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An immunoassay based on bioluminescent sensors for rapid detection of African swine fever virus antibodies

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ABSTRACT Serological assays for antibody detection have contributed significantly to the diagnosis and control of infectious diseases. African swine fever is the most devastating infectious disease of domestic pigs and wild boars, severely threatening the global pig industry in recent years. Here, we developed a rapid, simple, and sensitive immunoassay based on the split-luciferase system to detect IgG antibodies against African swine fever virus (ASFV). In this assay, the p30 protein of ASFV was genetically coupled to the LgBiT and SmBiT subunits of nanoluciferase, which were used as fusion probes for specific antibodies. Target engagement of the probes results in the reconstitution of a functional nanoluciferase, which further catalyzes bioluminescent reactions. Different orientations of the LgBiT and SmBiT-p30 fusion sensors were designed and investigated, and N-LgBiT/p30 and N-SmBiT/p30 were identified as a promising sensor pair for reforming active nanoluciferase in the presence of specific antibodies. After optimization, this split-luciferase complementation assay showed high sensitivity and specificity for the detection of ASFV antibodies. The analytical sensitivity of the assay was 16 times greater than that of the blocking enzyme-linked immunosorbent assay (ELISA) by the detection of serial dilutions of serum, and no cross-reaction was observed with other swine pathogens. As demonstrated in clinical samples, its performance is highly consistent with that of a commercial ELISA kit, with a concordance rate of 98.19%. This assay is simple and easy to perform, providing a more flexible and efficient approach for the measurement of ASFV antibodies in clinical applications.

IMPORTANCE The study is about a homogeneous split-luciferase assay for antibody detection. Split nanoluciferase biosensors for the detection of ASFV antibodies were designed. This sensor platform enables the sensitive and specific detection of antibodies. The split-luciferase assay is simple, rapid, and easy to use.

KEYWORDS luminescent biosensor, African swine fever, antibodies, split-luciferase complementation assay, point-of-care test

In recent years, increasing outbreaks and incidences of emerging infectious diseases have posed great threats to human and animal health worldwide; the ongoing threat of these diseases highlights the importance of laboratory testing for timely treatment and the implementation of control measures (1). The analytical methods for appropriate laboratory testing involve the detection and identification of specific nucleic acids, antigens and antibodies of infectious agents, or other disease biomarkers in clinical samples (2). Serological tests for antibody detection are critical for diagnosing and combating epidemics of infectious diseases. These tests can reflect current infection and disease progression but, more importantly, provide valuable information concerning the immune states of individuals and populations after exposure or vaccination (3). Numerous serological assays with different reaction formats, such as indirect immunofluorescence, lateral flow immunoassay (LFIA), enzyme-linked immunosorbent assay

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(ELISA), and Western blot, have been described for antibody measurements (4). Among them, ELISA currently is the most widely used technique and permits large-scale screening and interpretation of samples with high specificity and sensitivity (2). However, this affinity-based immunoassay is labor-intensive and always requires specialized personnel, expensive instruments, and tedious procedures, impeding its application in point-of-care practices. In addition, the need for sample dilution and indispensable enzyme-conjugated antibodies, coupled with multiple washing and incubation steps, potentially increases the error and variability of ELISA. LFIA is considered a simple and relatively easy-to-use technique, but its application is limited by low sensitivity, and it is generally applied in qualitative assays (5). These shortcomings highlight the need for rapid, simple, yet reliable serological diagnostics that require no specialized apparatus or technical assistance to meet the demand for point-of-care tests.

Bioluminescence systems have been exploited as "reporters" and are extensively used in various fields of biotechnology (6). Bioluminescence does not require an external source for excitation and, thus, results in a low background signal (7). Several promising bioluminescence-based sensors have been designed for the investigation of protein–protein interactions, bioluminescent imaging *in vivo*, and quantitative measurement of various analytes (8). Notably, the luciferases are the most widely used bioluminescence reporters and represent highly sensitive tools in bioanalytical applications (9). Recently, nanoluciferase (NanoLuc) derived from the deep-sea shrimp *Oplophorus gracilirostris* has attracted widespread attention from researchers, offering apparent advantages over traditional luciferases (10). A split-luciferase complementation-based system based on NanoLuc binary technology (NanoBiT) was further established and considered an attractive bioanalytical tool (11). NanoBiT consists of a large fragment (LgBiT) and an 11-amino-acid complementary peptide (SmBiT) of NanoLuc. The LgBiT and SmBiT subunits exhibit low affinity for each other, but they can reconstitute a functional luciferase when brought into proximity, generating a bright luminescent signal with furimazine as the substrate (11, 12). The NanoBiT reporter system is robust, highly adaptable, and particularly well adapted for detecting ternary complex formation (12). Due to their small size, the LgBiT and SmBiT subunits can be genetically fused to binding elements without ablating target recognition such that target binding results in the formation of a ternary complex and guides the reconstitution of an active enzyme. Based on the principle of antigen–antibody-specific interactions, this split-NanoLuc complementation system can be adapted for the rapid, sensitive, and quantitative detection of antigens or their associated antibodies in clinical specimens.

