# Specific High-sensitivity Multiple-probe-assisted DNA Capture and Amplification Technology for Direct Detection of African Swine Fever Virus without DNA Extraction

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**Abstract**

African Swine Fever (ASF) is one of the most devastating infectious diseases affecting domestic pigs and wild boar. The grave socio-economic impact of African Swine Fever infection at a global level makes large-scale rapid and robust diagnosis a critical step towards effective control. However, the nucleic acid purification required in most molecular detection methods is time- and labor-intensive, prone to nucleic acid loss or contamination, and impractical for massive active screening or for use in resource-limited areas. Here we describe multiple-probe-assisted DNA capture and amplification technology (MADCAT) - a novel sensitive, simple, and reliable method for detecting ASFV directly from whole blood or other complex matrices. Through the unique DNA capture method which specifically capture only the target DNA onto the well for subsequent amplification, MADCAT abandons the complicated extraction protocol and achieves ultrafast and high-throughput detection. The sample-to-result time for 96 samples is about 100 min, as compared with the 3 - 4 h time of the standard real time qPCR method. The limit of detection (LOD) is 0.5 copies/μL and is 10 times more sensitive than an OIE-recommended qPCR assay when testing serially diluted whole blood samples. The assay is 100% specific against other common swine pathogens. In clinical diagnosis of 48 field samples, all 22 positive samples were correctly identified with lower Ct values than OIE-recommended qPCR, confirming its high diagnostic sensitivity (100%). Owing to its high-throughput, specific high-sensitivity, and cost-efficient features, MADCAT shows great potential for future use in clinical ASFV active screening.

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**Abstract**

African Swine Fever (ASF) is one of the most devastating infectious diseases affecting domestic pigs and wild boar. The grave socio-economic impact of African Swine Fever infection at a global level makes large-scale rapid and robust diagnosis a critical step towards effective control. However, the nucleic acid purification required in most molecular detection methods is time- and labor-intensive, prone to nucleic acid loss or contamination, and impractical for massive active screening or for use in resource-limited areas. Here we describe multiple-probe-assisted DNA capture and amplification technology (MADCAT) - a novel sensitive, simple, and reliable method for detecting ASFV directly from whole blood or other complex matrices. Through the unique DNA capture method which specifically capture only the target DNA onto the well for subsequent amplification, MADCAT abandons the complicated extraction protocol and achieves ultrafast and high-throughput detection. The sample-to-result time for 96 samples is about 100 min, as compared with the 3 - 4 h time of the standard real time qPCR method. The limit of detection (LOD) is 0.5 copies/μL and is 10 times more sensitive than an OIE-recommended qPCR assay when testing serially diluted whole blood samples. The assay is 100% specific against other common swine pathogens. In clinical diagnosis of 48 field samples, all 22 positive samples were correctly identified with lower Ct values than OIE-recommended qPCR, confirming its high diagnostic sensitivity (100%). Owing to its high-throughput, specific high-sensitivity, and cost-efficient features, MADCAT shows great potential for future use in clinical ASFV active screening.

**KEYWORDS**

African swine fever, DNA capture, laboratory diagnosis, without DNA extraction

**Introduction**

African swine fever (ASF) is an extremely contagious disease of wild boar and domestic pigs with an almost 100% mortality rate, causing significant economic trauma to the pig industry in affected countries (Dixon et al., 2020). The clinical symptoms of ASF infection can be manifested from subclinical infection to sudden death with few other signs. This fact, together with the great similarities of clinical presentations and lesions between ASF and other hemorrhagic pig diseases (Dixon et al., 2020; Schulz et al., 2017), make differential laboratory diagnosis compulsory. Since there is no vaccine commercially available as of 2020 (Revilla et al., 2018; Teklue et al., 2020), control and eradication strategies are timely and comprehensive culling of infected pigs, relying on accurate and rapid laboratory diagnostic screening of ASFV-positive or suspected cases before the large-scale outbreak (Arias et al., 2018).

