



A fluorometric lateral flow assay for visual detection of nucleic acids using a digital camera readout

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Received: 15 March 2018 / Accepted: 26 May 2018 / Published online: 4 June 2018
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Abstract

A fluorometric lateral flow assay has been developed for the detection of nucleic acids. The fluorophores phycoerythrin (PE) and fluorescein isothiocyanate (FITC) were used as labels, while a common digital camera and a colored vinyl-sheet, acting as a cut-off optical filter, are used for fluorescence imaging. After DNA amplification by polymerase chain reaction (PCR), the biotinylated PCR product is hybridized to its complementary probe that carries a poly(dA) tail at 3' edge and then applied to the lateral flow strip. The hybrids are captured to the test zone of the strip by immobilized poly(dT) sequences and detected by streptavidin-fluorescein and streptavidin-phycoerythrin conjugates, through streptavidin-biotin interaction. The assay is widely applicable, simple, cost-effective, and offers a large multiplexing potential. Its performance is comparable to assays based on the use of streptavidin-gold nanoparticles conjugates. As low as 7.8 fmol of a ssDNA and 12.5 fmol of an amplified dsDNA target were detectable.

Keywords Biosensor · DNA · PCR · Fluorescence · Phycoerythrin · Fluorescein isothiocyanate · Strip

Introduction

Fluorometric lateral flow assays have received great acceptance, since they effectively improve the detection limits and offer quantitative data compared to colored nanoparticles, while they retain the advantages of simplicity, rapidity and portability [1]. Organic fluorescent labels have been widely used as reporters in lateral flow assays. They have been proven to be useful for antibodies, peptides and nucleic acids, due to their unique optical properties, strong fluorescence, efficient labeling of biomolecules, along with low cost [2]. Novel nanomaterials, despite their increased cost, have been also applied to lateral flow assays, as an effort to improve the performance of the assays.

Only a few reports have been published, so far, regarding the use of fluorescent molecules/nanoparticles combined with lateral flow assays for the detection of nucleic acids. Deng et

al. (2018) have developed a fluorescent lateral flow assay based on quantum dots for the detection of synthetic HIV-DNA target (0.76 pM) in reaction buffer and human serum [3]. Fluorescent carbon nanoparticles have been then utilized by Takalkar et al. (2017) for lateral flow assay development and DNA detection. The detection limit was 0.4 fM of synthetic single-stranded target DNA in assay buffer. Fluorescence was measured by a lateral flow reader [4]. Sapountzi et al. (2015) exploited quantum dots for visual detection of nucleic acids and single nucleotide polymorphisms (SNPs) using a common digital camera for fluorescence imaging. The limit of detection of this method was as low as 1.5 fmol of double-stranded DNA and was applied to real sample analysis with multiplexing potential [5]. A fluorescent probe-based lateral flow assay was developed by Xu et al. (2014) for multiplex nucleic acid detection. Fluorescence-labeled specific probes were used for the simultaneous detection of four types of HPV virus. The fluorescence was again monitored by a portable fluorescence reader, while the detection limit was $10\text{--}10^2$ copies plasmid DNA/ μL [6]. Wang et al. (2013) used fluorescent $\text{Ru}(\text{bpy})_3^{2+}$ -doped silica nanoparticles labeled with a reporter probe for the detection of synthetic single-stranded DNA sequence with a detection limit of 0.066 fmol. The intensity of the fluorescence signal was measured by a fluorescent microtiter plate reader using a custom

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s00604-018-2856-9>) contains supplementary material, which is available to authorized users.

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holder which fits eight strips per reading [7]. Finally, up-converting phosphor technology particles were also used as reporters in lateral-flow assays to detect as low as 0.1 fmol of single-stranded asymmetric PCR product. The analysis (IR scanning) of the strips was performed in an adapted microtiter plate reader provided with a 980-nm IR laser for excitation of the phosphor particles [8].

A cost-effective lateral flow assay that exploits widely used organic fluorophores and a digital camera read-out was developed for the visual detection of nucleic acids. The assay was applied for the detection of specific DNA sequences. Biotinylated single-stranded (ssDNA) and biotinylated PCR products, were initially hybridized to complementary dA-tailed probes and then applied to the lateral flow strips. The hybrids are captured by immobilized dT-sequences at the test zone of the strip and detected by streptavidin-fluorescein or streptavidin-phycoerythrin conjugates via streptavidin-biotin interaction forming a visual fluorescent line. The excess of streptavidin-fluorescein or streptavidin-phycoerythrin conjugates is captured by immobilized biotinylated BSA at the control zone of the strip, producing a second fluorescent line to ensure the proper function of the strip. As low as 7.8 fmol of ssDNA target and 12.5 fmol of PCR product were detectable by the fluorometric assay.

