



Enhancement of lateral flow immunoassay by alkaline phosphatase: a simple and highly sensitive test for potato virus X

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Abstract

Alkaline phosphatase (ALP) was used as an amplification tool in lateral flow immunoassay (LFIA). Potato virus X (PVX) was selected as a target analyte because of its high economic importance. Two conjugates of gold nanoparticles were applied, one with mouse monoclonal antibody against PVX and one with ALP-labeled antibody against mouse IgG. They were immobilized to two fiberglass membranes on the test strip for use in LFIA. After exposure to the sample, a substrate for ALP (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium) was dropped on the test strip. The insoluble dark-violet diformazan produced by ALP precipitated on the membrane and significantly increased the color intensity of the control and test zones. The limit of detection (0.3 ng mL^{-1}) was 27 times lower than that of conventional LFIA for both buffer and potato leaf extracts. The ALP-enhanced LFIA does not require additional preparation procedures or washing steps and may be used by nontrained persons in resource-limited conditions. The new method of enhancement is highly promising and may lead to application for routine LFIA in different areas.

Keywords Lateral flow immunoassay enhancement · Enzyme amplification · High sensitive lateral flow immunoassay · Potato virus X · Gold nanoparticles conjugates · Non-laboratory diagnostics · Signal enhancement

Introduction

Lateral flow immunoassay (LFIA) is an easy analytical method that is widely used for routine analysis. Despite the advantages of LFIA (rapidity, low cost, simplicity, and data interpretation), its applicability is often limited because of LFIA's low sensitivity in comparison to laboratory analytical methods [1]. Thus, developing highly sensitive LFIAs is of great importance. Approaches to decreasing LFIA's limit of detection (LOD) should not complicate the analysis procedure, be time consuming, or require special equipment.

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Previous developments for reaching higher sensitivity in LFIA may be divided into three major groups: 1) more efficient labels (e.g., quantum dots, dye-doped nanoparticles, etc.) [2]; 2) catalytic enhancement (e.g., amplification by enzymes, silver enhancement) [3]; and 3) label aggregation (e.g., gold enhancement) [2, 3]. Various detection techniques are used for LFIA: colorimetry/fluorometry [4, 5], chemiluminescence, amperometry, magnetometry [5], SERS registration [6], and so on. Colorimetric detection (determination of color intensity) is the simplest and most frequently used detection technique because it may be performed by visual inspection or by easily accessible and cheap detectors (smartphone or scanner) [5, 6].

One of the most promising and least considered tools for signal enhancement in LFIA is enzymes. Enzymes, particularly horseradish peroxidase (HRP; EC 1.11.1.7) and alkaline phosphatase (ALP; EC 3.1.3.1), are traditionally used in enzyme-linked immunosorbent assay and other types of immunoassays because of their commercial availability, high stability, and catalytic properties, which provide high sensitivity [7].

Enzymes have limited application in LFIA, focused mainly on HRP [8–16]. The decrease of the LOD of HRP-enhanced LFIA in comparison with that of conventional LFIA varies

from 5-fold [15] to 100-fold [16] for different antigens. However, the effectiveness of HRP enhancement may be low for real samples because of interference with their matrix components or the presence of HRP inhibitors [10]. Also, the endogenous peroxidase activity of some matrixes (e.g., plant extracts [17]) limits the use of HRP enhancement for LFIA.

Replacement of HRP with another enzyme, such as ALP, may be a prospective approach to LFIA enhancement. ALP is the second most frequently used enzyme in commercial ELISA kits [7]. Previously, Lathwal and Sikes [18] demonstrated that ALP provides the lowest LOD in dot immunoassay in comparison to HRP, silver enhancement, and polymerization-based amplification. The paper-based ELISA developed by Lathwal and Sikes [18] is time consuming (requiring more than 1 h) and requires washing steps. For practical use in routine analysis, these factors are drawbacks. This further confirms the prospects of ALP for fast and easy-to-use LFIA. However, ALP has been applied to LFIA in only a few studies, which used ALP-IgG conjugates [19–21]. ALP-IgG conjugates were used in liquid form and mixed with the sample before analysis. However, the application of liquid immunoreactants complicated the analysis procedure. Also, in these works, the authors did not show the decrease in the LOD compared to conventional LFIA. Thus, ALP was not used as a tool for decreasing the LOD in GNP-based LFIA.