Currently, molecular methods are recommended for the diagnosis and containment of ASFV (Fernandez-Pinero et al., 2013; OIE, 2018; Wang et al., 2020; Ye et al., 2019). Conventional PCR methods which require post-amplification manipulation (Aguero et al., 2003; OIE, 2018) are being broadly replaced by the real-time PCR system. Significantly, the World Organisation for Animal Health (OIE) -recommended real-time qPCR method amplifies a viral DNA fragment of 250 bp long (King et al., 2003; OIE, 2018), which is considered now as non-optimal size for a real-time PCR system (Fernandez-Pinero et al., 2013). Although various real-time PCR methods give good sensitivity and specificity rates, the robustness of the methods are decreased when weak ASFV-positive samples are analyzed (Gallardo et al., 2019).

One of the bottlenecks for PCR-based assays to detect DNA targets from clinical samples is that the presence of inhibitors suppresses the activity of DNA polymerase, necessitating a DNA purification prior to amplification. Various methods for DNA extraction have been developed (Thatcher, 2015). However, these methods are generally labor-intensive and time-consuming. Furthermore, the multiple sample processing steps involved in these methods increase the risk of cross-contamination and human error, making them sub-

optimal for high-throughput application and widespread deployment (Fernandez-Pinero et al., 2013; King et al., 2003; Wang et al., 2020). The "direct PCR" was then developed to make the analysis method less sensitive to interference by using mutants of *Taq* DNA polymerase that are more resistant to inhibitors from complex sample backgrounds so that the preparation procedure could be bypassed (Kermekchiev et al., 2009; Leelawong et al., 2019; Li et al., 2019; Zhang et al., 2010), but sometimes at the expense of removing the exonuclease activity required for cleavage of hydrolysis probes and increasing cost (Leelawong et al., 2019). PCR can also be optimized through the use of various PCR enhancer cocktail (Li et al., 2019; Zhang et al., 2010) and specific PCR buffers (Bu et al., 2008; Liu et al., 2018) to reduce the inhibitory effect of blood components. However, the application of these methods in practice is challenging as the fluorescent signal is quenched by compounds such as heme and hemoglobin (Kang et al., 2014), so that only a very small volume of sample can be assayed directly, and the sensitivity is often compromised compared with using equivalent amount of purified DNA. Automated extraction instrument provides another potential solution (Flannery et al., 2020), but significantly increasing the assay cost.

Herein, we describe the development of a specific high-sensitivity multiple-probe-assisted DNA capture and amplification technology (MADCAT) for direct detection of African swine fever virus DNA molecule in blood or tissue homogenates without DNA extraction to effectively overcome the challenges mentioned above.

## 2. Materials and Methods

### 2.1. Standard DNA, Primers and Probes

Plasmid carrying a 415 bp highly conserved sequence of ASFV VP72 gene based on the sequence alignment of 137 strains using MAFFT version 7 was synthesized (BioTeke) covering regions of the designed capture probes and the TaqMan amplicon. The plasmid was quantified with UV-Vis spectrophotometers (Thermo Scientific NanoDrop 2000) and linearized by QuickCut *EcoR* I (Takara). All primers and probes sequences, as listed in Table S1, were synthesized by Invitrogen.

### 2.2. Clinical Samples

Clinical samples, including serum, EDTA-blood, spleen, heart, kidney, lymph and lung were collected from domestic pigs in China. A panel of 22 positive and 26 negative samples were included in this study and provided by African Swine Fever Regional Laboratory of China (Lanzhou), Lanzhou Veterinary Research Institute, CAAS. The tissue samples were ground in a tissue homogenize and prepared a cell suspension at 10% with phosphate-buffered saline (1g tissue/10 ml sterile PBS).

