# Innovative molecular diagnostics of African swine fever virus and other transboundary animal diseases

Von

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# Innovative molecular diagnostics of African swine fever virus and other transboundary animal diseases

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**Contents**

## 1. Introduction

In recent years, transboundary animal diseases (TADs) have caused significant epidemics in human and animal populations resulting in immense suffering and economic losses. Almost all recent pandemics originated from animal reservoirs, and viral zoonoses are the most likely cause for the next pandemic [1, 2]. The World Health Organization (WHO) publishes a list of priority diseases annually to facilitate rapid research and development and shorten the interval between the designation of a public health emergency and availability of appropriate diagnostic tests and control tools (e.g., vaccines, antivirals) [World Health Organization. A research and development Blueprint for action to prevent epidemics 2018]. The following animal viruses are included, based on the lists of the World organisation for Animal Health (WOAH, previously OIE) and the Food and Agriculture Organisation of the United Nations (FAO): African swine fever virus (ASFV), Influenza A virus (IAV), Lumpy skin disease virus (LSDV), Bluetongue virus (BTV), and Peste des petits ruminants virus (PPRV) [3]. As a result of globalization and increased travel, these pathogens are TADs and can spread rapidly across the globe. The economic repercussions of infectious animal and zoonotic diseases can be severe and far-reaching, as evidenced by the global COVID-19 pandemic—a zoonotic virus believed to have originated in wildlife [4]. Another example is the current outbreaks of ASF in Asia and Europe which cause significant losses in agriculture and trade, and impact the day-to-day lives of millions of consumers, livestock producers, and stakeholders worldwide [5].

Effective diagnostic tools for TADs are needed to provide accurate and timely measures for case identification, surveillance, rapid elimination or appropriate treatment [6]. Identifying the cause of a disease is the first step in initiating disease surveillance and control. Accurate and timely detection of infectious animal diseases in the field is a major requirement for early detection and management of infected animals (e.g., cure, seclusion, or culling), which can avert a future epidemic and also reduce the potential costs. Accordingly, the simplification of reliable cost-effective methods would promote the application of a wide range of diagnostic tools in a variety of laboratories, especially those with limited facilities and resources. Several factors should be optimized to ensure rapid and

reliable diagnosis, such as the collection of samples, sample matrix, storage of samples, transport from the field to a central laboratory, nucleic acid extraction, and/or the amplification process, could affect the successful application of molecular diagnostic workflows. Therefore, it is important to consider the different conditions between the traditional workflows of laboratory-based methods and the application of molecular diagnostics in the field and to optimize the workflow accordingly. For instance, in addition to the manual nucleic acid extraction methods, the use of portable or electricity-free extraction instruments and methods would be useful in rapid diagnostics. Likewise, the implementation of portable real-time PCR kits, assays, and thermocyclers would promote the usability of diagnostic methods, and direct qPCR amplification would further accelerate the diagnostic processes.

In this dissertation, I addressed several factors, which affect the rapid, sensitive, specific and reliable detection of viral nucleic acids including the development and application of different DNA/RNA extraction and releasing methods, qPCR techniques, choice of appropriate sample matrices, storage conditions and the use of molecular applications in the field with transportable diagnostic tools.

# 2. Literature review

## 2.1. Model viruses used for the establishment and validation of molecular diagnostic systems

The emergence and re-emergence of transboundary animal diseases, such as ASF, highly pathogenic avian influenza (HPAI), and foot-and-mouth disease (FMD), necessitates the development of powerful and reliable diagnostic procedures. In our study, we investigated five important emerging and re-emerging animal diseases as stated by the International Committee on Taxonomy of Viruses (ICTV, see Table 1) [7].

**Table 1** Model viruses and their classification according to the ICTV.

| Virus name | Family | Genus | Main hosts | References |
|---|---|---|---|---|
| African swine fever virus (ASFV) | *Asfaviridae* | *Asfivirus* | Domestic pigs, wild boar | [8, 9] |
| Lumpy skin disease virus (LSDV) | *Poxviridae* | *Capripoxvirus* | Goats, sheep, cattle, domestic buffaloes | [10] |
| Influenza A virus (IAV) | *Orthomyxo-viridae* | *Alphainfluenza virus* | Human, pigs, horses, cats, dogs, marine mammals, birds (wild birds and poultry) | [11-13] |
| Peste des petits ruminants virus (PPRV) | *Paramyxo-viridae* | *Morbillivirus* | Goats, sheep | [14] |
| Bluetongue virus (BTV) | *Reoviridae* | *Orbivirus* | Domestic and wild ruminants | [15] |