In this article, a combination of gold nanoparticles (GNP) and ALP was used to enhance LFIA for highly sensitive detection of the economically important pathogen potato virus X (PVX) [22]. It is especially important to use ALP in LFIA of plant samples because HRP cannot be used due to the high peroxidase activity of plant extracts. The ALP catalyzes the reaction of the insoluble diformazan colored product formation. The accumulation of this product leads to a significant increase of the control and test zones' color intensity. Colorimetric detection can be achieved by visual inspection and performed in laboratories with limited resources [5]. ALP-enhanced LFIA preserves the major advantages of conventional LFIA: all components are in a dry form and rehydrated during the analysis, preserving their functional activity. Additionally, dry components are more convenient for practical use because no additional manipulation and preparation is required.

Materials and methods

Reagents

PVX was propagated in *Nicotiana tabacum* and purified from infected leaves following Goodman [23]. Monoclonal mouse and polyclonal rabbit antibodies specific to PVX were described in our previous work [24]. Goat-anti-mouse IgG-

ALP conjugate was purchased from MP Biochemicals (Santa Ana, CA, USA, www.mpbio.com).

Tris(hydroxymethyl)aminomethane (Tris), sucrose, bovine serum albumin (BSA), biotinamido hexanoyl-6-aminohexanoic acid N-hydroxysuccinimide ester, streptavidin – peroxidase polymer, BCIP/NBT SigmaFast tablets, and p-nitrophenyl phosphate were purchased from Sigma-Aldrich (St. Louis, MO, USA, www.sigmaaldrich.com). Chloroauric acid was purchased from Fluka (Taufkirchen, Germany). 3,3',5,5'-Tetramethylbenzidine (TMB) was purchased from Thermo Fisher (Waltham, MA, USA, www.thermofisher.com). All acids, alkali, salts, and solvents were purchased from Chimmed (Moscow, Russia, www.chimmed.ru/). All chemicals were of analytical reagent or chemical reagent grade. Solutions were prepared using water deionized by a Milli-Q system produced by Millipore (Billerica, MA, USA, www.merckmillipore.com). Lateral flow test strips were fabricated using nitrocellulose membranes (CNPC 12), conjugate release matrix (PT-R5), sample (GFB-R4), and absorbent pads (AP045) produced by Advanced Microdevices (Ambala Cantt, India, www.mdmembrane.com).

Gold nanoparticle synthesis

A modified Frens method [25] was used for GNP synthesis. First, 1 mL of 1% HAuCl₄ was added to 95 mL of deionized water and heated to boiling point, and then 4 mL of 1% sodium citrate was added while the mixture was stirred. The mixture was boiled for 25 min, cooled, and stored at 4 °C.

Gold nanoparticle conjugate synthesis

Two GNP conjugates were synthesized by physical adsorption as described by Hermanson [26] with modifications [27]. The first was a conjugate of GNP–monoclonal antibodies to PVX (GNP–mAb), and the second was a GNP–anti-mouse IgG–ALP conjugate (GNP–anti-mAb–ALP). Monoclonal antibodies and anti-mouse IgG–ALP conjugate were dialyzed against 10 mM Tris-HCl, pH = 9.0. For conjugate synthesis, the pH of the GNP solution was adjusted to pH 9.0 and mixed with monoclonal antibodies to PVX (14 µg mL⁻¹) or anti-mouse IgG–ALP (15 µg mL⁻¹). The mixtures were stirred at room temperature for 1 h, and then BSA was added to the final concentration of 0.25%. GNP conjugates were separated by centrifugation at 18000 g for 30 min. The conjugates were stored at 4°C in a Tris buffer containing 0.25% BSA, 0.25% Tween-20, 1% sucrose, and 0.03% sodium azide. The characteristic of GNP and GNP conjugate is presented in the Supplementary materials, section 1, Fig. S1.

