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Development of a gold nanoparticle-based lateral flow immunochromatographic assay for the rapid and quantitative detection of thymidine kinase 1 in human serum

Liya Ye, Xinxin Xu, Aihua Qu, Hua Kuang, Liqiang Liu, Chuanlai Xu *

International Joint Research Laboratory for Biointerface and Biodetection, and School of Food Science and Technology, Jiangnan University, Wuxi, People's Republic of China

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ABSTRACT

Thymidine kinase 1 (TK1) is a marker of cell proliferation that can be used for early screening, treatment monitoring, and evaluating the prognosis of patients with tumors. The main purpose of this study was to develop clinically applicable TK1 antibodies, establish an appropriate detection method, and provide material and technical support for the research and clinical application for different types of tumors. Experimental mice were immunized with the C-terminal 31 peptide of human TK1 to screen monoclonal cell lines capable of stably secreting specific antibodies. Monoclonal antibodies were then prepared, purified and screened for optimal pairing following the identification of purity and isotype. Finally, based on the principles adopted by the double-antibody sandwich detection method, we constructed a lateral flow immunochromatographic assay (LFIA) to quantify the concentration of TK1 in serum samples when using a gold nanoparticle-labeled anti-TK1 monoclonal antibody as a probe. The limit of detection for TK1 in serum was 0.31 pmol/L with a detection range of 0.31–50 pmol/L. The spiked recoveries ranged from 97.7% to 109.0% with an analytical precision of 5.7–8.2%; there was no cross-reactivity with common proteins in the serum. The established LFIA also exhibited good consistency with commercially available chemiluminescent immunoassay kits for the detection of clinical samples. The LFIA developed in this study has the advantages of high sensitivity, accuracy, reproducibility and strong specificity, and provides a new technical tool for the quantitative detection of TK1.

1. Introduction

Thymidine kinase 1 (TK1; ATP: thymidine 5′-phospho-transferase; EC 2.7.1.21) is an enzyme that plays a key role in DNA synthesis by catalyzing the phosphorylation of thymidine (Tdr) to thymidine monophosphate (TMP), which then forms thymidine triphosphate (TTP), one of the four obligatory deoxyribonucleotides involved in DNA synthesis [1]. A form of TK1 is also present at high levels in the serum of humans and animals with malignant tumors; consequently, serum TK1 activity measurements have been used for surveillance and prognostic purposes for a number of different malignant diseases [2], but mainly for leukemia and lymphoma [3]. Some studies have found that TK1 activity assays are not suitable for monitoring and evaluating patients with solid tumors [4,5]. In the meantime, TK1 activity assays have not yet been introduced on widely used diagnostic platforms and exhibit low sensitivity in samples from patients with solid tumors. The detection of serum TK1 protein levels has been extensively studied in serum samples for the evaluation of hematological tumors (e.g., leukemia and lymphoma) and for the monitoring and evaluation of solid tumors such as breast cancer [6], gastric cancer [7], lung cancer [8], and other tumors [9]. These studies have shown that the detection of TK1 protein level is significantly better than the detection of its activity [4]; consequently, the discovery of this phenomenon has significantly facilitated the detection of TK1 levels in serum samples.

Currently, the main method utilized by TK1 test kits is enzyme-linked immunosorbent assays (ELISAs) [10–12]. These immunoassay-based methods are used to determine the levels of TK1 protein in samples and rely primarily on specific anti-TK1 antibodies generated against the C-terminal portion of TK1 [13]. The main reason for adopting this strategy for antibody production is because the C-terminal region of TK1 plays a key role in cell cycle regulation [14]. This C-terminal region contains recognition sequences that trigger the degradation of TK1

* Corresponding author.
E-mail address: xcl@jiangnan.edu.cn (C. Xu).

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during mitosis and researchers have hypothesized that this region may produce antibodies [15]. The most likely factor responsible for the difficulty in establishing a sensitive and stable method for the determination of TK1 in serum is the presence of complex and variable forms of TK1 in the blood. Several studies have been conducted using human and canine TK1, thus resulting in the characterization of recombinant, cellular and serum forms of TK1 [16]. TK1 has been proven to exist in a variety of oligomeric complexes; importantly, only a small proportion of these complexes are enzymatically active. This is particularly the case for serum TK1 in patients with solid tumors. Oligomerization appears to be associated with the formation of disulfide cross-links in the blood but may also occur when tumor cells disintegrate and TK1 is transported into the blood. The fact that the major portion of TK1 protein in the blood is not enzymatically active could explain why measurements of TK1 enzyme activity are not very effective in patients with solid tumors [17]. Therefore, there is an urgent need to establish convenient and rapid *in vitro* diagnostic procedures that can determine the levels of serum TK1 with adequate sensitivity for clinical application.

The application of Enzyme-linked Immunosorbent Assay (ELISA) technology in the detection of serum proteins is limited by poor reproducibility, susceptibility to false positives and cumbersome operation. Furthermore, ELISA technology can be costly and requires a specific instrument. Collectively, these factors have restricted the application of ELISA for the clinical detection of TK1. Point-of-care testing (POCT) refers to tests that can be performed on-site or in the vicinity of a patient according to the medical and individual needs of each patients without the limitations imposed by location, time, the testing environment, and testing facilities; this technique is also referred to as immediate testing [18]. POCT can facilitate the detection of various biological indicators *in vitro*, reduce volume of sample required for testing, and significantly reduces the turnaround time for testing [19]. Furthermore, POCT can be applied at the patient's bedside, in the doctor's office, or at home for the rapid analysis of blood, urine, or other biological samples and can rapidly provide quantitative data.