### 2.1.1. African swine fever virus

In light of recent rapidly spreading outbreaks in Europe, and in particular the first outbreak in Germany in 2020, we focused mainly on the ASF virus (ASFV) [16]. ASF is a contagious viral haemorrhagic disease that affects domestic pigs and wild boar. The disease is notifiable to the World Organization for Animal Health (WOAH) and causes significant death rates and economic losses. ASFV is the only member of the genus *Asfivirus* in the family *Asfarviridae* [7, 17]. The virion has an icosahedral shape [18] and is relatively large with a size of 175–215 nm. A schematic presentation of the virus structure is illustrated in Figure 1. The linear double-stranded DNA genome—170 to 190 kbp in length—contains between 160 and 175 open reading frames (ORFs) and it encodes six multigene families [19]. Until today, there are 23 of ASFV distinctive common genotypes based on a partial sequence of the gene encoding the p72 protein [20]. It is the only known DNA arthropod-borne virus (ARBO) due to the sylvatic transmission cycle in Africa involving soft ticks of the genus *Ornithodoros* [21].

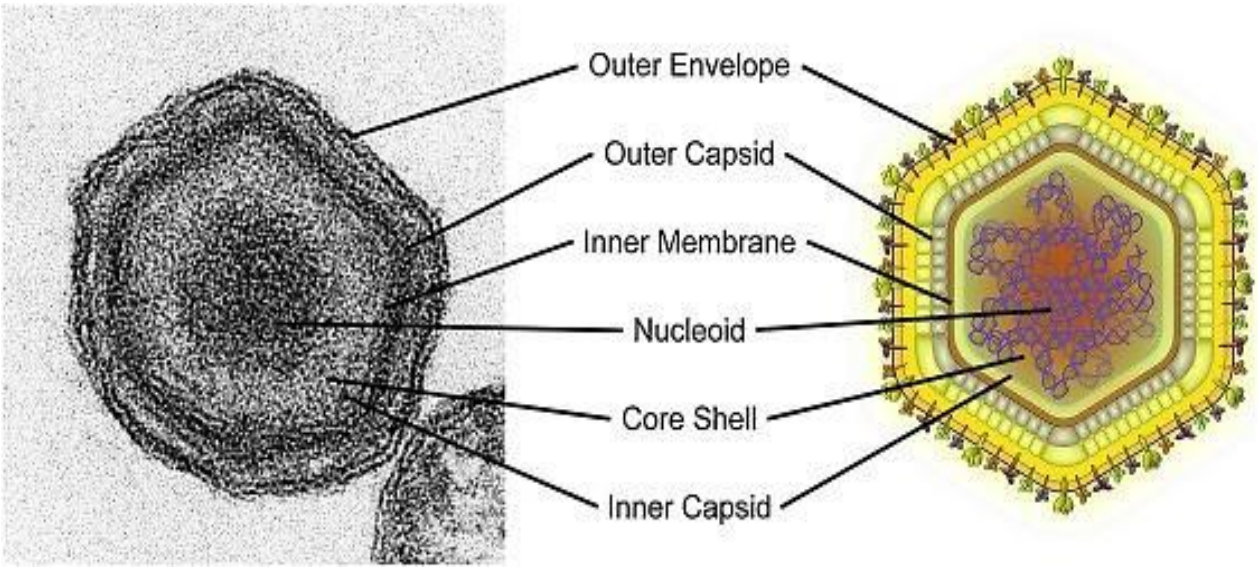


**Figure 1**: The morphological structure of the ASFV, permission was granted under the terms of the Creative Commons Attribution-Non-Commercial-No Derivatives Licence (CC BY NC ND). (Source: Blome et al., 2020 [22].)

The clinical findings of ASF vary depending on the virulence of the pathogen strain as well as on the immunological status of the host. ASFV in Europe (with the exception of Sardinia) and Asia belong to genotype II, which are highly interrelated and demonstrate high pathogenicity

for both domestic pigs and wild boar under experimental conditions [23-29]. Highly virulent strains of ASFV cause acute to peracute illness with up to 100% death rate within 7–10 days (d). Clinical signs are usually non-specific and include high fever, anorexia, dyspnoea, gastrointestinal disorders, and peracute death. After an incubation period of 2 to 7 d [30], infected animals exhibit the usual high fever as well as other nonspecific clinical signs, such as lassitude, reddened skin (especially on acral appendages), anorexia, and conjunctivitis.