Preparation of test strips

Two LFIA test strips were made: one for conventional LFIA with one GNP–mAb conjugate, and one for LFIA with signal

amplification and two GNP-mAb and GNP-anti-mAb-ALP conjugates. Test and control lines were applied by an IsoFlow dispenser (Imagene Technology, Hanover, NH, USA, www.imagenetechnology.com). Polyclonal antibodies against PVX (0.9 mg mL^{-1} in phosphate buffer (PB) with 0.5 mg mL^{-1} BSA and 5% glycerol, $0.15 \text{ }\mu\text{L}$ per 1 mm membrane width) were immobilized in the test zone. Protein A (0.4 mg mL^{-1} in PB with 5% glycerol, $0.15 \text{ }\mu\text{L}$ per 1 mm membrane width) was immobilized in the control zone. Two fiberglass membranes (3 mm length and 5 mm width) were soaked ($1.6 \text{ }\mu\text{L}$ of the conjugate to 1 mm of the fiberglass membrane length) with GNP conjugates solutions of different optical density (GNP-mAb: OD = 4.0; GNP-anti-mAb-ALP: OD = 2.0, 1.0, 0.5, 0.4, 0.2). All the membranes were dried at 32°C for 8 h. After drying, multimembrane composites were assembled. Fiberglass membrane impregnated with GNP-anti-mAb-ALP was applied at the bottom of the test strip, and fiberglass membrane impregnated with GNP-mAb was applied above in contact with a nitrocellulose membrane. There was a distance of 5 mm between the fiberglass membranes (Supplementary materials, section 4, Fig. S3). After assembly, the multimembrane composites were cut into strips (3 mm width) using an Index Cutter-1 (A-Point Technologies, Gibbstown, NJ, USA) and hermetically packed into bags composed of laminated aluminum foil and silica gel as a desiccant using an FR-900 continuous band sealer (Wenzhou Dingli Packing Machinery, Wenzhou Shi Zhejiang, China).

Lateral flow immunoassay

Three formats of LFIA were used (Fig. 1). The first format was common LFIA with one GNP-mAb conjugate (hereafter referred to as LFIA-1). The second format was LFIA with two conjugates (GNP-mAb, GNP-anti-mAb-ALP) and without the addition of ALP substrate solution (hereafter referred to as LFIA-2). The third format was LFIA with two conjugates and with the addition of ALP substrate solution (hereafter referred to as LFIA-3).

For all LFIAs, 50 mM Tris-HCl containing 0.05% Triton X-100 and 100 mM NaCl (Tris-T) was used as a running buffer. 100 mM NaCl was added to reduce nonspecific background.

For LFIA-2, the sample pad of the test strip was vertically submerged in the sample for 10 min. Each measurement was done in triplicate, and the qualitative results were estimated visually. For quantitative analysis, the test strips were scanned by a Canon 9000F Mark II scanner. The color intensity (relative units, RU) was determined by TotalLab TL120 (Nonlinear Dynamics, Newcastle upon Tyne, UK, www.totallab.com). The dependence of test zone color intensity (RU) on PVX content (ng mL^{-1}) was approximated using OriginPro 9.0 (Origin Lab, Northampton, MA, USA, www.originlab.com).

The first stage of LFIA with ALP enhancement was the same as described above. After 10 min of incubation, the strip was removed from the sample and placed on a horizontal surface, and $10 \text{ }\mu\text{L}$ of substrate solution was dripped on the test zone. The solutions of p-nitrophenyl phosphate (2 mg mL^{-1} in 100 mM sodium carbonate buffer; pH = 9.8) and BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate, 0.15 mg mL^{-1} ; nitro blue tetrazolium chloride, 0.30 mg mL^{-1}) in 100 mM Tris-HCl buffer with 5 mM MgCl_2 (pH = 9.5) were used as substrate solutions. After 5 min, the coloration of the test and control zones was visually observed, and the strips were scanned as described above. The PVX concentration that accords the visual test line appearance (color intensity ~ 1 RU) is considered the limit of visual detection.

Leaf extract preparation

Infected (11 samples) and healthy (2 samples) potato leaves were used to prepare the extracts. The potato leaves were homogenized in Tris-T (1:10 *w/v*) in a porcelain mortar and stored at 4°C before use.

Results and discussion

Lateral flow immunoassay design

The analysis procedure requires only the dipping of the test strip into the extract and the substrate addition. The analyst is not required to synthesize the nanomaterial or perform any other sophisticated procedures. Three formats of LFIA were used (Fig. 1). The first format was conventional LFIA with one GNP-mAb conjugate (hereafter referred to as LFIA-1). It is based on the formation of complexes between the antigen and antibodies conjugated to GNP and the capture of these complexes in the test line.