Lateral flow immunoassays (LFIAs) are solid-phase immunoassays that combine thin-layer chromatography and immunorecognition to provide a simple platform for POCT [20]. LFIA was first used for the detection of serum proteins in the 1960 s; since then, this technique has been used widely in clinical testing, food testing, and environmental monitoring based on its rapidity, simplicity, reduced cost, and ease of operation [21–23]. The gold nanoparticle lateral flow immunoassays (AuNP-based LFIA) is one of the most widely used forms of the POCT assay [24]. The principle underlying the application of colloidal gold immune-quantitative detection is predominantly based on the proportionality between the depth color indicated by a test line on a nitrocellulose (NC) membrane and the concentration of the substance to be detected. In this assay, reagent strips are scanned by an optical sensor and the resultant data is then compared with standard curves, thus providing an efficient form of quantitative analysis [25]. However, LFIAs have yet to be evaluated for their potential to detect TK1 in serum. Consequently, in the present study, we aimed to establish an LFIA method to achieve sensitive, quantitative, rapid and accurate detection of TK1 in serum.

When generated by recombinant human TK1, monoclonal antibodies (mAbs) are capable of binding to human recombinant TK1 (rTK1) and, to varying degrees, the cellular form of human TK1 (sometimes denoted as cytoplasmic TK1). However, these mAbs have been shown not to bind efficiently to the human form of TK1 in a clinical sample, such as serum and blood samples [17]. In serum, human TK1 can be present in the form of high molecular weight complexes which exhibit TK1 activity such as oligomers, as well as dimers and tetramers with very low or even an absence of TK1 activity. Therefore, in this study, we selected the critical 31 amino acid sequence (aa 195–225) exposed to the C-terminal region of the antigenic determinant cluster on the surface of the TK1 protein to generate anti-human TK1 mAbs. Next, the mAbs were coupled to AuNPs and the optimal experimental antibody combination was determined by antibody pair screening. Finally, under optimal experimental conditions, we established a standard curve for the detection of the TK1 by the novel AuNP-LFIA and evaluated its sensitivity, specificity, reproducibility and accuracy.

2. Materials and methods

2.1. Main reagents and apparatus

The pET28a (+) plasmid, *Escherichia coli* BL21 (DE3) cells, and SP 2/0 myeloma cells used in this study were already held by the laboratory. Freund's complete adjuvant, Freund's incomplete adjuvant, polyethylene glycol (PEG), isopropyl-beta-D-thiogalactopyranoside (IPTG), keyhole limpet hemocyanin (KLH), bovine albumin (BSA) and goat anti-mouse IgG were purchased from Sigma-Aldrich (St. Louis, MO, USA). The general reagents used in the experiments described herein were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Ni-NTA and Protein G agarose gel resins were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). RPMI Medium 1640 and fetal bovine serum were purchased from Thermo Fisher Scientific (Shanghai, China). Nitrocellulose membranes (CN95, Sartorius), glass fiber membranes, absorbent pads and PVC plates were purchased from Jieyi Biotech (Shanghai, China). Kinbio Tech Co. Ltd. (Shanghai, China) provided all of the instruments used to produce tests strips, including a three-dimensional spraying and scribing device, a CTS 300 chop cutter device and a strip-cutting device. The ultra-micro spectrophotometer (Nano Drop 2000) was purchased from Thermo Fisher Scientific (Shanghai, China) and the ultrasonic cell pulverizer (JY92-IIN) was purchased from Scientz Biotechnology Co., Ltd. (Ningbo, China).

2.2. Approval for animal research

Specific Pathogen Free-grade BALB/c female mice, 6–8 weeks of age, were purchased from Charles River (Beijing, China). All of the mice used in this experiment were raised and used for research purposes, and all procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Jiangnan University and approved by the Animal Ethics Committee of Jiangnan University (JN. No20230228b1800928 [034]).

2.3. Preparation of human recombinant TK1

The full length human TK1 cDNA (GenBank accession number: NM_003258.4) was analyzed by bioinformatics and the encoding gene sequence was synthesized by Talen Biotechnology Co., Ltd. Next, this sequence was ligated into the pET28a (+) plasmid to construct an expression plasmid (pET28a-TK1). Recombinant TK1 protein was then prepared according to a method described previously [26,27] with minor modifications. The pET28a-TK1 plasmid was first transformed into *E. coli* BL21 (DE3) cells, inoculated with Luria-Bertani medium, and cultured at 37℃ with rotation at 220 r/min until the A_{600} value was 0.6. At this point, IPTG was added to a final concentration of 0.7 mmol/L and expression was induced for more than 12 h at 16℃ with rotation at 180 r/min. Next, the bacteria were pelleted from the culture medium, lysed ultrasonically and the supernatant was then passed through a 0.45 μm filter membrane. Finally, the expressed protein was purified by affinity chromatography on a Ni column, and the eluent was analyzed by 12% SDS-PAGE.

2.4. Preparation and characterization of mAbs

First, we selected a C-terminal 31 peptide from human TK1 (aa 195–225; sequence GQPAGPDNKENCPVPGKPGEAVAARKLFAPQ) as the immunogen. The peptide (TK1-PP) was synthesized by Royobiotech Co., Ltd. (Shanghai, China) and cross-linked with the carrier protein KLH in glutaraldehyde (2.5%). The conjugate and Freund's complete

