

AperTO - Archivio Istituzionale Open Access dell'Università di Torino

Detection of urinary prostate specific antigen by a lateral flow biosensor predicting repeat prostate biopsy outcome

This is the author's manuscript

Original Citation:

Availability:

This version is available <http://hdl.handle.net/2318/1755115> since 2025-01-20T10:03:32Z

Published version:

DOI:10.1016/j.snb.2020.128812

Terms of use:

Open Access

Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(Article begins on next page)

1 **Detection of urinary prostate specific antigen by a lateral flow biosensor predicting repeat prostate**
2 **biopsy outcome**

3 Fabio Di Nardo^{1,3,*}, Sergio Occhipinti^{2,3}, Paolo Gontero⁴, Simone Cavalera¹, Matteo Chiarello¹, Claudio
4 Baggiani¹, Laura Anfossi¹

5
6 ¹Department of Chemistry, University of Turin, Via Pietro Giuria 5, 10125, Turin, Italy

7 ²Department of Molecular Biotechnologies and Health Sciences, Experimental Research and Medical
8 Studies Centre, University of Turin, Via Santena 5, 10126, Turin, Italy

9 ³NIB biotec srl, Via Gioacchino Quarello 15/A, 10135, Turin, Italy

10 ⁴Department of Urology, Città della Salute e della Scienza—Molinette Hospital, University of Turin, Turin,
11 Italy

12
13 **Corresponding Author**

14 * E-mail: fabio.dinardo@unito.it Tel: +39 011 6705252

15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52

53 **Abstract**

54 This work describes the development and the application of a lateral flow biosensor for the detection of the
55 prostate specific antigen in urine (uPSA). The biosensor allowed uPSA detection in 10 minutes with a limit
56 of detection and a range of quantification respectively of 20 ng mL⁻¹ and 37 – 420 ng mL⁻¹, showing within
57 and between-day coefficients of variation ≤ 13%. It showed 92% of accuracy and an almost perfect
58 concordance with the reference electrochemiluminescence immunoassay method. The biosensor design
59 provides the disappearance of the Test line signal at the cut-off concentration. This was achieved with a
60 double layer sensing strategy, in which gold nanoparticles were functionalized with Staphylococcal protein
61 A – a mediator – instead of anti-PSA antibody. This strategy allow making a fine-tune on the concentration
62 of the specific antibody, obtaining an on/off switch of the Test line at the cut-off value. The cut-off value
63 was also established in this work, based on the distribution of uPSA levels from 140 patients, who were
64 suspected of prostate cancer and who underwent to first biopsy. The clinical application of the biosensor to
65 predict repeat biopsy outcome in 28 patients showed sensitivity, specificity, positive and negative
66 predictive values of 100%, 64%, 74% and 100%, respectively.

67 **Keywords:** Lateral Flow Immunoassay, Paper-based biosensors, Immunochromatographic strip test,
68 Prostate cancer, Repeat biopsy, Urinary prostate specific antigen

69

70

71

72

73

74

75

76

77

78

79

80

81

82

83

84

85

86 **1. Introduction**

87 Prostate cancer (PCa) is the second most common tumor among men worldwide [1] and, respectively, the
88 second and the third most frequent cause of cancer-related death in Europe and United States [2,3]. PCa is
89 generally asymptomatic until it reaches an advanced stage. Therefore, the possibility to screen for its early
90 detection is of utmost importance in order to reduce the chance of death from PCa.

91 Routine screening for PCa relies on the measurement of the prostatic specific antigen (PSA) level in blood
92 and on the digital rectal examination (DRE) [4]. Men with a positive PSA test result (serum PSA higher than
93 4 ng mL⁻¹) usually undergo a transrectal ultrasound-guided biopsy of the prostate, which is the gold
94 standard for PCa diagnosis [5].

95 Results of several clinical trials suggested that PSA screening can reduce prostate cancer mortality, thanks
96 to its high diagnostic sensitivity in detecting PCa at an early stage [6-8]. However, PCa diagnosis based on
97 serum PSA lacks sufficient diagnostic specificity [9] because elevated PSA levels in blood may be related to
98 other conditions, e.g., benign prostatic hyperplasia (BPH), prostatitis and infections of the urinary tract [10].
99 For this reason, screening based on PSA is still controversial as can be observed from the different
100 recommendations for and against, among the several guidelines existing on the subject [11]. All the
101 guidelines agree that the identification of asymptomatic cancer that would never cause symptoms or
102 contribute to death is one of the most important damage of PSA-based screening programs [12].

103 Another important issue in PCa diagnosis is related to the different aggressiveness of the tumor. According
104 to aggressiveness, the physician and the patient will decide for the immediate treatment, for monitoring
105 the progression of the tumor by re-measuring PSA level and by DRE (observation), or looking for changes in
106 cancer growth through biopsies on a regular basis (active surveillance). Usually the aggressiveness of PCa is
107 assessed by means of the Gleason score (GS), which requires the execution of the biopsy and the
108 assignment by a pathologist. GS ranges from 2 to 10, but most PCa are scored 6 to 10 [13]. Because biopsy
109 does not guarantee 100% accuracy, in case of first negative biopsy the subject is still considered at risk and
110 the European guidelines suggest recurring to rebiopsy after six-twelve months [9].

111 Overtreatment of indolent PCa, which aggressiveness is overestimated negatively impacts patients' life,
112 causing urinary retention, urinary incontinence and erectile dysfunction. Therefore, the availability of
113 reliable PCa biomarkers and accurate and non-invasive tests to reduce the number of unnecessary biopsies
114 and avoid overtreatment is highly demanded. Recently, the possible use of multi-parametric Magnetic
115 Resonance Imaging (MRI) for screening for PCa has been suggested, since the very high specificity showed
116 by this technique [14]. However, difficulty of results interpretation combined with high cost and limited
117 access are major obstacles to the widespread implementation of MRI as a viable screening tool.

118 As an alternative, several risk prediction models based on blood and urine biomarkers (e.g., Prostate Cancer
119 Antigen 3 [15], Prostate Health Index [16], 4KScore [17], Stockholm3 test [18] and iXip index [19]) and risk

120 calculators based on the European Randomized Study of Screening for Prostate Cancer trial data [20], have
121 been proposed in the last decades. Some of these models increased the accuracy in predicting PCa and
122 reduced the number of false-positive results. However, no unanimous consensus has been expressed about
123 the use of these tests in routinely clinical practice, some of them are extremely expensive and time-
124 consuming and therefore hardly applicable on a large scale. For this reason, the level of serum PSA is still
125 widely used by the health care system as the standard screening test for PCa early diagnosis, during
126 observation and in the active surveillance. PSA has also been revalued in combination with other
127 parameters for the risk stratification in the PCa management. In its last update (March 2020 [21]), the
128 National Comprehensive Cancer Network® Guideline on PCa reported 10 ng mL⁻¹ as the PSA upper limit to
129 identify subjects with low risk of PCa progression.

130 Physiologically, PSA is present in urine due to the anatomic proximity of the prostate to the bladder and
131 urethra [22]. Tremblay et al. measured a urinary excretion of PSA (μg/24 hr, mean ± standard error of the
132 mean) equals to 195 ± 26, 410 ± 87, 97 ± 29 in normal subjects (10-45 years old), patients with BPH and
133 patients with PCa, respectively [23]. Bolduc and co-workers [24] measured urinary PSA (uPSA) in patients
134 that have been diagnosed PCa and BPH, through an enzyme-linked immunosorbent assay (ELISA). They
135 supported uPSA as a useful marker in the differential diagnosis of PCa and BPH, especially when serum PSA
136 is between 2.5 ng mL⁻¹ and 10 ng mL⁻¹. Moreover, they stated that low uPSA points toward prostate cancer
137 and that an uPSA threshold of > 150 ng mL⁻¹ may be used to decrease the number of prostatic biopsies.

