Applied Microbiology and Biotechnology (2022) 106:4695–4704
https://doi.org/10.1007/s00253-022-11999-8

METHODS AND PROTOCOLS

Non-nucleic acid extraction and ultra-sensitive detection of African swine fever virus via CRISPR/Cas12a

Gaihua Cao[1] · Yifan Xiong[1] · Fuping Nie[2] · Xiaolong Chen[1] · Lan Peng[3] · Yingguo Li[2] · Mei Yang[1] · Danqun Huo[1,4] · Changjun Hou[1]

Received: 31 March 2022 / Revised: 19 May 2022 / Accepted: 26 May 2022 / Published online: 18 June 2022

Abstract

Early diagnosis of the African swine fever virus (ASFV) is the main preventive measure for ASFV. Here, we developed a fluorescent biosensor and lateral flow assay (LFA) strip based on direct PCR combined with CRISPR/Cas12a system for ASF. Direct PCR can simultaneously split samples and efficiently amplify without sacrificing sensitivity, which eliminated the steps of nucleic acid extraction. Furthermore, by the CRISPR/Cas12a, the biosensor addressed false positives caused by non-specific amplification and had high sensitivity with the actual limit of detection (LOD) of 7.6×10^{-4} ng·μL^{-1} (4 copies·μL^{-1}). In addition, the strategy was built on the lateral flow assay (LFA) strip to achieve visual and portable detection for point-of-care testing. Moreover, the biosensor by a fluorometer and LFA strip showed a high accuracy to rival qPCR in actual sample detection. Therefore, the biosensor is an ultra-sensitive and specific tool that can replace traditional methods.

Key points

- *No nucleic acid extraction, direct PCR-simplified steps, and reduced time and cost*
- *CRISPR/Cas12a solved the false positives caused by nonspecific amplification*
- *The combination of the LFA strip and biosensor is more convenient for POC detection*

Keywords African swine fever · Direct PCR · CRISPR/Cas12a · Lateral flow assay (LFA) strip · Non-nucleic acid extraction

Introduction

African swine fever (ASF), an infectious disease caused by the *African swine fever virus* (ASFV), causes high fever, hemorrhaging, pulmonary edema, and severe lymphoid tissue necrosis with a 100% fatality rate (Yoon et al. 2021; Zhai et al. 2019). Moreover, ASFV has strong environmental stability and there are many ways of transmission, such as feed dust, transportation, and soft ticks (Khanal et al. 2021; Yoo et al. 2021). In addition, due to no commercially available vaccine or treatment method at present, all the pigs will be slaughtered once the ASFV is found on the farm. As a consequence, the spread of ASFV brings huge economic losses to the farm,

Gaihua Cao and Yifan Xiong contributed equally to this work.

✉ Mei Yang
yangmei@cqu.edu.cn

✉ Danqun Huo
huodq@cqu.edu.cn

✉ Changjun Hou
houcj@cqu.edu.cn

[1] Key Laboratory for Biorheological Science and Technology of Ministry of Education, State and Local Joint Engineering Laboratory for Vascular Implants, Bioengineering College of Chongqing University, Chongqing 400044, People's Republic of China

[2] State Key Laboratory of Cattle Diseases Detection (Chongqing), Diagnosis and Testing Laboratory of Lumpy Skin Disease, Chongqing Customs Technology Center, Chongqing 400044, People's Republic of China

[3] Chongqing Medical and Pharmaceutical College Basic Department, Chongqing 401331, People's Republic of China

[4] Chongqing Key Laboratory of Bio-perception & Intelligent Information Processing, School of Microelectronics and Communication Engineering, Chongqing University, Chongqing 400044, People's Republic of China

which demands a rapid and early diagnosis method of ASFV. The detection methods are mainly divided into two categories: immunological assay (Zhang et al. 2021) and nucleic acid detection. For example, Chen built AlphaLISA (amplified luminescent proximity homogenous assay-linked immunosorbent assay) method using two monoclonal antibodies M-5 and M-6 to specifically identify ASFV P72 with a detection limit of 0.78 $ng{\cdot}mL^{-1}$ (Chen et al. 2021b). Chen established a time-resolved fluorescence immunoassay (TRFIA) method to detect P30 recombinant antigen based on double antibodies sandwich mechanism with the detection limit of 0.015 $ng{\cdot}mL^{-1}$ (Chen et al. 2021a). But there are some drawbacks to antibody testing, such as during early infection they are so low that it is difficult to detect. Furthermore, a study showed that the antibody-based lateral flow assay is not suitable for detecting infection in wild boar carcasses, as proteins are susceptible to denaturation in frozen and thawed samples (Deutschmann et al. 2021). By contrast, the nucleic acid detection is timely and accurate, for example, polymerase chain reaction (PCR) (Bisimwa et al. 2021; Liu et al. 2021), quantitative real-time polymerase chain reaction (qPCR) (Chen et al. 2021c; Chen et al. 2021d; Trinh et al. 2021), droplet digital PCR (ddPCR) (Jia et al. 2020), loop-mediated isothermal amplification (LAMP) (Wang et al. 2021a; Yang et al. 2021a; Zhu et al. 2020), and recombinase polymerase amplification (RPA) (Ren et al. 2021; Wang et al. 2021b; Zhang et al. 2020a). Among them, qPCR is the gold standard due to its accuracy and sensitivity, but its equipment and reagents are more expensive and require professional personnel to operate, limiting its popularization in resource-poor areas. Moreover, the gray area where the Ct value is between 35 and 40 is difficult to distinguish between negative and positive (Chen et al. 2021d). LAMP and RPA have good amplification ability, but they are vulnerable to cause aerosol pollution, which is difficult to eliminate and easy to cause false positives, while PCR has shown significant stability in detection. However, all of the above methods require nucleic acid extraction and purification and they have high requirements on the purity of the DNA template. In general, the lost of nucleic acid during the extraction and purification process as well as the presence of residual reagents could affect the activity of the amplification enzyme. Although various DNA extraction kits, magnetic beads extraction kits, and one-step extraction kits (Cho et al. 2007) are developed to simplify the steps, these extraction approaches are still time-consuming, tedious, and laborious, especially for large samples. And magnetic beads extraction needs a specialized instrument. Moreover, it is inevitable to lose DNA in the extraction process, which increases the difficulty of detection. Therefore, it is of great significance to develop a detection method without nucleic acid extraction for ASFV assay.