African swine fever (ASF), caused by African swine fever virus (ASFV), is one of the most complex infectious viral diseases affecting domestic pigs and wild boars (13, 14). The ongoing pandemic of ASF has caused substantial economic losses in many affected countries and has posed an unprecedented threat to the global pig industry (13, 15). Currently, an effective vaccine or treatment against the disease has not yet been available; control measures mainly rely on rapid laboratory diagnosis and culling (16). A strong humoral immune response against ASFV can be elicited in infected animals, and the identification of specific antibodies against the virus suggests infection (16). Here, a serological assay for the detection of ASFV-specific antibodies was developed based on the split-luciferase system. In this assay, the LgBiT and SmBiT fragments of NanoLuc are genetically fused to the antigen of ASFV, which retains both antigenicity and enzymatic activity. ASFV-specific IgG induces the formation of a sandwich immunocomplex through the two Fab arms simultaneously binding to the SmBiT and LgBiT fusion sensors, mediating the complementation of the two subunits and thereby reconstituting the luciferase activity. This proposed assay is rapid, sensitive, and specific for the detection of ASFV antibodies and provides an attractive tool for the diagnosis and surveillance of ASF in affected areas.

MATERIALS AND METHODS

Reagents and materials

The pBiT1.1-N [TK/LgBiT], pBiT1.1-C [TK/LgBiT], pBiT2.1-N [TK/SmBiT], and pBiT2.1-C [TK/SmBiT] vectors containing the LgBiT/SmBiT tag at the N- or C-terminus of the cloning sites were obtained from MiaoLingBio (China). All restriction enzymes included in this study were acquired from New England Biolabs (USA). The plasmid purification kit NucleoBond Xtra Midi Plus was purchased from Macherey-Nagel (Germany). Lipofectamine 3000 Transfection Reagent was obtained from Invitrogen (USA). The protease inhibitor cocktail and protein A/G Magnetic Beads were acquired from MCE (USA). The Mag-Beads His-Tag protein purification was purchased from BBI (China). The Pierce BCA Protein Assay Kit and Pierce ECL Western Blotting Substrate were obtained from Thermo Fisher Scientific (USA). The horseradish peroxidase (HRP)-conjugated anti-His tag antibody and HRP-conjugated goat anti-pig secondary antibody were acquired from Abcam (USA). The NanoLuc substrate provided in the Nano-Glo Live Cell Assay System was purchased from Promega (USA). The commercial kit for ASFV antibody detection was obtained from Ingenasa (Ingezim PPA Compac K3, Spain). The ASFV-positive and negative sera were provided by the ASF Regional Laboratory of China (Lanzhou). Sera positive for classical swine fever virus (CSFV), porcine circovirus type 2 (PCV2), pseudorabies virus (PRV), and porcine reproductive and respiratory syndrome virus (PRRSV) were obtained from the China Veterinary Culture Collection Center. The positive serum against foot-and-mouth disease virus serotype O (FMDV-O) was provided by the WOAH/National Foot-and-Mouth Diseases Reference Laboratory.

Plasmid construction

The DNA fragment of the *CP204L* (p30) gene of the ASFV genotype II strain was synthesized by GenScript (Nanjing, China). It was inserted into pBiT1.1-N [TK/LgBiT] and pBiT1.1-C [TK/LgBiT] vectors at the *Sac* I and *Nhe* I restriction enzyme sites, pBiT2.1-N [TK/SmBiT] vectors at the *Xho* I and *EcoR* I sites, and pBiT2.1-C [TK/SmBiT] vectors at the *Nhe* I and *EcoR* I sites. The LgBiT and SmBiT of NanoLuc were separately fused to the ASFV p30 protein either at the N- or C-terminus across the GS linker (Gly-Ser-Ser-Gly-Gly-Gly-Gly-Ser-Gly-Gly-Gly-Gly-Ser-Ser), and a His-tag was appended to facilitate the purification of the p30 derivatives. The recombinant plasmids were transformed into *Escherichia coli* (*E. coli*) DH5α and cultured in lysogeny broth supplemented with ampicillin (100 µg/mL). The resulting plasmids were purified by a NucleoBond Xtra Midi Plus kit (Macherey-Nagel, Germany) following the manufacturer's protocol and subjected to sequencing to confirm the integrity of the constructs.