138 In this study, we retrospectively evaluated the use of uPSA as a biomarker for predicting outcomes of
139 repeat biopsies and developed a lateral flow biosensor for the point-of-care detection of uPSA. Urinary
140 levels of PSA from 168 subjects who were scheduled for biopsy according to Prostate Cancer Guidelines of
141 European Association of Urology [25] were quantified by means of an electrochemiluminescence
142 immunoassay employing a Cobas® laboratory-based platform. All the subjects underwent to biopsy and
143 were classified as PCa positive (PCa+) or negative (PCa-) according to the GS. Twenty eight patients
144 classified as PCa-, after the first biopsy, were still considered at risk and were scheduled for repeat biopsy.

145 Based on the receiver operating characteristic (ROC) curve from 140 patients that were not rescheduled for
146 biopsy, we calculated a threshold value for uPSA corresponding to 90% diagnostic sensitivity. According to
147 the established cut-off value, we developed a simple and fast biosensor for the detection of PSA in urine
148 and verified its suitability for predicting repeat biopsy outcomes. Results were validated on the group of 28
149 subjects who were submitted to the second biopsy.

150 The lateral flow immunoassay (LFIA) technology is widely recognized as a convenient and efficient tool for
151 monitoring and self-monitoring health status. The advantages of point-of-care diagnosis based on LFIA are
152 rapidity, cost-effectiveness, affordability, and capability of providing accurate identification and/or
153 quantification of different kinds of biomarkers, including pathogens, proteins, hormones, metabolites, etc.

154 [26]. Therefore, we designed a biosensor based on the LFIA technology for detecting PSA in urine. The LFIA
155 biosensor comprised: a reactive membrane where two lines were drawn, one (Test) line responsive to the
156 uPSA level and the second (Control) line to confirm validity of the device; a sample membrane suitable for
157 the direct application of the urine sample; and an absorbent membrane to guide the capillary flow from the
158 sample to the reactive membrane (Fig. 1a). Gold nanoparticles (AuNPs) were employed as the colorimetric
159 probe and linked to Staphylococcal protein A (Au-SpA), which is able to bind immunoglobulins from various
160 species (in this case, a specific mouse monoclonal anti-PSA antibody), while the anti-PSA antibody was
161 added to the sample. To comply with the clinical significance of the uPSA biomarker, the assay format was
162 designed to provide a positive outcome (i.e. the appearance of a visible signal at the Test line) for those
163 subjects with increased risk of developing PCa and a negative outcome (i.e. no signal in correspondence of
164 the Test line) for patients with low risk of evolving the malignancy. In fact, according to the inverse
165 relationship found between urinary PSA and the risk of evolving the tumor, uPSA exceeding the cut-off
166 value corresponded to healthy or low-risk subjects (negative outcome) while subjects with uPSA below the
167 cut-off value should be tested as positive. The parameters of the assay (AuNp-SpA, anti-PSA antibody and
168 antigen amount, capillary flow rate, etc.) were tailored to reach the disappearance of the Test line color in
169 correspondence of the cut-off value established for urinary PSA.

170 The uPSA measurement through the proposed POCT device as an affordable and rapid tool for the follow
171 up of patients considered at risk of developing PCa would dramatically cut operating costs and times to
172 treatment and would be a great ally in the management and monitoring of subjects with different levels of
173 risk to incur PCa.

174

175 **2. Materials and methods**

176 **2.1 Immunoreagents, chemicals and materials**

177 Staphylococcal protein A (SpA), bovine serum albumin (BSA), rabbit immunoglobulins (r-IgG) and Tween
178 20[®] were obtained from Sigma–Aldrich (St. Louis, MO, USA). Urea, creatinine and other chemicals were
179 purchased from VWR International (Milan, Italy). Synthetic urine was prepared as follows: 0.65 g L⁻¹
180 CaCl₂·2H₂O, 0,65 g L⁻¹ MgCl₂·H₂O, 4.6 g L⁻¹ NaCl, 2.3 g L⁻¹ Na₂SO₄, 0.65 g L⁻¹ Na₃ citrate·2H₂O, 2.8 g L⁻¹
181 KH₂PO₄, 1.6 g L⁻¹ KCl, 1.0 g L⁻¹ NH₄Cl, 25 g L⁻¹ urea and 1.1 g L⁻¹ creatinine, with pH solution adjust to 6.5.
182 PSA and mouse monoclonal antibodies against PSA (anti PSA Ab) were kindly supplied by Nib biotec Srl
183 (Turin, Italy). Nitrocellulose membranes (HF180 plus card), glass fiber conjugate pad and cellulose
184 absorbent pads were obtained from Merck Millipore (Billerica, MA, USA). Glass fiber sample pads Standard
185 14 were obtained from Whatman (Little Chalfont, UK). Plastic cassettes were furnished by Kinbio (China).

186

187 **2.2 Study population**

188 One hundred and sixty-eight men scheduled for prostate biopsy at the urology clinic, Città della Salute e
189 della Scienza di Torino - Molinette Hospital, Turin, Italy were included in the study. Subjects received biopsy
190 indication based on PSA level, DRE, prostate volume, and family history.

191 A group of 140 men who were classified for suspected prostate cancer according to the result of the first
192 biopsy were considered as the training dataset. In this population, 53 subjects (38%) were diagnosed PCa+
193 (clinically significant PCa, GS \geq 7), while 87 (62%) were PCa- (no cancer and indolent PCa, GS=6).

194 The validation set included 28 men whose first biopsy was negative (PCa-), but were still considered at risk
195 basing on other parameters (i.e. family history, PSA level, etc.) and who had one repeat biopsy. According
196 to the second biopsy 14 (50%) subjects were confirmed as PCa- while 14 subjects (50%) were re-classified
197 as PCa+.

198 The clinical characteristics of the study population (training and validation data set) are shown in Table 1.

199 **2.3 Quantification of urinary PSA**

200 Urine samples were collected before the biopsy after a standardized DRE. After a gentle shake of the
201 sample, 15 mL were collected and stored in Falcon tubes at -80 °C within 5 minutes from collection.

202 Urinary measurements of PSA were performed by means of an electrochemiluminescence immunoassay
203 (ECLIA) employing a Cobas[®] laboratory-platform (Roche Diagnostic GmbH, Mannheim, Germany) in the
204 Biochemical and Clinical Laboratory (Baldi and Riberi), Città della Salute e della Scienza di Torino - Molinette
205 Hospital, Turin, Italy. The measuring range of the ECLIA is 0.003 to 100 ng mL⁻¹ (defined by the lower limit of
206 quantification, LLOQ, and the upper limit of quantification, ULOQ). Samples with PSA concentrations above
207 the measuring range can be diluted with Elecsys Diluent Universal (furnished within the kit assay) up to a
208 recommended dilution of 1:50. Values below the detection limit are reported as < 0.003 ng/mL, while
209 values above the measuring range are reported as > 100 ng mL⁻¹ (or up to 5000 ng mL⁻¹ for 50-fold diluted
210 samples).

211 Considering the physiological levels of uPSA, urine samples were diluted 1:50 with the recommended
212 diluent and analyzed.

213 **2.4 Statistical data treatment**

214 SigmaPlot v.14.0 (Systat Software, Inc) was used to perform the statistical analyses. For statistical
215 comparisons, a p value < 0.05 was accepted as statistically significant. Samples with urinary PSA above the
216 ULOQ (5000 ng mL⁻¹) were assigned the ULOQ value. No samples contained uPSA below the LLOQ. For the
217 definition of the cut-off level 140 subjects were considered (training set). Statistic descriptive of the PCa-

218 and PCa+ groups for the training set are shown in Table S1. Normality was tested by the Kolmogorov-
219 Smirnov and the Shapiro-Wilk tests. Both distributions deviated from the normal distribution, then groups
220 were compared using the Wilcoxon rank-sum test (Mann-Whitney U test) and differences were shown
221 using the Box-Whisker plot. To evaluate the diagnostic performance of uPSA, the ROC curve was
222 constructed and the area under the curve (AUC) was calculated.