adjuvant were mixed at a ratio of 1:1 by volume, fully emulsified, and then injected subcutaneously into the backs of mice at multiple points (100 μg/injection). The immunization was performed 3–5 times, separated by intervals of 21 days. Except for the initial immunization with Freund's complete adjuvant, subsequent immunizations were performed with Freund's incomplete adjuvant by intraperitoneal injection [28]. One week after the last immunization, we acquired blood samples from the tail vein of each mouse and the potency of the anti-TK1 antibody was detected by ELISA. Cell fusion was performed by an artificial induction method that utilized PEG [29]. Successfully fused cells were then incubated in 96-well plates in a cell culture incubator at 37℃ for 7 days. The potency of the antibody in each cell supernatant was then detected by indirect ELISA, and the positive wells in each 96-well plate were initially screened with TK1-PP coupled with BSA as the coating antigen. Then, the positive wells were re-screened with recombinant human TK1 protein as the coating antigen, in order to identify hybridoma cell lines that were capable of secreting antibodies in a continuous and stable manner. Next, we used the *in vivo* ascites induction method to produce large amounts of the monoclonal antibodies. Then, the hybridoma cells were injected into the peritoneal cavities of experimental mice. After 7 days, we collected ascites and the supernatant was centrifuged; finally, mAbs were purified by Protein G affinity chromatography. Subsequently, the concentrations of the antibodies were determined with a ultraviolet-visible (UV-Vis) spectrometer (HITACHI UH5300, Tokyo, Japan) and purity was determined by 12% SDS-PAGE., In addition, isotypes were identified by an Antibody Isotype Determination Kit (Biodragon, Suzhou, China).

2.5. Synthesis of gold nanoparticles

Colloidal gold was prepared in accordance with a previously reported technique [30] with slight modifications. First, 140 mL of ultrapure water was heated in a three-necked flask at 137°C in an oil bath with continuous stirring at 900 × g/min. When the temperature of the oil bath reached 100°C, we added l0 mL of 1% sodium citrate solution. The samples were then heated further for 40 min and 1 mL of 1% chloroauric acid solution was added; the reaction was allowed to continue for 60 s before adding 5 mL of 0.1 mol/L Tris-base solution into the center of the vortex. Next, the temperature was set to 100°C, the reaction was allowed to continue for 30 min, and then 1 mL of 1% chloroauric acid solution was rapidly injected into the center of the vortex. The reaction was allowed to continue for another 30 min, and the previous step was repeated. At the end of the reaction, heating was stopped, and the sample was allowed to cool naturally to room temperature under stirring; finally, the sample was stored at 4°C in the dark. Absorption profiles were scanned using an UV-Vis spectrometer and transmission electron microscopy (TEM) was used to investigate uniformity and particle size.

2.6. Preparation of test strips

2.6.1. Preparation process of test strips

The AuNP-labeled TK1 detection mAb was re-dissolved in resuspension buffer (0.01 M PBS, pH=7.4, containing 0.05% PC-950 Antimicrobial Preservative, 1% polyvinylpyrrolidone, 1% sucrose, 0.1% BSA, 1% PEG 20,000 and 5% additive), uniformly sprayed onto a conjugate pad and dried at 37°C for 12 h. The TK1 capture mAb and goat anti-mouse IgG were coated onto the NC membrane with a three-dimensional spraying and scribing device to create the test line (T line) and the control line (C line), respectively; this was followed by drying at 37°C for 12 h. Fig. 1 provides a schematic diagram depicting the quantitative detection of TK1 by test strips. A sample pad, a conjugate pad featuring an immobilized AuNP-labeled mAb, a NC membrane coated with T and C lines, and an absorbent pad were assembled onto a PVC backing plate, cut into 3.0 mm wide test strips [31], and stored under dry conditions in the dark.

2.6.2. Antibody pairwise screening

The antibodies selected for this experiment were mAbs that had been raised against the TK1 31-peptide. We used the checkerboard method to combine these mAbs [27]. The mAb was coupled with AuNPs to generate a probe, and the antibodies were then coated onto a NC membrane. Afterwards, the mAbs were assembled according to their pairwise combinations to generate the test strips. The optimal antibody pair was then determined based on the T/C value detected for each combination based on a 5 pmol/L concentration of recombinant TK1.

2.6.3. Determination of the optimal pH value for labeling

The pH of AuNP solution was adjusted by adding 10, 20, 30, 40, 50, 60, 70 and 80 μL of K_2CO_3 (0.1 mol/L) in a 2 mL tube; then, 15 μg of specific antibody was added. After 45 min of incubation, we added 100 μL of 10% BSA; this was then mixed, incubated for 2 h[32], and centrifuged at 8000 × g for 45 min. Next, the solution was re-dissolved in 100 μL of resuspension buffer, uniformly immobilized on a conjugate pad, and dried at 37°C for 12 h. The optimal labeling pH was determined based on data arising from the detection of negative and positive

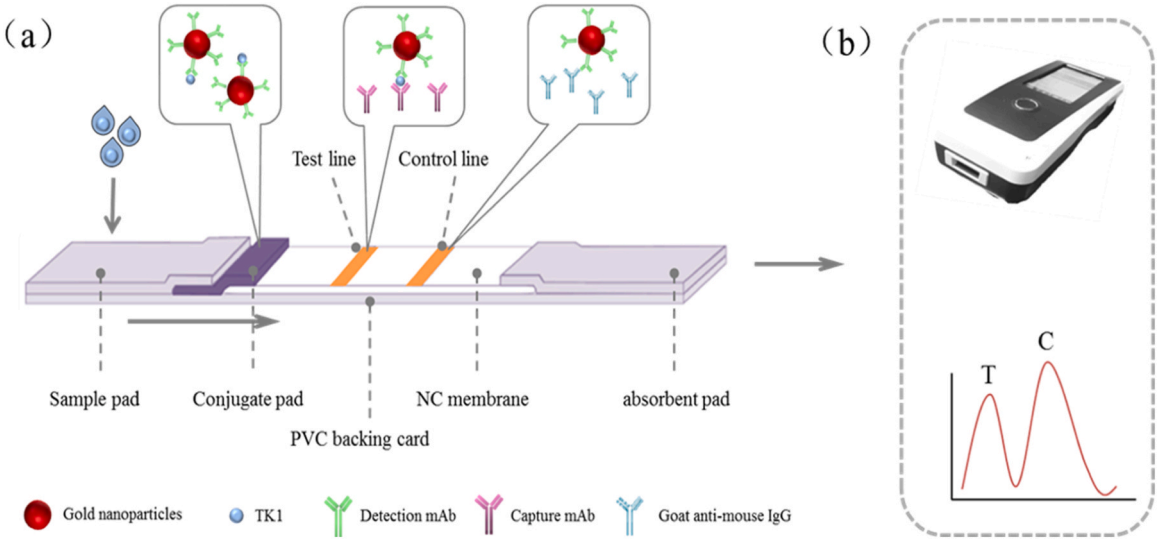

Fig. 1. A schematic diagram of the AuNP-LFIA. (a) Structure and (b) the principle of TK1 quantitative detection in serum.

samples.