### 2.3. MADCAT Procedures

Up to 12 μL sample (plasmid, gDNA, whole blood, serum or tissue homogenates) was lysed with 16.7 μL of 3 × lysis mixture, 0.5 μL of capture probes (0.1 μM), 2.5 μL of proteinase K (20 mg/mL, Tiangen), and 18.3 μL of deionized water at 56 for 5 min with vigorous shaking in customized 96-well capture plate. Thermal denaturation was performed at 98 for 5 min, followed by 10-min target capture at 55 . Both the lysis and capture processes were carried out under the condition of shaking at 1200 rpm with 96-well Thermomixer (Eppendorf), while the denaturation was performed in a 96-well PCR thermocycler. After washing three times with washing solution to remove all unbound probes and irrelevant nucleic acids, the captured targets were then amplified with a 25 μL PCR reaction mix containing 0.2 μM of each primer, 0.1 μM of Taqman probe (VP-FP), and 12.5 μL of 2 × probe qPCR premix (Takara). The PCR reaction was carried out on the CFX96 Real-Time PCR Detection System (Bio-Rad) or LightCycler® 96 System (Roche Life Science), with 95  for 30 s, followed by 40 cycles of 95  5 s, 54  10 s and 72  20 s. A positive result was called if the Ct value $< 40$ and a sigmoidal plot is observed.

### 2.4. Reference Real-time PCR

The OIE-recommended qPCR method (King et al., 2003; OIE, 2018) for ASF diagnosis was used as the reference technique in comparative assays. Briefly, genomic DNA was extracted from 200 μL clinical sample (blood, serum and tissue homogenates) with the TIANamp Genomic DNA Kit (QIAGEN), and 5 μL of

genomic DNA was amplified in a 25 μL reaction, consisting of 0.2 μM of each primer (VP-UP and VP-DP), 0.1 μM of Taqman probe (VP-FP), and 12.5 μL of 2 × probe qPCR premix (Takara). The thermocycling parameters and positive judgment criteria are the same as the MADCAT method.

### 2.5. Capture Efficiency

The capture efficiency (Ec) was used to evaluate whether there is nucleic acid loss during the capture process by comparing the Ct shift between MADCAT and standard real-time PCR method using plasmid DNA. The two methods shared the same PCR composition and condition, except that the MADCAT method has additional capture and washing steps before amplification. The Ct values generated by standard real-time PCR ($Ct_0$) and MADCAT (Ctc) were then used to calculate the capture efficiency, according to the following formula: Ec=2 ^ ($Ct_0$- Ctc) * 100%.