Experimental

Materials and apparatus

The Immunopore FP membrane from Whatman (Florham Park, NJ, www.gelifesciences.com) was used for the construction of the strips. The sample, absorbent, and glass-fibered conjugation pad were from Schleicher&Schuell (Dassel, Germany, www.gelifesciences.com). Streptavidin-phycoerythrin (SA-PE) conjugate was from Molecular Probes (Eugene, OR, www.thermofisher.com/gr/en/home/brands/molecular-probes.html), whereas streptavidin-fluorescein isothiocyanate (SA-FITC) conjugate was from Sigma (St Louis, MO, www.sigmaldrich.com). Gold nanoparticles (AuNPs), 40 nm in diameter, were from BBI Solutions (Cardiff, UK, www.bbisolutions.com). Monohydrate and dihydrate sodium phosphate were from Lachner (Neratovice, Czech Republic, www.lach-ner.com). Sephadex G-25 was from Sigma and Triton X-100 were from Sigma-Aldrich (St Louis, MO, www.sigmaldrich.com), Bovine Serum Albumin (BSA) was purchased from Serva Electrophoresis GmbH (Heidelberg, Germany, www.serva.de), Streptavidin (SA) was from Roche (Basel, Switzerland, www.roche.com) and sucrose was from AppliChem (Maryland Heights, MO, [\[applichem.com\]\(http://www.applichem.com\)\). Sulfosuccinimidyl-6-\(biotin-amido\) hexanoate \(EZ-Link sulfo-NHS-LC-Biotin\), used for the biotinylation of BSA, was from Thermo Fisher Scientific \(Waltham, MA, USA, \[www.thermofisher.com\]\(http://www.thermofisher.com\)\). The terminal transferase reaction pack, containing the enzyme, the terminal transferase buffer and 2.5 mM CoCl₂ solution, was purchased from New England BioLabs \(Ipswich, MA, \[www.neb.com\]\(http://www.neb.com\)\). Deoxythymidine triphosphate \(dTTP\) and deoxyadenosine triphosphate \(dATP\) were purchased from Invitrogen \(Carlsbad, CA, \[www.thermofisher.com\]\(http://www.thermofisher.com\)\). A 2× Super Hot Master Mix, containing a mixture of *Taq* DNA polymerase, PCR buffer, MgCl₂ and dNTPs was purchased from Bioron GmbH \(Rhein, Germany, \[www.bioron.net\]\(http://www.bioron.net\)\). Agarose and DNA molecular weight markers \(*Φ*X174 DNA, *Hae*III digest\) were obtained from HT Biotechnology Ltd. \(Cambridge, UK\). Oligonucleotides and primers were from Eurofins Scientific \(Brussels, Belgium, \[www.eurofinsgenomics.eu\]\(http://www.eurofinsgenomics.eu\)\). The sequences of all the probes and primers used in this study are summed up in Table 1. Phosphate-buffered saline \(PBS\), pH 7.4, consisted of 137 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄ and 2 mM KH₂PO₄, 6× Saline-Sodium Citrate \(SSC\) buffer pH 7.0 consisted of 900 mM sodium chloride and 90 mM sodium citrate and 0.1 M phosphate Buffer \(PB\) pH 6.8 consisted of 46.3 mM Na₂HPO₄ and 53.7 mM NaH₂PO₄. The hybridization buffer consisted of 0.22 M NaCl, 0.1 M Tris \(pH 8.0\) and 0.88 mL·L⁻¹ Triton X-100. The developing solution consisted of 20 mL·L⁻¹ glycerol, 10 mg·L⁻¹ BSA and 2 mL·L⁻¹ Tween-20 in 1× PBS pH 7.4.](http://www.</p></div><div data-bbox=)

PCR amplification and denaturation reactions were performed in a TP 600 Gradient thermal cycler obtained from TaKaRa Bio (Shiga, Japan, www.clontech.com). The TLC applicator CAMAG Linomat 5 (Muttentz, Switzerland, www.camag.com) was used for the construction of the strip zones. All the membranes were dried in a Dedalos – 4c oven obtained from KEL (Patras, Greece, kel.com.gr). An ultraviolet (UV) lamp VL-4C, 254 nm, 4 W, from Vilber Lourmat (Marne La Vallee, France, www.vilber.com) was used for the excitation of the fluorescent dyes. A home-made black box and the Sony DSC-H 300 (Tokyo, Japan, www.sony.com) digital camera were used for fluorescence image capturing. The open source program ImageJ (<https://imagej.net/Welcome>) was used for image processing.