Expression and protein purification

The fusion proteins LgBiT-14aa-p30-8xHis tag, 8xHis tag-p30-14aa-LgBiT, SmBiT-14aa-p30-8xHis tag, and 8xHis tag-p30-14aa-SmBiT were expressed and purified from human embryonic kidney (HEK)293T cells. Briefly, 10 µg of recombinant plasmids encoding these p30 derivatives was transfected into HEK293T cells in a cell culture plate (10 cm^2) via the Lipofectamine 3000 Transfection Reagent (Invitrogen, USA) according to the manufacturer's protocol. The cells were incubated at 37°C with 5% (vol/vol) CO_2, and 48 h post-transfection, the cells were harvested via centrifugation and resuspended in phosphate-buffered saline (PBS) containing a protease inhibitor cocktail (MCE, USA). After sonication and centrifugation, the His-tagged proteins were purified via Mag-Beads His-Tag Protein Purification (BBI, China) according to the manufacturer's instructions.

Gel electrophoresis and Western blotting

The purity and integrity of the target proteins were evaluated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS−PAGE), and the protein concentration was determined with a Pierce BCA protein assay kit (Thermo Fisher Scientific, USA). The

fusion proteins were further verified by Western blot with specific antibodies. Briefly, 5 µg of purified protein was resolved by SDS–PAGE and subsequently transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, USA). The membrane was blocked with 5% (wt/vol) skim milk in PBS containing 0.05% Tween 20 (PBST) for 1 h at room temperature. After being washed five times with PBST (10 min each), the membrane was probed with an HRP-conjugated anti-His tag antibody (1:3,000, Abcam, USA). Moreover, the reactivity of the p30 fusion proteins was detected by Western blot with appropriate dilutions of ASFV-positive serum as the primary antibody and the corresponding HRP-conjugated goat anti-pig secondary antibody (1:10,000, Abcam, USA). Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific, USA) was used to develop the immunoreactive bands, which were subsequently detected with a ChemiDoc imaging system (Bio-Rad, USA).

Pairwise selection of fusion protein sensors

The LgBiT and SmBiT subunits appended at either the N- or C-terminus of the p30 protein to detect specific antibodies via the split-luciferase complementation assay resulted in four different combinations, including N-LgBiT/p30 + N-SmBiT/p30, N-LgBiT/p30 + p30/SmBiT-C, p30/LgBiT-C + N-SmBiT/p30, and p30/LgBiT-C + p30/SmBiT-C. To test the efficiency of the different orientations of the LgBiT- and SmBiT-p30 fusion probes, 20 µL of ASFV-positive serum was incubated with 30 µL of protein A/G magnetic beads (MCE, USA) for 30 min, and ASFV-negative serum was used as a control. The IgG-protein A/G magnetic beads were washed with PBS and then incubated with 20 or 40 ng of both LgBiT- and SmBiT-p30 fusion probes for 30 min at 37°C in a total volume of 80 µL. The NanoLuc substrate provided in the Nano-Glo Live Cell Assay System (Promega, USA) was prepared according to the manufacturer's protocol, and 20 µL was added for signal development. Luminescence signals were measured using GloMax Navigator Microplate Luminometer (Promega, USA). The LgBiT- and SmBiT-p30 fusion sensor pair that provided the highest signal-to-noise ratio was selected for further analysis.

Split-NanoLuc complementation assay for the detection of ASFV antibodies

The selected LgBiT- and SmBiT-p30 fusion sensors were employed in a split-NanoLuc complementation assay for detecting ASFV antibodies. To maximize the performance and signal-to-noise ratio of the assay, different amounts of fusion sensors were tested by detecting ASFV-positive and -negative sera. Briefly, serial dilutions of ASFV-positive serum were incubated with protein A/G magnetic beads for 30 min. After washing with PBS, a wide concentration range (5~80 ng) of LgBiT- and SmBiT-p30 fusion sensors was added, and the mixture was incubated for 30 min at 37°C in a total volume of 80 µL. The NanoLuc substrate was added, and luminescence signals were developed and measured as described above. ASFV-negative serum was used as a control, and each assay was carried out in duplicate. The optimal concentrations of the LgBiT- and SmBiT-p30 fusion sensors were determined at the highest ratio of luminescence units between ASFV-positive and -negative sera.