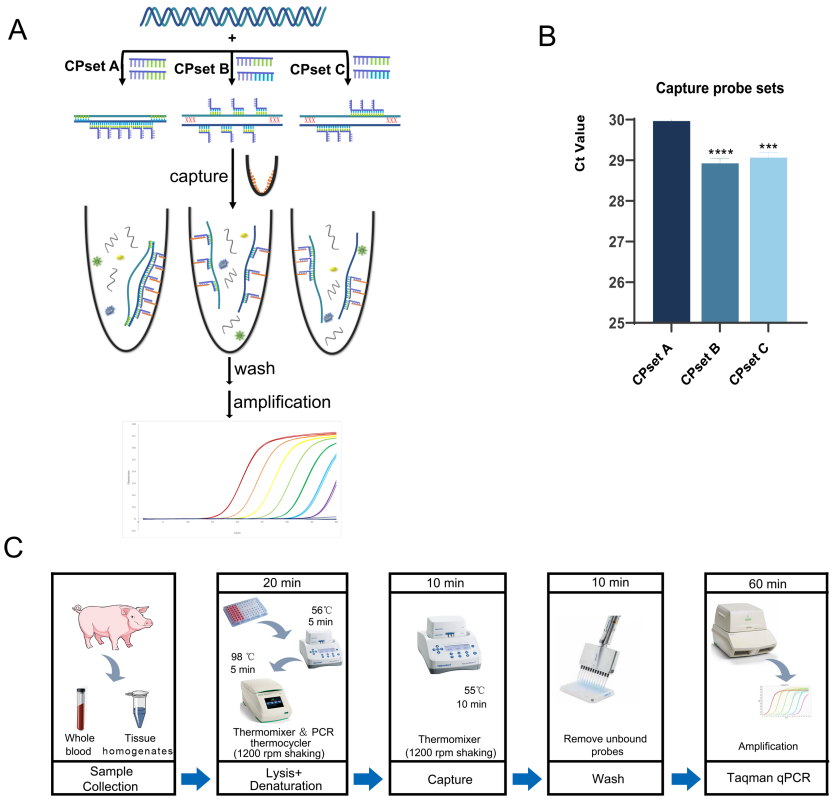
### 2.6.Statistical Analysis

Basic statistical analysis, including the calculation of means, standard deviations and coefficient of variation was performed using Excel software (Microsoft, Bellevue, WA). Two-tailed Fisher's exact tests and paired t tests were performed using GraphPad software version 8.1.1 (GraphPad, Inc., USA), with statistical significance set at $P$ -value $< 0.05$.

### 2.7. Ethics Approval

Animal treatment and sample preparation complied with the Animal Ethics Procedures and Guidelines, and was approved by the Animal Ethics Committee of Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences (Approval No. LVRIAEC2020–06).

## 3. Results



### 3.1. Design of the MADCAT System

The principle of the multiple-probe-assisted DNA capture and amplification technology (MADCAT) is illustrated in Figure 1A. Target DNA of lysed samples are captured on a 96-well plate by a series of capture probes, each of which incorporates a target-specific sequence and an additional "tail" sequence that can interact with the oligonucleotide conjugated on the surface of each well in 96-well plate. After washing off all unbound probes and irrelevant nucleic acids, the captured targets were then amplified with the target-specific primers and probes.

Based on this multiple-probe-per-strand design principle, three sets of capture probe (CPsets A, B and C) were designed for the same region by utilizing different design schemes (Figure 1A). The CPset B and CPset C proved to be more conducive to capturing double-stranded DNA targets than CPset A (Figure 1B). The candidate three sets of primers, Primerset1 (AV-F1 and AV-R1), Primerset2 (AV-F2 and AV-R2) and OIE-recommended primers (VP-UP and VP-DP) (King et al., 2003), were computationally filtered to eliminate potential homology to the swine genome and to a panel of related viruses. Experimentally, among the three schemes, Primerset2 showed better performances (lower Ct values) than OIE-recommended primers and Primerset1 (Figure S1), and was used for the subsequent tests consequently.

### 3.2. Laboratory Validation of the MADCAT Assay

To systematically ascertain the diagnostic performance of MADCAT assay for ASFV detection, we used plasmids and clinical whole blood sample to assess the sensitivity, reproducibility and specificity of this assay.

A standard curve was constructed with a significant linear relationship ($R^2$=0.9980) and a linear dynamic range across seven orders of magnitude. The limit of detection (LOD) was 0.5 copies/μL of DNA sample (Figure 2A, B). There was no statistical difference ($P> 0.05$) of Ct values between the MADCAT, which underwent capture before real-time PCR amplification, and standard real-time PCR (Figure 2B), indicating that all the target DNA released after lysis can be captured without any loss. When we spiked plasmid DNA of the same series of concentrations in porcine blood and tested with our method, the same LOD and linear dynamic range were observed. For the clinical whole blood sample, a 10-fold dilutions series were tested with MADCAT. In comparison, the DNAs extracted from the same blood samples were tested with the OIE-recommended qPCR. The results showed that the MADCAT method had increased