Construction of the lateral flow assay

The lateral flow assembly (strip), 4 mm × 70 mm in size, was composed by a wicking pad, a glass fiber conjugate pad, a nitrocellulose membrane, an absorbent pad and a plastic adhesive backing pad to obtain inflexibility. The four parts were placed onto the backing pad with overlapping ends, ensuring the continuous flow of the developing solution. Biotinylated bovine serum albumin (b-BSA) and poly(dT) sequences (dT(30) probe tailed with dTTP) were used for the preparation

Table 1 Oligonucleotide sequences used as primers and probes

Oligonucleotide	Name	Sequence (5' → 3')
PCR primers	HRPT1 F-primer	TGGAAAGGGTGTATTATTCCTCAT
	HRPT1 b-R-primer	b-ATGTAATCCAGCAGGTCAGCAA
Probes	HRPT1 IS probe	GTTAGTTAGATTATTGTTAGTTAG
	b-dA(30)	b-AAAAAAAAAAAAAAAAAAAAAAAAAAAAA
	dT(30)	TTTTTTTTTTTTTTTTTTTTTTTTTTTTT

of the control and the test zone of the strip, respectively. More specifically, a solution containing 300 ng/ μL BSA, diluted in 5% (v/v) methanol, 2% (w/v) sucrose and $1 \times$ PBS buffer was loaded at a density of 150 ng/4 mm onto the control zone of the membrane using the TLC applicator. Then, a solution containing 10 pmol/ μL poly(dT) sequences, diluted in 2% (v/v) methanol, 2% (w/v) sucrose and $6 \times$ SSC buffer was also loaded onto the membrane, 5 mm below the newly loaded control zone, at a density of 30 pmol/4 mm, for the formation of the test zone. The membranes were then dried in a common oven for 2 h at 80 °C and the strips were finally assembled.

Preparation of streptavidin-functionalized gold nanoparticles (SA-AuNPs)

A volume of 200 μL of gold nanoparticles, 40 nm in diameter, was pH-adjusted to 6.0 with $\sim 70 \mu\text{L}$ of 20 mM phosphate Buffer (pH 6.8). Then, 3 μL of streptavidin (SA) $1 \text{ mg}\cdot\text{mL}^{-1}$ in H_2O , were added to the gold nanoparticles and the mixture was incubated for 2 h at room temperature with frequent hand-stirring. The unreacted parts of the gold nanoparticles were blocked with $10 \text{ mg}\cdot\text{L}^{-1}$ BSA for 30 min at room temperature. SA-AuNPs were then isolated by centrifugation at 3300 g for 20 min, resuspended in 20 μL of 10 mM phosphate Buffer pH 6.8 and stored at 4 °C. An aliquot of 3 μL of SA-AuNPs was directly applied onto the conjugation pad of the strip for visual detection of DNA sequences.

Hybridization assay of the PCR product to the complementary dA-tailed probe

The molecular recognition of the DNA target was accomplished by hybridization to a specific oligonucleotide probe that is complementary to a small segment of the DNA target. More specifically, the PCR product (diluted properly in a solution of $0.1 \text{ g}\cdot\text{L}^{-1}$ BSA) was denatured in the thermal cycler for 3 min at 98 °C and rapidly cooled down on an ice bath for 2 min. A 5- μL volume of the denatured PCR product was then added to a pre-heated at 37 °C solution, containing 0.5 pmol of the dA-tailed specific probe and a proper volume of hybridization buffer to reach a final volume of 10 μL . The mixture was finally incubated for 15 min at 37 °C.

Fluorometric lateral flow assay

A 5- μL aliquot of biotinylated single-stranded dA(30) sequence or the pre-hybridized double-stranded PCR product, along with a 5- μL drop of a SA-PE or a SA-FITC conjugate, $0.02 \text{ mg}\cdot\text{mL}^{-1}$ in PBS pH 7.4 containing $1 \text{ mg}\cdot\text{L}^{-1}$ BSA, were applied onto the conjugate pad of the strip. The strip was then immersed into 300 μL of the developing solution. After 15 min, the strip was removed from the developing solution and placed into a home-made black box to avoid ambient light. Fluorescent green and orange zones were formed on the membrane of the strip due to streptavidin-biotin interaction after excitation of the fluorescent dyes by an ultraviolet lamp. The emitted fluorescence was captured by a 20.1-megapixel digital camera, well attached at the top of the box, at 12-cm distance above the strip, using a low-cost green or orange transparent vinyl sheet as a cut-off optical filter, respectively (Fig. 1). The images were further processed using the open source image processing program ImageJ. For comparison, the same solutions of single-stranded DNA sequences were analyzed by a 3- μL aliquot of streptavidin-functionalized gold nanoparticles (SA-AuNPs).

