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RESEARCH

A broadly applicable split-luciferase biosensor approach for rapid antibody detection in emerging infectious diseases

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Abstract

Conventional immunoassays such as ELISA are widely used in serological testing, yet their reliance on multi-step workflows and labeled reagents limits diagnostic scalability and speed. Bioluminescence-based biosensors are attractive alternatives that offer high sensitivity, operational simplicity, and cost efficiency for detecting diverse analytes. Here, we present a broadly compatible bioluminescent biosensor for antibody detection based on analyte-mediated reconstitution of split-Nanoluciferase (NanoLuc). The platform utilizes engineered bifunctional probes, comprising the protein G C2 domain fused to split-NanoLuc subunits (LgBiT and SmBiT), which serve dual functions in antibody binding and signal generation. Upon immunocomplex formation with multi-epitope antigens, the probes colocalize LgBiT and SmBiT, reconstituting NanoLuc activity and producing a quantifiable bioluminescent signal. We implemented this Fc-binding split-NanoLuc complementation assay to detect antibodies against African swine fever virus (ASFV). The optimized system showed high sensitivity, a wide linear dynamic range, and no cross-reactivity with sera positive for other common swine viruses. Clinical validation exhibited 97.11% agreement with a commercial ELISA kit, confirming its practicality and reliability. Furthermore, the sensor platform was seamlessly adapted to detect antibodies against SARS-CoV-2 and Chikungunya virus (CHIKV) without requiring additional molecular design or reconfiguration, highlighting its inherent versatility. By leveraging the broadly applicable Fc-binding capacity of protein G and the intrinsic modularity of split-NanoLuc, this strategy streamlines assay development and eliminates the need for species-specific secondary antibodies. Together, our findings demonstrate a proof-of-concept biosensor approach that could be further developed into a useful tool for rapid antibody detection in emerging infectious disease settings.

Key points

- *Fungal biological processes alter upon illumination, also under the microscope*
- *Red shifted fluorescent protein toolboxes decrease interference by illumination*
- *Innovations like two-photon, lightsheet, and near IR microscopy reduce phototoxicity*

Keywords Biosensor · Split-luciferase system · Emerging infectious diseases · African swine fever · COVID-19 · Chikungunya fever · Antibody detection

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Introduction

Laboratory testing is essential for the diagnosis, monitoring, control, and treatment of infectious diseases. Antibodies generated in response to specific antigens or other foreign molecules serve as vital biomarkers across a broad spectrum of conditions, including infectious, autoimmune, and neoplastic diseases (Napodano et al. 2021). Serologic antibody testing plays a critical role in various clinical and public health applications, such as precise diagnosis of infections and autoimmune disorders, assessment of humoral immune status after vaccination or natural infection, and the support of large-scale sero-epidemiological surveys that track herd immunity and outbreak dynamics (Burbelo et al. 2023; Dimech 2021). Immunoassays that exploit antigen–antibody recognition are widely used for antibody detection because of their high specificity and reliability (Lim et al. 2023). These methods utilize various tracer reagents and signal reporting systems, enabling implementation across multiple platforms including enzyme-linked immunosorbent assays (ELISA), chemiluminescence immunoassays (CLIA), Western blot, and lateral flow immunoassay (LFIA) (Azam et al. 2023). However, conventional immunoassays involve multiple processing steps and require independent labeled reagents for the recognition and signal generation, limiting their broad adoption (Azam et al. 2023).

Biosensors have rapidly advanced and emerged as promising analytical tools for detecting diverse analytes owing to their high sensitivity, operational simplicity, and cost-effectiveness (Xiao et al. 2023). In particular, bioluminescence-based optical biosensors are well-established and broadly applicable to medical diagnostics, environmental monitoring, and biomedical research (Xu et al. 2019). These biosensors convert specific biomolecular interactions directly into measurable optical signals via conformational changes and signal-transduction mechanisms, without external excitation (Xu et al. 2019). Among bioluminescent reporters, Nanoluciferase (NanoLuc) is the brightest luciferase known to date and holds great potential for biosensing in biological samples (Hall et al. 2012). NanoLuc can be split into two catalytically incomplete subunits, LgBiT (159 amino acids, 17.6 kDa) and SmBiT (11-amino acid complimentary peptide, 1.3 kDa), which reconstitute an active enzyme when brought into close proximity, catalyzing furimazine to produce intense luminescence (Biewenga et al. 2020; Dixon et al. 2016). The two fragments weakly associate with each other; however, when fused separately to interacting partner proteins, LgBiT and SmBiT are colocalized and reconstitute functional NanoLuc (Dixon et al. 2016). This split-NanoLuc report system can be flexibly engineered to detect antigens or antibodies based on their specific binding interactions.

Over recent decades, the incidence of emerging and re-emerging infectious diseases has risen considerably across many countries and regions, posing substantial threats to global public health and food security (Brooks et al. 2022). Notable recent examples include the COVID-19 pandemic, which profoundly affected human health, and widespread outbreaks of African swine fever (ASF), which continue to threaten animal health and the global pig industry (Dixon et al. 2020; Proal et al. 2025). More recently, the endemic emergence and local transmission of Chikungunya fever (CF) in China has garnered significant concern as another pressing public health challenge (Feng et al. 2025). Beyond direct health impacts, cross-border and intercontinental spread have provoked political emergencies and substantial socio-economic disruptions in affected areas. Although vaccination remains a cornerstone of disease control, no effective vaccines are yet available for ASF and CF (Feng et al. 2025; Gallardo et al. 2019). Even for COVID-19, where vaccines have reduced mortality, breakthrough infections among vaccinated individuals continue to sustain viral transmission (Planas et al. 2022). Moreover, many infections can progress to chronic or subclinical forms, often persisting lifelong with minimal or no symptoms. Emerging variants that cause asymptomatic infections are of particular concern, as they enable silent transmission and complicate containment efforts (Gallardo et al. 2019; Pasquini et al. 2025). Effectively managing current pandemics and preventing future outbreaks of these diseases urgently require advanced serological assays that enhance diagnostic efficiency and facilitate targeted intervention strategies (Ni et al. 2025; Proal et al. 2025).