Another barrier in the detection is specificity. The amplification products are usually detected by fluorescent dyes, such as SYBR green I (Zhang et al. 2020a) or gel electrophoresis. While gel electrophoresis has low sensitivity leading to false negatives, the specificity of the fluorescent dye is affected by nonspecific amplification and primer dimers, which are prone to false positives. Hence, it is vital to improve the sensitivity and specificity of the ASFV assay. Recently, clustered regularly interspaced short palindromic repeats/CRISPR-associated (CRISPR/Cas) systems have opened the door to new biotechnological applications, such as genome editing (Hubner et al. 2018) and detection (Fu et al. 2021; Lin et al. 2021). Particularly, Cas12a has both cis and trans-cleavage activity, which has become the basis of the new diagnostic platform (Chen et al. 2018), which provides a convenient, sensitive, and specific method for nucleic acid detection. To be specific, the Cas12a specifically combines a DNA by recognizing the protospacer adjacent motif (PAM) site and complementarity to the CRISPR RNA (crRNA). By specifically identifying the targets, Cas12a improves the specificity of detection. After combining the DNA targets, the cis-cleavage activity of the Cas12a is triggered to shear the targets. Besides, Cas12a shows a nonspecific endonuclease activity (trans-cleavage activity) against the surrounding single stranded DNA (ssDNA), such as fluorophore-labeled ssDNA reporters (Yan et al. 2019). The trans-cleavage activity is the important mechanism of CRISPR/Cas12a for signal amplification. For example, based on DNA endonuclease-targeted CRISPR trans reporter (DETECTR) method combined with RPA, Ma developed a method for ASFV detection with a detection limit as low as 8 copies (Li et al. 2020a). Kim developed a high-throughput instrument coupled with the Cas12a system to identify ASFV with detection limits as low as 1 pM (Gao et al. 2020). The above exhibited that CRISPR/Cas12a system is a highly promising candidate for detecting applications.

Here, we used direct PCR combined with CRISPR/Cas12a system for detecting ASFV without nucleic acid extraction. The method without nucleic acid extraction realized the direct detection of blood and tissue from pigs, which greatly saved manpower and material resources. In addition, CRISPR/Cas12a system was employed for specific recognition of amplified products, which improved the specificity and sensitivity of the biosensor. Furthermore, we developed two signal output modes, fluorescence with high sensitivity and lateral flow assay (LFA) strips for intuitive and portable detection. Direct detection of actual samples without nucleic acid extraction steps and CRISPR/

Cas12a system has a huge market and potential in practical applications.

Material and methods

Reagents and instrumentations

The nucleic acid sequences used in this study were ordered by Sangon Biotech (Shanghai, China) and shown in Table S1. QIAamp DNA Mini Kit was purchased from QIAGEN, Germany. Blood and Tissue Super Direct TM PCR kit (UNG)-EDTA was offered by FOREGEN, China. DL2000 DNA Marker and 2×Premix Ex Taq (Probe qPCR) with UNG were purchased from TaKaRa. LbCas12a (Cpf 1) was bought from New England Biotechnology Co., Ltd (Beijing, China). CRISPR-based lateral flow assay strips were purchased from Wobo Biological Co., Ltd, China. The nucleic acid of the *lumpy skin disease virus* wild strain (LSDV, NI 2490) was donated by Dr. Emmanuel Albina, France. *Goat pox virus* vaccine strain (GTPV, CVCC AV41) and *sheep pox virus* vaccine strain (SPPV, CVCC AV42) were bought from the China Veterinary Drug Administration. The nucleic acids of *bluetongue virus* (BTV) and *Peste des Petits Ruminants Virus* (PPRV) were gifted by Dr. Ai from Kunming Customs Technology Center. The negative blood samples of the pig as control (NC) and the nucleic acids of *Classical swine fever virus* (CSFV) were provided by Chongqing Customs Technology Center. Fluorescence thermostatic amplifier GS8 was offered by GenDx Co., Ltd, China. ABI Pro Flex ™ PCR Amplifier and ABI 7500 Fast fluorescence quantitative PCR instrument were purchased from Life Technology ™, USA. The electrophoresis apparatus was bought from Biometra GmbH, Germany.

Sample preparation

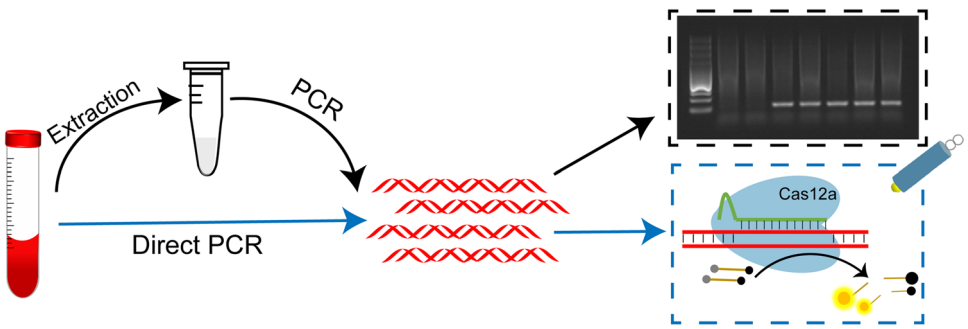
For serum or whole blood samples, they can be directly added to the PCR system without disposing. For tissue samples, 10-mg tissue was added to the EP tube containing 100 µL AL buffer and 4 µL Foregene protease, which was incubated at 65 °C for 10–30 min and 95 °C for 5 min. The EP tube was stored at 4 ℃ for subsequent testing, from which 2 µL of liquid was used for direct PCR.

The qPCR was used to verify results by extracting nucleic acids from serum, whole blood, and tissue according to the QIAamp DNA Mini Kit instructions (Section S1).

Detection by direct PCR-CRISPR/Cas12a

The genome of African swine fever is large double-stranded DNA, 150–170 kb, encoding more than 120 proteins (Quembo et al. 2018). Primers were designed based on the conserved sequence of *B646L* encoding P72 protein for accurately detecting ASFV as shown in Fig. S1. The system of PCR consists of 10 µL of 2×PCR Easy ™ Mix (UNG), 250 nM forward primer, 250 nM reverse primer, and 2 µL blood sample or 2 µL treatment liquid of the tissue sample. The PCR procedure was set as 37 °C for 5 min, 94 °C for 5 min, followed by 35 cycles of 94 °C for 10 s, 55 °C for 20 s, 72 °C for 30 s, and extending with 72 °C for 5 min.

CRISPR/Cas12a was applied to specifically detect PCR products in Scheme 1. The reaction system contained 1×NEB 2.1 buffer, 100 nM LbCas12a, 100 nM crRNA, 500 nM DNA reporters, and 4 µL products. Then, it was put into the fluorescence thermostatic amplifier GS8 and set at 37 °C for 30 min. And it was observed in the gel imager after incubating at 37 °C for 30 min.



Scheme 1 Principle of detecting ASFV by the biosensor. The black path represents the gel electrophoresis. The blue path represents the biosensor based on the direct PCR combined with CRISPR/Cas12a method for blood or tissue samples. The direct PCR reagent completes both split samples to release the nucleic acid and amplification reaction. And then, Cas12a forms a binary complex with the crRNA to identify PCR products, which activates the collateral cleavage activity to arbitrarily slice the ssDNA reporters, resulting in an increase of fluorescence intensity

LFA strips for detecting ASFV

In order to satisfy the point-of-care test, the LFA strip as the most promising tool combined with direct PCR and CRISPR/Cas12a system was applied for ASFV assay in Scheme 2. The process of direct PCR and CRISPR/Cas12a reaction was the same as above. It was noteworthy that the DNA reporter was replaced DNA reporter-LFA strip, which modified FAM and biotin at both ends. After reaction by CRISPR/Cas12a, 80 μL ddH_2O was added and the strip was inserted into the tube, which was kept at room temperature for 2 min. If the target was present, red bands would appear on both the test line (T line) and control line (C line) or only on the T line. Otherwise, red bands would appear only on the C line.