The second format was LFIA with two conjugates (GNP-mAb, GNP-anti-mAb-ALP) and without the addition of ALP substrate solution (hereafter referred to as LFIA-2). Because the sorption capacity of GNP is limited, we used separate GNP for ALP and mAb. The GNP-anti-mAb-ALP conjugate does not interact with antigen but binds GNP-mAb. The effect of the interaction of the two GNP conjugates on the LOD was studied using LFIA-2.

The third format was LFIA with two conjugates and the addition of ALP substrate solution (hereafter referred to as LFIA-3). The advantage of GNP-anti-mAb-ALP is its universal applicability to different types of LFIA with the GNP-monoclonal antibodies. The influence of ALP enhancement on the LOD was studied. We compared two commonly used ALP substrates, p-nitrophenyl phosphate (yellow soluble product) and BCIP/NBT (violet-black insoluble product) for signal enhancement. The two substrates were added in liquid

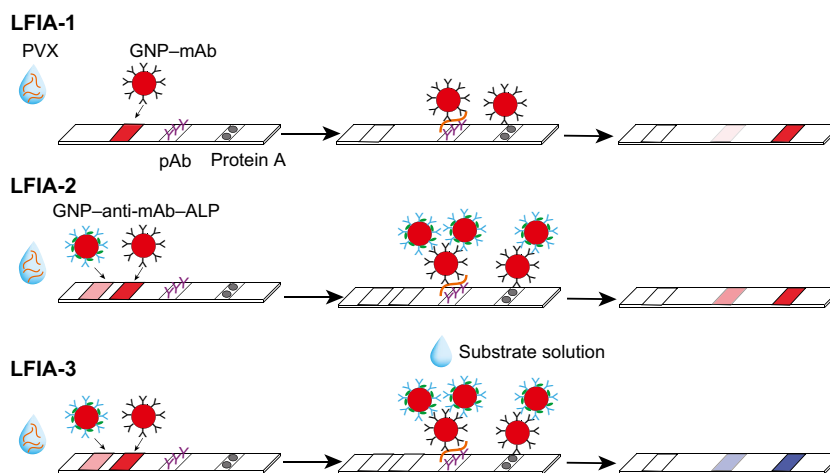


Fig. 1 LFIA-1 is a convenient LFIA based on the capture of the immune complexes PVX- GNP-mAb by pAb in the test zone. The color intensity of the test zone is related to the concentration of PVX in the sample. LFIA-2 is based on the interaction between PVX-GNP-mAb and GNP-anti-mAb-ALP. The capture of these complexes by pAb in the

test zone. LFIA-3 is based on the same principle as the LFIA-2, but after the analysis, the substrate solution (BCIP/NBT) was added. The formation of the insoluble colored diformazan products catalyzed by ALP leads to a significant increase in color intensity

form (10 μL) and then spread across the membrane by capillary forces.

Staphylococcal protein A was used to form the control zone. As shown in Fig. S4, protein A binds both the mouse IgG (GNP-mAb) and goat IgG (GNP-anti-mAb-ALP). Literature data [28] confirm that protein A binds mouse as well as goat IgG. During the assay, the analyst visually controls the migration of GNP conjugates across the membrane (or checks for evidence of the conjugate on the conjugate pad after the assay). For more thorough control of functional activity of the two GNP conjugates, a scheme utilizing separate control zones for each conjugate was proposed (data about this scheme are given in the Supplementary Materials, section 5).

Lateral flow immunoassay without enhancement

The first set of experiments aimed to compare LOD in LFIA-1 and LFIA-2 and study the effect of the second GNP conjugate. The GNP-anti-mAb-ALP conjugate acts not only as an enzyme carrier but also as an enhancement tool because of its interaction with the GNP-mAb conjugate and the formation of larger GNP labels. The GNP-anti-mAb-ALP conjugate solution with different OD_{520} was applied to a glass fiber membrane. An increase of OD_{520} to 2.0 results in high non-specific coloration of the test zones of blank samples (data not shown), whereas a decrease of OD_{520} to 1.0 or lower results in clear test zones. Thus, a concentration of $\text{OD}_{520} = 1.0$ was used in further experiments because it provides the highest number of ALP molecules, leading to high signal enhancement and the absence of nonspecific background.