2.6.4. Analysis of immune reaction kinetics

Next, we determined the optimal time for the quantitative detection of TK1 on test strips according to the kinetics of the immunoreaction involved. First, 5 pmol/L of rTK1 was added dropwise to the prepared test strip, and the T/C value of the test strip was recorded by an analyzer at 1-min intervals for 20 min. Next, kinetic data relating to the immunoreaction were plotted with the reaction time as the horizontal coordinate and the T/C value as the vertical coordinate. The optimal reading time for the quantitative detection of TK1 on the test strips was then determined by considering the stabilization period of the T/C value.

2.7. Performance analysis of the AuNP-LFIA assay

2.7.1. Sample detection and sensitivity

The recombinant TK1 protein was diluted with serum from healthy subjects to prepare seven standards at different concentrations (0, 0.02, 0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10, 50 and 100 pmol/L) to establish a standard curve. The test was performed by adding samples to spiked wells for qualitative observation. Under normal experimental conditions (excluding false positives and false negatives), red bands on both the T and C lines if the sample was positive; if the sample was negative, no band appeared on the T line, but a band on the C line. Regardless of whether the sample was positive or negative, the test was considered invalid if there no band was generated on the C line. For quantitative testing, test strips were inserted into the test port of a quantitative analyzer; the target was quantified based on T/C values from the relative intensities of the T- and C-lines. Negative samples of sera were assayed with the established AuNP-LFIA (n=20), and the mean and standard deviation (SD) of T/C values were calculated for 20 determinations. The sensitivity of the AuNP-LFIA for the detection of serum TK1 was determined by the limit of detection (LOD) which was calculated by substituting the mean T/C (plus 3 times the SD) into the standard curve.

2.7.2. Precision and recovery

A known concentration of TK1 protein standard was added to sera to prepare spiked concentrations of serum samples (1 pmol/L, 5 pmol/L, and 20 pmol/L). Based on an established AuNP-LFIA, three batches of test strips were randomly selected to assay (n=10) three concentrations of the prepared samples. The mean and SD of the T/C values were calculated, and the precision of the experiments were evaluated by considering the intra- and inter-coefficient of variation (CV). In addition, spiked recoveries were calculated to evaluate the accuracy of the method.

2.7.3. Evaluation of interference and specificity

Three interfering substances that are commonly found in serum (triglyceride, cholesterol and bilirubin) were used for interference and specificity testing. A certain concentration of each interfering substance was added to the serum samples to be tested for TK1. These samples were referred to as the 'interfering substance' samples. We ensured that the volume of the interfering substances added did not exceed 5% of the total volume; the control group was mixed with the same volume of PBS. Samples from the two groups were tested separately, and each concentration was tested in triplicate. Finally, the interference rate was calculated according to Eq. (1).

$$\text{interference rate} = (\text{measured concentration of interfering substance sample} - \text{measured concentration of control sample}) / \text{measured concentration of control sample} \times 100\% \quad (1)$$

Next, TK1 standard (20 pmol/L), 50 pmol/L of human serum albumin (HSA), C-reactive protein (CRP), carcinoembryonic antigen (CEA), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α) were simultaneously detected by AuNP-LFIA to determine specificity. These proteins are commonly found in human serum.

2.7.4. The detection of serum TK1 levels in clinical samples

To validate the accuracy of the AuNP-LFIA for the detection of serum TK1, we used the AuNP-LFIA to analyze 25 clinical serum samples and then compared this data to that derived by a Norman fully automated chemiluminescence system (Nanjing Norman Biotechnology Co., Ltd.). Serum samples were provided by Yixing People's Hospital.

3. Results and discussion

3.1. Expression and purification of recombinant TK1 protein

Expression of the TK1 protein was induced overnight with 0.7 mmol/L of IPTG at 16°C at 180 r/min. Fig. 2(a) shows that the protein was expressed well under these induction conditions and produced a band of the expected molecular weight of the TK1 protein (26.8 kD) in SDS-PAGE. Since the recombinant TK1 protein contained a 6×His tag at the N-terminal, we used a Ni column affinity chromatography to purify the protein. Subsequently, the protein was eluted with imidazole at different concentration gradients (20, 50, and 500 mmol/L). The purity of the recombinant protein was analyzed by SDS-PAGE; analysis revealed that the optimal concentration of imidazole for elution was 500 mmol/L. It was highly likely that the target protein obtained by affinity chromatography was mixed with heterogeneous proteins. Therefore, the recombinant protein was purified further by gel filtration chromatography; this technique is used to separate and purify proteins according to differences in relative molecular mass. Fig. 2(b) demonstrates that following gel filtration chromatography, the purified recombinant TK1 protein band was more homogeneous and exhibited higher levels of purity; consequently, this protein was utilized in subsequent studies.