223 The discriminating value of uPSA was established as the concentration allowing to obtain 90% diagnostic
224 sensitivity on the training data set. Then, we checked its predictivity on the validation set of data (n=28),
225 considering the response of the second biopsy to classify subjects.

226 **2.5 Preparation and characterization of gold nanoparticles and of the colorimetric probe**

227 Gold nanoparticles (AuNPs) with a diameter of about 30 nm were synthesized through the tetrachloroauric
228 acid reduction with sodium citrate, as previously reported [27, 28]. Briefly, 1 mL of 1% w/v sodium citrate
229 was added to 100 mL of boiling 0.01% tetrachloroauric acid under vigorous stirring. The color of the
230 solution changed gradually from light yellow to ruby red, indicating the successful formation of AuNPs.
231 Finally, the colloidal gold was cooled down to room temperature and stored at 4 °C for subsequent
232 conjugation to SpA.

233 The AuNPs were used to label SpA (AuNPs-SpA), through passive adsorption, according to previous reports
234 [27, 29]. Briefly, 12 µg of SpA and 1 ml of borate buffer (20 mM, pH 7.4) were mixed with 10 ml of colloidal
235 gold and incubated for 30 min at 37 °C. Next, 1 ml of 1% BSA in borate buffer was added and reacted for
236 10 min at 37 °C to saturate the uncovered AuNPs surface. AuNPs-SpA conjugates were recovered by
237 centrifugation (13000 g, 15 min) and washed twice with borate buffer supplemented with 0.1% BSA.
238 Finally, the pellet was reconstituted in AuNPs storage buffer (borate buffer 20 mM pH 7.4 with 1% BSA,
239 0.25% Tween 20®, 2% sucrose and 0.02% sodium azide) and stored at 4°C until use.

240 AuNPs and AuNPs-SpA were characterized by visible spectroscopy, transmission electron microscopy (TEM)
241 and dynamic light scattering technique (DLS). Spectrophotometric analyses were carried out by Varian Cary
242 1E spectrophotometer (Agilent Technologies, USA). For TEM imaging, a drop of the GNPs aqueous
243 suspension was put on a copper grid covered with a lacey carbon film for the analysis using a Jeol 3010-
244 UHR (Jeol Ltd, Japan) high resolution transmission electron microscope (HR-TEM) equipped with a LaB₆
245 filament operating at 300 kV and with an Oxford Inca Energy TEM 300 X-ray EDS analyzer. The
246 hydrodynamic diameter distribution of the suspensions prepared was evaluated using a Zetasizer
247 instrument (Zetasizer Nano-ZS, Malvern Instruments, U.K.). The measurements were performed after
248 checking that the automatic attenuator was between 6 and 9. The results were expressed as hydrodynamic
249 diameter distribution in intensity (average of mean values of 5 measurements).

250 **2.6 Lateral flow biosensor fabrication and uPSA detection**

251 The biosensor developed in this work to detect uPSA is composed by i) a glass fiber sample pad, ii) a glass
252 fiber conjugate pad, iii) a nitrocellulose membrane (NC), iv) a cellulose absorbent pad and v) a plastic
253 cassette.

254 PSA (0.5 mg mL^{-1}) and rabbit IgG (1 mg mL^{-1}) solutions prepared in 20 mM phosphate buffer pH 7.4, were
255 applied on the NC card, by means of XYZ3050 dispenser platform (Biodot, Irvine, CA, USA), to form the Test
256 and the Control line, respectively. The reagents were non-contact dispensed at $1 \text{ } \mu\text{L cm}^{-1}$, keeping a
257 distance of 4 mm between the lines. The NC card were dried at 37°C for 45 minutes under vacuum. The
258 conjugate pad was saturated with the AuNPs-SpA conjugate (optical density 1) and dried for 3 hours at
259 room temperature. Finally, we cut the laminated cards by means of CM400 guillotine (Biodot, Irvine, CA,
260 USA) to obtain 5-mm width strips, inserted them into plastic cassettes and stored the final biosensor at
261 room temperature in the dark in plastic bags containing silica.

262 To perform the test, 70 μL of solution, containing patient urine (or PSA standard solution) and anti-PSA
263 antibody (prepared in 20 mM phosphate buffer pH 7.4 with 1% BSA and 0.1% Tween 20[®]) in 1:1 ratio, was
264 applied to the sample pad. The immunoreactions take place and the immunocomplexes between anti-PSA
265 and PSA immobilized on NC were revealed by the AuNPs-SpA probe that is capable to bind to
266 immunoglobulins from various species with high affinity (Fig. 1b). If the sample contained PSA, the binding
267 of the antibody to the immobilized PSA was increasingly inhibited, until reaching the complete inhibition
268 when PSA level is above the cut-off value (Fig. 1c). The result was assessed after 10 minutes by the naked-
269 eye as the absence/presence of the Test line. Strip images were also acquired by a scanner and processed
270 by a software (QuantiScan 3.0, Biosoft, Cambridge, UK) in order to quantify the Test line intensity (for
271 biosensor development).

272 The assay was considered invalid in the absence of the Control line. The Control line signal was generated
273 by the interaction between AuNPs-SpA and the rabbit IgG.

274 **2.7 Set-up of the lateral flow biosensor parameters for uPSA detection**

275 It is well known that several parameters deeply affect the analytical performance of lateral flow
276 immunoassay devices. The parameters evaluated to tune the lateral flow biosensor were: a) PSA and rabbit
277 IgG concentrations to form Test and Control lines, respectively; b) the amount of anti-PSA; c) the optical
278 density of AuNPs-SpA and d) the volume of sample to be analyzed. The modulation of these parameters
279 was conducted to obtain a lateral flow biosensor in which the Test line signal disappeared when uPSA was
280 equal or greater than the established cut-off level.

281 **2.8 Analytical evaluation of the lateral flow biosensor for uPSA detection**

282 The biosensor was calibrated between 0 and 5000 ng mL^{-1} by using PSA-fortified synthetic urine [30] and
283 measuring each analyte concentration in triplicate. The limit of detection (LOD) was calculated as the

284 concentration of the analyte that corresponds to the signal of the blank minus three standard deviation of
285 the blank. The range of quantification was considered as the PSA level giving signals between 80% (IC₈₀) and
286 20% (IC₂₀) of the signal of the blank.

287 For comparing with the reference ECLIA, the 168 samples were also classified as positive/negative on the
288 basis of the measured uPSA below /above the cut-off value. We calculated the biosensor accuracy in
289 comparison to the reference ECLIA as the number of agreeing results divided by the number of samples
290 measured (multiplied by 100). Concordance between the two methods was estimated by the Cohen's K.

291 We evaluated the within-day, between-day, within-batch and between-batch reproducibility of the uPSA
292 biosensor by analyzing 10 different samples (5 samples with uPSA < cut-off and 5 samples with uPSA > cut-
293 off) on 3 different days and using strips from 3 different batches (n=3).

294 Finally, we evaluated the shelf life of the biosensor performing real-time stability experiments. For stability
295 experiments, fully assembled test strips were sealed in a foil pouch with desiccant and stored at room
296 temperature. On each testing day, the necessary strips were removed from storage and used to test 3 uPSA
297 concentrations. The shelf life was studied during a 6 months period, testing the strips on month 0, 1, 3 and
298 6.

299 **2.9 Diagnostic validity of the lateral flow biosensor for repeat prostate biopsy outcome**

300 The biosensor was applied to detect uPSA in the whole study population. Results were visually judged by
301 three operators, who provided agreeing results for all tested samples. Samples were assigned as
302 positive/negative according to the presence/absence of the test line.

303 The diagnostic sensitivity (Se), specificity (Sp), positive predictive value (PPV) and negative predictive value
304 (NPV) of the biosensor were calculated on the validation set (n=28) by considering the diagnosis from the
305 second biopsy.