The comparison of LOD for LFIA-1 and LFIA-2 was performed in buffer (Fig. 2). Both assays demonstrated similar

color intensity in the test zone. However, the LOD of LFIA-2 was about 1.5 times lower (5 ng mL^{-1}) than that of LFIA-1 (8 ng mL^{-1}). The same LOD (8 ng mL^{-1}) in conventional LFIA for the same antibodies and GNP with a size of about 20 nm was shown by Safenkova et al. [29]. Previous studies have shown that the application of two GNP conjugates specifically interacting with each other (gold enhancement) has varying effects on LOD, resulting in a 100-fold [30] decrease, a 2.5-fold [31] decrease, or even no effect [32].

The use of additional GNP carriers for ALP provides not only a high number of adsorbed ALP molecules for high signal enhancement but also a higher number of colored GNPs in the detected immune complexes.

The cross-reactivity of mAb and pAb against other widespread potato viruses Y^{O} , Y^{N} , S, M, A and potato leafroll virus were studied by enzyme-linked immunosorbent assay (see the Supplementary Materials, section 2). The monoclonal antibody detected only PVX, and cross-reactivity was less than 0.1%. The polyclonal antibody showed 0.2% cross-reactivity with PVY^{O} and PVS. In the LFIA, immune complexes with PVX were formed between pAb in the test zone and mAb in the GNP-mAb. Considering the absolute specificity of the mAb, LFIA detected only PVX.

Alkaline phosphatase-enhanced lateral flow immunoassay

The next set of experiments compared the LOD of LFIA-2 and LFIA-3 and studied ALP enhancement. The ALP substrates that produce soluble (p-nitrophenyl phosphate) and insoluble (BCIP/NBT) products were compared.

ALP enhancement was performed on lateral flow test strips with noncolored test zones (PVX concentration: 4 ng mL^{-1}).

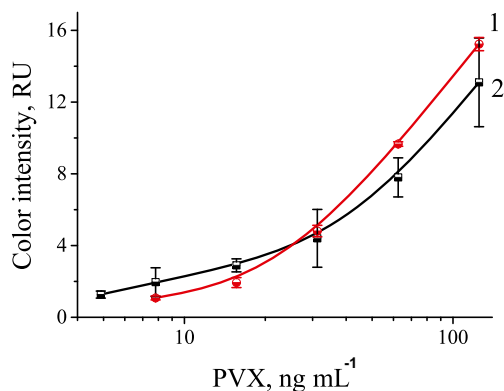
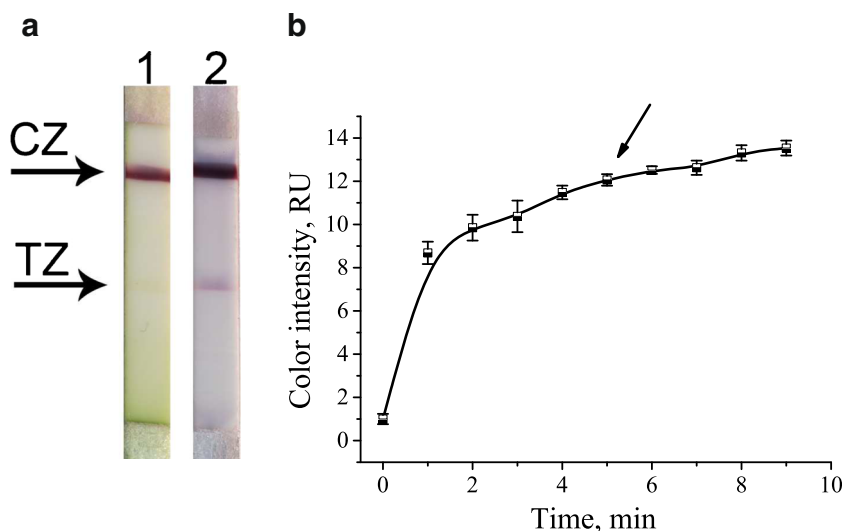


Fig. 2 Calibration curves for LFIA-1 (1) and LFIA-2 (2) for the detection of PVX in buffer. The color intensity of the test zone in relative unit RU (Y-axis) versus PVX amount ng mL⁻¹ (X-axis)

The number of fourth immunocomplexes (GNP–anti-mAb–ALP/GNP–mAb/PVX/pAb) captured in the test zone was not sufficient to produce a visually perceptible red coloration. After ALP enhancement, soluble products spread along the membrane via capillary forces. The blurred colors complicated interpretation of the results (Fig. 3a, strip 1). Thus, a substrate producing insoluble products is more applicable to LFIA. The colored dark-violet products accumulated in test and control zones, and narrow high-contrast bands were formed. The BCIP/NBT substrate was used for further experiments.