3.2. Characterization of TK1 antibodies

We used specific carrier proteins (KLH and BSA) to bind to TK1-PP to prepare the immunogen and coating antigen, respectively. All antigen conjugates were characterized by UV-Vis spectrometry, and evaluated based on changes in the shape and position of the characteristic absorption peak. Figure S1 shows that KLH and BSA exhibited a characteristic absorption peak that was close to 280 nm. The peaks for TK1-PP was located at 260 nm, while the characteristic peaks of the coupled antigens, TK1-PP-KLH (Figure S1 (a)) and TK1-PP-BSA (Figure S1 (b)), were both located at around 270 nm, thus suggesting that the antigens had been successfully synthesized. After multiple fusions, we finally selected ten hybridoma cell lines that exhibited the highest affinities. Samples of ascites from experimental mice were purified by Protein G affinity chromatography and analyzed by SDS-PAGE; Fig. 3(a) demonstrates that the mAbs generated were of high purity, and that light and

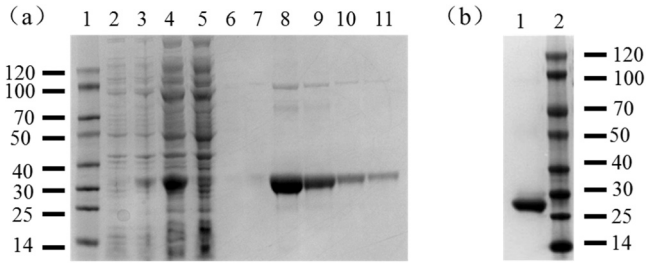

Fig. 2. SDS-PAGE of purified recombinant TK1, Lane 1: protein marker; Lane 2: cellular protein without induction using IPTG; Lane 3: cellular protein after induction with 0.7 mM IPTG; Lane 4: cellular protein in supernants of the sonicated cell lysates; Lane 5: flow through solution; Lane 6: wash buffer; Lane 7–11: interest protein eluent by elution buffer; (b) lane1: secondary purified protein; lane 2: protein marker.

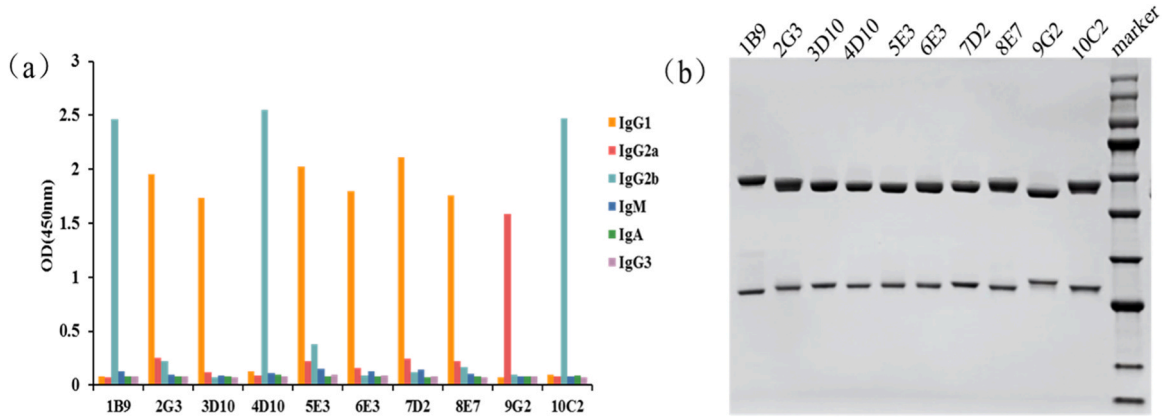

Fig. 3. (a) Isotypes and (b) SDS-PAGE of mAbs.

heavy chains were clearly evident. Isotype identification of the 10 mAbs revealed that the isotype of the 9G2 mAb was IgG2a; the isotope of the 1B9, 4D10 and 10C2 mAbs was IgG2b, and the isotype of the remaining mAbs was IgG1 (Fig. 3(b).

3.3. Characterization of gold nanoparticles

By naked eye, the prepared AuNPs adopted a translucent burgundy coloration. TEM revealed that the prepared colloidal gold nanoparticles exhibited a uniform particle size, a spherical shape, and that the average particle size was 20 nm (Figure S2 (a)). The UV–Vis absorption spectrogram shown in Figure S2(b) demonstrates that the maximum absorption peak of the colloidal gold was 520 nm; this peak exhibited a small width, thus indicating that the particle sizes within the colloidal gold solution were uniformly distributed[33].

3.4. Optimal antibody pairing

In this experiment, we coupled ten detection mAbs against TK1 epitopes with AuNPs to prepare detection probes. Next, the conjugate pads coated with AuNP-mAbs were combined with an NC membrane that had been coated with the capture mAbs. Then, we identified the antibody with the best binding affinity to the antigenic epitope, as determined by the T/C value of the chromatographic assay for each combination based on a high concentration of standard (5 pmol/L). Table S1 shows the data arising from experiments with ten checkerboard combinations of mAbs. Analysis revealed that the highest T/C value was obtained for the combination of 2G3 with 4D10, thus indicating that the highest specific binding efficiency was between AuNP-labeled mAb 4D10 and the NC membrane-encapsulated mAb 2G3. Therefore, we chose the combination of 2G3 and 4D10 as the best antibody pair for subsequent experiments to improve the detection signal.

3.5. Optimization of test strip parameters

Current LFIA probes commonly wrap proteins by electrostatic adsorption on AuNPs; however, the pH of the labeling reaction buffer and the surface chemistry of the AuNPs can influence the adsorption efficiency and bioactivity of the antibodies produced[34]. In the present study, we adjusted the pH of the labeling buffer with 0.1 mol/L of K_2CO_3 solution; results are depicted in Figure S3 (a). When labeling was performed under low pH conditions (pH $\leq$ 7), we found that AuNPs appeared agglomerated and precipitated; Furthermore, it was difficult to re-solubilize after centrifugation, and the test strips were very light in color (Figure S3 (b)). In general, protein molecules can be maximally adsorbed onto the surface of gold nanoparticles when their labeling pH is close to the isoelectric point of the protein; Fig. 4(a) shows that the highest Positive/Negative (P/N) value occurred at a pH of 8.5.