Serum is a complex biological matrix and contains numerous endogenous proteins, which may complicate the discovery of specific antibodies for disease diagnosis (17). To avoid clumping proteins that result in a detectable signal, the serum should be diluted to an appropriate level for antibody detection. Herein, with the optimal dilutions of LgBiT- and SmBiT-p30 fusion sensors, the dilution factor of serum (20, 10, 5, 2.5, and 1 µL) was determined by detecting ASFV-positive and negative sera according to the highest signal-to-noise ratio, as described above. Similarly, different amounts of protein A/G magnetic beads (5, 10, 20, 40, and 50 µL), incubation times for IgG capture (5, 10, 15, 20, and 25 min), and sample testing (5, 10, 15, 20, and 25 min) were tested accordingly in this assay to determine the optimal conditions.

Cutoff value of the split-NanoLuc complementation assay

The cutoff value of the split-NanoLuc complementation assay was determined by testing sera that were known to be positive and negative for ASFV. Three hundred and twenty-four swine serum samples (216 negative and 108 positive samples) collected individually were provided by the ASF Regional Laboratory of China (Lanzhou). The samples were analyzed with the proposed split-NanoLuc complementation assay using the optimal conditions and procedures mentioned above. The luminescence of each sample was interpreted as the signal-to-noise (S/N) ratio. The cutoff value of the split-NanoLuc complementation assay was evaluated using a receiver operating characteristic (ROC) curve.

Cross-reaction and sensitivity

The specificity of the split-NanoLuc complementation assay was evaluated by testing sera positive for CSFV, PRRSV, PRV, PCV2, and FMDV-O. The detection sensitivity of the assay was defined and compared with that of a commercial ELISA kit in which serial dilutions of ASFV-positive serum were tested.

Comparison tests

The performance of the split-NanoLuc complementation assay was compared with that of a commercial ELISA kit (Ingezim PPA Compac K3, Ingenasa, Spain) by testing clinical swine sera. A total of 166 serum samples were collected from pig farms affected by ASF in Gansu and Henan provinces from May to December 2022. These samples were analyzed simultaneously by the proposed split-NanoLuc complementation assay and the blocking ELISA method described in the commercial kit for the detection of specific antibodies against ASFV. The consistency between the split-NanoLuc complementation assay and the blocking ELISA was determined based on the test results for each serum sample.

Statistical analyses

Graphing and statistical analysis were conducted with GraphPad Prism (version 8.0; GraphPad Software, Inc., La Jolla, CA, USA) or Microsoft Excel (Microsoft, Inc., USA). The kappa index value was calculated to determine the agreement between the split-NanoLuc complementation assay and the blocking ELISA by SPSS software (version 25.0, SPSS, Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

Functional principle of split-NanoLuc mediated the detection of specific antibodies and sensor design

The small size and extremely bright luminescence of NanoLuc make it ideal for use as a biosensor in many applications in biological research fields (12). The NanoLuc complementation assay has been proven to be an attractive bioluminescent building block and is widely used to detect protein/protein and protein/small molecule interactions in live cells (18, 19). This NanoLuc reporter system has been further exploited to enable the sensitive bioluminescent detection of disease biomarkers, pathogens, and their specific antibodies in clinical specimens (20, 21). In these approaches, the two small subunits LgBiT and SmBiT of NanoLuc are engineered as fusion probes to bind elements such that the target of interest binding facilitates the complementation of the two subunits to reconstitute a functional luminescent enzyme that can generate a detectable signal. Here, this innovative NanoBiT split-luciferase sensing platform was adapted for the detection of specific antibodies against ASFV (Fig. S1). In this approach, LgBiT and SmBiT of NanoLuc are individually fused to the p30 antigen as a pair of sensors that can bind to specific IgG molecules against ASFV. The free immunoglobulin IgG in the tested samples was captured primarily by protein A/G magnetic beads. The complementation

strategy based on NanoBiT utilizes the bivalent Fab arms of IgG that involve two antigen epitope binding sites. One Fab arm of the p30-specific antibody binds to the LgBiT-p30 fusion probe, and the other binds to the SmBiT-p30 fusion probe. Bringing LgBiT/SmBiT into proximity contributes to the enzymatic reconstitution of NanoLuc and generates a luciferase signal output. The free unbound LgBiT- and SmBiT-tagged fusion probes are removed by washing steps to minimize background noise (Fig. S1a). Theoretically, the specific IgG molecules would also bind only two LgBiT or SmBiT fusion probes that do not produce luciferase activity, which would result in 50% reconstitution of the functional enzyme for split-NanoLuc-mediated antibody detection in this assay (Fig. S1b).