306 **3. Results and discussion**

307 **3.1 Urinary PSA cut-off level**

308 Urine samples were collected from patients suspected of PCa at the time of the biopsy. The uPSA levels
309 were measured by means of a quantitative *in vitro* diagnostic test based on the ECLIA method routinely
310 employed in a major clinical laboratory of Torino (Italy). The measuring range of the ECLIA is 0.003 to 100
311 ng mL⁻¹ (0.15-5000 ng mL⁻¹ considering that urine samples were 50-fold diluted before analysis). Samples
312 with PSA concentrations above the measuring range were assigned to 5000 ng mL⁻¹ (22 samples out of
313 140). No samples were measured below the LLOQ. Urinary PSA mean, median and interquartile range of
314 the training population were 1708 ng mL⁻¹, 1024 ng mL⁻¹ and 202 ng mL⁻¹ - 2871 ng mL⁻¹, respectively.

315 The uPSA levels distribution of patients diagnosed PCa+ and PCa- at the biopsy were compared using the
316 Mann-Whitney U test and differences were shown using the Box-Whisker plot (Fig. 2).

317 The difference in the median values between PCa+ and PCa- groups is greater than would be expected by
318 chance ($P < 0.001$), thus confirming a statistically significant difference between uPSA levels in the PCa+ and
319 PCa- population.

320 To evaluate the diagnostic performance of uPSA in distinguish PCa+ and PCa-, the ROC curve was
321 constructed, and the area under the curve (AUC) was calculated as 0.70 (Fig. 3). The AUC confirmed the
322 diagnostic power of the uPSA that was significantly higher than serum PSA (AUC 0.54) and similar to the
323 combination of serum PSA, age and DRE (AUC 0.73), which is the model currently in use for PCa screening
324 [25].

325 Considering the use of uPSA as a first level screening tool, the diagnostic threshold to distinguish the group
326 was set at 90% diagnostic sensitivity. The corresponding level of uPSA was calculated as ca. 2800 ng mL⁻¹.

327 To evaluate the diagnostic performance of uPSA in predicting re-biopsies outcomes, we compared the ROC
328 curves for serum PSA, uPSA (from ECLIA) and the combination of age, serum PSA and DRE for the validation
329 set of subjects and the AUCs were calculated as 0.65, 0.97 and 0.76, respectively (Fig. 3b). The comparison
330 pointed out an outstanding improvement in the diagnostic performances of uPSA, which showed Se, Sp,
331 PPV and NPV of 100%, 50%, 64% and 100%, respectively.

332 **3.2 Characterization of gold nanoparticles and of the colorimetric probe**

333 The AuNPs resulted almost spherical in shape as reported by TEM analysis that allowed visualizing AuNPs
334 morphology (Fig.4 b).

335 Visible spectrophotometric measurements were used to verify the effective adsorption of SpA onto AuNPs,
336 since the formation of a protein layer onto AuNPs surface causes the modification of the refractive index
337 and a shift of the localized surface plasmon resonance (LSPR) band. A λ_{\max} shift from 525 nm to 529 nm was
338 recorded and was considered as indicating successful conjugation (Fig. 4a).

339 The effective adsorption of the protein layer was also confirmed by DLS measurements resulted in a greater
340 hydrodynamic size for AuNPs-SpA (Fig. 4c).

341 **3.3 Principle of the lateral flow biosensor**

342 Ideally, the sensitive detection of PSA can be achieved by applying a sandwich-type immunoassay format,
343 using a pair of specific antibodies (i.e. a capture Ab on the NC and a detection Ab labelled to provide the
344 signal when the ternary complex forms). In this format, the signal formed in correspondence of the Test
345 line means that PSA is present in the sample and increases proportionally to the increase of the analyte

346 amount. The coloring of the Test line is commonly attributed to a positive status, e.g., confirmed
347 pregnancy, positivity to infections, etc.

348 In the case of uPSA, the positivity, intended as the high risk of evolving the cancer, is associated with
349 decreasing levels of the biomarker that can lead to a misleading interpretation of the result. In order to
350 facilitate result interpretation, we opted for a 'reverse' format to detect uPSA (i.e. the indirect competitive
351 format). In this format, the antigen (PSA) is used as the capture reagent and one PSA-specific antibody is
352 used (detection antibody). PSA immobilized on the NC and the one in the urine sample compete for binding
353 to the detection antibody, resulting in inverse correlation between the signal and the analyte level. The
354 immunocomplexes are revealed thanks to the AuNPs-SpA probe. The strategy to functionalize AuNPs with a
355 mediator (SpA) and not directly with the anti-PSA antibody, allowed us to tailor the amount of the specific
356 antibody to achieve the disappearance of the test line due to the complete binding inhibition of the anti-
357 PSA antibody to the immobilized PSA, since the available anti-PSA sites were saturated by the PSA in the
358 sample at the exact cut-off value.

359

360 **3.4 Set-up of the lateral flow biosensor parameters for uPSA detection**

361 Amounts of the antigen used to form the Test line, the anti-PSA antibody and the AuNPs-SpA optical
362 density were established by the checkerboard titration approach, based on requirements of reaching
363 intense signals for uPSA below 2800 ng mL^{-1} and a sharp decrease of the Test line color in correspondence
364 of the cut-off value. We prepared calibrators by fortifying simulated urine [30] with known amount of PSA
365 in the interval between 0 and 5000 ng mL^{-1} and analyzed them with different AuNPs-SpA and anti-PSA
366 combinations (Fig. S1). We also checked the optimal sample volume to be used and the time required to
367 achieve clearly visible and stable colors at the Test and Control lines. The best performance was obtained
368 using the following conditions: 0.5 mg mL^{-1} PSA and 1 mg mL^{-1} rabbit IgG as Test and Control line,
369 respectively; 50 ng anti PSA Ab; AuNPs-SpA optical density 1; $70 \text{ }\mu\text{L}$ as sample volume; 10 min as the
370 running time. Extensive discussion about the study on parameters is reported in the supplementary
371 materials (table S2, Fig. S1 and S2).

372 As already mentioned, several parameters affect the analytical performance of lateral flow assay devices.
373 One of the most important, especially for competitive immunoassay based on a cut-off readout, is the
374 amount of the specific antibody used. We decided to apply a double layer sensing strategy, in which gold
375 nanoparticles were functionalized with Staphylococcal protein A and used to stain the anti-PSA antibody.
376 The double layer sensing strategy has several advantages and allowed us to i) stabilize AuNPs with a protein
377 different from the specific antibody and therefore avoiding restriction regarding the anti-PSA amount; ii)
378 use a high amount of probe without affecting the immunoreactions and ensuring the revelation of the
379 immunocomplexes even using low amount of specific antibody; iii) use a precise and accurate amount of

380 anti-PSA; iv) have superior capacity of giving specific binding since the antibodies are well oriented thanks
381 to the SpA high affinity for the Fc portion of IgG; v) avoid loss in specific binding capacity due to possible
382 steric hindrance and antibodies multilayer formation phenomena when AuNPs are directly functionalized
383 with specific antibodies.

384 The aforementioned advantages enabled the calibration of the specific antibody in order to switch the
385 signal off/on at the exact cut-off value.

386 **3.5 Analytical performance of the lateral flow biosensor for uPSA detection**

387 A typical calibration curve using the optimal conditions is shown in Fig. 5. We obtain a LOD and a
388 quantification range of 20 ng mL^{-1} and $37 - 420 \text{ ng mL}^{-1}$, respectively. However, our main goal was to
389 obtain the disappearance of the Test line signal at the cut-off level (Fig. 5a, b). Accordingly, the parameters
390 were defined to switching off the color at the Test line in correspondence of the clinically relevant uPSA
391 level.