In this study, we developed a broadly compatible bioluminescent sensor platform for antibody detection based on analyte-mediated reconstitution of split-NanoLuc (Hall et al. 2012). A pair of broadly applicable bifunctional sensor probes was engineered, each comprising a protein G C2 domain and a split-NanoLuc subunit. These probes function simultaneously as antibody binding and signal amplification elements, thereby eliminating the need for labeled secondary antibodies. By leveraging multi-epitope antigens, the assay supports the binding of target antibodies to antigens, after which the probes bind to the Fc regions of the captured antibodies. This docking event initiates reconstitution of the luminescent enzyme, producing a strong signal output (Hall et al. 2021; Ni et al. 2021). The resulting bioluminescent immunoassay requires only the target antigen immobilized on a solid surface plus two standardized probe components (LgBiT/C2 and SmBiT/C2), streamlining workflow and enabling a versatile, efficient approach to antibody detection. We demonstrated the feasibility and high performance of this sensor platform by sensitively detecting antibodies against ASFV, and we further showcased broad applicability by rapidly adapting

the platform to detect antibodies against SARS-CoV-2 and chikungunya virus.

Materials and methods

Materials and regents

All materials and reagents used in this study are listed in the Supplementary Material.

Vector construction

The split NanoLuc fragments LgBiT and SmBiT were each genetically fused to either the N- or C-terminus of the protein G C2 domain via a flexible glycine–serine (G/S) linker (Zhang et al. 2025). An 8 × His tag was placed opposite the LgBiT or SmBiT moiety to enable affinity purification. The resulting fusion probes contained the protein G C2 domain and one NanoLuc fragment, and were designated as: LN-C2 (LgBiT–G/S linker–C2–His_8), LC-C2 (His_8–C2–G/S linker–LgBiT), SN-C2 (SmBiT–G/S linker–C2–His_8), and SC-C2 (His_8–C2–G/S linker–SmBiT), representing alternative fusion orientations of C2 and NanoLuc (Figure S1). Synthetic gene fragments (GenScript, Nanjing, China) were subcloned into pET-30a for cytoplasmic expression using *Nde* I/*Hin*d III digestion. All constructs were sequence-verified by Sangon Biotech (Shanghai, China).

Protein expression and verification

Recombinant plasmids were transformed into *Escherichia coli* competent cells and cultured at 37 °C in lysogeny broth (LB) containing 100 μg/mL kanamycin at 200 rpm. Protein expression was induced with 0.5 mM IPTG at an OD600 of ~ 0.6, followed by incubation for 6 h at 37 °C, 200 rpm. Cells were harvested (8,000 × g, 20 min, 4 °C) and lysed by sonication. His-tagged proteins were purified with MagBeads His-Tag Protein Purification (BBI, China) according to the manufacturer's instructions. Expression and purity were assessed by SDS–PAGE and Western blotting using an HRP-conjugated anti-His-tag antibody (1:3,000; Proteintech, USA). Protein concentrations were determined with the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, USA), and final products were aliquoted and stored at −80 °C for subsequent use.

Selection of sensor combination

To investigate the orientation influences of the LgBiT and SmBiT on the ability of the fusion probes to reconstitute the active NanoLuc in the presence of target antibodies, four sensor combinations (LN-C2 + SN-C2, LN-C2 + SC-C2, LC-C2 + SN-C2, and LC-C2 + SC-C2) were tested. Briefly, white 96-well plates (Corning, USA) were coated overnight at 4 °C with recombinant p30 protein of ASFV (0.1 or 0.2 μg/well; GenBank accession no. FR682468) prepared previously in our laboratory (Zhao et al. 2023), washed three times with PBST [PBS with 0.05% (v/v) Tween-20], and blocked with 2% (w/v) BSA in PBST at 37 °C for 1 h. After washing, ASFV-positive serum (1 μL) and each LgBiT/C2 and SmBiT/C2 fusion probe (10 ng each) were diluted in PBST containing 1% BSA to a final volume of 100 μL and added per well. After incubation at 37 °C for 30 min, wells were washed six times, 80 μL furimazine substrate (Nano-Glo® Live Cell Assay System, Promega, USA) was added, and bioluminescence was read on a GloMax® Navigator microplate luminometer (Promega, USA). ASFV-negative serum served as a control. All assays were performed in duplicate. The probe pair with the highest signal-to-noise (S/N) ratio was selected for subsequent experiments.