Expression and verification of the p30 fusion probes

The ASFV phosphoprotein p30 is one of the most antigenic structural proteins and plays an important role in virus entry (22). p30 is expressed in the early stage of infection and persists throughout the virus lifecycle (23). Due to the robust antibody response induced by p30 during infection, it is considered an ideal antigen and is widely used in the serological diagnosis of ASFV (24, 25). In this assay, p30 was selected as a probe and fused with LgBiT and SmBiT of NanoLuc in mammalian cells, which were further exploited as biosensors for the detection of antibodies against ASFV. SDS−PAGE analysis revealed that four p30 derivatives (LgBiT-14aa-p30-8xHis tag, 8xHis tag-p30-14aa-LgBiT, SmBiT-14aa-p30-8xHis tag, and 8xHis tag-p30-14aa-SmBiT) were successfully expressed and purified with specific bands of the expected molecular weight. Western blot assays verified the identity of the four p30 derivatives with the anti-His tag antibody. Moreover, the reactivity of these p30 derivatives to the ASFV antibody was further evaluated by Western blot, and the results revealed that all p30 derivatives could be specifically recognized by positive serum against ASFV, indicating that the binding activities of the p30 fusion sensors will not be interfered with appending the NanoLuc fragments (Fig. S2).

Pairwise selection of the LgBiT- and SmBiT-p30 fusion sensors

As the LgBiT and SmBiT of NanoLuc can be fused at either the N- or C-terminus of the p30 probe, four possible combinations of sensor pair (N-LgBiT/p30 + N-SmBiT/p30, N-LgBiT/p30 + p30/SmBiT-C, p30/LgBiT-C + N-SmBiT/p30, and p30/LgBiT-C + p30/SmBiT-C) were proposed and evaluated for their ability to generate functional NanoLuc in the presence of the specific antibody. The results revealed that all the combinations generated luminescence signals and differentiated serum samples positive and negative for ASFV to varying degrees (Fig. 1). These results indicated that the p30 fusion probes are adequately flexible to allow proximity of the two subunits, resulting in the reconstitution of the active NanoLuc. Among them, the N-LgBiT/p30 and N-SmBiT/p30 sensor pair (1:1, at both 20 and 40 ng) provided a higher signal-to-noise ratio than the other sensor pairs did, suggesting that the orientation of LgBiT and SmBiT fused to the probe could affect the complementation of NanoLuc and sensor sensitivity (Fig. 1). Compared with the C-terminus, the subunit fusion at the N- terminus of p30 favored the complementation of NanoLuc in this assay, which might have been caused by the dimerization domain at the C-terminus of the probe or improper protein folding (2, 26). A previous report revealed that NanoLuc fragment fusion at the N-terminal region of the nucleocapsid of SARS-CoV-2 provided high sensitivity and specificity in a Fab-binding immunoassay because the nucleocapsid contains a dimerization domain at the C-terminus (21). These results highlight the importance of the LgBiT and SmBiT fusion sensor design in the split-NanoLuc complementation assay.

Optimization of the split-NanoLuc complementation assay for the detection of ASFV antibodies

The sensor pair N-LgBiT/p30 + N-SmBiT/p30 provided the highest signal-to-noise ratio in this IgG-driven NanoLuc complementation assay and was, thus, used for further