The BCIP/NBT incubation time was optimized to achieve high coloration and minimal background. Lathwal and Sikes [18] showed that the optimal incubation time for ALP with BCIP/NBT was 4 min because prolonged incubation leads to an increase in background staining. As shown in Fig. 3b, incubation for 5 min is sufficient for the development of high color intensity. During this period, the color intensity increased from 1 RU to 12 RU, while further incubation (8–10 min) led to a nonsignificant increase in color intensity

Fig. 3 ALP enhancement. **a** Test strips after ALP enhancement using different substrates. 1: p-nitrophenyl-phosphate; 2: BCIP/NBT. CZ – control zone; TZ – test zone. PVX: 4 ng/mL; **b** Increase in color intensity during incubation of ALP with BCIP/NBT. Optimal incubation time (5 min) is indicated by the arrow



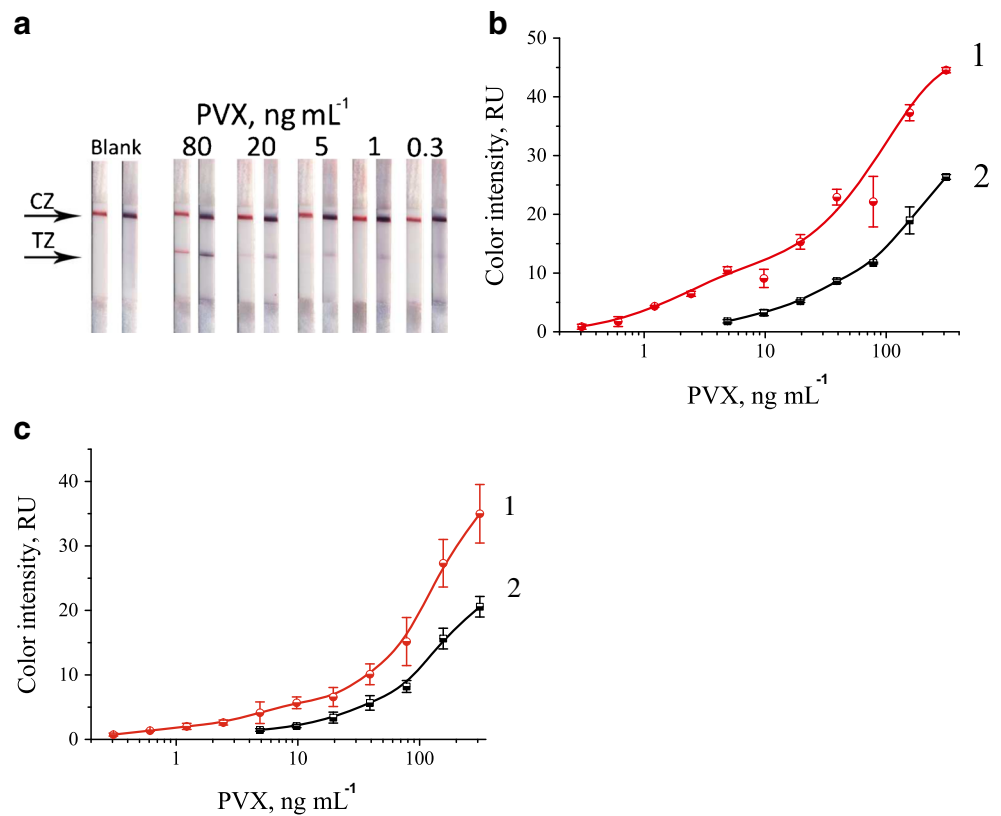
(from 12 to 14 RU). After 2–3 min of incubation, the substrate completely soaked into the membrane. Thus, 5 min incubation was used in further experiments.

Buffer solutions and leaf extracts containing a certain amount of PVX (0.3–250 ng mL⁻¹) were used to determine the LOD of LFIA-2 and LFIA 3. To study the effect of the components of the potato leaf extracts on sensitivity, the LODs in buffer and extract were compared. Strips before and after ALP enhancement are presented in Fig. 4a. The calibration curves for PVX LFIA without and with ALP enhancement in buffer and potato leaf extract are presented in Fig. 4b and c, respectively.