One-step bioluminescent immunoassay for ASFV antibodies

The selected sensor pair was applied in a one-step bioluminescent enzyme immunoassay for detection of ASFV antibodies. The optimal concentration and dilution factors of each component of the bioluminescent immunoassay were tested. Briefly, recombinant p30 protein (0.8, 0.4, 0.2, 0.1, 0.05, 0.025 μg/well) diluted in carbonate buffer was adsorbed onto white 96-well plates at 4 °C overnight, washed three times with PBST, and blocked with 2% BSA at 37 °C for 1 h. After washing, ASFV-positive serum (1 μL) and equimolar LgBiT/C2 and SmBiT/C2 probes (total 160, 80, 40, 20, or 10 ng per well; 1:1 mass ratio) were diluted in PBST with 1% BSA to 100 μL, added to wells, and incubated for 30 min at 37 °C. Wells were washed six times with PBST and 80 μL of the furimazine substrate was added to the plate per well. The luminescence signals were developed and recorded as described above. ASFV-negative serum was used as a control. The optimal amounts of p30 protein and the fusion sensors were defined by the highest luminescence ratio of positive to negative serum.

Next, the LgBiT/C2: SmBiT/C2 mass ratio (range 8:1 to 1:8) was optimized by testing ASFV-positive and -negative sera. To determine the appropriate dilution factor for serum, different dilutions of serum samples (20, 10, 5, 1, 0.5, and 0.25 μL) were tested. Likewise, the incubation time for sample testing (5, 10, 15, 20, 30, 45, and 60 min) was also optimized. For each parameter, conditions yielding the highest S/N were selected.

The assay cutoff value was established by testing 263 ASFV-negative pig serum samples, which were collected and verified by the ASF Regional Laboratory of China (Lanzhou). The luminescence signal and S/N ratio of each

sample were recorded, and the mean S/N ratio ($\overline{X}$) of the negative samples and the standard deviation (SD) were calculated using the SPSS software (version 25.0, SPSS, Inc., Chicago, IL, USA). The cutoff value of the assay was defined as $\overline{X}+3SD$.

Specificity and analytical sensitivity

Specificity of the bioluminescent immunoassay was evaluated using polyclonal sera positive for porcine circovirus type 2 (PCV2), respiratory syndrome virus (PRRSV), porcine reproductive and pseudorabies virus (PRV), foot-and-mouth disease virus serotype O (FMDV-O), and classical swine fever virus (CSFV); ASFV-positive and -negative sera were included as controls. Analytical sensitivity was assessed using fivefold and twofold serial dilutions (1:5, 1:25, 1:50, 1:100, 1:200, 1:400, 1:800, 1:1,600, 1:3,200, 1:6,400) of three ASFV-positive sera with different antibody titers. Specificity and the limit of detection (LoD) were determined using the cut-off defined above.

Comparison of the bioluminescent enzyme immunoassay with a commercial kit

The bioluminescent immunoassay was applied to the detection of ASFV antibodies in 242 clinical pig serum samples, which were collected routinely from Gansu and Henan Province (March 2020–December 2023) by the ASF Regional Laboratory of China (Lanzhou). Results were compared with those of a commercial ASFV antibody detection ELISA kit (JN60915, JNT, China). Notably, the commercial kit is an indirect ELISA that uses the same p30 antigen and has been authorized by the Ministry of Agriculture and Rural Affairs of China (MARA). The agreement rate between the bioluminescent immunoassay and ELISA method was assessed according to the tested result of each serum sample using SPSS software (version 25.0, SPSS, Inc., Chicago, IL, USA).

Compatibility with SARS-CoV-2 and chikungunya virus antibody detection

To demonstrate the feasibility of the sensor platform for detecting antibodies against other infectious diseases, the broadly applicable sensor probes (LgBiT/C2 and SmBiT/C2) were applied to detect antibodies against SARS-CoV-2 and Chikungunya virus (CHIKV). Sensor-pair screening (LN-C2 + SN-C2, LN-C2 + SC-C2, LC-C2 + SN-C2, LC-C2 + SC-C2) followed the procedures as described in the previous section. Plates were coated with SARS-CoV-2 spike RBD (P08128, GenBank accession no. NC_045512; Solarbio, China) or CHIKV E2 (strain SL-CK1; 40,440-V08B; GenBank accession no. ADG95913; Sino Biological, China) at 0.1 μg/well at 4 °C overnight, washed with PBST, and blocked with 2% BSA for 1 h at 37 °C. The affinity-purified rabbit polyclonal antibodies against SARS-CoV-2 Spike RBD (K110843P, Solarbio, China) or against CHIKV E2 (40,440-T46; Sino Biological, China) (0.1 μL) were mixed with 40 ng of the LgBiT/C2 and SmBiT/C2 fusion sensors in dilution buffer (PBST containing 1% BSA); then, 100 μL of the mixture was added to plates and incubated for 30 min at 37 °C. After six final washes with PBST, the furimazine substrate (80 μL/well) was added and the luminescence signals were recorded in a microplate luminometer. Normal rabbit serum was used as the negative control. The optimal sensor pair for each target was determined according to the highest S/N ratio. Furthermore, with the optimal sensor combination, serially diluted polyclonal antibodies against SARS-CoV-2 Spike RBD and CHIKV E2 were analyzed by the bioluminescent immunoassay.

Statistical analysis

Data analysis and curve fitting were performed using GraphPad Prism version 8.0 (GraphPad Software, Inc. LA Jolla, CA, USA) and SPSS software (version 25.0, SPSS, Inc., Chicago, IL, USA). The kappa coefficient determined assay agreement between the bioluminescent immunoassay and the commercial ELISA kit.