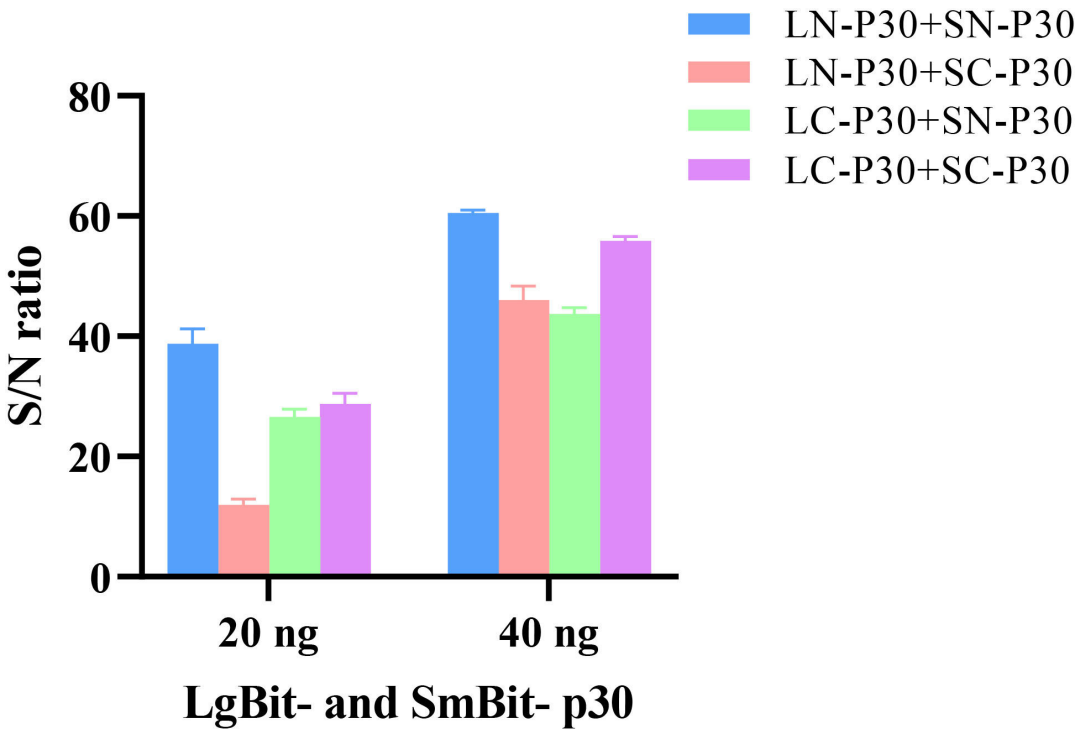

FIG 1 Selection of the LgBiT or SmBiT fusion sensor pair. Four sensor combinations (LN-P30 + SN-P30, LN-P30 + SC-P30, LC-P30 + SN-P30, and LC-P30 + SC-P30) were tested for their capacity to reform the active luciferase in the presence of ASFV antibodies. LN represents N-terminal fusion with LgBiT, LC represents C-terminal fusion with LgBiT, SN represents N-terminal fusion with SmBiT, and SC represents C-terminal fusion with SmBiT. For all assays, 20 or 40 ng of both the LgBiT- and SmBiT-p30 fusion sensors was tested by detecting ASFV-positive and -negative sera. The signal-to-noise (S/N) ratios of the ASFV-positive serum and the negative serum were calculated. Different colors represent different sensor combinations. Data are presented as mean of duplicate, and error bars indicate the standard deviations of duplicate tests.

analysis. To improve the performance and sensitivity of the assay, the optimal amounts of N-LgBiT/p30 and N-SmBiT/p30 fusion sensors were evaluated by testing different concentrations of IgG that were captured by protein A/G magnetic beads from dilutions of the ASFV-positive serum. The results revealed that the S/N ratio increased gradually with increasing amounts of probes at different dilution endpoints (Fig. S3). Moreover, lower concentrations of the sensors minimized the background signal but simultaneously decreased the amount of IgG-driven reconstituted NanoLuc, resulting in a lower S/N ratio (Fig. S3). In this assay, 80 and 40 ng of sensors provided ideal performance in detecting dilutions of ASFV-positive serum and displayed a comparable S/N ratio at a high dilution endpoint (1:6,400; Fig. S3). To build on these results and minimize the consumption of the reagents, 40 ng of N-LgBiT/p30 and N-SmBiT/p30 fusion sensors was selected as the near-optimal dose for the split-NanoLuc complementation assay for the detection of ASFV antibodies.

As mentioned above, the complexity of serum may interfere with the luminescence signal generated in the NanoLuc complementation assay; consequently, the optimal dilution of serum was determined (17). With the optimal doses of sensors, 5 µL (1:20) of serum resulted in a large increase in luminescence and minimal background noise, resulting in the highest S/N ratio (Fig. S4a). Moreover, the optimal amount of protein A/G magnetic beads for IgG capture was evaluated. The results revealed that 10 µL (IgG binding capacity: 0.5 mg/mL) of protein A/G magnetic beads resulted in the highest S/N ratio (Fig. S4b). These results suggested that excessive amounts of IgG or magnetic beads could generate high background noise and decrease the S/N ratios. The incubation time for IgG capture by magnetic beads was subsequently determined to be 15 min to improve the performance (Fig. S4c). Likewise, the reaction time for the analyte-driven

reconstitution of NanoLuc was further estimated, and the S/N ratios increased gradually in a time-dependent manner (Fig. S4d). A robust luminescence signal was produced after 20 min of incubation and reached a steady state at 25 min, with an S/N ratio close to 300. Therefore, 25 min was adopted as the suitable incubation time to assess the reconstitution of luciferase in subsequent assays.

Cutoff value of the split-NanoLuc complementation assay

The suggested cutoff value of the split-NanoLuc complementation assay is crucial for its overall performance. Under the optimal conditions, 216 ASFV-negative and 108 ASFV-positive swine serum samples were tested using the proposed assay. The S/N ratios of the samples varied but clustered well between the positive and negative groups; the majority of the negative samples were close to the baseline, which may be due to the removal of the free sensors by the washing process (Fig. 2a). ROC curve analysis revealed that the area under the curve (AUC) was 0.9969 (standard error = 0.0017), with a 95% confidence interval of 0.9935–1.0 (Fig. 2b), indicating the outstanding discrimination power of the split-NanoLuc complementation assay. The S/N ratio of 4.167, which corresponds to the maximum AUC, was assigned the threshold to differentiate ASFV infections, and the diagnostic sensitivity and specificity of the assay were 98.15% and 97.69%, respectively.