As shown in Fig. 4b and c, the visual LOD of LFIA-2 was 5 ng mL⁻¹ in both buffer and extract. The potato leaf extracts appeared to have no effect on the LOD for LFIA-3. The color intensity of the test zone after ALP enhancement increased by 40% to 400%. The potato leaf extracts did not feature endogenous phosphatase activity (clear test zones in blank samples). ALP enhancement resulted in a 27-fold reduction of the LOD of LFIA-3 (to 0.3 ng mL⁻¹ for buffer and extract) compared to LFIA-1. The color of the test zone not only increased in intensity but also changed from red to dark violet (Fig. 4a). Dark colored zones on the white nitrocellulose membrane provide more contrast, enabling better visual detection in comparison with red GNP coloration.

The LODs of the LFIA-2 and LFIA-3 were compared with ELISA with the same immunoreagents (see the Supplementary Materials, section 3). The limit of the detection for ELISA was 3 ng mL⁻¹. ELISA is a classical laboratory method that requires stationary equipment and takes about 4 h. The LFIA-3 was 10-fold more sensitive than ELISA, does not require sophisticated sample preparation, and takes only 15 min.

Fig. 4 Application of ALP enhancement to LFIA of PVX. **a** Test strips after LFIA-2 (left) and LFIA-3 (right) of different PVX concentrations in Tris-T. CZ – control zone; TZ – test zone. **b** Calibration curves for LFIA-3 (1) and LFIA-2 (2) of PVX in Tris-T. **c** Calibration curves for LFIA-3 (1) and LFIA-2 (2) of PVX in potato leaf extracts. The color intensity of the test zone in relative unit RU (Y-axis) versus PVX amount ng mL^{-1} (X-axis)



The closest analogues of the ALP-enhanced LFIA are HRP-enhanced LFIA, which demonstrated significantly varied decreases in LOD for different antigens (from 5-fold [15] to 100-fold [16]). However, as indicated above, the applicability of HRP enhancement to LFIA of plant extracts is limited because of the high endogenous peroxidase activity [17].

In the previous published immunoassays of PVX, different LODs were reported: 60 ng mL^{-1} for immunofiltration assay with magnetic detection [33], 2 ng mL^{-1} for LFIA with silver enhancement [34], $1\text{--}3 \text{ ng mL}^{-1}$ for magnetic microsphere enzyme immunoassay [35], 2.2 ng mL^{-1} for surface-enhanced Raman spectroscopy immunoassay [36], 1 ng mL^{-1} for multiplexed LFIA [37], 0.5 ng mL^{-1} for LFIA with magnetic concentration [38], and from 2 to 0.3 ng mL^{-1} for ELISA for different antibodies [39]. LFIA-3 provides the lowest LOD in comparison with the previously published assays. LFIA-3 is a fast method (15 min) that does not require additional stages of washing and concentration [33, 34, 38] or the use of expensive stationary equipment [36] as were needed in previous assays. LFIA-3 does not complicate the analysis procedure and provides an easy-to-use method for the non-laboratory detection of PVX in early stages of infection. Table S2 (Supplementary Materials, section 7) summarizes the LOD decreases for different LFIA enhancement strategies and indicates their advantages and