**Table 1.** Intra- and inter-assay variability of this method.

| | Intra-assay Variability (C.V of Ct; n=4) | Intra-assay Variability (C.V of Ct; n=4) | Inter-assay Varia |
|---|---|---|---|
| | LC-96[a] | Bio-Rad[b] | |
| $5 \times 10^4$ copies/μL | 0.53 % | 0.65 % | 1.15 % |
| $5 \times 10^2$ copies/μL | 0.48 % | 1.17 % | 1.25 % |
| $5 \times 10^{-1}$ copies/μL | 1.17 % | 2.51 % | 2.13 % |

[a] LightCycler® 96 of Roche

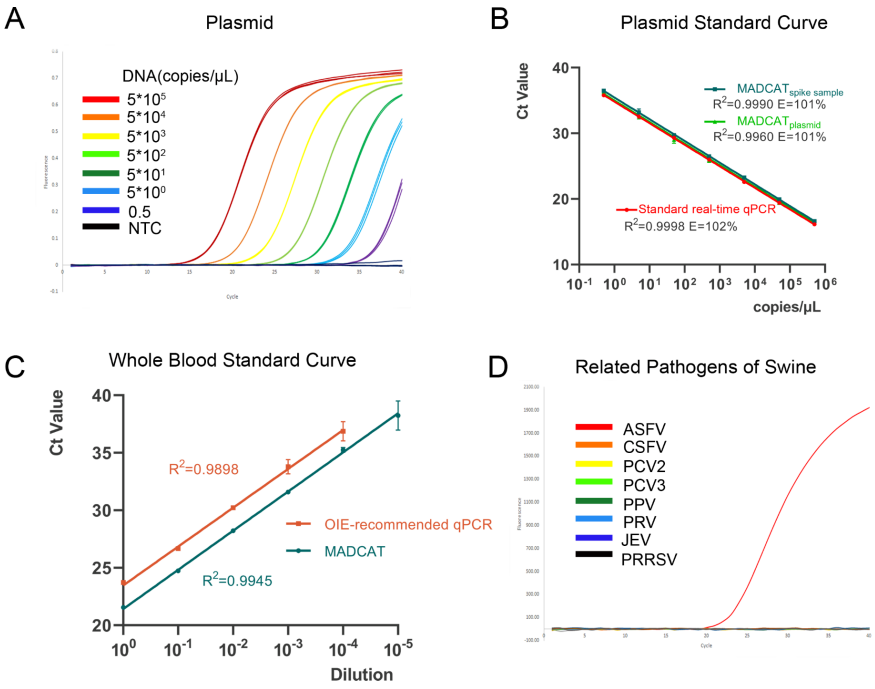
[b] CFX96 of Bio-Rad

analytical sensitivity by an order of magnitude than the OIE-recommended qPCR (Figure 2C), indicating a better ability of MADCAT assay to detect virus in clinical samples.

The reproducibility of this method was determined by their intra-assay variability and inter-assay variability using high ($5 \times 10^4$ copies/μL), medium ($5 \times 10^2$copies/μL) and low (0.5 copies/μL) concentrations of plasmid DNA. Six independent runs on two real-time quantitative detection systems (CFX96 of Bio-Rad and LightCycler® 96 of Roche) were acquired with each sample tested in quadruplicates on every run. Both intra-assay variability (C.V of Ct value) and inter-assay variability was $< 3\%$, showing reproducible detection and good precision at different viral loads (Table 1).

The specificity of MADCAT was evaluated by testing closely related classical swine fever virus (CSFV), as well as other pathogens of swine: porcine parvovirus (PPV), porcine circovirus type 2 (PCV2), porcine circovirus type 3 (PCV3), pseudorabies virus (PRV), porcine reproductive and respiratory syndrome virus (PRRSV), and Japanese encephalitis virus (JEV). The clinical sample was obtained from previously confirmed infected donor pigs with above-mentioned virus. Fluorescence signal was observed exclusively in ASFV-positive control (Figure 2D).

