay development and may help better understand the cancer biology of *TMPRSS2-ERG* subtypes. Using FusBLU, we found that *TMPRSS2-ERG* mRNA was most abundant in the sediment fraction of urine (Fig. 5). *TMPRSS2-ERG* mRNA in circulating free fraction was the second most abundant source while minimal fusion mRNA was detected in the exosomal fraction. A possible explanation for our observation could be the shedding of tumor cells from the primary (and metastastic) tumors in the prostate into the urine through the prostatic urethra [32, 33]. It could also be from ingested tumor cells that are present in urine within white blood cells of the immune system [34]. The presence of the *TMPRSS2-ERG* mRNA in circulating free fraction was likely from the spontaneous lysis of cells since minimal RNA was detected in the exosomal fraction. Nevertheless, the data is still preliminary and warrants further investigation. Notwithstanding, the data did suggested that although *TMPRSS2-ERG* was detectable in whole urine, the sediment fraction is most likely the best choice for sampling *TMPRSS2-ERG* mRNA due to ease of preparation and high abundance. Additionally, the use of our assay as a rapid and convenient research tool for biological studies was also demonstrated through this set of successful experiments to investigate the source of *TMPRSS2-ERG* mRNA in urine.

FusBLU shows promise as a faster, simpler, and more cost-effective solution for gene fusion biomarkers detection as compared to current techniques. Nevertheless, there remain some aspects of the assay which could be further investigated in

future studies. Firstly, our main aim in this report is to demonstrate FusBLU as a successful proof-of-concept technology which could be applied to real patient samples. While FusBLU detected *TMPRSS2-ERG* levels in 12 clinical urinary samples as a demonstration, it would be ideal to apply the method to a larger pool of patients and also of samples at various stages of PC. This would allow a better evaluation of the clinical utility of the assay for PC diagnosis. Secondly, we envisaged an improved version of FusBLU assay with multiplexing capabilities to detection multiple biomarkers. It has been suggested that detecting a combination of gene fusion isoforms or other PC biomarkers could improve PC diagnostic and predictive accuracy [14]. In this regard, we feel that an electrochemical readout would be better suited for this purpose as performing the assay on an array of miniaturized electrodes could greatly increase throughput.

In summary, we have developed FusBLU; a quantitative *TMPRSS2-ERG* assay with single-cell level detection sensitivity and highly specific for the most common gene fusion isoform in PC. This assay was achieved by the novel combination of isothermal RT-RPA and HRP-catalyzed colorimetric readout of RT-RPA products. Our assay was successfully applied to cell lines and patient urine samples with minimal equipment and at a low cost in approximately 75 minutes. Through the use of this assay, we also determined that total RNA isolated from urinary sediments as the main source for *TMPRSS2-ERG* mRNA detection from urinary samples. We believe that our colorimetric assay is potentially useful for POC detection of *TMPRSS2-ERG*, or other oncogenic fusion genes.

Abbreviations

PC: prostate cancer; PSA: prostate specific antigen; RT-qPCR: quantitative reverse-transcription PCR; HRP: horseradish peroxidase; DRE: digital rectal examination; TMB: tetramethyl benzidine; H_2O_2: hydrogen peroxide; RT-RPA: reverse-transcription recombinase polymerase reaction; SPRI: Solid Phase Reversible Immobilization; SA: streptavidin; LOD: limit-of-detection; NTC: no-template control; Ct: threshold cycle.

Acknowledgements

We thank Matthias Nees, Gregor Tevz, and Michelle Hill for providing the cell lines used in our experiments.

Competing Interests

The authors have declared that no competing interest exists.

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