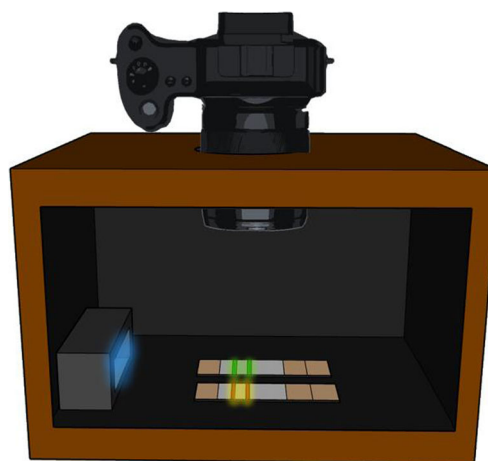
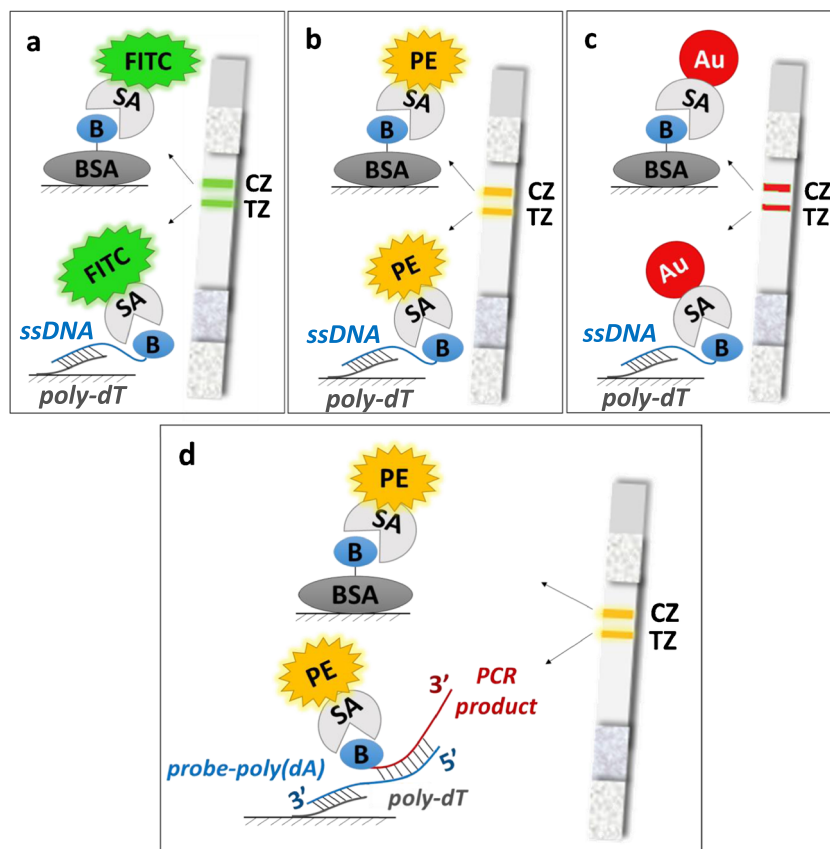


Fig. 1 Schematic illustration of fluorescence imaging using a common digital camera and a home-made UV black box

Fig. 2 Principle of the lateral flow assay for the detection of single-stranded DNA sequence using **a** SA-FITC, **b** SA-PE and **c** SA-AuNPs. **d** Principle of the lateral flow assay for the detection of double-stranded DNA target (PCR product) using SA-PE. CZ: control zone, TZ: test zone, SA: streptavidin, B: biotin, BSA: bovine serum albumin, FITC: fluorescein, PE: phycoerythrin, Au: gold nanoparticles, ssDNA: single-stranded DNA



Results and discussion

A low-cost fluorometric lateral flow assay was developed for the detection of nucleic acids using a common digital camera and a regular colored vinyl-sheet as optical filter. The double-stranded DNA target that corresponds to the DNA sequence used as the Internal Standard (IS) of the HPRT1 gene as previously described [9] was used as a model for the construction of the assay. In more details, the target DNA is amplified by PCR using a biotinylated primer. The biotinylated PCR product is then denatured at high temperature and let to hybridize to its complementary oligonucleotide probe that carries a poly-dA tail at its 3' end through tailing reaction. The hybrids are placed on the conjugate pad. Immediately, a SA-PE or a SA-FITC conjugate are also added to the conjugate pad of the assembly just under the DNA sample and the strip is immersed into the developing solution. As the developing solution flows up to the strip, all the reagents are forced to move upwards due to capillary forces. The hybrids are captured at the test zone of the strip by immobilized poly-dT sequences and detected by the SA-PE or SA-FITC conjugate due to streptavidin-biotin interaction forming a visual orange or green fluorescent line, respectively. A second fluorescent line is also formed at the control zone of the strip as the excess of the SA-PE or SA-FITC conjugates are bound to

the immobilized b-BSA. The control zone is always formed to confirm the proper function of the lateral flow assay (Fig. 2). FITC has excitation and emission maximum at 495 and 519 nm, respectively, while PE has an excitation peak at 496 nm and a fluorescence emission peak at 578 nm.