Agarose gel electrophoresis

To further demonstrate the feasibility of the method, gel electrophoresis was employed as an auxiliary tool. After direct PCR, the amplified product was mixed with 6× loading buffer and added into 2% agarose gel for 120 V for 20 min. An Azure Biosystems C150 (USA) was applied to observe the gel images.

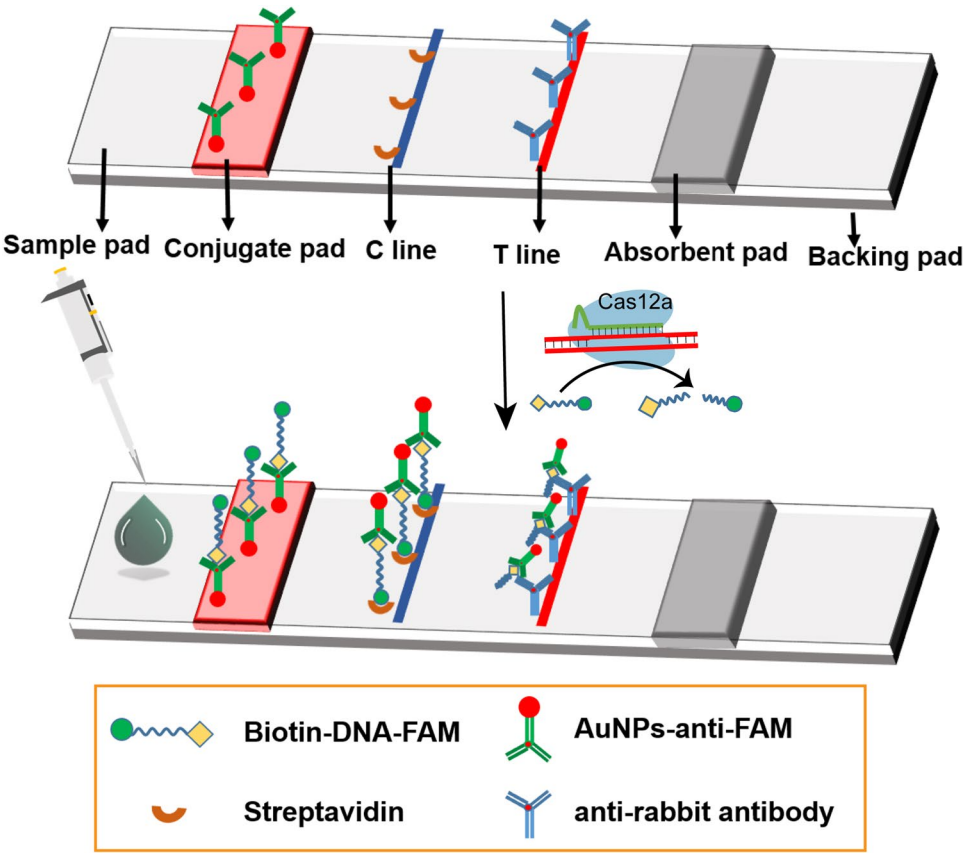
The qPCR for detecting ASFV

The qPCR was employed to verify the results according to SN/T 1559-2010. After extracting from the sample, 5 μL sample were added into a 2-μL mixture containing 0.4 μM SN/T-R primers and SN/T-F primers, 0.2 μM SN/T-probes, and 12.5 μL of 2×Premix Ex Taq (Probe qPCR) with UNG. It was carried out by ABI 7500 Fast fluorescence quantitative PCR instrument with the procedure of 37 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 10 s and 58 °C for 34 s.

Results

The specificity of the method for ASFV assay

As shown in Fig. 1, the LSDV, SPPV, GTPV, CSFV, BTV, and RPPV at the same concentration of 7.6 $ng{\cdot}\mu L^{-1}$, and NC were used as interference for verifying the specificity of the biosensor. The results showed that only ASFV had strong fluorescence and the fluorescence intensities of other viruses were the same as the control group. Fluorescence brightness and LFA strip revealed the same results in Fig. 1(b) and (c),



Scheme 2 Principle of LFA strip based on the biosensor. The DNA reporters bind AuNPs on the absorption pad through the affinity of FAM and anti-FAM antibody. And then, the intact DNA reporters are captured on the C line through biotin binding to the streptavidin, leading to a red band on the C line, while the DNA reporters are cut by Cas12a in the presence of ASFV. The cracked DNA reporters with AuNPs are captured by the anti-rabbit antibodies on the test line (T line) appearing a red band on the T line, but only a band will emerge on the C line in the absence of ASFV

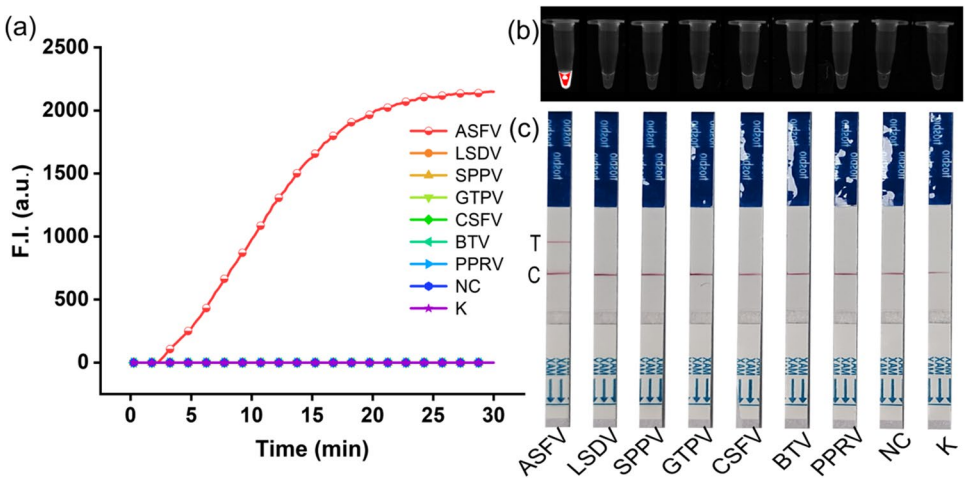


Fig. 1 The specificity detection of the biosensor for ASFV detection. **a** shows the fluorescence intensity corresponding to the nucleic acids of ASFV, LSDV, SPPV, GTPV, CSFV, BTV, and RPPV at the same concentration of 7.6 ng·μL^{-1}, NC, and K (K referred to the ddH_2O as the target). **b** presents the fluorescent brightness of different viruses. **c** displays the LFA strip results of the different viruses

indicating that only ASFV was amplified and activated the trans-cleavage activity of Cas12a. Furthermore, 2% agarose gel electrophoresis was applied for verifying specific amplification in Fig. S2. Only ASFV had an obvious band at 257 bp, while others had no bands. Additionally, the viruses were added to the pig serum for exploring the ability of the biosensor in Fig. S6. The results showed that ASFV had strong fluorescence and others did not. This indicated that the biosensor combined direct PCR with CRISPR/Cas12a system has strong specificity for ASFV detection.