ASF was initially reported in East Africa in the early 1900s and was thought to kill almost all pigs infected with acute haemorrhagic fever. A sylvatic cycle in ticks was identified as the source of infection [31, 32]. Introduction via Georgia into the Caucasus in 2007 led to the rapid spread of ASFV to Russia, Ukraine, and Belarus in the following years, with cases emerging in the European Union (EU), particularly in the Baltic states and Poland during 2014. Within a few years, the disease spread to Belgium, Serbia, Romania, Bulgaria, the Czech Republic, Hungary, and Germany [16, 33-36] (Figure 2).

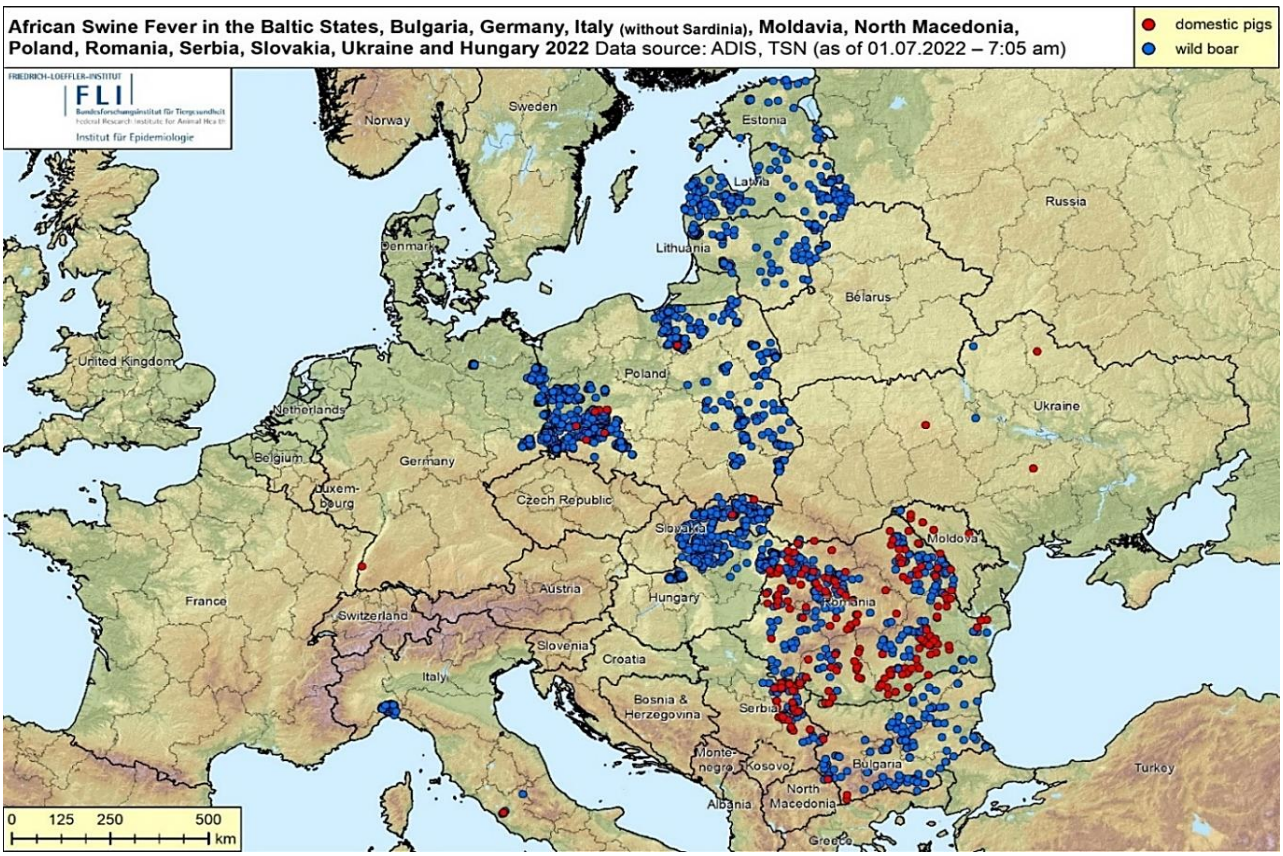

**Figure 2**: Epidemiological distribution of African swine fever in Europe 2022. Reproduced with permission from the Institute of Epidemiology, Friedrich-Loeffler-Institut, PD. Dr. Carola Sauter-Louis and Stephan Eichenberg. (red dots: domestic pigs, blue dots: wild boar.)