### 3.3. MADCAT Detection of ASFV in Porcine Clinical Sample.




A panel of 48 clinically validated field samples (22 of serum, 21 of whole blood, and 5 of tissue homogenates from heart, spleen, kidney, lymph, and lung) were tested to further verify the clinical performance of the MADCAT assay. The experiment was performed at African Swine Fever Regional Laboratory of China (Lanzhou), Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences under ethical approval. There was 100% agreement between the results of MADCAT method and OIE-recommended qPCR method (Table 2). In addition, lower Ct values were obtained with our method compared to OIE-recommended qPCR when detecting the ASFV-infected samples (Table 3).

**Table 2.** Comparison between the OIE-recommended qPCR and the MADCAT assay for the detection of ASFV among real clinical samples

| | | OIE-recommended qPCR Positive | OIE-recommended qPCR Negative | OIE-recommended qPCR Total |
|---|---|---|---|---|
| MADCAT | Positive | 22 | 0 | 22 |
| | Negative | 0 | 26 | 26 |
| | Total | 22 | 26 | 48 |

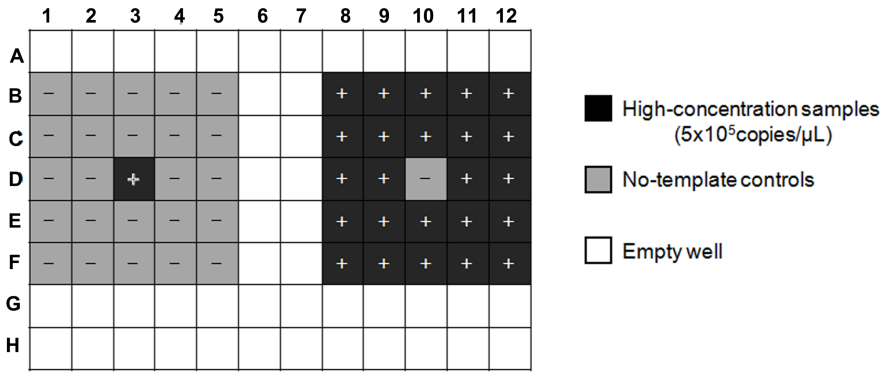
**Table 3.** . Ct values of positive clinical samples.

| Sample number | Sample type | MADCAT | OIE qPCR | Sample number | Sample type | MADCAT | OIE qPC |
|---|---|---|---|---|---|---|---|
| NO.1 | Serum | 17.22 | 19.79 | NO.12 | Blood | 19.94 | 25.93 |
| NO.2 | | 24.59 | 27.32 | NO.13 | | 22.16 | 24.67 |
| NO.3 | | 17.12 | 20.77 | NO.14 | | 19.32 | 23.61 |
| NO.4 | | 34.20 | 37.50 | NO.15 | | 23.16 | 27.80 |
| NO.5 | | 21.85 | 25.60 | NO.16 | | 20.99 | 22.83 |
| NO.6 | | 20.04 | 24.68 | NO.17 | | 22.83 | 24.74 |
| NO.7 | | 20.87 | 24.33 | NO.18 | Heart homogenates | 25.41 | 30.74 |
| NO.8 | | 28.43 | 32.25 | NO.19 | Spleen homogenates | 28.58 | 29.53 |
| NO.9 | | 23.61 | 27.69 | NO.20 | Kidney homogenates | 29.45 | 30.45 |
| NO.10 | | 34.61 | 34.58 | NO.21 | Lymph homogenates | 30.43 | 33.65 |
| NO.11 | Blood | 21.56 | 23.72 | NO.22 | Lung homogenates | 22.40 | 27.10 |

### 3.4. Contamination Assessment Test

Since multi-well plate format was used, it is possible the additional washing steps may introduce cross-contamination. We assessed the contamination risk of MADCAT assay (Figure 3). The results showed that even if 24 high-concentration samples were processed in the same plate, they did not contaminate a negative well surrounded by them, nor did a high-concentration sample well bring contamination to any negative well surrounding it. Given the high sensitivity of the assay, such results indicate very low probability of cross-contamination.