The performance of the biosensor for ASFV assay

To verify the sensitivity of the biosensor, different concentrations of nucleic acid were detected. As shown in Fig. 2(a), the fluorescence intensity increased continuously with the increase of concentration from 7.6 ×10^{-5} ng·μL^{-1} to 7.6 ng·μL^{-1}. By fitting the fluorescence intensity at 25 min and the logarithm of concentration from 7.6 ×10^{-4} ng·μL^{-1} to 7.6 ng·μL^{-1}, Fig. 2(b) shows a good linear relationship, F =1864.67+509.81 Lg C, R^2=0.9557. In addition, the fluorescence brightness decreased with the decrease of concentration in Fig. 2(d). It made visual detection by naked eyes

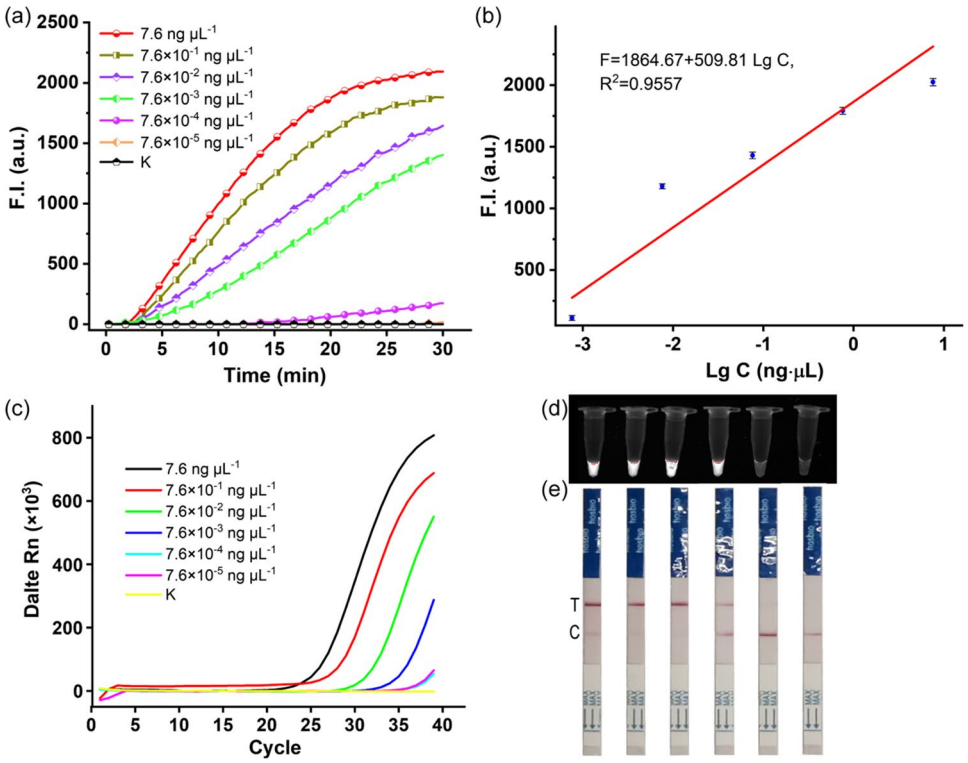


Fig. 2 The sensitivity detection of ASFV by the biosensor. **a** shows the fluorescence spectra of the nucleic acids of ASFV with the concentration from 7.6×10^{-5} ng·μL^{-1} to 7.6 ng·μL^{-1}; **b** illustrates the linear relationship between F and logarithm of the concentration from 7.6×10^{-4} ng·μL^{-1} to 7.6 ng·μL^{-1}. **c** shows the sensitivity detection for the nucleic acid of ASFV by qPCR. **d** from left to right represents fluorescence brightness at different concentrations from 7.6 ng·μL^{-1} to 7.6×10^{-5} ng·μL^{-1}; **e** displays the LFA strip test for the nucleic acid of ASFV with the different concentrations from 7.6 ng·μL^{-1} to 7.6×10^{-5} ng·μL^{-1} (from left to right)

possible under excitation of 488 nm ultraviolet light. Therefore, the actual detection limit by fluorescence detection was 7.6 $\times10^{-4}$ ng·μL^{-1} (about 4 copies·μL^{-1} quantified by ddPCR in Section S2, Table S3, and Fig. S7). Furthermore, the LFA strip was used to test the sensitivity of the biosensor. Figure 2(e) revealed as the concentration decreased from 7.6 ng·μL^{-1} to 7.6 $\times10^{-5}$ ng·μL^{-1}, the T line became weaker and weaker. Until the concentration was as low as 7.6 $\times10^{-4}$ ng·μL^{-1}, the band on the T line was blurred. Therefore, the actual detection limit by the LFA strip was 7.6 $\times10^{-3}$ ng·μL^{-1} (about 40 copies·μL^{-1}). Compared to other methods (Table S2), the biosensor has excellent performance to detect ASFV.

By 2% agarose gel to verify PCR products, it was found that the brightness of the bands gradually decreased with the decrease of concentration in Fig. S3. And the result of 7.6 $\times10^{-2}$ ng·μL^{-1} was similar to the control group, indicating that the detection limit of gel electrophoresis was 7.6 $\times10^{-2}$ ng·μL^{-1}. In addition, targets at different concentrations were detected by qPCR, and it was found that the Ct value increased with the decrease of concentration in Fig. 2(c). When the concentration was above 7.6 $\times10^{-2}$ ng·μL^{-1}, the Ct value was less than 35 cycles which can be determined as positive; but below that, the Ct value was more than 35 cycles which was hard to judge properties in the actual diagnosis. As a result, the biosensor had a high signal-to-noise ratio and was more sensitive than gel electrophoresis and qPCR.

Detection of ASFV in the blood sample

As shown in Fig. 3(a), the nucleic acids with concentrations of 7.6 ng·μL^{-1}, 7.6×10^{-1} ng·μL^{-1}, 7.6×10^{-2} ng·μL^{-1}, 7.6×10^{-3} ng·μL^{-1}, and 7.6×10^{-4} ng·μL^{-1} were added into the whole blood for verifying the performance of the biosensor. Obviously, the fluorescence intensity increased with the increase of concentration. Furthermore, the fluorescence brightness was also dimmed as the concentration decreased in Fig. 3(c). A good linear relationship was obtained by linear fitting between the fluorescence intensity at 25 min and the logarithms of concentration from 7.6×10^{-4} to 7.6 ng·μL^{-1} in Fig. 3(b). That was $F' = 2071.25 + 420.44 \text{ Lg } C$, R^2=0.974. This showed that the biosensor had a strong anti-interference ability in blood samples assay. Moreover, blood mixed with different concentrations of ASFV was amplified by direct PCR and detected by agarose gel electrophoresis in Fig. S4. It was found that there were obvious bands when the concentration was more than 7.6×10^{-2} ng·μL^{-1}, and the bands gradually dimmed with the decrease of concentration. In addition, Fig. 3(d) showed the detection limit can be as low as 7.6×10^{-3} ng·μL^{-1} by the LFA strip.