Cross-reaction and sensitivity

The specificity of the split-NanoLuc complementation assay was assessed based on cross-reactivity in sera positive for several swine disease viruses, including CSFV, PCV2, PRV, PRRSV, and FMDV-O. As displayed in Fig. 3a, the optimized assay is specific for ASFV, and no cross-reaction was observed with other swine viruses. This split enzyme-based assay requires LgBiT and SmBiT fusion sensors to bind simultaneously to the same IgG molecules specific for p30 and form a sandwich immunocomplex to mediate IgG-driven NanoLuc reconstitution, which results in superior specificity. The detection sensitivity of the split-NanoLuc complementation assay was evaluated and compared to that of a commercial ELISA kit by testing serial dilutions of ASFV-positive serum under optimal detection conditions. As shown in Fig. 3b, the maximum dilution with a positive result of the proposed assay was 1:51,200, whereas that of the commercial ELISA kit was 1:3,200, suggesting that the detection sensitivity of the split-NanoLuc complementation assay is approximately 16 times greater than that of the ELISA kit. This Fab-binding NanoBiT immunoassay can detect specific antibodies over a wide range of serum dilutions with

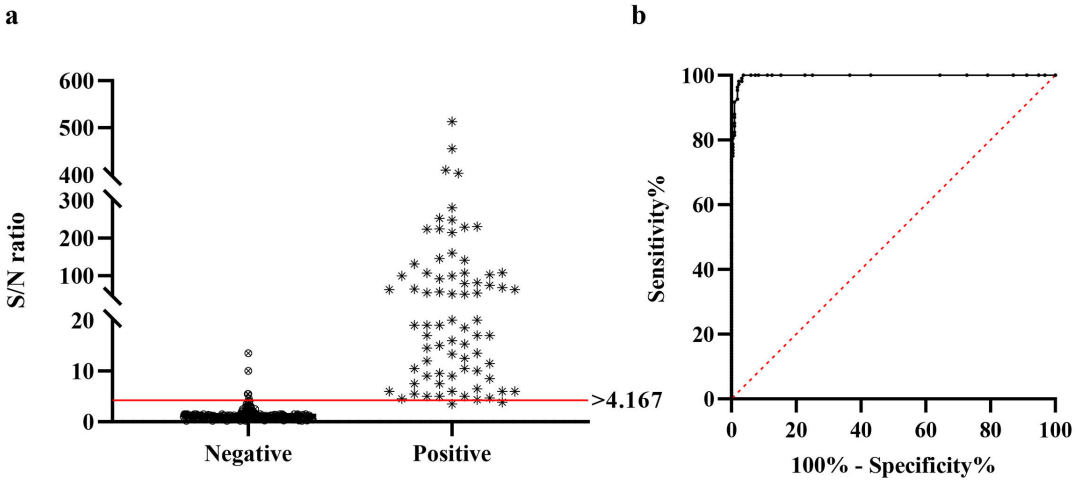

FIG 2 Determination of the cutoff value of the split-NanoLuc complementation assay for ASFV antibody detection through ROC curve analysis. (a) The signal-to-noise (S/N) ratios obtained by the assay from a panel of sera that are classified as positive or negative. Interactive dot plot diagram displaying the S/N ratios of sera when the cutoff value was set to 4.167. (b) ROC curve based on the data obtained.