392 The ability of adequately measuring PSA level in human urine was assessed by comparing the results
393 obtained from the biosensor with those furnished by the reference ECLIA method. Two out of the 168 urine
394 samples provided invalid results, as they were unable to flow along the device and to complete the run in
395 the due time (1.2% invalid test rate). Then, the following analyses were conducted on 166 sample. We
396 obtained 92.2 % of accuracy and an almost perfect concordance between the two methods, expressed as
397 the Cohen's kappa coefficient ($\kappa = 0.82$) [27].

398 The reproducibility of the test was assessed by analyzing 10 urine samples (five scoring as negative and 5 as
399 positive) in triplicate on each day, on three days, and by using three batches of strips. Each sample was
400 analyzed 9 times in total each day. The analytical response of the test was qualitatively the same: no
401 samples were misclassified, confirming the reliability of the biosensor (Fig. 6). To quantitatively evaluate
402 the reproducibility of the test we compared the mean values of the signals (T/C) by a one way analysis of
403 variance (ANOVA). For each sample, we compared the mean values obtained in the same day using strips
404 from the same batch and strips from two other batches. For each batch, we compared the mean values
405 obtained in different days using the same strips batch. We also compared the overall means value for each
406 sample using three different batches on three different days. No significant statistical difference was
407 observed and we concluded that the precision of the test was adequate to allow the correct classification
408 of samples, considering within-day, between-day, within-batch and between-batch variability.

409 We assessed the real-time stability of the strips for 6 months at room temperature. On each testing day, we
410 measured in triplicate PSA-fortified synthetic urine (0, 100 and 1000 ng mL^{-1}). The measurements
411 evidenced good stability since the results were acceptably comparable to those of the freshly prepared
412 device, with coefficient of variation ranging from 2 to 12 % (Fig. S3). In detail, we calculated the mean

413 values of the signal (T/C) obtained as measurements for each uPSA concentration on the various months
414 (n=3) and compared the mean values with the ones obtained using freshly prepared strips (month 0) by a
415 one way ANOVA. The differences in the mean values among the groups were not statistically significant (P
416 ranging from 0.086 to 0.977) even if the powers of performed tests with alpha = 0.050 were below the
417 desired power of 0.800.

418 **3.6 Clinical application of the lateral flow biosensor**

419 The lateral flow biosensor was applied to detect uPSA in samples belonging to all subjects involved in the
420 study (training and validation data set).

421 The diagnostic validity of the developed test was assessed calculating the diagnostic Se, Sp and accuracy,
422 PPV and NPV for the validation data set. Se is the proportion of subjects with confirmed PCa+ who were
423 tested positive; Sp is the proportion of subjects with PCa- who were tested negative; the diagnostic
424 accuracy is the proportion of true results over the total samples of the validation data set; PPV is the
425 probability that an individual with a positive test result is PCa+; and NPV is the probability that an individual
426 with a negative test result is PCa-. Both PPV and NPV are related to Se and Sp through the disease
427 prevalence. The diagnostic figures of merit of the lateral flow biosensor, with the corresponding 95%
428 confidence interval (CI), is reported in Table 2.

429 The application of the lateral flow biosensor to measure uPSA would have avoided 64% of unnecessary re-
430 biopsies (9 out 14 PCa- subjects), saving adverse effects of the invasive procedure and reducing cost for the
431 health system. More relevant, 100% of subjects who were re-classified as PCa+ by the repeated biopsy
432 were correctly identified by the lateral flow biosensor as positive, based on the urinary PSA level measured
433 at the time of the first biopsy. This finding also confirmed the lack in accuracy of the gold standard in PCa
434 diagnosis (biopsy).

435 The results of this preliminary study allow us to make a speculative, but fairly well founded consideration
436 about the possible use of uPSA as a biomarker in the management process of PCa diagnosis, not only as a
437 tool to predict the outcome of repeat biopsy, but also to classify patients even before the first biopsy,
438 allowing for the non-invasive and rapid stratification of PCa risk (Fig. 7a, b).

439 In this broader context, the availability of a point-of-care test for uPSA measurement would further enable
440 screening analyses in an easy and rapid way, at low cost and directly at the physician's office.

441

442 **4. Conclusion**

443 We evaluated uPSA as possible biomarker for predicting re-biopsies outcomes. We established a threshold
444 for uPSA value to distinguish PCa+ and PCa- on a training population of 140 subjects scheduled for biopsy

445 because of the high risk of PCa. Based on the established cut-off value, we developed and reported the first
446 POCT to detect uPSA exploiting an intuitive immunoassay format for the straightforward result
447 interpretation. The lateral flow biosensor showed a LOD and a range of quantification respectively of 20 ng
448 mL⁻¹ and 37 – 420 ng mL⁻¹. In the analysis of human urine samples, the biosensor showed coefficients of
449 variation and test invalid rate of ≤ 13% and 1.2%, respectively. We applied the lateral flow biosensor for
450 PCa diagnosis in a validation population and we found that the extremely simple, non-invasive and cost-
451 effective detection of uPSA by a portable and rapid biosensor can be used to improve accuracy of PCa
452 detection and to avoid 64% unnecessary re-biopsies without losing diagnostic sensitivity. Future work is
453 focusing on increasing the study population in order to improve the diagnostic validity of the test and to
454 confirm the use of the uPSA biosensor as a convenient tool to guide clinical decision-making in assessing
455 PCa risk stratification.

456 **Ethics statement**

457 The study was conducted according to the Declaration of Helsinki and approved by the Scientific Ethics
458 Committee of A.O.U. Città della Salute e della Scienza di Torino, A.O. Mauriziano, A.S.L. TO1 (Prot. No.
459 0110644). All patients provided written informed consent and specimens were anonymized.

460 **Acknowledgments**

461 The authors are grateful to the Biochemical and Clinical Laboratory (Baldi and Riberi), Città della Salute e
462 della Scienza di Torino - Molinette Hospital, Turin, Italy, for the quantification of urinary PSA. S.O.
463 acknowledges Fondazione Umberto Veronesi for “Postdoctoral Fellowship 2018, 2019 and 2020”. The
464 authors acknowledge the Ministry of Education, University and Research of Italy (PRIN: 2017Y2PAB8) for
465 financial support.

466 **Competing Interests**

467 The authors declare the following competing interests: S.O and F.D.N are shareholders of NIB biotec srl.

468

469

470

471

472

473

474

475
476
477
478
479
480
481
482
483
484
485
486
487
488
489
490
491
492
493
494
495
496
497
498
499
500
501
502
503
504
505
506
507

References

- [1] F. Bray, J. Ferlay, I. Soerjomataram, R. L. Siegel, L. A. Torre, A. Jemal, Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries, *CA Cancer J. Clin.* 68 (2018) 394–424.
<https://doi.org/10.3322/caac.21492>.
- [2] J. Ferlay, E. Steliarova-Foucher, J. Lortet-Tieulent, S. Rosso, J. W. Coebergh, H. Comber, D. Forman, F. Bray, Cancer incidence and mortality patterns in Europe: estimates for 40 countries in 2012, *Eur. J. Cancer* 49 (2013) 1374–1403.
<https://doi.org/10.1016/j.ejca.2012.12.027>.
- [3] R. L. Siegel, K. D. Miller, A. Jemal, Cancer statistics, 2018, *CA Cancer J. Clin.* 68 (2018) 7–30.
<https://doi.org/10.3322/caac.21442>.
- [4] O. W. Brawley, D. P. Ankerst, I. M. Thompson, Screening for prostate cancer, *CA Cancer J. Clin.* 59 (2009) 264-273.
<https://doi.org/10.3322/caac.20026>.
- [5] N. Mottet, J. Bellmunt, M. Bolla, et al., EAU-ESTRO-SIOG Guidelines on prostate cancer. Part 1: screening, diagnosis, and local treatment with curative intent, *Euro. Urol.* 71 (2017) 618–629.
<https://doi.org/10.1016/j.eururo.2016.08.003>.
- [6] F. H. Schröder, J. Hugosson, M. J. Roobol, et al., Screening and prostate cancer mortality in a randomized European study, *N. Engl. J. Med.* 360 (2009) 1320–1328.
<https://doi.org/10.1056/NEJMoa0810084>.
- [7] F. H. Schröder, J. Hugosson, M. J. Roobol, et al., Prostate-cancer mortality at 11 years of follow-up, *N. Engl. J. Med.* 366 (2012) 981–990.
<https://doi.org/10.1056/NEJMoa1113135>.
- [8] F. H. Schröder, J. Hugosson, M. J. Roobol, et al., Screening and prostate cancer mortality: results of the European Randomised Study of Screening for Prostate Cancer (ERSPC) at 13 years of follow-up, *Lancet* 384 (2014) 2027–2035.
[https://doi.org/10.1016/S0140-6736\(14\)60525-0](https://doi.org/10.1016/S0140-6736(14)60525-0).
- [9] M. L. Jr. Blute, E. J. Abel, T. M. Downs, F. Kelcz, D. F. Jarrard, Addressing the need for repeat prostate biopsy: new technology and approaches, *Nat. Rev. Urol.* 12 (2015) 435-444.
<https://doi.org/10.1038/nrurol.2015.159>.