Detection of ASFV in the clinical sample

Thirty clinical samples from canned pork, sausage, preserved pork, and raw pork were tested by the biosensor. Among them, twelve samples were positive, seventeen were negative, and one sample was weakly positive which had weak fluorescence intensity in Fig. 4(a). Notably, the fluorescent picture and LFA strip showed the same phenomena in Fig. 4(b) and Fig. 4(c). The positive samples had the obvious red band on the T line, while the negative samples only had the red band on the C line. In addition, qPCR was employed for verifying the results. The results showed that the Ct value was 100% consistent with the results of the biosensor in Fig. S5.

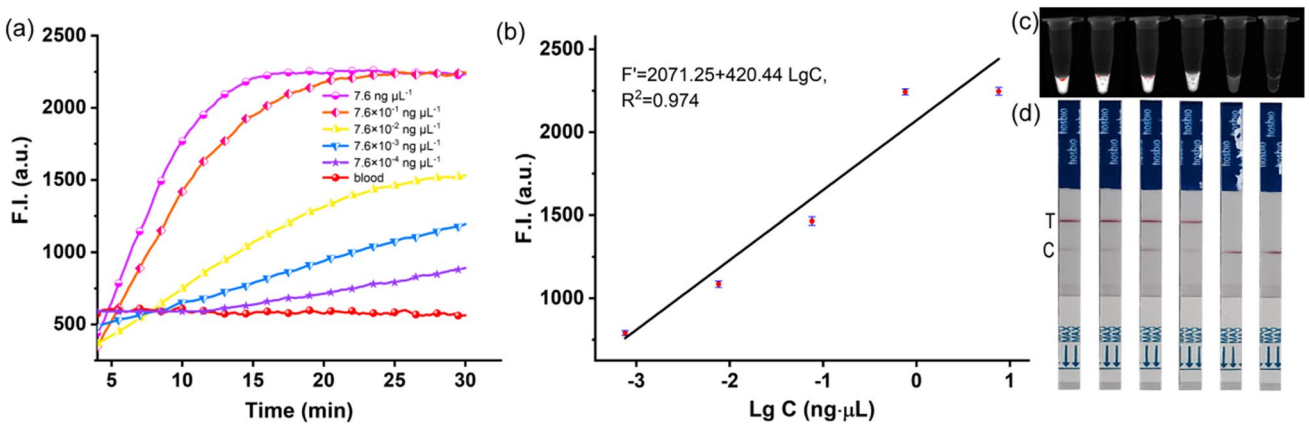


Fig. 3 Blood sample detected by the biosensor. **a** represents the corresponding fluorescence spectra of samples which mixed different concentrations nucleic acid of ASFV with pig blood; **b** illustrates the linear relationship between F' and the logarithm of concentration from 7.6×10^{-4} to 7.6 ng·μL^{-1}. **c** presents the fluorescence brightness and **d** shows the results of the mixed samples tested by the LFA strip

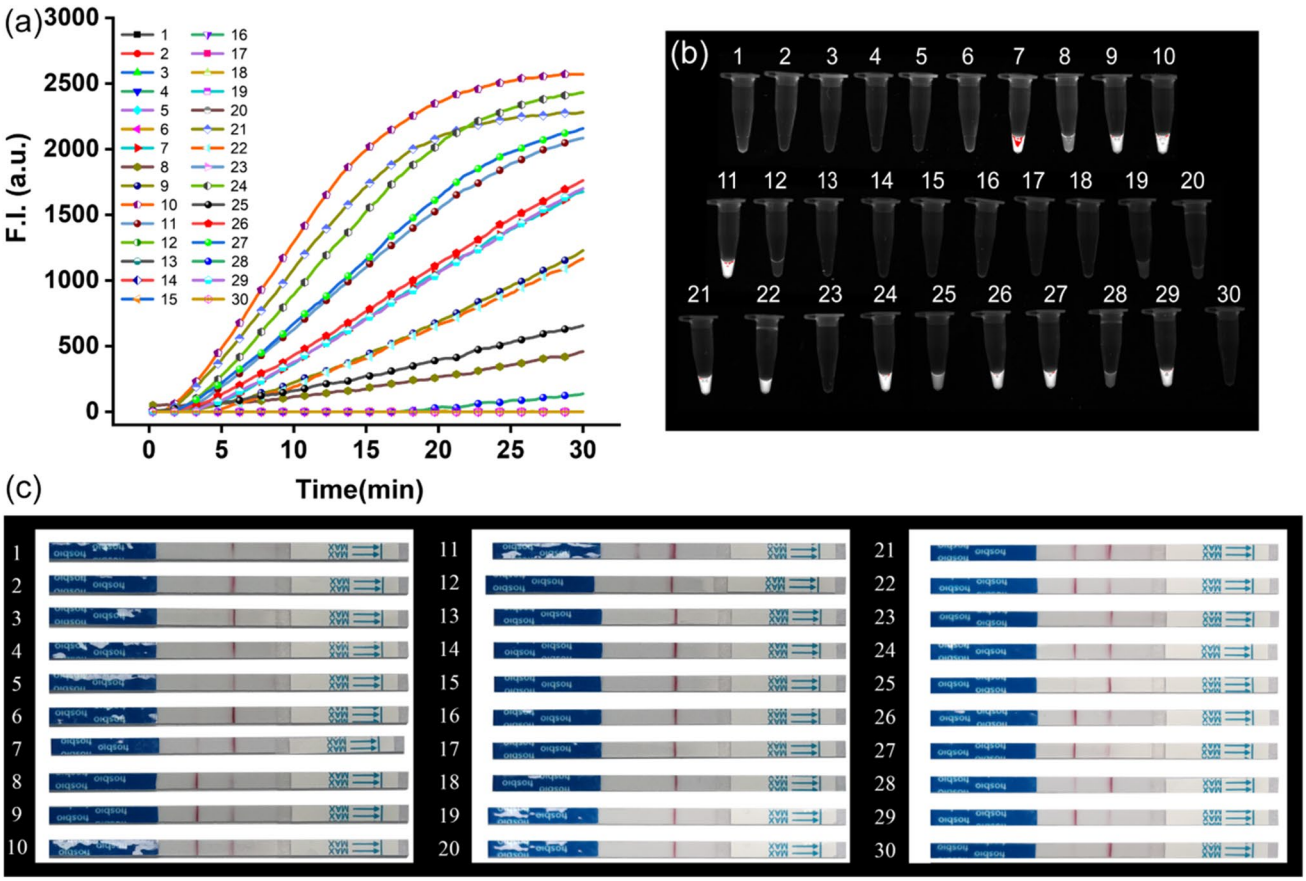


Fig. 4 Tissue samples detected by the biosensor. **a** is the fluorescence spectrum of the samples. **b** shows the fluorescence brightness of the samples. **c** demonstrates that tissue samples were detected by the LFA strip