Optimization of fluorescence imaging

The optimum concentration of streptavidin-fluorescein (SA-FITC) and streptavidin-phycoerythrin (SA-PE) conjugates was initially determined to obtain high fluorescence signal. All the optimization studies of the lateral flow assay development were made using a biotinylated single-stranded dA(30) sequence (b-dA(30)) as DNA target. For this purpose, a 5- μL aliquot of three different concentrations of both fluorescent conjugates, 0.01, 0.02 and 0.1 $\text{mg}\cdot\text{mL}^{-1}$ were used for the detection of 50 μL of b-dA(30). All fluorescent images were processed in grayscale and the density of each fluorescent zone was determined by using the image processing software ImageJ. The concentration of 0.02 $\text{mg}\cdot\text{mL}^{-1}$ of both SA-PE and SA-FITC was selected as the optimum concentration that gave the higher fluorescence signal, along with the lower background signal.

The settings of the digital camera, that was placed onto the home-made black UV box for fluorescence imaging, were then investigated. The ISO values were set at the lowest

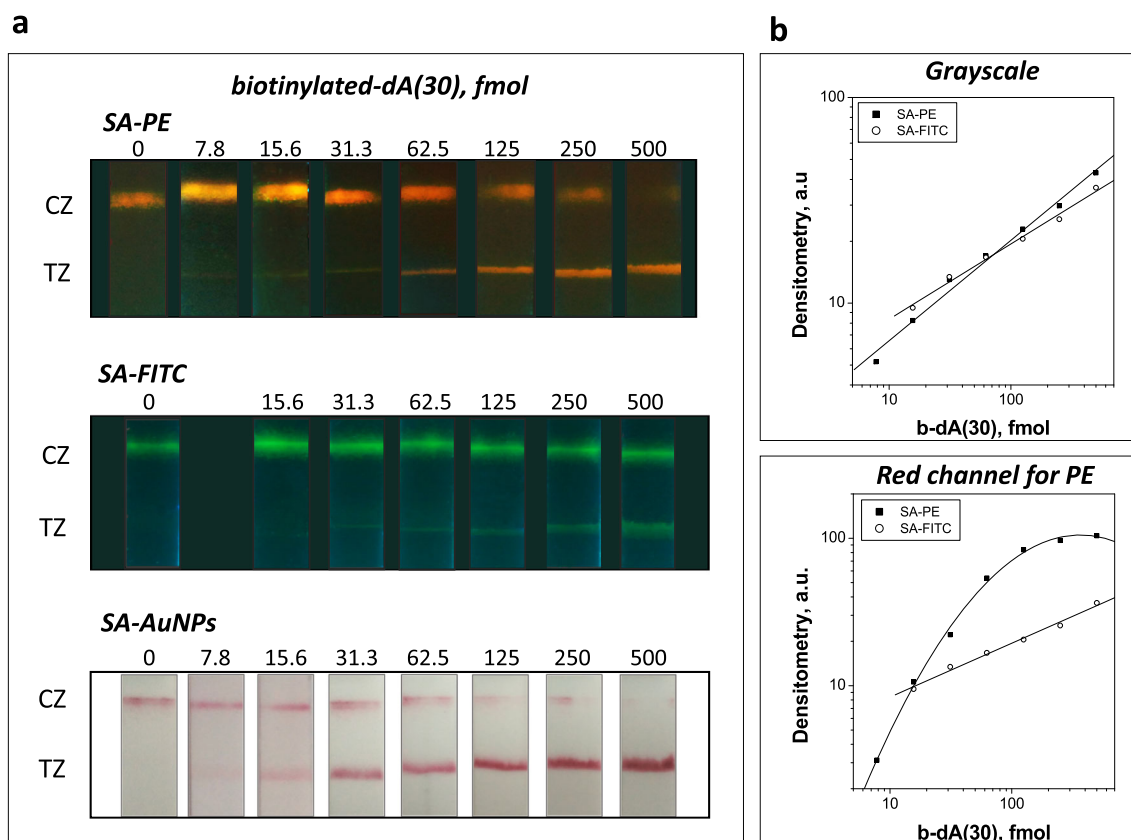


Fig. 3 **a** Lateral flow assay for the detection of ssDNA sequence (biotinylated-dA(30)) based on streptavidin-phycoerythrin, streptavidin-fluorescein and streptavidin-gold nanoparticles conjugates. **b** Calibration graph of the fluorometric lateral flow assay: the fluorescence intensity of fluorescein and phycoerythrin. Respectively, measured in grayscale mode