- 508 [10] D. Reinhardt, W. J. Catalona, Prostate cancer: The growing evidence supporting mid-life PSA
509 testing, *Nat. Rev. Urol.* 10 (2013) 436-438.
510 <https://doi.org/10.1038/nrurol.2013.144>.
- 511 [11] K. A. O. Tikkinen, P. Dahm, L. Lytvyn, et al., Prostate cancer screening with prostate-specific antigen
512 (PSA) test: a clinical practice guideline, *BMJ* 362 (2018) k3581.
513 <https://doi.org/10.1136/bmj.k3581>.
- 514 [12] US Preventive Services Task Force. Screening for Prostate Cancer: US Preventive Services Task
515 Force Recommendation Statement, *JAMA* 319 (2018) 1901-1913.
516 <https://doi.org/10.1016/j.eururo.2013.12.062>.
- 517 [13] National Comprehensive Cancer Network. Prostate Cancer for patients (2019).
518 <https://www.nccn.org/patients/guidelines/content/PDF/prostate-patient.pdf> (Last accessed
519 12.05.2020).
- 520 [14] H. U. Ahmed, A. El-Shater Bosaily, L. C. Brown, et al., Diagnostic accuracy of multi-parametric MRI
521 and TRUS biopsy in prostate cancer (PROMIS): a paired validating confirmatory study, *Lancet* 389
522 (2017) 815–822.
523 [https://doi.org/10.1016/S0140-6736\(16\)32401-1](https://doi.org/10.1016/S0140-6736(16)32401-1).
- 524 [15] J. T. Wei, Z. Feng, A. W. Partin, et al., Can urinary PCA3 supplement PSA in the early detection of
525 prostate cancer? *J. Clin. Oncol.* 32 (2014) 4066-4072.
526 <https://doi.org/10.1200/JCO.2013.52.8505>.
- 527 [16] S. Loeb, W. J. Catalona, The Prostate Health Index: a new test for the detection of prostate cancer,
528 *Ther. Adv. Urol.* 6 (2014) 74-77.
529 <https://doi.org/10.1177/1756287213513488>.
- 530 [17] R. J. Bryant, D. D. Sjoberg, A. J. Vickers, et al., Predicting high-grade cancer at ten-core prostate
531 biopsy using four kallikrein markers measured in blood in the ProtecT study, *Natl. Cancer Inst.* 107
532 (2015) djv095.
533 <https://doi.org/10.1093/inci/djv095>.
- 534 [18] H. Grönberg, J. Adolfsson, M. Aly, et al., Prostate cancer screening in men aged 50–69 years
535 (STHLM3): a prospective population-based diagnostic study, *Lancet Oncol.* 16 (2015) 1667–1676.
536 [https://doi.org/10.1016/S1470-2045\(15\)00361-7](https://doi.org/10.1016/S1470-2045(15)00361-7).
- 537 [19] A. Gallotta, G. Giannarini, L. Laurini, D. Zani, A. Garbeglio, S. Guazzieri, M. Plebani, G. Fassina, F.
538 Zattoni, Clinical validation of the iXip index in avoiding unnecessary prostate biopsy: results from a
539 prospective multicenter study involving 426 patients, *Cancer Treat. Res. Comm.* 10 (2017) 40–45.
540 <https://doi.org/10.1016/j.ctarc.2017.01.002>.

- 541 [20] V. Cavadas, L. Osório, F. Sabell, F. Teves, F. Branco, M. Silva-Ramos, Prostate Cancer Prevention
542 Trial and European Randomized Study of Screening for Prostate Cancer risk calculators: a
543 performance comparison in a contemporary screened cohort, *Eur. Urol.* 58 (2010) 551–558.
544 <https://doi.org/10.1016/j.eururo.2010.06.023>.
- 545 [21] National Comprehensive Cancer Network. NCCN Guidelines Version 1.2020, Prostate Cancer
546 (2020).
547 https://www.nccn.org/professionals/physician_gls/pdf/prostate.pdf (Last accessed 12.05.2020).
- 548 [22] J. N. Eskra, D. Rabizadeh, C. P. Pavlovich, W. J. Catalona, J. Luo, Approaches to urinary detection of
549 prostate cancer, *Prostate Cancer Prostatic Dis.* 22 (2019) 362–381.
550 <https://doi.org/10.1038/s41391-019-0127-4>.
- 551 [23] J. Tremblay, G. Frenette, R. R. Tremblay, A. Dupont, M. Thabet, J. Y. Dubé, Excretion of three major
552 prostatic secretory proteins in the urine of normal men and patients with benign prostatic
553 hypertrophy or prostate cancer, *The prostate* 10 (1987) 235-243.
554 <https://doi.org/10.1002/pros.2990100306>.
- 555 [24] S. Bolduc, L. Lacombe, A. Naud, M. Grégoire, Y. Fradet, R. R. Tremblay, Urinary PSA: a potential
556 useful marker when serum PSA is between 2.5 ng/mL and 10 ng/mL, *CUAJ* 1 (2007) 377-381.
557 <https://doi.org/10.5489/cuaj.444>.
- 558 [25] <https://uroweb.org/guideline/prostate-cancer/> (Last accessed 12.05.2020).
- 559 [26] W. C. Mak, V. Beni, A. P. F. Turner, Lateral-flow technology: From visual to instrumental, *TrAC* 79
560 (2016) 297-305.
561 <https://doi.org/10.1016/j.trac.2015.10.017>.
- 562 [27] L. Anfossi, F. Di Nardo, M. Profiti, C. Nogarol, S. Cavalera, C. Baggiani, C. Giovannoli, G. Spano, E.
563 Ferroglio, W. Mignone, S. Rosati, A versatile and sensitive lateral flow immunoassay for the rapid
564 diagnosis of visceral leishmaniasis, *Anal. Bioanal. Chem.* 410 (2018) 4123–4134.
565 <https://doi.org/10.1007/s00216-018-1067-x>.
- 566 [28] L. Anfossi, F. Di Nardo, A. Russo, S. Cavalera, C. Giovannoli, G. Spano, S. Baumgartner, K. Lauter, C.
567 Baggiani, Silver and gold nanoparticles as multi-chromatic lateral flow assay probes for the
568 detection of food allergens, *Anal. Bioanal. Chem.* 411 (2019) 1905–1913.
569 <https://doi.org/10.1007/s00216-018-1451-6>.
- 570 [29] F. Di Nardo, S. Cavalera, C. Baggiani, C. Giovannoli, L. Anfossi, Direct vs Mediated Coupling of
571 Antibodies to Gold Nanoparticles: The Case of Salivary Cortisol Detection by Lateral Flow
572 Immunoassay, *ACS Appl. Mater. Interfaces* 11 (2019) 32758-32768.
573 <https://doi.org/10.1021/acsami.9b11559>.
- 574 [30] E. Miró-Casas, M. Farré Albaladejo, M. I. Covas, J. O. Rodríguez, E. Menoyo Colomer, R. M. Lamuela
575 Raventós, R. De La Torre, Capillary gas chromatography-mass spectrometry quantitative