Discussion

ASV caused huge economic losses. However, there is no commercial vaccine or treatment, so an early and sensitive diagnosis is crucial to minimize the damage. At present, in nucleic acid detection, extraction of nucleic acid is an important step and the premise of detection. Many researchers are making efforts to reduce the steps, time, and loss of nucleic acid in the extraction process and improve the purity of nucleic acids, such as magnetic bead enrichment (Emaus et al. 2020; Pearlman et al. 2020; Wolfe et al. 2002). However, this step is tedious and time-consuming, especially in the case of a large sample size. The extraction process prolongs the detection time and increases the cost, which is not conducive to rapid detection. Here, we constructed the biosensor based on direct PCR and CRISPR/Cas12a for the ASFV assay. Direct PCR can complete cleavage and amplification at the same time, and samples can be directly amplified without the step of nucleic acid extraction, which greatly reduced labor, material costs, and time. In addition, CRISPR/Cas12a system has specific cis-cleavage and trans-cleavage activity with strong signal amplification capability (Yan et al. 2019). In other words, Cas12a recognizes the target by pairing with the crRNA, and Cas12a cleaves the target through its cis-cleavage activity. At the same time, the target activates its trans-cutting activity, which arbitrarily slices nearby ssDNA. This is a new and specific method of signal amplification (Wang et al. 2020). Therefore, the CRISPR/Cas12a with the high amplification efficiency improved sensitivity and specificity, providing a more convenient and sensitive method for the detection of ASFV.

Specificity is the basic element of virus detection. Some methods used RPA, LAMP, or other amplification techniques, and modified chemical groups on primers for LFA strip detection (James et al. 2010; Miao et al. 2019; Xu et al. 2018). This method was simple but not specific enough. To be specific, there was non-specific amplification in the amplification process. On the other hand, primer dimers can cause false positives because primer dimers also displayed results on the strip. Similarly, a lot of methods using fluorescent dyes for DNA assay also had false positive results (Dixit et al. 2018; Tsai et al. 2016). Therefore, it is necessary to eliminate the influence of these factors to improve the specificity. CRISPR/Cas12a can specifically identify targets, which avoids the effects of false positives

generated by nonspecific amplification and primer dimers. Therefore, CRISPR/Cas12a has stronger specificity in the field of LFA strips.

In the environment or the early stage of infection, the level of ASFV is low. This constantly requires sensors or detection methods to improve sensitivity. For instance, Zhang built a biosensor based RPA combined with SYBR Green I to detect ASFV with the LOD of 10^3 copies (Zhang et al. 2020b). Wang used RPA combined with the lateral flow strip for portable detection and the LOD was as low as 10^2 copies (Wang et al. 2021c). Although these methods were easy to operate, they were not sensitive enough. The CRISPR/Cas12a system had greatly improved sensitivity due to the trans-cleavage. Such as, Li made use of CRISPR/Cas12a coupled with RPA for ASFV assay with a LOD as low as 8 copies (Li et al. 2020b). And Yang developed a biosensor based on LAMP and CRISPR/Cas12a to detect ASFV and the LOD was 7 copies (Yang et al. 2021b). Isothermal amplification methods combined with CRISPR/Cas12a are better detection methods because of the lower instrument cost, but they still require nucleic acid extraction, and they are more prone to aerosol contamination which is difficult to remove. Based on the direct PCR which had a great tolerance to pollution, the biosensor coupled with CRISPR/Cas12 had a greater advantage in sensitivity with the LOD as low as 4 copies·μL^{-1} of fluorescence and 40 copies·μL^{-1} of the LFA strip. This not only provided a more convenient signal output mode, but also improved the sensitivity. Therefore, the biosensor was a more accurate method to detect ASFV in the early stage of infection.

In general, the lost of nucleic acid during the extraction and purification process as well as the presence of residual reagents could affect the activity of the amplification enzyme. Ingeniously, the reagent of direct PCR not only digested the sample to release the nucleic acid, but also had high amplification efficiency. As a result, the biosensor did not lose any sensitivity compared to the detection of high-purity nucleic acids, indicating that the biosensor based on direct PCR combined with CRISPR/Cas12a had the excellent anti-interference capability for ultra-sensitive detecting actual samples. For blood sample testing, the fluorescence spectrum had some fluctuation in Fig. 3(a) since the whole blood contained some trace fluorescent substances, such as endogenous porphyrins, tryptophan, reduced nicotinic purine dinucleotides, and xanthine adenine dinucleotides. Furthermore, the biosensor took full advantage of the strong signal amplification capability of CRISPR/Cas12a, improving sensitivity by two orders of magnitude compared with agarose gel electrophoresis. Hence, it was an accurate and potential tool that can replace the gel electrophoresis and qPCR method.

In summary, ASFV has brought huge losses in the pig industry. Due to its high transmission, high fatality rate, and no good treatment, rapid and accurate detection of ASFV becomes a fundamental countermeasure. To simplify the procedure and improve the precision of the nucleic acid test, we constructed a biosensor-based direct PCR combined with CRISPR/Cas12a to detect ASFV. Direct PCR did not require complex and time-consuming nucleic acid extraction, which can directly detect blood and tissue. What is more, the experiment proved that it did not lose sensitivity. In addition, CRISPR/Cas12a system has both strong specificity and high signal amplification capability. By the specific binding of the crRNA to the target, the biosensor solved the problem of false positives caused by non-specific amplification. And its trans-cleavage activity realized the second signal amplification with the detection limits of 7.6×10^{-4} ng·μL^{-1} (4 copies·μL^{-1}). Moreover, the LFA strip achieved visual detection, which was portable and suitable for ASFV detection in remote areas. The biosensor had good performance in actual sample detection. Hence, the biosensor-based CRISPR/Cas12a combined with direct PCR is a highly sensitive and specific tool that can be popularized in practical applications.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00253-022-11999-8.

Data availability All data generated or analyzed during this study are included in this published article (and its supplementary information files).

Author contribution GC: conceptualization, methodology, writing—original draft, writing—review and editing
YX: methodology and formal analysis
FN: resources and funding acquisition
XC: methodology and formal analysis
LP: validation, software
YL: methodology
MY: project administration, funding acquisition
DH: supervision, project administration
CH: writing—review and editing, project administration, funding acquisition
All authors read and approved the manuscript.

Funding This work was supported by the National Natural Science Foundation of China (NO. 31171684, 81772290), Graduate Scientific Research and Innovation Foundation of Chongqing, China (CYB21070), Chongqing Technology Innovation and Application Development Special Project (CSTC2019jscx-gksbX0132), Research Project of Chongqing Customs (2020CQKY10), The Chongqing Science and Technology Commission (CSTC2015shmszxl20097), Chongqing Graduate Tutor Team Construction Project, Analytical and Testing Center of Chongqing University, and the sharing fund of Chongqing University's large equipment.