Next, we further optimized the concentration of the capture mAb sprayed on the T line of the test strip. As shown in Fig. 4(b), the T/C value of the test strip was gradually enhanced as the spraying concentration of the mAb increased; the P/N value was highest when 1.5 mg/mL of mAb was sprayed onto the T line. However, the P/N value decreased as the concentration of mAb continued to increase. Thus, we chose a concentration of 1.5 mg/mL when spraying mAbs onto the T line. To improve the accuracy and reproducibility of quantification by the test strip, we plotted an immunokinetic curve showing how the T/C of the test strips changed with immunoreaction time. Then, we used the

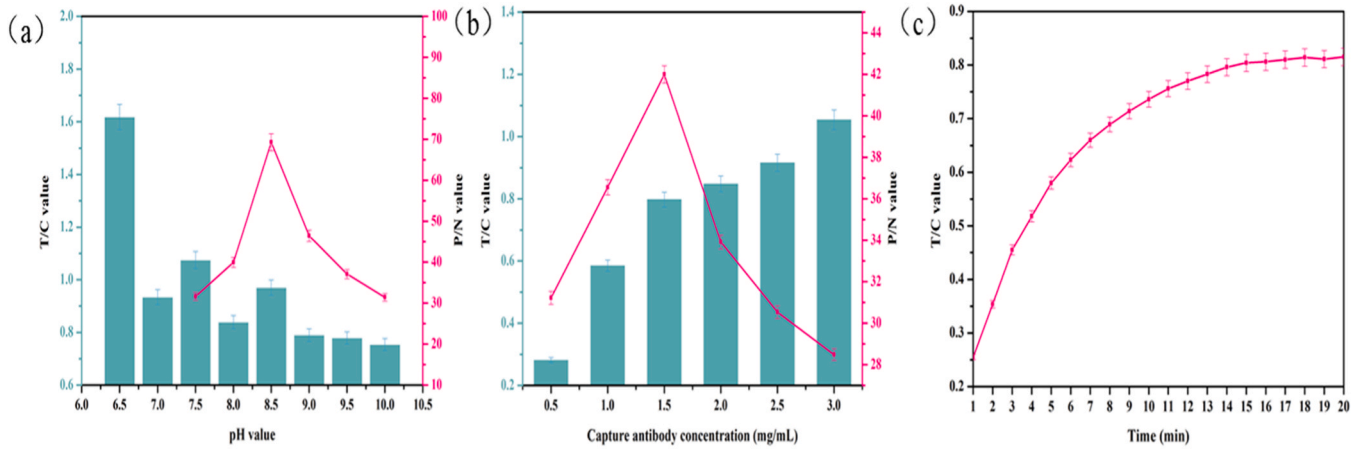

Fig. 4. Optimization of the test strip parameters. (a) The pH of antibody labeling; (b) capture mAb concentration; (c) kinetic reaction curve.

immunokinetic curve to determine the optimal reading time of the test strips for the quantitative detection of TK1, as shown in Fig. 4(c). With an increase in immunoreaction time, the T/C ratio of the test strips gradually decreased and then stabilized after 15 min. Therefore, we decided that the optimal immunoreaction time was 15 min after the addition of samples.

3.6. Sensitivity of the test strips for the quantitative detection of TK1

Following optimization, serum samples containing different concentrations of TK1 were assayed in triplicate with the AuNP-LFIA. A calibration curve was plotted using the logarithm of TK1 concentration as the horizontal coordinate and the T/C value as the vertical coordinate. As shown in Fig. 5(b), when the serum concentration of TK1 increased from 0.5 to 50 pmol/L, the T/C values of the test strips showed a good linear relationship with the logarithmic value of TK1 concentration, as follows: y=0.2109lnx+0.2959 (R^2=0.993), where x represents the concentration of TK1 and y represents the T/C value. Next, we tested a batch of negative serum samples (n=20); the mean T/C value from the 20 tests was 0.021 with a SD of 8.5×10^{-3}. Then, the average T/C value (plus 3 times the SD) was aligned with the standard curve; this identified the LOD as 0.31 pmol/L. And our LOD value is much lower than 2, which will not cause false negatives or false positives during the actual testing, and can meet the needs of clinical detection. TK1 value in healthy human blood has a normal reference value of less than 2 pmol/L, and our LOD value is much lower than it, which will not cause false negatives or false positives during the actual testing; therefore, this LFIA can meet the clinical needs. As shown in Fig. 5(a), when the serum concentration of TK1 was > 50 pmol/L, the T/C value showed a decreasing trend, and the test strip developed a "hook" effect. Therefore, it is necessary to dilute samples when using test strips to detect ultra-high concentrations (> 50 pmol/L) of TK1.

3.7. Assessment of accuracy and precision

Data arising from analysis and the intra- and inter-assay coefficients of variation for the AuNP-LFIA assay for the detection of serum TK1 are summarized in Table 1. For high, medium, and low TK1 standards, the intra-assay CV ranged from 5.7% to 7.3% for the same batch of test while the inter-assay CV ranged from 7.6% to 8.2% for a different batch of strips. Overall, the intra- and inter-assay CVs of the AuNP-LFIA for the detection of TK1 calibrators were < 10%. These results indicated that the newly established AuNP-LFIA showed good reproducibility and stability for the quantitative detection of TK1 in serum samples. Recovery ranged from 97.7% to 109.0% (Table 1), thus indicating that the method exhibited good accuracy.