Results

Design of the one-step bioluminescent enzyme immunoassay

Based on the split-luciferase complementation system, a one-step bioluminescent enzyme immunoassay was proposed for detecting antigen-specific antibodies using two broadly applicable sensor probes. In this biosensing platform, the NanoLuc fragments LgBiT and SmBiT were genetically fused to the C2 domain of protein G, creating two fusion constructs LgBiT/C2 and SmBiT/C2, which function as versatile antibody probes. Target-specific IgGs recognizing different epitopes present in the tested samples are captured by the solid-phase immobilized antigen, and the broadly applicable fusion probes LgBiT/C2 and SmBiT/C2 then bind to the Fc region of the captured IgG antibodies, bringing the LgBiT and SmBiT into proximity, restoring NanoLuc luciferase activity and producing a luminescence signal in the presence of furimazine substrate (Yao et al. 2021). Non-specific antibodies, free unbound LgBiT/C2 and SmBiT/C2 fusion probes, and their conjugates are removed by washing steps to ensure assay specificity (Fig. 1). This design simplifies the assay workflow, reduces complexity, and improves the efficiency of immunocomplex formation.

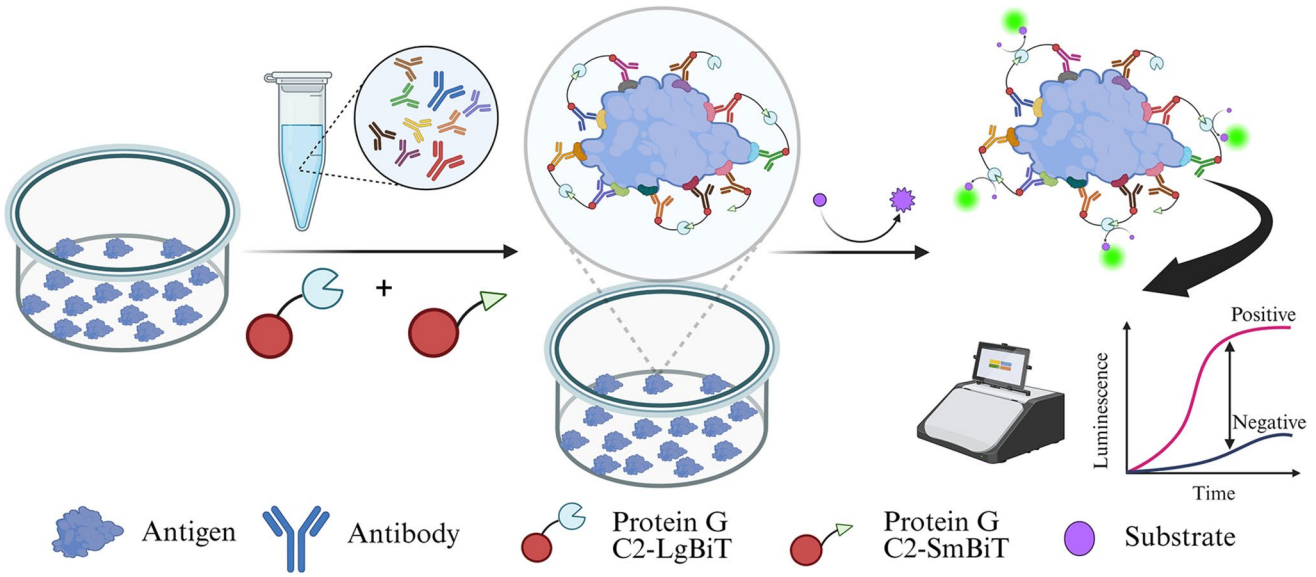

Fig. 1 Schematic illustration of the bioluminescence immunosensor for antibody detection

Expression of the fusion sensors

To generate broadly applicable sensor probes, LgBiT or SmBiT was fused to either the N- or C-terminus of the protein G C2 domain, creating four fusion sensors (LN-C2, LC-C2, SN-C2, and SC-C2). Recombinant plasmids encoding the NanoLuc fragments and the protein G C2 domain were constructed using standard cloning procedures. Following the conventional protocols, the fusion sensors containing two functional domains were expressed in *E. coli* and purified by Ni–NTA affinity chromatography. SDS-PAGE analysis showed that four fusion sensors were observed as dominant bands with expected molecular sizes in the purified products (Figure S2), and Western blotting with an HRP-conjugated anti-His-tag antibody confirmed their identities (Figure S3).

Characterization of the fusion sensors

The LgBitT and SmBiT fragments fused to the C- or N-terminus of the probes may influence their binding activities, resulting in variable bioluminescent signals and background noise when binding to target IgGs. Thus, we assessed all sensor pairings for their ability to reconstitute NanoLuc in the detection of ASFV antibodies. The results showed that the performance of those sensor combinations differed significantly (Fig. 2). Three sensor pairs (LN-C2 + SN-C2, LC-C2 + SN-C2, and LC-C2 + SC-C2) produced relatively high S/N ratios (> 10) when differentiating ASFV-positive and -negative sera. Of those, pairing LN-C2 with SN-C2 yielded the highest S/N (> 90) at both 0.1 and 0.2 μg/well of p30 antigen (Fig. 2). Therefore, the LN-C2 and SN-C2 sensor pair was selected for subsequent assays.