## Discussion




Here we develop MADCAT for high-throughput, large-scale ASFV screening application, which is based upon a series of capture probes hybridizing alternate regions on each strand of the target DNA (Figure 1A). Initially we used our previously established method for capturing single-stranded RNA to capture the denatured DNA (Xu & Zheng, 2016). However, this single-strand capture method used in DNA capture was shown to have a low capture efficiency (Figure 1B), probably due to the competitive interference of the complementary strand of the target DNA. In this study, two other kinds of capture probes were used, each targeting both DNA strands with a multiple-probe-per-strand design (Figure 1A, B). By simultaneously capturing both strands they achieved near 100% capture efficiency (Figure 2B). It is possible the steric hindrance after probe binding makes it difficult for the two stands to reanneal. Compared with another capture-based biosensor method, which exploited a triplex formation with ssDNA/LNA chimeric probes (Biagetti et al., 2018), the MADCAT approach does not require modified nucleotide for the probes and will not be affected by potential blood impurities, which may produce non-specific responses and non-interpretable results in biosensor method. In addition, the simultaneous action of multiple capture probes ensures that all ASFV genotypes can be captured, tolerating genetic drift or point mutations.

Notably, unlike any current molecular technology, which extracts all the DNA into a solution followed by selective amplification, our method specifically captures the ASFV target on the solid support, and can completely separate the target from irrelevant sequences, impurities and inhibitors through a simple washing step, leaving the target DNA as the only amplifiable template in the subsequent PCR reaction. This can not only reduce non-specific signal interference, but also increase the efficiency of amplification, and can even test pooled samples in large-scale screening without losing sensitivity. When spiked plasmid DNA in porcine blood was tested, the Ct value was almost identical to that of DNA without blood, and the same LOD was observed (Figure 2B), demonstrating a much better ability against blood interference than most "direct PCR" methods. By circumventing the extraction processes, DNA loss and laboratory personnel error may be minimized, and overall sample processing time and cost can be reduced. Due to reduced steps, this method not only saves labor, but also increases the assay reproducibility with a coefficient of Ct value variation (C.V) $< 3\%$ (Table 1). Despite the additional wash step, our method can handle high-concentration samples without any cross-contamination (Figure 3), probably because the target DNAs are bound at the bottom of the well not in the solution during the handling. Considering that only 12 μL of sample input is required, the MADCAT method greatly reduces the need for sample volume and eases sampling pressure, whereas a DNA extraction workflow often consumes 200 μL of precious samples (Aguero et al., 2003; Gallardo et al., 2015; King et al., 2003; OIE, 2018). Although less sample volume is required, this method amplifies more sampled DNA than extraction-based method, which can only amplify a portion of the extracted DNA in the sampled volume.

Since the routine virus testing of infected pigs has become a key element of control strategy for ASF (Dixon et al., 2020), the high-throughput MADCAT has a potential to meet the needs of active surveillance system with its ELISA-like workflow on 96-well plate format. Throughout the procedure only one plate was used, and 96 samples can be easily assayed with minimal user input and a sample-to-result time within 100 min (Figure 1 C). Coupled with an ultra-high sensitivity of 0.5 DNA copies/μL or 6 DNA copies/reaction, MADCAT offers the potential of large-scale epidemiological screening, with sample pooling strategy, for early ASFV infections or even asymptomatic infections.

In conclusion, we have developed a specific high-sensitivity, easy to use, inexpensive, and accurate molecular assay suitable for high-throughput ASFV screening. This new DNA detection platform holds great application potential in detecting other types of nucleic acid targets, such as other infectious pathogens and disease genetic markers.

**Conflicts of interest**

There are no conflicts to declare.

**Acknowledgements**

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**Data Availability Statement**

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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