3.8. Analysis of interference and specificity

Different concentrations of triglyceride, cholesterol and bilirubin were added to test samples as potential interfering factors; the same volume of system buffer was added to control samples. The results shown in Table 2 demonstrated that triglyceride, cholesterol and bilirubin did not exert the same influence on the samples at different concentrations. When the concentrations of triglyceride, cholesterol and bilirubin did not exceed 30 g/L, 5 g/L and 0.2 g/L, respectively, the rate of interference was < 10%. In other words, no obvious interference was evident when these three interfering factors were used at concentrations below these specific concentrations.

Next, we determined the specificity of the test strips by evaluating their cross-reactivity with proteins that are commonly found in serum. As shown in Fig. 6, when the concentrations of HSA, CRP, CEA, IL-6, and TNF-α were 50 pmol/L, there was almost no signal response on the T line of the test strip; furthermore, the T/C value of the test strip was zero, thus indicating that the test strip had a high degree of specificity of the detection of TK1 in serum.

3.9. Validation of clinical samples

Next, we used the AuNP-LFIA assay to detect the levels of TK1 in 25 clinical serum samples; the T/C values of each sample were then aligned with the standard curve to calculate the corresponding concentrations, which were then compared with those derived from Norman chemiluminescence assays, and the results of the two methods were shown in

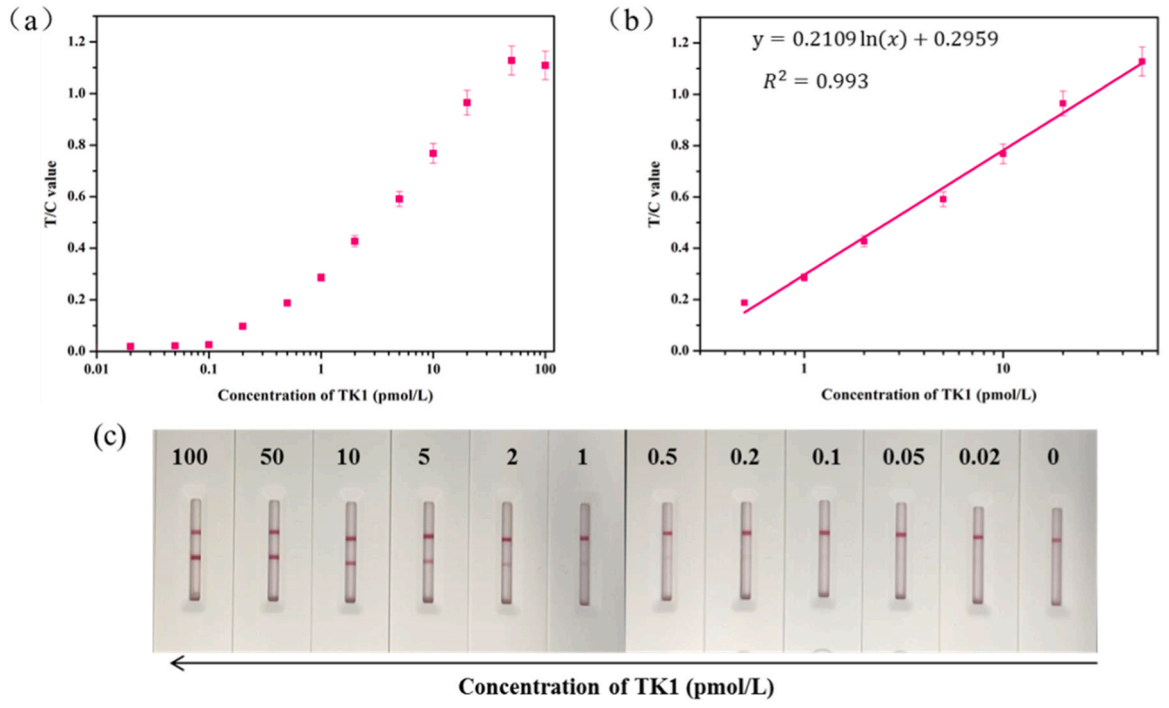

Fig. 5. (a) Dose-response relationship between T/C and the TK1 concentration; (b) Calibration curve of quantitative detection of TK1 in serum with AuNP-LFIA strips.

Table 1
Precision and recovery of the AuNP-LFIA strips in serum.

TK1 concentration (pmol/L)	Intra–assay				Inter–assay[a]			
	Mean[b]	Recovery (%)	SD	CV (%)	Mean	Recovery (%)	SD	CV (%)
0.31	0.33	106.4	0.02	6.1	0.35	112.9	0.03	8.5
1	1.09	109.0	0.08	7.3	0.98	98.0	0.08	8.2
5	5.12	102.4	0.29	5.7	5.36	107.2	0.41	7.6
20	20.23	101.2	1.34	6.6	19.57	97.7	1.52	7.8

[a] Assay was completed every one day for three days continuously.
[b] Mean value of ten replicates at each diluted concentration.

Table 2
The result of interferences analysis.