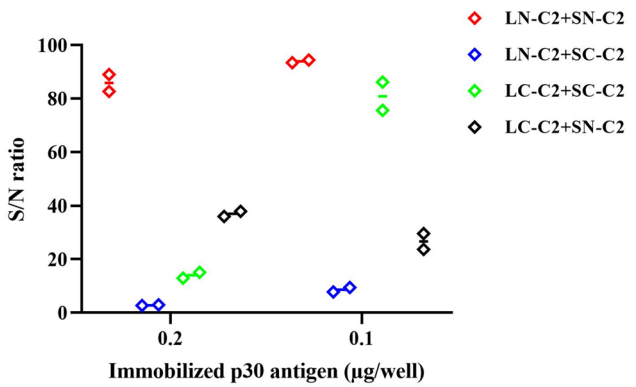

Fig. 2 Selection of the optimal sensor pair for detection of ASFV antibodies. Different sensor combinations were evaluated based on their ability to reconstitute functional luciferase in the presence of ASFV antibodies. The pair consisting of LN-C2 and SN-C2 yielded the highest signal-to-noise (S/N) ratio at both 0.1 μg and 0.2 μg of p30 protein, and was therefore selected for all subsequent assays. Data represent means of duplicate tests; error bars indicate standard deviations

One-step bioluminescent enzyme immunoassay for detection of antibodies against ASFV

A one-step bioluminescent enzyme immunoassay was developed to detect ASFV antibodies, which involved several essential components, including immobilized antigen, tested serum samples, and broadly applicable sensor probes (LN-C2 and SN-C2). The optimal amounts of immobilized

p30 antigen and two broadly applicable sensor probes (at a fixed ratio of 1:1) were initially determined by checkerboard titrations. The results showed that combining 0.1–0.2 μg/well of p30 protein with 20–40 ng of sensor probes yielded comparable and relatively high S/N ratios without significant differences (Fig. 3a). For robustness, we selected 0.2 μg/well of p30 with 40 ng of sensor probes for subsequent experiments. Considering that the LN-C2 and SN-C2 can bind randomly to the specific IgGs targeting distinct p30 epitopes, we optimized their mixing ratio. We observed that S/N ratios increased progressively with the relative excess of SN-C2, peaking at a 1:4 LN-C2:SN-C2 mass ratio (40 ng:160 ng) with an S/N ratio of ~ 400 (Fig. 3b). Titration of serum input showed that 5 μL (1:20 dilution) gave the highest S/N ratio (Fig. 4a). In addition, incubation time for IgG-mediated reconstitution of NanoLuc was also evaluated by incubating the LN-C2/SN-C2/serum mixture for varying durations. The results showed that the bioluminescent signals and S/N ratios exhibited time-dependent increases, plateauing at 30 min with almost stable readings thereafter (Fig. 4b). Based on these results, 40 ng of LN-C2 and 160 ng of SN-C2 mixed with 5 μL of tested serum samples and incubated with 0.2 μg of immobilized p30 protein for 30 min at 37 °C were determined to be the optimal conditions for the bioluminescent immunoassay.

Two hundred sixty-three ASFV-negative pig serum samples were analyzed by the bioluminescent immunoassay with the optimal conditions and procedures. The results showed that the mean S/N ratio ($\overline{X}$) of those samples was 1.2626, with a standard deviation (SD) value of 0.9348. Therefore, the cutoff value of the proposed assay for ASFV antibody detection was defined to be 4.0668 ($\overline{X}+3SD$). Consequently, serum samples with S/N ratios ≥ 4.07 were classified as positive, while those below were classified as negative.

Specificity and analytical sensitivity

Specificity was evaluated using polyclonal sera positive for PCV2, PRRSV, PRV, FMDV-O, and CSFV; all tested negative by this assay, whereas ASFV-positive sera were clearly above the cut-off (Fig. 5a), confirming the specificity of the proposed assay for ASFV antibody determination. Analytical sensitivity, assessed with three ASFV-positive serum samples of differing antibody titers (S1–S3), showed limits of detection of 1:3,200 (S1), 1:800 (S2), and 1:200 (S3) (Fig. 5b), demonstrating its ability to reliably identify ASFV antibodies across a range of concentrations.

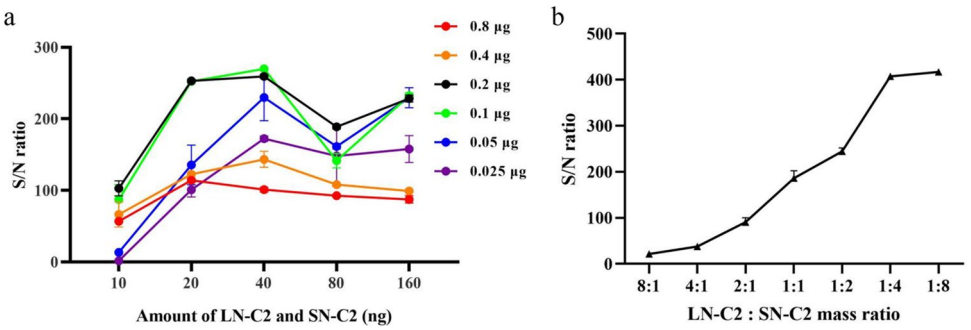

Fig. 3 Determination of the optimal doses of antigen and the sensor pair for ASFV antibody detection. **a** Checkerboard titration was used to determine the optimal amounts of immobilized p30 antigen and broadly applicable sensor probes (LN-C2 and SN-C2; fixed at a 1:1 ratio). **b** The optimal molecular ratio between LN-C2 and SN-C2 for ASFV antibody detection was identified. Data represent means of duplicate tests; error bars indicate standard deviations