### 2.1.2. Other emerging and re-emerging viruses

Four other socio-economically important diseases were considered for the studies, specifically HPAI, BT, LSD and PPR. The disease-causing pathogens represent both DNA and RNA viruses (single and double-stranded) and have affected animal production in almost all European or bordering countries in the last decade.

#### 2.1.2.1 Lumpy skin disease virus (LSDV)

LSDV is one of the three species of the genus *Capripoxvirus* in the family *Poxviridae* [7, 10]. LSDV is the most serious poxvirus of cattle livestock industries [37-39], affecting mainly cattle, but in a few cases also sheep and goats [10, 40]. LSDV outbreaks can lead to severe production losses, including milk production and muscle mass, permanent or temporary sterility of bulls, and degradation the tissue and skin. It has a severe influence on the economy at both national and global scales [40-44]. Therefore, the WOAH classified this virus as a notifiable disease [7]. The morphological structure of LSDV is illustrated in Figure 3. Capripox virus-induced diseases are characterized by general clinical manifestations, such as fever [37, 39, 40, 45], depression, diarrhoea, emaciation, and coughing [37]. Diseased animals mostly develop skin lesions, pustules, and tumours [39, 40, 46].

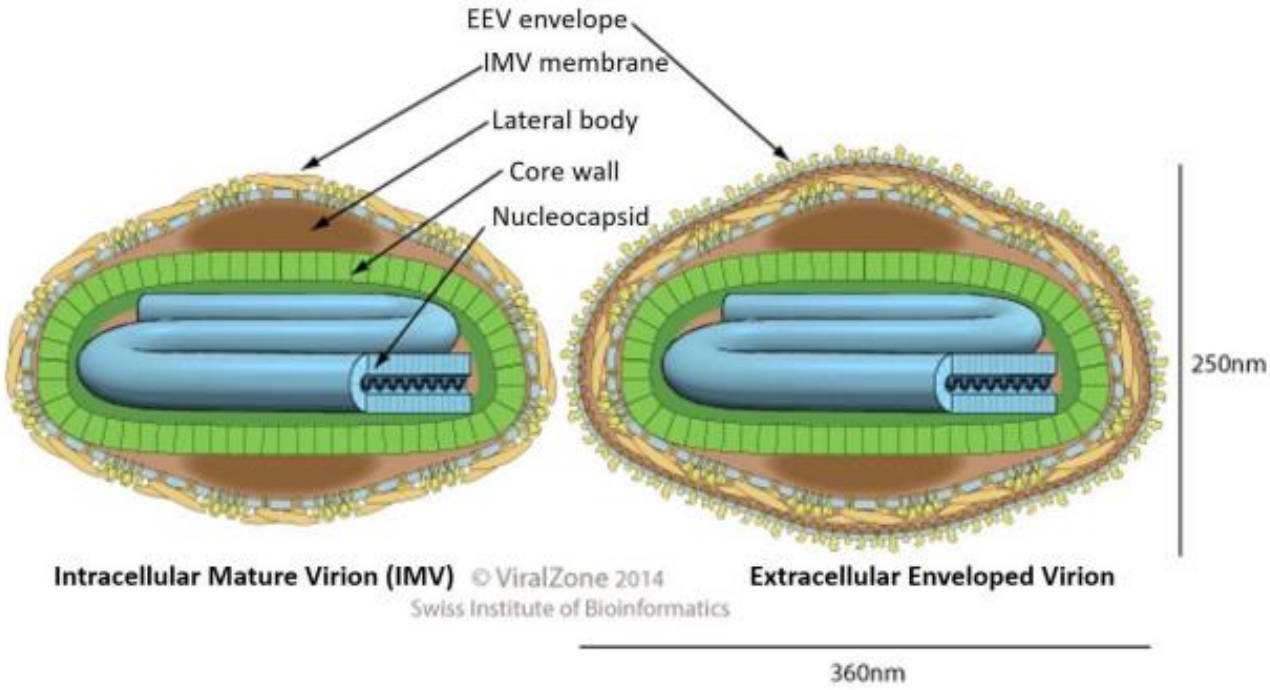

**Figure 3**: Morphological structure of poxvirus virions. (Source: ViralZone 2014, Swiss Institute of Bioinformatics ©2008, [47])