576 determination of hydroxytyrosol and tyrosol in human urine after olive oil intake, *Anal. Biochem.*
577 294 (2001) 63–72.
578 <https://doi.org/10.1006/abio.2001.516>.
579
580

Table 1. Clinical characteristic of the training and validation data set

Parameters	patients (total enrolled 140)	Patients (total enrolled 28)
mean age, yrs (median, IQR)	68 (69, 64-75)	70 (71, 65-75)
mean serum PSA, ng/ml, (median, IQR)	13 (5.9, 4.5-8.5)	6.2 (5.7, 4.5-8.3)
DRE abnormal, n (%)	61 (44)	10 (36)

Table 2. Diagnostic figures of merit of the biosensor

Parameters	Value (95% CI)
Se	100.0% (76.8% - 100.0%)
Sp	64.3% (35.1% - 87.2%)
LR	2.8 (1.4 - 5.6)
PPV	73.7% (58.1% - 85.0%)
NPV	100.0% (100.0% - 100.0%)
accuracy	82.1% (63.1% - 93.9%)

LR: Likelihood ratio

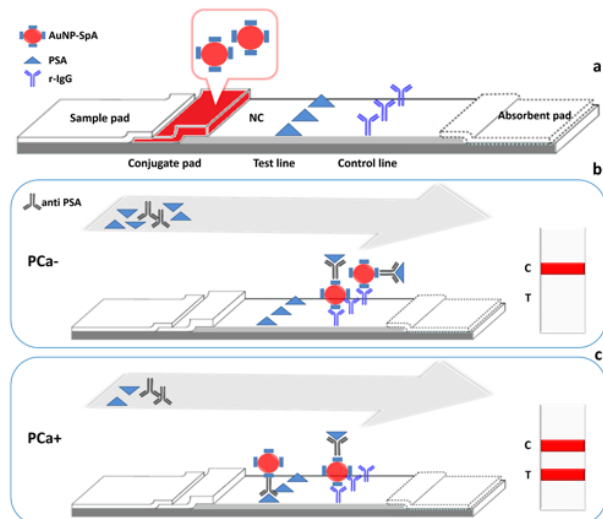


Fig. 1. Schematic diagram of the lateral flow biosensor. (a) Components of the lateral flow biosensor. (b) Negative test outcome for uPSA > cut-off level ('low risk PCa'). (c) Positive test outcome for uPSA < cut-off level ('high risk PCa').

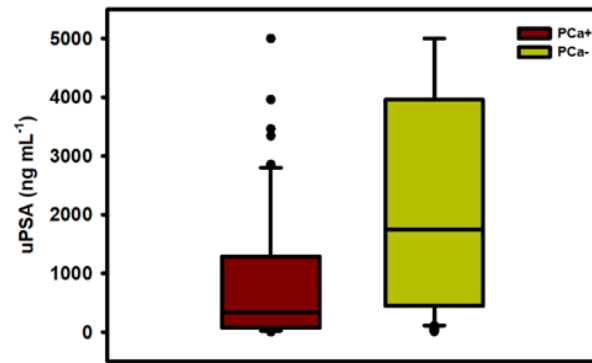


Fig. 2. Box-Whisker plot for uPSA levels of PCa+ and PCa- groups. The whisker above the box includes the data within up to $Q_3 + 1.5$ IQR while the whisker below the box includes data down to $Q_1 - 1.5$ IQR. The data outside the whiskers are potentially outliers and are displayed as dots.

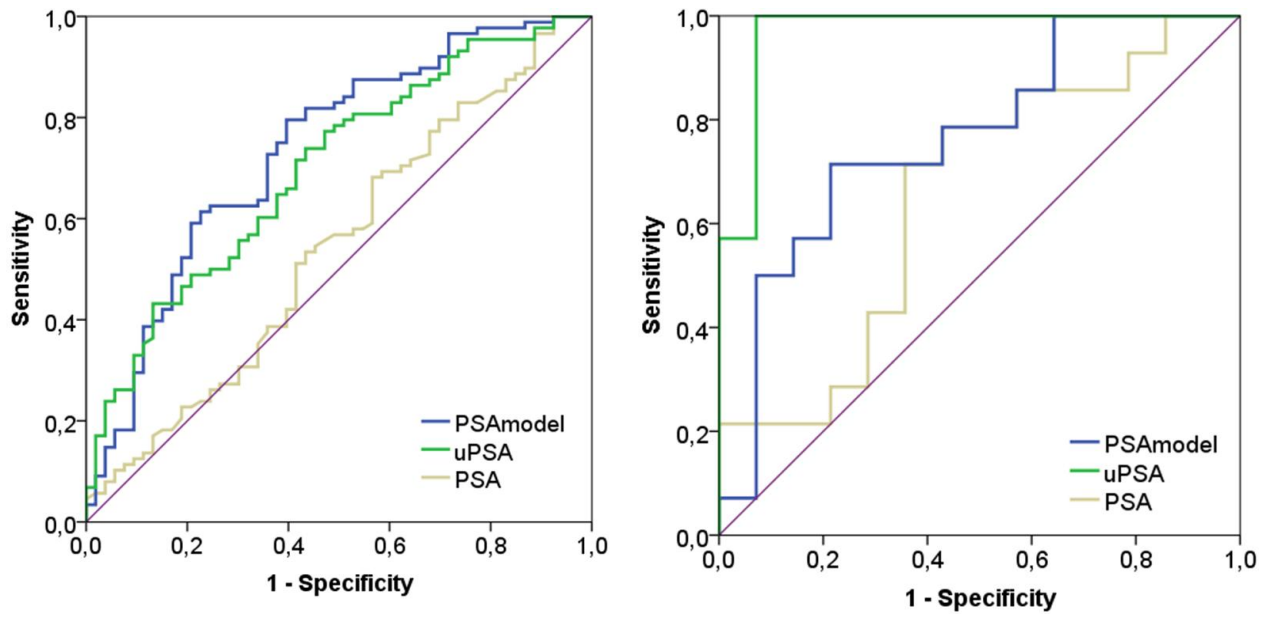


Fig. 3. ROC curves for uPSA, serum PSA and the combined model (age, serum PSA and DRE) recommended as diagnostic indicator [25] from: (a) the training set (n=140, subjects classified according to the first biopsy) and (b) the validation set (n=28, patients classified according to the repeat biopsy).