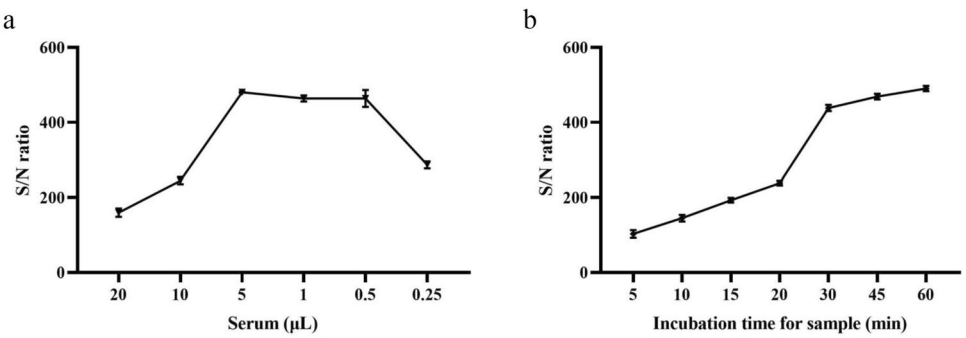

Fig. 4 Optimization of reaction conditions for ASFV antibody detection. **a** Optimal serum volume. **b** Optimal incubation time for sample detection. Data represent means of duplicate tests; error bars indicate standard deviations

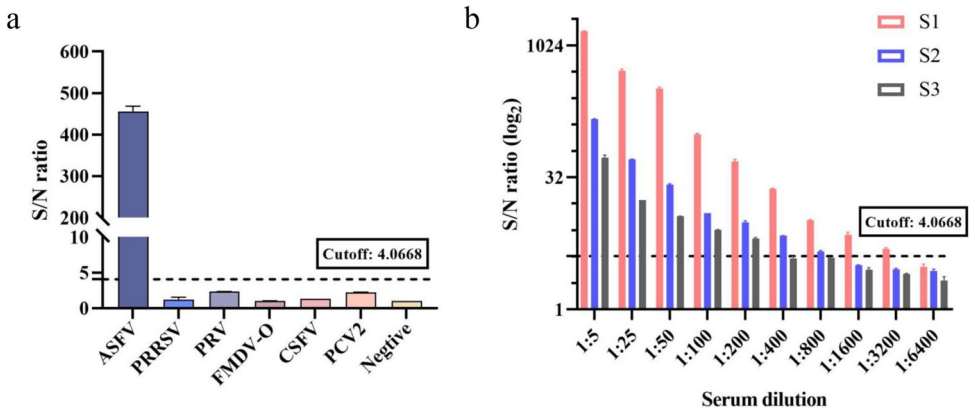

Fig. 5 Specificity and analytical performance of the bioluminescent immunoassay for ASFV antibody detection. **a** Specificity was assessed using sera positive for PCV2, PRRSV, PRV, FMDV-O, and CSFV. Except for ASFV-positive samples, all signal-to-noise (S/N) ratios fell below the defined cut-off value. **b** Serial dilutions of three ASFV-positive serum samples (S1, S2, S3) with varying antibody titers were analyzed to evaluate assay performance. Data represent means of duplicate tests; error bars indicate standard deviations

Comparison of the bioluminescent enzyme immunoassay with a commercial kit

To evaluate the performance of the bioluminescent immunoassay, 242 clinical pig serum samples were analyzed in parallel using both the proposed assay and a commercial ELISA kit. The bioluminescent immunoassay identified 66 positives and 176 negatives, whereas ELISA detected 67 positives and 175 negatives. Among these, 235 samples yielded consistent results between the two methods, corresponding to an agreement rate of 97.11% (235/242) with a kappa value of 0.9272 (Table 1), indicating excellent concordance between the two assays.

Compatibility of the sensor platform

The broadly applicable sensor platform employing LgBiT/C2 and SmBiT/C2 probes was utilized to detect antibodies targeting SARS-CoV-2 and Chikungunya virus as a proof-of-concept. The assay was adapted by incorporating the SARS-CoV-2 spike RBD and CHIKV E2 proteins into a bioluminescent immunoassay format. Following a strategy similar to that described earlier, optimal sensor pairs were screened for specific antibody detection, and LC-C2 + SN-C2 was identified as the best pair for both targets (Fig. 6a, b). In addition, anti-SARS-CoV-2 Spike RBD and anti-CHIKV E2 antibodies were serially diluted and analyzed by the bioluminescent immunoassay. The results revealed concentration-dependent increases in luminescence signals and S/N ratios. Detection limits were 1:1600 for anti-SARS-CoV-2 spike RBD and 1:400 for anti-CHIKV E2 antibodies (S/N > 2.1) (Fig. 7). Maximum fold changes in bioluminescence reached 658 for SARS-CoV-2 RBD and 289 for CHIKV E2. These findings demonstrate that the sensor platform and its design strategy enable rapid development of bioluminescent immunoassays for detecting antibodies against infectious agents, with potential applicability to other molecular targets.

Discussion

NanoLuc luciferase produces intense and stable luminescence, and the split-NanoLuc complementation assay has become a valuable tool for the determination of protein–protein interactions, cellular signaling events, and biomolecular localization (Biewenga et al. 2020; Kuramoto et al. 2025; Niles et al. 2025). This strategy has extended to diagnostic applications, where it enables the detection of antigen–antibody immunocomplexes with performance superior to traditional methods (Adamson et al. 2022; Elledge et al. 2021; He et al. 2023; Karaaslan et al. 2025; Kim et al. 2021). A

Table 1 Comparison of the bioluminescent immunoassay and a commercial ELISA kit by detecting clinical pig serum samples

Methods and determination indexes		Commercial ELISA kit			Agreement (%)	Kappa
		Positive	Negative	Total		
The bioluminescent immunoassay	Positive	63	3	66	97.11	0.9272
	Negative	4	172	176		
	Total	67	175	242		