(up) and in the red channel for phycoerythrin (down), is plotted against the amount of the ssDNA sequence. **b**: biotin, SA-PE: streptavidin-phycoerythrin, SA-FITC: streptavidin-fluorescein and SA-AuNPs: streptavidin-gold nanoparticles, CZ: control zone, TZ: test zone

possible value (80 and 100), to reduce the background noise. To increase the luminosity of the image, different aperture times were tested (2, 5, 10, 20 and 30 s) in both ISO values (photographic sensitivity in light, named after the International Organization for Standardization). The lateral flow assay was tested using 50 fmol b-dA(30) and SA-PE conjugate as reporter. The results are presented in Fig.S1. The lower ISO value 80 and the acquisition time of 30 s were finally selected to get the higher signal-to-background ratio.

Fluorometric lateral flow assay for single-stranded DNA (ssDNA) sequences

Three calibration graphs were constructed to assess the detectability of the assay. Biotinylated single-stranded DNA sequences were initially used for the development of the assay. Seven different amounts from 7.8–500 fmol of single-stranded b-dA(30) were analyzed using (a) streptavidin-phycoerythrin conjugate (SA-PE), (b) streptavidin fluorescein conjugate (SA-FITC) and (c) streptavidin-functionalized gold nanoparticles (SA-AuNPs), as reporters. The fluorescence images were taken by the digital camera with the following settings: ISO 80

and aperture time of 30 s. The fluorescent intensity was further determined by densitometric analysis using the ImageJ software. We compared the color information in four different dimensions: a) in grayscale mode and b) in red, green and blue channels of the original RGB (red-green-blue) images. The signal-to-background ratios of the intensity values of each channel was then plotted versus the amount of ssDNA. The optimum ratios were obtained in grayscale mode for FITC and in the red channel for PE (Fig.S2). Finally, for the construction of calibration curves, the density values of each test zone (measured both in grayscale mode and in the red channel for PE) were plotted against the amount of b-dA(30) oligonucleotide. As low as 7.8 fmol of single-stranded DNA target was detectable with a signal-to-background ratio of 1.7. Moreover, the density values in the red channel were increased, but they provided a narrower linear range. We observed that the fluorescent dye PE is more sensitive and provided higher detectability than FITC. This may be attributed to the fact that PE has greater brightness (a combination of molar extinction coefficient and quantum yield) and photostability than FITC. Also, PE-based lateral flow assay exhibited similar performance with gold

nanoparticles (Fig. 3). Finally, we observed that the fluorescent zones were stable for more than 24 h.

Fluorometric lateral flow assay for double-stranded DNA sequences

The detectability of double-stranded DNA sequences was then determined. Different amounts ranging from 12.5 to 100 fmol of double-stranded biotinylated PCR product were analyzed by the strip using SA-PE as the optimum reporter. After densitometric analysis by the ImageJ software (in grayscale mode and in the red channel), two calibration graphs were constructed by plotting the density of each fluorescent test zone versus the amount of PCR product. As low as 12.5 fmol of PCR product was detected by this assay, with a signal-to-background ratio of 1.8. The results are presented in Fig. 4. Again, density values in the red channel were higher than in grayscale mode, but both measurements gave equal analytical range. The specificity of the assay, in addition, depends on the specificity of the probes used in the hybridization reaction with the DNA target prior to the application to the lateral flow strip, as previously reported [10].

Reproducibility of the assay

The reproducibility of the assay was finally defined by analyzing, in triplicate, 50 fmol of the PCR product (Fig. 4). After image processing and densitometric analysis using the ImageJ

software, the % Coefficient of Variation (CV) calculated was equal to 6.2%.

Conclusions

Lateral flow assays represent a powerful tool in biosensing. An overview on recently reported lateral flow assays for the determination of specific DNA sequences is summarized in Table 2. Apart from fluorescence-based approaches, different advanced methods using various reporters and sensing systems have been explored for lateral flow assays development. Wang et al., reported a method for eliminating the carryover contamination of loop-mediated isothermal amplification combined to simultaneous treatment with uracil-DNA-glycosylase. DNA isolated from *Streptococcus pneumoniae* was subjected to isothermal amplification using fluorescein-dUTP and biotin-dUTP. The amplified products were detected by a lateral flow strip using anti-fluorescein immobilized antibodies and colored polymer beads conjugated to streptavidin. The limit of detection was 25 fg/ μ L in cultures and 470 cfu/mL of spiked blood samples [11]. Surface-enhanced Raman scattering (SERS)-based lateral flow assays have been also developed using a Raman microscope for Raman spectra acquisition. This system offers spatial discrimination of multiple targets with a detection limit of 0.043 pM and 0.24 pg/mL of single-stranded synthetic DNA oligonucleotide [12, 13]. Moreover, a formamidopyrimidine DNA glycosylase probe chemistry combined to lateral flow assay