Declarations

Ethics approval This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest The authors declare no competing interests.

References

Bisimwa PN, Ishara LK, Wasso DS, Bantuzeko F, Tonui R, Bwihangane AB (2021) Detection and genetic characterization of *African swine fever virus* (ASFV) in clinically infected pigs in two districts in South Kivu province. Democratic Republic Congo. Heliyon 7(3):e06419. https://doi.org/10.1016/j.heliyon.2021.e06419

Chen C, Lai H, Liang H, He Y, Guo G, Li L (2021a) A new method for detection *African swine fever virus*: time-resolved fluorescence immunoassay. J Fluoresc 31(5):1291–1296. https://doi.org/10.1007/s10895-021-02754-9

Chen D, Wang D, Wang C, Wei F, Zhao H, Lin X, Wu S (2021b) Application of an AlphaLISA method for rapid sensitive detection of *African swine fever virus* in porcine serum. Appl Microbiol Biotechnol 105(11):4751–4759. https://doi.org/10.1007/s00253-021-11339-2

Chen JS, Ma E, Harrington LB, Da Costa M, Tian X, Palefsky JM, Doudna JA (2018) CRISPR-Cas12a target binding unleashes indiscriminate single-stranded DNase activity. Science 360(6387):436. https://doi.org/10.1126/science.aar6245

Chen L, Wen K, Chen FE, Trick AY, Liu H, Shao S, Yu W, Hsieh K, Wang Z, Shen J, Wang TH (2021c) Portable magnetofluidic device for point-of-need detection of African swine fever. Anal Chem 93(31):10940–10946. https://doi.org/10.1021/acs.analchem.1c01814

Chen Y, Wu S, Wu H, Cheng P, Wang X, Qian S, Zhang M, Xu J, Ji F, Wu J (2021d) CRISPR/Cas12a-based versatile method for checking quantitative polymerase chain reaction samples with cycles of threshold values in the gray zone. ACS Sens 6(5):1963–1970. https://doi.org/10.1021/acssensors.1c00515

Cho YK, Lee JG, Park JM, Lee BS, Lee Y, Ko C (2007) One-step pathogen specific DNA extraction from whole blood on a centrifugal microfluidic device. Lab on a Chip 7(5):565–573. https://doi.org/10.1039/b616115d

Deutschmann P, Pikalo J, Beer M, Blome S (2021) Lateral flow assays for the detection of *African swine fever virus* antigen are not fit for field diagnosis of wild boar carcasses. Transbound Emerg Dis. https://doi.org/10.1111/tbed.14248

Dixit KK, Verma S, Singh OP, Singh D, Singh AP, Gupta R, Negi NS, Das P, Sundar S, Singh R, Salotra P (2018) Validation of SYBR green I based closed tube loop mediated isothermal amplification (LAMP) assay and simplified direct-blood-lysis (DBL)-LAMP assay for diagnosis of visceral leishmaniasis (VL). Plos Neglect Trop D 12(11):10.1371/journal.pntd.0006922

Emaus MN, Varona M, Eitzmann DR, Hsieh SA, Zeger VR, Anderson JL (2020) Nucleic acid extraction: fundamentals of sample preparation methodologies, current advancements, and future endeavors. Trac-Trend Anal Chem 130. https://doi.org/10.1016/j.trac.2020.115985

Fu J, Zhang Y, Cai G, Meng G, Shi S (2021) Rapid and sensitive RPA-Cas12a-fluorescence assay for point-of-care detection of *African swine fever virus*. PLoS One 16(7):e0254815. https://doi.org/10.1371/journal.pone.0254815

Gao Y, Xu SY, He T, Li JW, Liu LS, Zhang YY, Ge SG, Yan M, Liu HY, Yu JH (2020) Ultrasensitive and specific microRNA detection via dynamic light scattering of DNA network based on rolling circle amplification. Sensor Actuat B-Chem 324. https://doi.org/10.1016/j.snb.2020.128693

Hubner A, Keil GM, Kabuuka T, Mettenleiter TC, Fuchs W (2018) Efficient transgene insertion in a *pseudorabies virus* vector by CRISPR/Cas9 and marker rescue-enforced recombination. J Virol Methods 262:38–47. https://doi.org/10.1016/j.jviromet.2018.09.009

James HE, Ebert K, McGonigle R, Reid SM, Boonham N, Tomlinson JA, Hutchings GH, Denyer M, Oura CAL, Dukes JP, King DP (2010) Detection of *African swine fever virus* by loop-mediated isothermal amplification. J Virol Methods 164(1-2):68–74. https://doi.org/10.1016/j.jviromet.2009.11.034

Jia R, Zhang G, Liu H, Chen Y, Zhou J, Liu Y, Ding P, Wang Y, Zang W, Wang A (2020) Novel application of nanofluidic chip digital PCR for detection of *African swine fever virus*. Front Vet Sci 7:621840. https://doi.org/10.3389/fvets.2020.621840

Khanal P, Olcha M, Niederwerder MC (2021) Detection of *African swine fever virus* in feed dust collected from experimentally inoculated complete feed using quantitative PCR and virus titration assays. Transbound Emerg Dis. https://doi.org/10.1111/tbed.14176

Li Z, Wei J, Di D, Wang X, Li C, Li B, Qiu Y, Liu K, Gu F, Tong M, Wang S, Wu X, Ma Z (2020a) Rapid and accurate detection of *African swine fever virus* by DNA endonuclease-targeted CRISPR trans reporter assay. Acta Biochim Biophys Sin (Shanghai) 52(12):1413–1419. https://doi.org/10.1093/abbs/gmaa135

Li ZJ, Wei JC, Di D, Wang X, Li CX, Li BB, Qiu YF, Liu K, Gu F, Tong ML, Wang SM, Wu XD, Ma ZY (2020b) Rapid and accurate detection of *African swine fever virus* by DNA endonuclease-targeted CRISPR trans reporter assay. Acta Bioch Bioph Sin 52(12):1413–1419. https://doi.org/10.1093/abbs/gmaa135

Lin W, Tian T, Jiang Y, Xiong E, Zhu D, Zhou X (2021) A CRISPR/Cas9 eraser strategy for contamination-free PCR end-point detection. Biotechnol Bioeng 118(5):2053–2066. https://doi.org/10.1002/bit.27718

Liu H, Shi K, Sun W, Zhao J, Yin Y, Si H, Qu S, Lu W (2021) Development a multiplex RT-PCR assay for simultaneous detection of *African swine fever virus*, *classical swine fever virus* and *atypical porcine pestivirus.* J Virol Methods 287:114006. https://doi.org/10.1016/j.jviromet.2020.114006

Miao FM, Zhang JY, Li N, Chen T, Wang LD, Zhang F, Mi LJ, Zhang JX, Wang SC, Wang Y, Zhou XT, Zhang YY, Li M, Zhang SF, Hu RL (2019) Rapid and sensitive recombinase polymerase amplification combined with lateral flow strip for detecting *African swine fever virus*. Front Microbiol 10. https://doi.org/10.3389/fmicb.2019.01004