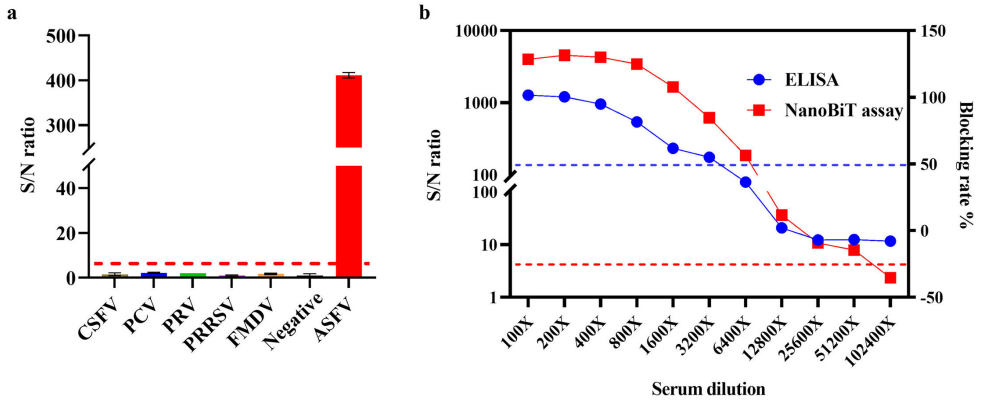

FIG 3 Specificity and detection sensitivity of the split-NanoLuc complementation assay. (a) Specificity analysis of the split-NanoLuc complementation assay by testing sera positive for CSFV, PRRSV, PRV, PCV2, and FMDV-O. The signal-to-noise (S/N) ratios of sera positive for these pig viruses were calculated. Data are presented as the mean of duplicate, and error bars indicate the standard deviations of duplicate tests. (b) Comparison of the detection sensitivity between the split-NanoLuc complementation assay (red color) and the commercial Ingenasa ELISA kit (blue color) with dilutions of the ASFV-positive sample.

remarkable sensitivity, which is likely attributed to the high stability and brightness of reconstituted split NanoLuc and the very low background activity of the split fragments (12).

Sample analysis and validation

Robust antibody detection has contributed significantly to the diagnosis and control of infectious diseases (2). Because a qualified vaccine against ASFV currently is not available, the presence of specific antibodies identified by serological assays invariably suggests virus infection (25). The antibody response of ASF can be detected as early as 7 days post-infection, is durable, and may persist for years (27). Several ELISA methods and commercial kits are available for the detection of ASFV antibodies; among these kits, Ingezim PPA Compac K3, which is based on a blocking monoclonal antibody target p72, has been extensively validated and is already widely used (28). Therefore, the performance of the split-NanoLuc complementation assay was analyzed and compared with that of an ELISA kit by testing 166 clinical swine serum samples. Comparative studies revealed that the proposed assay produced an ideal diagnostic performance equivalent to that of the ELISA kit, with a concordance rate of 98.19% (Table 1). The correlation between the split-NanoLuc complementation assay and ELISA results was highly consistent, with a kappa value of 0.9229 (Table 1), suggesting the reliable detection capability of the proposed assay in clinical applications. In this comparative analysis, the two methods produced inconsistent results for three serum samples (Table 1). One serum sample was positive in the assay developed herein but negative by ELISA, possibly because of the superior detection sensitivity of the proposed assay. In addition, two serum samples were positive by ELISA but negative in the assay developed herein. These inconsistent results were most likely caused by hemolysis of the serum samples. Previous reports have demonstrated that the performance of Ingenasa ELISA is influenced by hemolyzed sera, resulting in significantly reduced specificity (28). Therefore, positive results should be validated by alternative methods, such as indirect immunofluorescence or indirect immunoperoxidase tests, as recommended by the WOAH.

As an immunoassay, the split-NanoLuc reporter can detect specific antibodies with high specificity and sensitivity (2). The detection duration was shortened by approximately 1 h, which may facilitate the rapid identification and implementation of control strategies for ASF in affected areas. Like the chemiluminescence immunoassay, this assay produces robust luminescence signals spanning a large dynamic measurement range,

TABLE 1 Comparison of the split-NanoLuc complementation assay and a commercial ELISA kit

Methods and determination indices		Commercial ELISA kit			Concordance rate %	Kappa
		Positive	Negative	Total		
NanoBiT assay	Positive	21	1	22	98.19	0.9229
	Negative	2	142	144		
	Total	23	143	166		

allowing easy differentiation between positive and negative samples. Furthermore, it refrains from several tedious steps required by ELISA and other serological techniques but does not compromise performance. These features suggest that the proposed split-NanoLuc immunoassay could be a valuable alternative technique for serological diagnosis and surveillance of ASF.

Conclusions

In summary, an immunoassay based on split-NanoLuc complementation was developed for the simple, rapid, and sensitive detection of ASFV antibodies. The LgBiT and SmBiT subunits coupled to the N-terminus of the p30 probe favored IgG-driven NanoLuc complementation in a sandwich format. Using the N-LgBiT/p30 and N-SmBiT/p30 sensor pair, this NanoBiT-mediated assay exhibited high specificity, sensitivity, and a wide measurement range of ASFV antibodies. The performance of the proposed assay was validated by testing clinical swine serum samples and was significantly consistent with that of a well-characterized ELISA kit. This split-NanoLuc sensing platform provides opportunities for designing appropriate biosensors to detect various macromolecules or biomarkers of diseases.

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ADDITIONAL FILES

The following material is available online.

Supplemental Material

Supplemental material (JCM00463-24-s0001.pdf). Figures S1 to S4.

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