Fig. 4 **a** Calibration graph of the fluorometric lateral flow assay for the detection of double-stranded DNA sequence (PCR product) based on streptavidin-phycoerythrin conjugate. **b** Reproducibility of the method. The PCR product is analyzed in triplicate by the fluorometric lateral flow assay (CV = 6.2%). The mean fluorescence intensity values ($n = 3$) of phycoerythrin were plotted versus the amount of the PCR product **c** measured in grayscale mode and **d** in the red channel of the RGB image. SA-PE: streptavidin-phycoerythrin, CZ: control zone, TZ: test zone

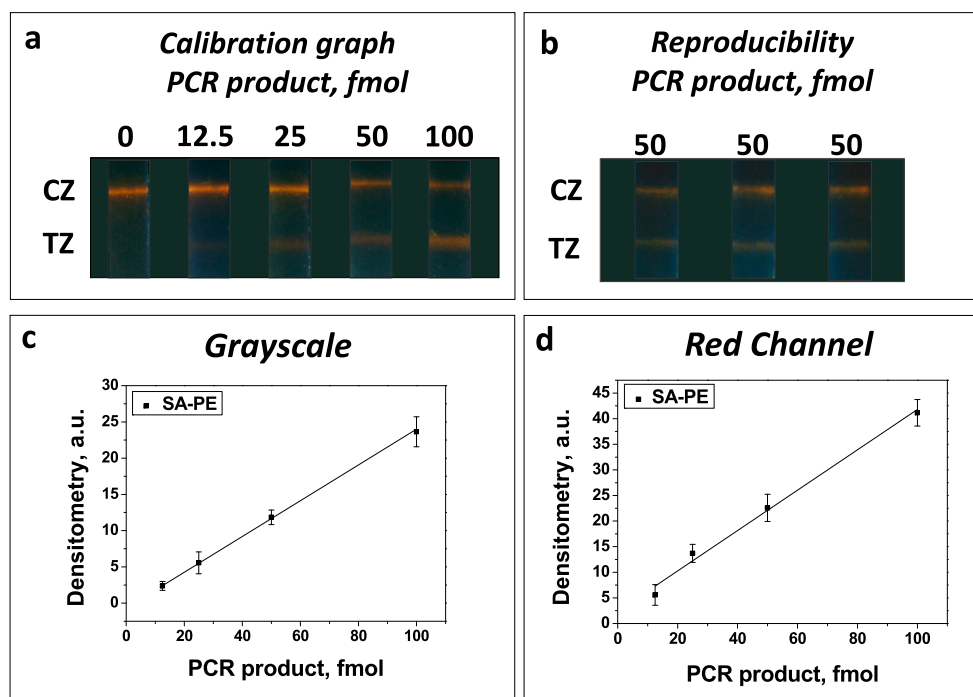


Table 2 An overview on recently reported methods for determination of DNA sequences using lateral flow assays (LFA)

Reporter	Analyte	LOD ¹	Multiplexing potential	Total analysis time ²	Limitations	Ref
Quantum dots	HIV single-stranded DNA target	0.76 pM ssDNA ⁴	***	4–4.5 h	Additional equipment is required It was not applied to real samples	3
Fluorescent carbon nanoparticles	Synthetic DNA target	0.4 fM ssDNA	**	20 min for LFA of synthetic target	Additional equipment is required It was not applied to real samples	4
Quantum dots	<i>S. epidermidis</i>	1.5 fmol	***	2.5 h	Demanding procedure for conjugation to biomolecules	5
Fluorescently-labeled probes	HPV virus	10–10 ² copies of plasmid DNA/ μ L	***	3.5 h	Additional equipment is required	6
Fluorescent Ru(bpy) ₃ ²⁺ -doped silica nanoparticles	<i>Trypanosoma</i> DNA target	0.066 fmol ssDNA	*	20 min for LFA of synthetic target	Additional equipment is required It was not applied to real samples	7
Up-converting phosphor particles	<i>Emiliana huxleyi</i>	0.1 fmol	*	3 h	Additional equipment is required	8
Colored polymer beads	<i>S. pneumoniae</i>	25 fg/ μ L	*	1.5 h	Risk of false-positive results	10
SERS tags ³ and gold nanoparticles	Kaposi's sarcoma-associated herpesvirus, Bacillary angiomatosis and HIV-1 DNA target	0.043 pM ssDNA 0.074 pM ssDNA 0.24 pg/mL ssDNA	**	20 min for LFA of synthetic target	Multiplexing by spatial discrimination Additional equipment is required It was not applied to real samples	11 12
Gold nanoparticles	<i>Human genomic target</i> <i>Campylobacter jejuni</i> <i>E. coli</i> O157:H7	10–10 ² copies of DNA	*	40 min	Multiplexing by spatial discrimination	13
3D network of gold nanoparticles	HPV single-stranded DNA target	0.01 pM ssDNA	*	40 min for LFA of synthetic target	Additional equipment is required It was not applied to real samples	14
Gold nanoparticles	Synthetic DNA target	0.2 fmol	*	1 h	It was not applied to real samples	15
Gold nanoparticles	DENV single-stranded target DNA	3×10 ³ copies ssDNA	*	1.5 h	It was not applied to real samples	16
Carbon nanotubes	Synthetic DNA target	40 pM ssDNA	*	20 min for LFA of synthetic target	It was not applied to real samples	17
Carbon nanoparticles	Invertase gene	2.5 fmol	*	1.5 h	More demanding procedure for conjugation of oligonucleotides than proteins	18
Gold nanoparticles	HCV virus	0.01 fM ssDNA	*	< 1.5 h	LOD was not determined for double-stranded DNA	19
Colored polymer beads	<i>B. anthracis</i> RNA	0.25 fmol	**	< 1.5 h	–	20
Fluorescent dyes	HPRT1 gene	7.8 fmol ssDNA 12.5 fmol PCR product	***	2.5 h	–	This report