Interferences concentration		Low concentration 2 (pmol/L)			High concentration 15 (pmol/L)		
		Control	Interferences	Rate (%)	Control	Interferences	Rate (%)
Triglyceride (g/L)	60	2.16	2.63	21.76	14.78	17.34	17.32
	30	2.16	2.34	8.33	14.78	16.09	8.86
	15	2.16	2.29	6.02	14.78	15.37	3.99
Cholesterol (g/L)	10	2.16	2.71	25.46	14.78	16.77	13.46
	5	2.16	2.35	8.80	14.78	15.94	7.85
	2.5	2.16	2.21	2.31	14.78	15.52	5.01
Bilirubin (g/L)	0.4	2.16	2.68	24.07	14.78	17.58	18.94
	0.2	2.16	2.31	6.94	14.78	16.11	8.99
	0.1	2.16	2.25	4.17	14.78	15.47	4.67

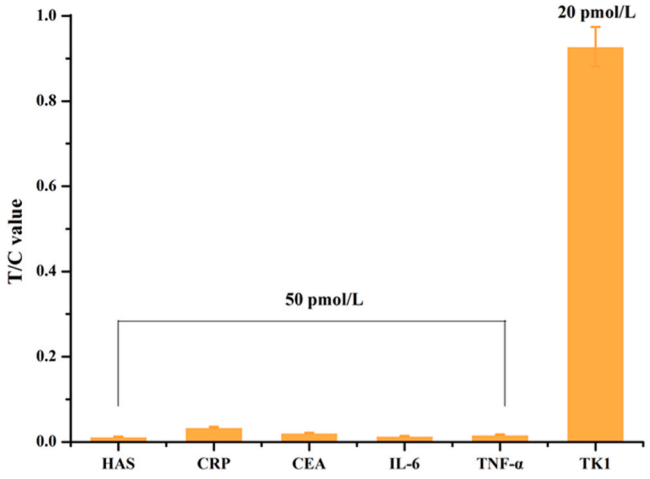

Fig. 6. Specificity evaluation of AuNP-LFIA strips.

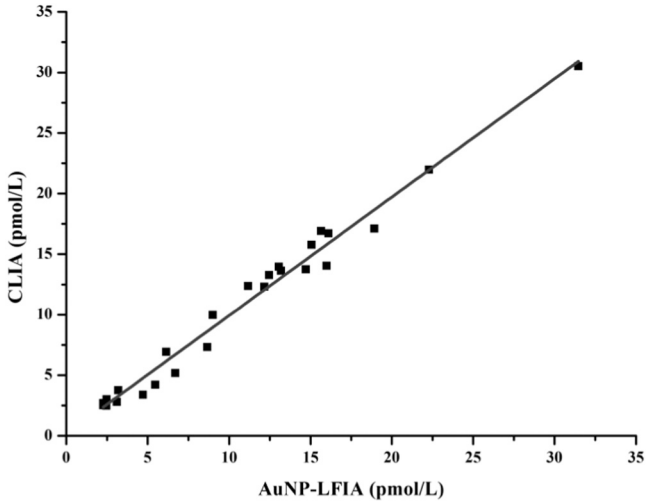

Fig. 7. Correlation of detection results of AuNP-LFIA strip and chemiluminescence immunoassay (CLIA) kit.

Table S2. The newly established AuNP-LFIA assay exhibited good accuracy for the detection of serum TK1, as indicated by a correlation coefficient (R^2) of 0.979 (Fig. 7). Consequently, this new assay represents a quantitative, rapid and accurate method for the detection of serum TK1. Although CLIA is characterized by high sensitivity and specificity, LFIA is one of the most powerful proteins sensing assay platform, offering the advantages of being fast, low cost, simple and user-friendly. Therefore, LFIA has been widely used in clinical diagnostics, especially for POCT. The performance of our established LFIA is comparable to that of CLIA in detecting serum TK1, thus making its use for home self-testing possible.

4. Conclusion

The sensitivity of test strips when determined by immunochromatographic protocols is usually determined only at the nanogram level; this represents a significant limitation to the widespread use of such techniques without depleting their advantages [35]. Furthermore, the products that are commonly used for the detection of TK1 in clinical serum samples are mainly chemiluminescent products which exhibit high levels of sensitivity and a wide dynamic range; however, the development of related products based on immunochromatographic technology has yet to be reported. Therefore, there is a clear need to develop test strips with similar levels of performance that meet the

requirements for rapid detection in a clinical setting.

In this study, we used amino acids 195–225 (GQPAGPDNKENCPVP GKPGEAVAARKLFAPQ) from a region close to the C-terminus of human TK1 to act as the immunizing antigen to generate specific murine anti-human TK1 mAbs; these mAbs were then screened and characterized. We selected two mAbs and developed a highly sensitive AuNP-LFIA for the detection of TK1 in samples of serum. Next, we determined the LOD, specificity, anti-interference ability, precision, and recovery of the test strip. The LOD of the test strip was determined to be 0.31 pmol/L, and the detection range was 0.31–50 pmol/L. The performance of the test strips was evaluated using three exogenous substances (triglyceride, cholesterol and bilirubin); the test strips remained specific even in the presence of interfering substances. The CV was $< 10\%$ for samples with high levels of precision, and recoveries ranged from 97.7% to 109.0%; collectively these findings implied that the assay performed and met the requirements of a clinical assay. Compared with an established chemiluminescence assay, the new LFIA exhibited a high level of consistency ($R^2 = 0.979$). Due to the limited number of clinical samples we have collected so far, the assay now needs to be tested in a larger number of clinical samples to further evaluate the performance of the technique in a clinical setting. In conclusion, the newly developed AuNP-LFIA is simple to perform and can accurately and reproducibly detect the levels of TK1 in clinical serum samples with high levels of specificity. Following further validation, this assay could be readily translated to the clinic as a more efficient alternative to existing methods.

Credit Author Statement

Liya Ye performed the experiments and drafted the manuscript. Xinxin Xu participated in sequence design and protein expression. Aihua Qu carried out the data analysis. Hua Kuang and Liqiang Liu guided experimental design. Chuanlai Xu reviewed and edited the manuscript, and all authors read and approved the manuscript prior to submission.

CRediT authorship contribution statement

liqiang liu: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software. **hua kuang:** Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **aihua qu:** Writing – original draft, Visualization, Validation, Project administration, Conceptualization. **xinxin xu:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources. **Liya Ye:** Writing – original draft, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation. **chuanlai xu:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Project administration, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jpba.2024.116146.

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