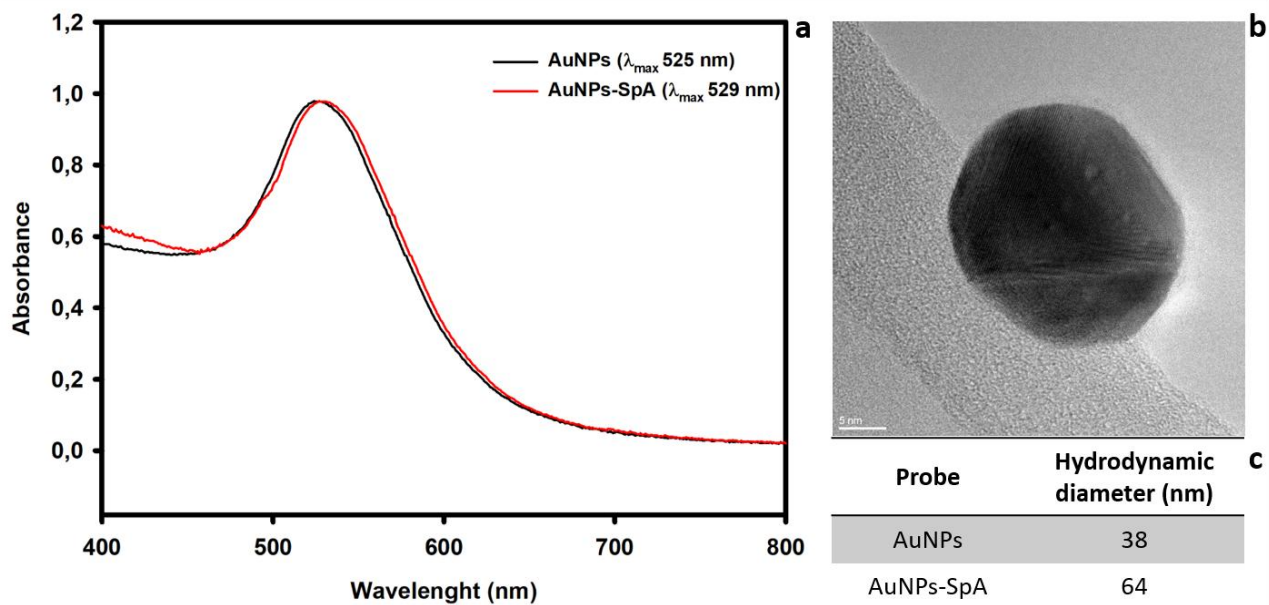


Fig. 4. Characterization of gold nanoparticles and of the colorimetric probe: (a) Vis spectra of AuNPs and AuNPs-SpA, (b) TEM image of AuNPs obtained through the citrate reduction method. (c) Hydrodynamic diameters of AuNPs and AuNPs-SpA.

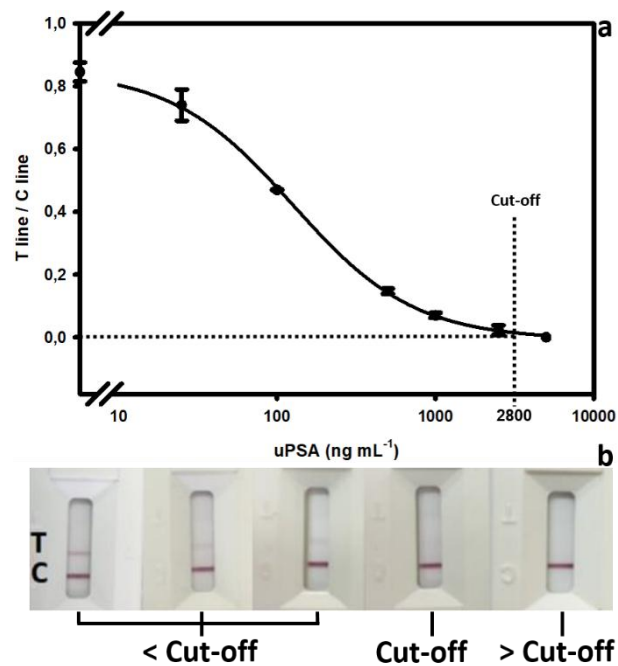


Fig. 5. uPSA detection by the lateral flow biosensors. (a) Plot of calibrator signals obtained at the Test line normalized by the color at the Control line towards uPSA concentrations in fortified synthetic urine. (b) The naked-eye readout, in which the color intensity of the Test line progressively decreased as uPSA increased and completely disappeared in correspondence of the cut-off level. The uPSA concentrations are 0, 500, 1000, 2800 and 5000 ng mL⁻¹.

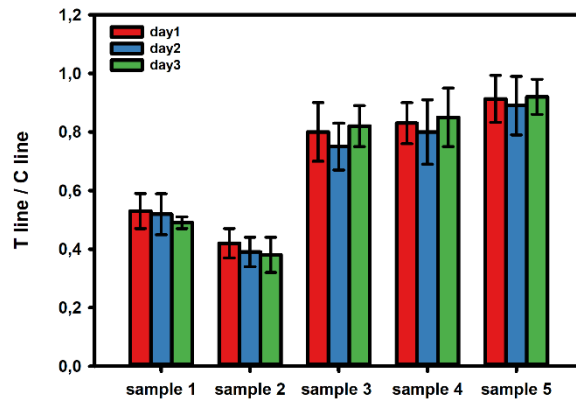


Fig. 6. Assessment of the reproducibility of the biosensor as measured on different days and from different strip batches. Bars represent standard deviation of nine measurements repeated on the same day (three for each batch). Samples 6 to 10 did not show the Test line signals as their uPSA concentrations were above the cut-off level.

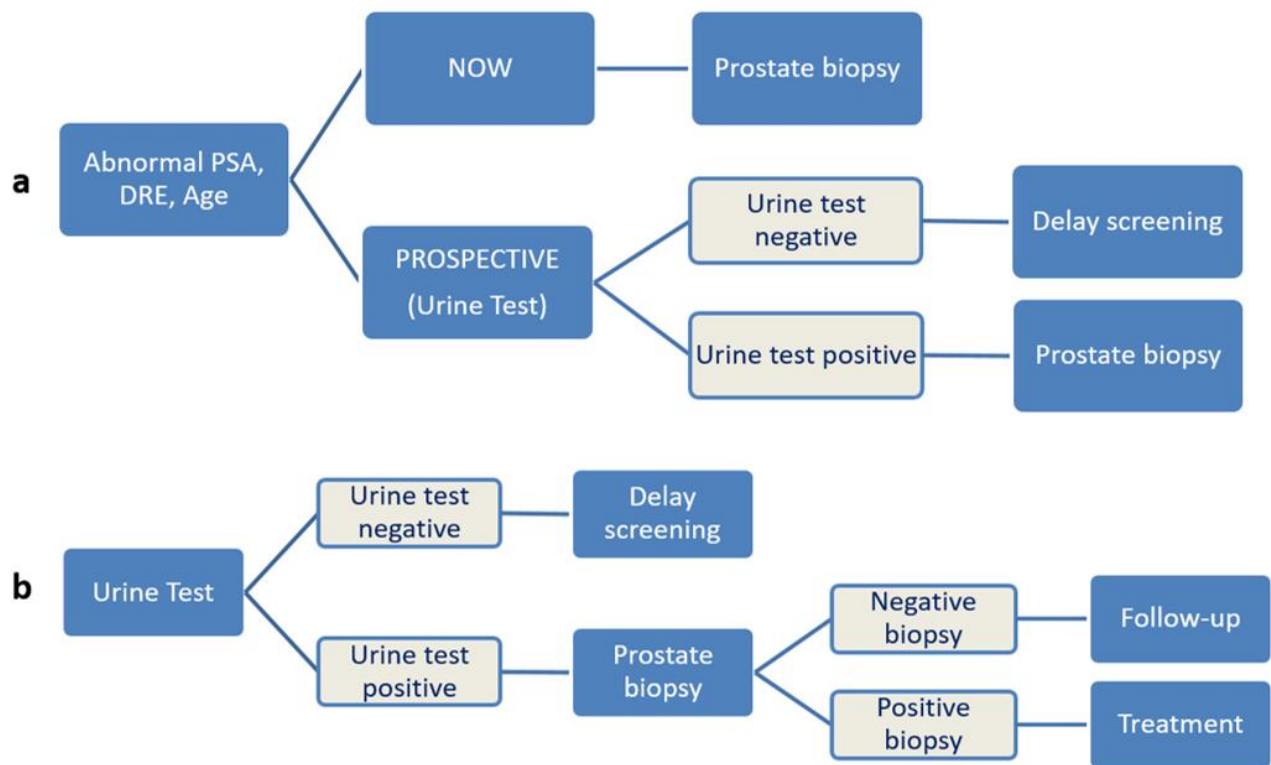


Fig. 7. Urine test will help to improve clinical decision making in detection and management of PCa. (a) Decision tree currently used to identify patients with significant prostate cancer and prospective introduction of the urine test to reduce repeat biopsy. (b) Alternative decision tree based on the use of uPSA as a first level screening biomarkers of PCa.