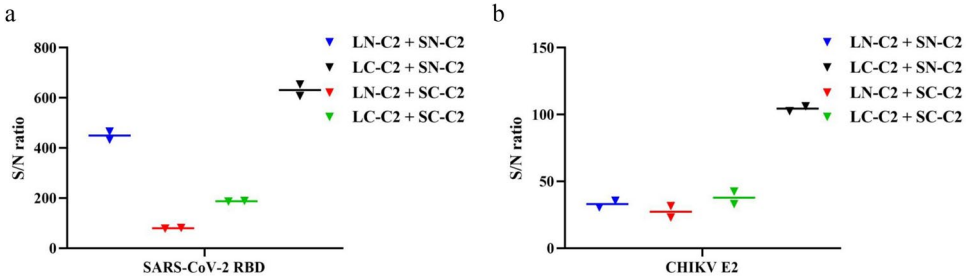

Fig. 6 Sensor pair selection for detection of SARS-CoV-2 and CHIKV antibodies. **a** Different sensor combinations were screened for their ability to reconstitute luciferase activity in the presence of anti-SARS-CoV-2 RBD antibodies. **b** Sensor pairs were similarly evaluated for luciferase complementation in the presence of anti-CHIKV E2 antibodies. Data represent means of duplicate tests; error bars indicate standard deviations

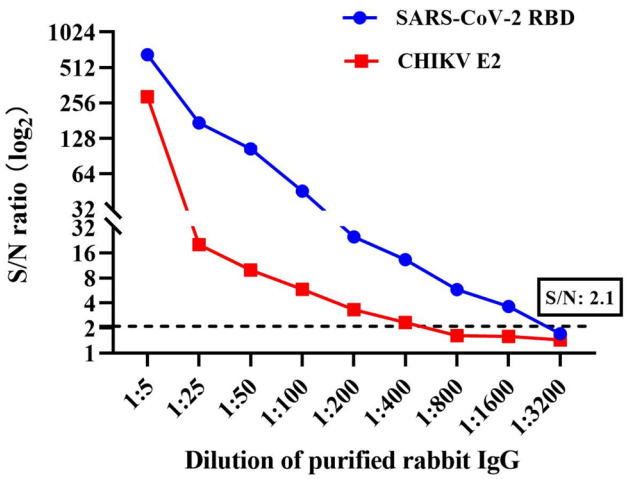

Fig. 7 Analytical performance of the bioluminescent immunoassay for detection of anti-SARS-CoV-2 RBD (6.39 mg/mL) and anti-CHIKV E2 antibodies (1.0 mg/mL). Data represent means of duplicate tests; error bars indicate standard deviations

typical luminescent biosensor based on the split-NanoLuc system has been established for rapid and quantitative detection of SARS-CoV-2 antibodies. In this design, the NanoLuc subunits LgBiT and SmBiT are separately fused to the same antigen (SARS-CoV-2 spike or nucleocapsid protein). By leveraging the two Fab arms of the target antibody, the simultaneous binding of the fusion probes to a single IgG molecule facilitates the reconstitution of luciferase enzymatic activity. This Fab-binding split-NanoLuc complementation assay achieves high sensitivity and specificity for anti-spike and anti-nucleocapsid antibodies in clinical samples (Elledge et al. 2021). Similar designs have been applied to antibodies against Nipah virus G glycoprotein, as well as Crimean Congo hemorrhagic fever virus nucleoprotein and GP38 glycoprotein (Bergeron et al. 2024; Karaaslan et al. 2025). Despite its powerful capabilities, Fab-binding split-NanoLuc sensors require considerable time and effort for the development of NanoLuc-antigen fusion probes, and in-vitro production of large antigen–NanoLuc fusions can be technically challenging, with increased risk of misfolding that may compromise the structure and function integrity of both fusion partners (Burbelo et al. 2023). In this study, we present an Fc-binding split-NanoLuc complementation assay utilizing broadly applicable protein G and split-NanoLuc adaptors for antibody detection, eliminating the need for target-specific molecular design and additional protein engineering.

To validate the principle and characterize the performance of the proposed bioluminescent sensor platform, we initially implemented this strategy for the detection of ASFV antibodies. A variety of antigens have been used in anti-ASFV ELISA to detect antibodies, including virus-infected cell lysates and recombinant p72, p54, p30, k205R, CD2v, and B602L proteins (Chen et al. 2025; Huang et al. 2023; Zhou et al. 2023b; Zhu et al. 2024). Among them, p30 is a highly immunogenic structural protein located in the viral inner envelope that mediates viral internalization (Lithgow et al. 2014; Petrovan et al. 2019). It elicits a robust antibody response in infected animals, making it a well-established antigen for ASFV serological testing (Gallardo et al. 2019; Zhou et al. 2023a). Therefore, p30 was chosen as the capture antigen to target antibodies, while the LgBiT/C2 and SmBiT/C2 sensors served as recognition elements for antibody binding and meanwhile tracers to enable signal amplification in the immunoassay. Different sensor combinations were evaluated for their ability to reconstitute functional luciferase and to achieve an optimal S/N ratio, and their performance varied significantly across constructs. Fusing the NanoLuc subunits to the N-terminus of the protein G C2 domain (LN-C2 and SN-C2) most effectively promoted complementation of LgBiT and SmBiT for detecting the p30-antibody immunocomplex, yielding the strongest signal intensity alongside the lowest background noise, and consequently, a higher S/N ratio. These findings suggest that the fusion sites of the NanoLuc subunits may affect the binding activity of the sensors or cause improper protein folding (Kim et al. 2021).