Pearlman SI, Leelawong M, Richardson KA, Adams NM, Russ PK, Pask ME, Wolfe AE, Wessely C, Haselton FR (2020) Low-resource nucleic acid extraction method enabled by high-gradient magnetic separation. Acs Appl Mater Inter 12(11):12457–12467. https://doi.org/10.1021/acsami.9b21564

Quembo CJ, Jori F, Vosloo W, Heath L (2018) Genetic characterization of *African swine fever virus* isolates from soft ticks at the wildlife/domestic interface in Mozambique and identification of a novel genotype. Transbound Emerg Dis 65(2):420–431. https://doi.org/10.1111/tbed.12700

Ren M, Mei H, Zhou M, Fu ZF, Han H, Bi D, Peng F, Zhao L (2021) Development of a super-sensitive diagnostic method for african swine fever using CRISPR techniques. Virol Sin 36(2):220–230. https://doi.org/10.1007/s12250-020-00323-1

Trinh TBN, Truong T, Nguyen VT, Vu XD, Dao LA, Nguyen TL, Ambagala A, Babiuk S, Oh J, Song D, Le VP (2021) Development of a novel real-time PCR assay targeting p54 gene for rapid detection of *African swine fever virus* (ASFV) strains circulating in Vietnam. Vet Med Sci. https://doi.org/10.1002/vms3.605

Tsai CC, Shih HC, Ko YZ, Wang RH, Li SJ, Chiang YC (2016) Direct LAMP assay without prior DNA purification for sex determination of *papaya*. Int J Mol Sci 17(10):10.3390/ijms17101630

Wang XJ, Shang XY, Huang XX (2020) Next-generation pathogen diagnosis with CRISPR/Cas-based detection methods. Emerg Microbes Infec 9(1):1682–1691. https://doi.org/10.1080/22221751.2020.1793689

Wang Y, Dai J, Liu Y, Yang J, Hou Q, Ou Y, Ding Y, Ma B, Chen H, Li M, Sun Y, Zheng H, Zhang K, Wubshet AK, Zaberezhny AD, Aliper TI, Tarasiuk K, Pejsak Z, Liu Z et al (2021a) Development

of a potential penside colorimetric LAMP assay using neutral red for detection of *African swine fever virus*. Front Microbiol 12:609821. https://doi.org/10.3389/fmicb.2021.609821

Wang Z, Yu W, Xie R, Yang S, Chen A (2021b) A strip of lateral flow gene assay using gold nanoparticles for point-of-care diagnosis of *African swine fever virus* in limited environment. Anal Bioanal Chem 413(18):4665–4672. https://doi.org/10.1007/s00216-021-03408-2

Wang ZY, Yu WJ, Xie RB, Yang SM, Chen AL (2021c) A strip of lateral flow gene assay using gold nanoparticles for point-of-care diagnosis of *African swine fever virus* in limited environment. Analytical and Bioanalytical Chemistry 413(18):4665–4672. https://doi.org/10.1007/s00216-021-03408-2

Wolfe KA, Breadmore MC, Ferrance JP, Power ME, Conroy JF, Norris PM, Landers JP (2002) Toward a microchip-based solid-phase extraction method for isolation of nucleic acids. Electrophoresis 23(5):727–733. https://doi.org/10.1002/1522-2683(200203)23:5<727::Aid-Elps727>3.0.Co;2-O

Xu YC, Wei YJ, Cheng N, Huang KL, Wang WR, Zhang L, Xu WT, Luo YB (2018) Nucleic acid biosensor synthesis of an all-in-one universal blocking linker recombinase polymerase amplification with a peptide nucleic acid-based lateral flow device for ultrasensitive detection of food pathogens. Anal Chem 90(1):708–715. https://doi.org/10.1021/acs.analchem.7b01912

Yan FC, Wang W, Zhang JQ (2019) CRISPR-Cas12 and Cas13: the lesser known siblings of CRISPR-Cas9. Cell Biol Toxicol 35(6):489–492. https://doi.org/10.1007/s10565-019-09489-1

Yang B, Shi Z, Ma Y, Wang L, Cao L, Luo J, Wan Y, Song R, Yan Y, Yuan K, Tian H, Zheng H (2021a) LAMP assay coupled with CRISPR/Cas12a system for portable detection of *African swine fever virus*. Transbound Emerg Dis. https://doi.org/10.1111/tbed.14285

Yang B, Shi ZW, Ma Y, Wang LJ, Cao LY, Luo JC, Wan Y, Song R, Yan YY, Yuan KH, Tian H, Zheng HX (2021b) LAMP assay coupled with CRISPR/Cas12a system for portable detection of *African swine fever virus*. Transbound Emerg Dis. https://doi.org/10.1111/tbed.14285

Yoo DS, Kim Y, Lee ES, Lim JS, Hong SK, Lee IS, Jung CS, Yoon HC, Wee SH, Pfeiffer DU, Fournie G (2021) Transmission dynamics of *African swine fever virus*, South Korea, 2019. Emerg Infect Dis 27(7):1909–1918. https://doi.org/10.3201/eid2707.204230

Yoon H, Hong SK, Lee I, Choi DS, Lee JH, Lee E, Wee SH (2021) Arthropods as potential vectors of *African swine fever virus* outbreaks in pig farms in the Republic of Korea. Vet Med Sci 7(5):1841–1844. https://doi.org/10.1002/vms3.545

Zhai S-L, Wei W-K, Sun M-F, Lv D-H, Xu Z-H (2019) African swine fever spread in China. Veterinary Record 184(18):10.1136/vr.l1954

Zhang S, Sun A, Wan B, Du Y, Wu Y, Zhang A, Jiang D, Ji P, Wei Z, Zhuang G, Zhang G (2020a) Development of a Directly visualized recombinase polymerase amplification-SYBR green I method for the rapid detection of *African swine fever virus*. Front Microbiol 11:602709. https://doi.org/10.3389/fmicb.2020.602709

Zhang S, Sun AJ, Wan B, Du YK, Wu YN, Zhang AK, Jiang DW, Ji PC, Wei ZY, Zhuang GQ, Zhang GP (2020b) Development of a directly visualized recombinase polymerase amplification-SYBR green I method for the rapid detection of *African swine fever virus*. Front Microbiol 11. https://doi.org/10.3389/fmicb.2020.602709

Zhang X, Liu X, Wu X, Ren W, Zou Y, Xia X, Sun H (2021) A colloidal gold test strip assay for the detection of *African swine fever virus* based on two monoclonal antibodies against P30. Arch Virol 166(3):871–879. https://doi.org/10.1007/s00705-020-04915-w

Zhu YS, Shao N, Chen JW, Qi WB, Li Y, Liu P, Chen YJ, Bian SY, Zhang Y, Tao SC (2020) Multiplex and visual detection of *African swine fever virus* (ASFV) based on Hive-Chip and direct loop-mediated isothermal amplification. Anal Chim Acta 1140:30–40. https://doi.org/10.1016/j.aca.2020.10.011