¹ limit of detection² after DNA isolation³ surface-enhanced Raman scattering⁴ single-stranded DNA

was reported for the detection of 10–100 copies of *E. coli* DNA amplified by recombinase polymerase amplification reaction [14], while three-dimensional DNA-gold nanoparticles conjugates were utilized for ultrasensitive paper-based detection of 0.01 pM of PCR product [15]. Gold nanoparticles were also exploited for the detection of as low as 0.2 fmol of recombinase amplified DNA product using tailed primers and 3×10^3 copies of isothermal amplified DNA target using a smartphone for signal acquisition [16, 17]. Other reporters, such as carbon nanotubes and carbon nanoparticles were used in lateral flow assays offering a detection limit of 40 pM and 0.8 fmol, respectively, showing greater stability in solutions with high ionic strength than gold nanoparticles [18, 19]. Subsequently, isothermal strand-displacement polymerase reaction was used in combination with gold nanoparticles to detect as low as 0.01 fM of single-stranded DNA target [20]. Finally, a “sandwich-type” hybridization on a lateral flow strip using colored polystyrene beads and nucleic acid sequence-based amplification (NASBA) was reported by Carter et al. The detection limit of this assay was 0.25 fmol of amplified product [21].

Several alternative labels have been explored and advanced methods have been developed for novel detection systems in lateral flow assays. Regarding real sample analysis, DNA isolation and amplification steps are crucial in order to achieve high detectability. Recombinase polymerase amplification reaction and isothermal amplification have proven to be faster than polymerase chain reaction and not at the expense of detectability. Fluorescence-based assays had shown higher sensitivity/detectability and wider dynamic range than conventional gold nanoparticles. Gold nanoparticles, on the other hand, are visualized by naked eye and they need no costly and complex readout device [15, 22, 23], while most of the aforementioned methods require special and expensive equipment for signal measuring. The use of a common digital camera or a smartphone device has been introduced as promising detectors in fluorescent-based lateral flow tests aiming to improve point-of-care testing [24–30]. This alternative sensing system has the advantages of developing low-cost, simple and highly portable lateral flow devices.

In conclusion, a new and low-cost fluorometric lateral flow assay was developed for the detection of specific DNA sequences. The detection was based on, commercially available, streptavidin-phycoerythrin and streptavidin-fluorescein conjugates, while a common digital camera and a regular colored vinyl-sheet, as cut-off optical filter, were used for fluorescence capturing. All images were processed properly using the free online software ImageJ. As low as 7.8 fmol of single-stranded target DNA and 12.5 fmol of PCR product were detected by this assay. This lateral flow assay is universal and can be applied for the detection of any DNA sequence, is low-cost, rapid, portable, reproducible and sensitive and exhibits similar performance compared to gold nanoparticles-based assay. Its

main advantages are the simplicity, along with the great multiplexing potential, as more than one fluorophores can be simultaneously applied on the same lateral flow strip. Moreover, no instrument is required for fluorescent measurement. In addition, the exploitation of more specialized cut-off optical filters may increase the sensitivity of the assay.

Acknowledgements We acknowledge the assistance of Dr. Sotirios Tragoulias with image acquisition.

Compliance with ethical standards The author(s) declare that they have no competing interests.

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