Utilizing the optimal sensor pair, we systematically optimized operating parameters for the Fc-binding split NanoLuc complementation assay to detect ASFV antibodies. A decrease in S/N ratios was observed at high probe concentrations, which is likely attributable to the "hook effect." This well-documented immunoassay phenomenon occurs when an excess of detection reagent leads to suboptimal immunocomplex formation and reduced signal output. Previous reports have similarly described this effect, highlighting the importance of probe titration for optimal assay performance (Adamson et al. 2022; Douglass et al. 2013; Ni et al. 2021). Furthermore, we found that an excess of SN-C2 over LN-C2 significantly enhanced the S/N ratio, achieving a maximum value of approximately 400. This suggests that an asymmetric probe ratio enhances split–NanoLuc complementation efficiency, possibly by facilitating the formation of immunocomplexes and/or reducing steric hindrance during antibody binding (Elledge et al. 2021). The assay showed no cross-reactivity with sera positive for other common swine viruses, supporting high analytical specificity for ASFV serodiagnosis. However, our specificity testing included sera against common swine pathogens; future studies are required to evaluate performance with more challenging clinical samples, such as those from animals with autoimmune conditions or other inflammatory states. Further evaluation of analytical sensitivity using serially diluted ASFV positive sera revealed dose-dependent luminescence across a wide range of antibody concentrations, indicating excellent assay linearity. The clinical practicality of the Fc-binding split–NanoLuc complementation assay was validated by testing clinical samples, showing a high concordance rate of 97.11% with a commercially available p30-based ELISA kit, which confirms the reliability of this assay. Unlike conventional two-step indirect ELISA, which requires sequential incubation to form antigen–antibody complexes followed by the addition of a labeled secondary antibody (Burbelo et al. 2023), our format uses bifunctional Fc-binding NanoLuc adaptors to enable a one-step workflow: serum and probes are added simultaneously, rapidly assembling the antigen–antibody–NanoLuc immunocomplex (Li et al. 2020). Additionally, this assay requires only a single 30-min incubation for IgG-mediated luciferase reconstitution, substantially reducing hands-on time and supporting rapid diagnostics.

This broadly compatible bioluminescence sensor platform presents a promising strategy for the rapid development of highly sensitive antibody assays against emerging infectious agents (e.g., SARS-CoV-2 and CHIKV) (Ni et al. 2025; Proal et al. 2025). Its modular design allows straightforward substitution of antigens without requiring extensive re-engineering of the core sensor components. With SARS-CoV-2 Spike RBD and CHIKV E2, the LC-C2 and SN-C2 sensor pair exhibited superior IgG-driven NanoLuc complementation, producing strong bioluminescent signals and high S/N ratios. In contrast to the p30-based assay, fusing LgBiT at the C-terminus and SmBiT at the N-terminus of the protein-G C2 domain improved performance for both RBD- and E2-based sensors, underscoring the importance of rational probe orientation. Although the reaction conditions for SARS-CoV-2 Spike RBD and CHIKV E2 antibody detection were not specifically optimized in this study, the assays enabled sensitive detection of both antibodies across a wide range of dilutions using a similar procedure, supporting the inherent reliability and robustness of the sensor platform. However, the assays utilized affinity-purified rabbit IgG as a well-defined proof-of-concept. Further validation with whole serum samples from multiple species is necessary to fully assess platform performance in realistic diagnostic settings.

Implementation of an Fc-binding split-NanoLuc complementation assay for antibody detection is straightforward and involves three steps: (1) selection of a suitable LgBiT/C2 and SmBiT/C2 sensor pair, (2) co-incubation of sensor components and samples with immobilized antigen, and (3) luminescent signal generation via substrate catalysis. We adopted a one-step format to streamline the workflow and minimize hands-on time. Although this format may permit nonspecific probe interactions, assay specificity is maintained by washing steps. In contrast, a sequential two-step approach (with separate serum and probe incubation) could theoretically reduce nonspecific binding but would also increase operational complexity and assay duration, rendering it less practical for user-friendly adoption. Furthermore, since protein G binds to IgG antibodies from humans and many other mammalian species (Ni et al. 2021), using protein G–based split-NanoLuc adaptors eliminates the need for multiple species-specific, labeled secondary antibodies. These features make the proposed biosensor as a versatile and efficient platform ideal for rapid and high-throughput antibody detection. While the sensor platform shows considerable promise, several aspects require attention. Notably, the binding affinity of Protein G varies across IgG subclasses and species, which may affect detection sensitivity and should therefore be considered when adapting the platform for clinical applications. Additionally, current validation has been conducted with relatively small antigens; extrapolating the platform's effectiveness to larger, multidomain antigens may present challenges, as epitope spacing and steric accessibility could influence the efficiency of NanoLuc reconstitution.

Conclusions

In summary, we describe a broadly compatible, Fc-binding split-NanoLuc complementation assay that utilizes broadly applicable protein G-based split-NanoLuc adaptors for

sensitive and species-flexible antibody detection. This bioluminescence immunosensor was first validated for detecting ASFV antibodies, displaying high sensitivity, specificity, and a broad linear detection range. The assay exhibited high concordance with a commercial ELISA kit when tested on clinical serum samples, while reducing hands-on time to a single 30-min incubation in a one-step format. Owing to its modular design, the system was seamlessly extended to detect SARS-CoV-2 and CHIKV antibodies without requiring sensor re-engineering. With its easy-to-implement workflow, the platform provides a versatile, efficient, and robust alternative to traditional immunoassays, suitable for rapid, high-throughput antibody detection across diverse serological applications in research and diagnostics.

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Data availability All data supporting the results of this study are included in the publication and the supplementary document.

Declarations

Ethics approval Not applicable.

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