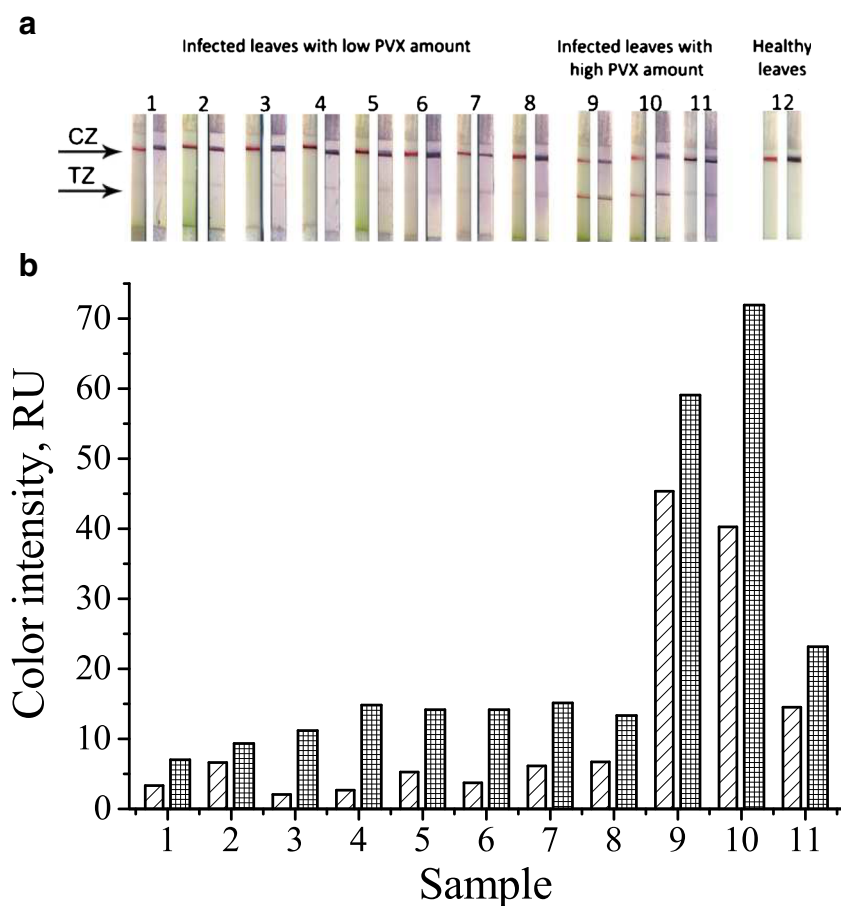
disadvantages for practical use. LFIA-3 provides high signal enhancement and demonstrates applicability for the routine analysis of PVX.

The ability of LFIA-3 to provide quantitative analysis results was confirmed by validation with ELISA (see the Supplementary Materials, section 6, Fig. S6). The correlation coefficient (R^2) was equal to 0.984, indicating the suitability of LFIA-3 for quantitative analysis. The ALP-enhanced LFIA is an easy-to-use method that requires only 5 additional minutes in comparison with conventional LFIA. The synthesized GNP-anti-mAb-ALP conjugate preserves enzymatic activity over at least 90 days of storage, modulating the acceleration of aging (for further information see the Supplementary Materials, section 8, Fig. S7). ALP enhancement with two dry GNP conjugates does not complicate the LFIA procedure and may be used without a laboratory infrastructure.

Alkaline phosphatase-enhanced lateral flow immunoassay of infected potato leaves

LFIA was used to detect PVX in infected potato leaves ($n = 11$). Strips before (LFIA-2, left) and after ALP amplification (LFIA-3, right) are presented in Fig. 5a, and the increase in color intensity after ALP amplification is presented in Fig. 5b.

Fig. 5 ALP-enhanced LFIA of PVX in potato leaf extracts. **a** Test strips after LFIA-2 (left) and LFIA-3 (right). CZ – control zone; TZ – test zone. **b** Color intensity of test zones after LFIA-2 and LFIA-3



There was no false positive coloration of test zones for healthy leaves (Fig. 5a, strip 12). ALP enhancement led to increased color intensity and a high-contrast test zone, which allows better visual detection. For some samples (1, 3, 4, 5, and 6), visual detection was hindered in LFIA-2. As shown in Fig. 5b, ALP enhancement dramatically increased color intensity by 30–400%.

Conclusion

The results demonstrate the effectiveness of applying ALP to enhance LFIA. The approach is based on the interaction between two conjugates, GNP–mAb and GNP–anti-mAb–ALP, and the ALP-catalyzed formation of insoluble product, which significantly increased the color intensity of the test and control zones. The ALP-enhanced LFIA for highly sensitive PVX detection showed a 27-fold decrease in LOD (to 0.3 ng mL^{-1}) in both buffer and extract. The matrix components of potato leaves did not affect the ALP reaction. Additionally, the enhancement did not complicate the LFIA procedure and can be performed in only 5 min without additional equipment. All immunoreagents and ALP are dried and rehydrated during the

analysis. However, an additional step, liquid substrate dropping, is required. In terms of analysis simplicity, this may be considered a drawback. We believe that the development of a full dry LFIA with ALP enhancement is in the future, which will make this test even more convenient for practical application and use by nontrained persons.

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Compliance with ethical standards

Conflict of interest The author(s) declare that they have no competing interests.

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