### 2.1.2.2 Influenza A virus (IAV)

Alphainfluenzavirus (IAV) is a genus of the RNA virus family *Orthomyxoviridae* [12]. IAV possess a single-stranded segmented RNA genome composed of eight gene segments, which encode nine structural proteins and up to five non-structural proteins. According to the variation of the membrane glycoproteins hemagglutinin (HA) and neuraminidase (NA), IAV are currently classified into 18 HA and 11 NA subtypes. Each virus must carry one HA and one NA subtype (e.g. H1N1, H5N1, H9N2). IAV is known for its rapid rate of mutation and continuous evolution of new viral strains [11]. IAV infects a wide range of mammals (including humans) and birds. IAV can be sub-classified based on its host of origin to e.g., human IAV, avian influenza virus (AIV) or swine IAV (swIAV) [48, 49]. Since 1918, human IAVs have resulted in four pandemics with millions of causalities in humans and annual epidemics worldwide. Moreover, IAV caused severe losses in poultry and pig production worldwide [11, 12, 50-52]. AIV of subtypes H5 and H7 can be further divided into two pathotypes based on virus virulence in poultry: Low pathogenicity AIV (LPAIV) and high pathogenicity AIV (HPAIV) [53]. Other non-H5/H7 viruses exhibit normally low virulence in poultry. The IAV virion with the HA and NA glycoprotein spikes is illustrated in Figure 4.

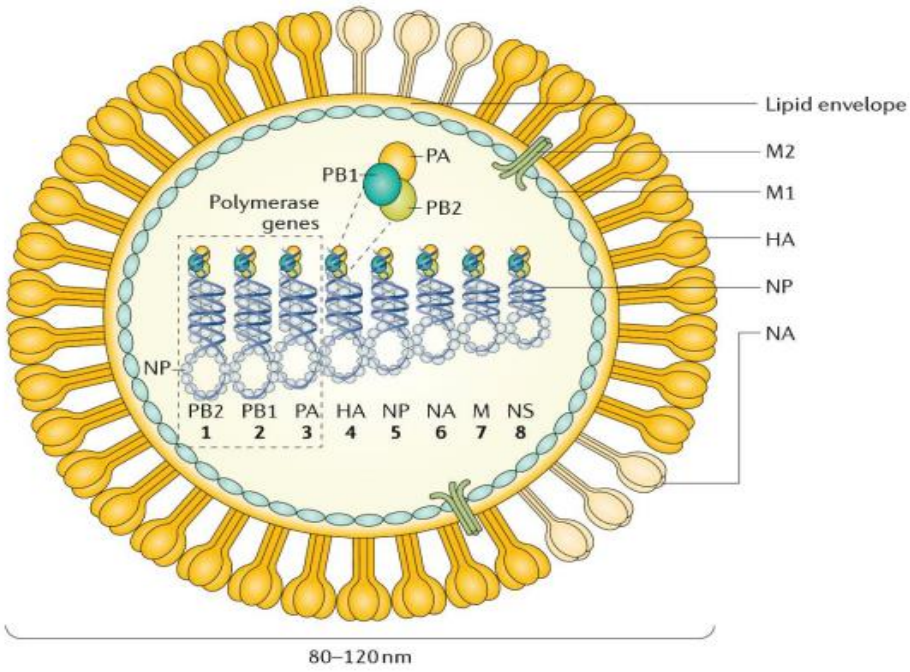

**Figure 4**: Structure of the IAV virus particle [12]. (Source and permission for reproduction was obtained from the Copyright Clearance Center's RightsLink® service).

#### 2.1.2.3 Peste des petits ruminants virus (PPRV)

The Peste des petits ruminants virus (PPRV) (species name: Small Ruminant Morbillivirus (SRMV)) is one of the most widespread diseases of small ruminants. It is highly contagious and has a high death rate [54, 55]. Goats are more affected than sheep. The PPRV causes excessive body temperature accompanied with a lethargy in general condition. The disease is distinguished by oculo-nasal discharges, erosive lesions of the nasal and oral mucous membranes, and respiratory abnormalities in conjunction with gastrointestinal difficulties [54]. PPRV belongs to the genus *Morbillivirus* that is a member of the *Paramyxoviridae* family [14]. The genome encodes six structural (nucleocapsid (N), phosphoprotein (P), matrix (M), fusion (F), hemagglutinin-neuraminidase (HN), large (L)) and two non-structural proteins (C and V) are identified [56]. The genomic RNA of PPRV is enveloped by the N protein, which is the most predominant viral protein produced among all PPRV genes [57, 58]. P and L proteins are the main elements of the viral RNA-dependent RNA polymerase, which is responsible for viral replication and transcription [57, 58]. The effective replication of the virus genome occurs after the "rule of six", which signifies that the RNA-dependent RNA polymerase will only function properly if the total number of nucleotides states a multiple of six nucleotides (6 n + 0) [59]. The schematic virion and genomic structure of PPRV are illustrated in Figure 5.

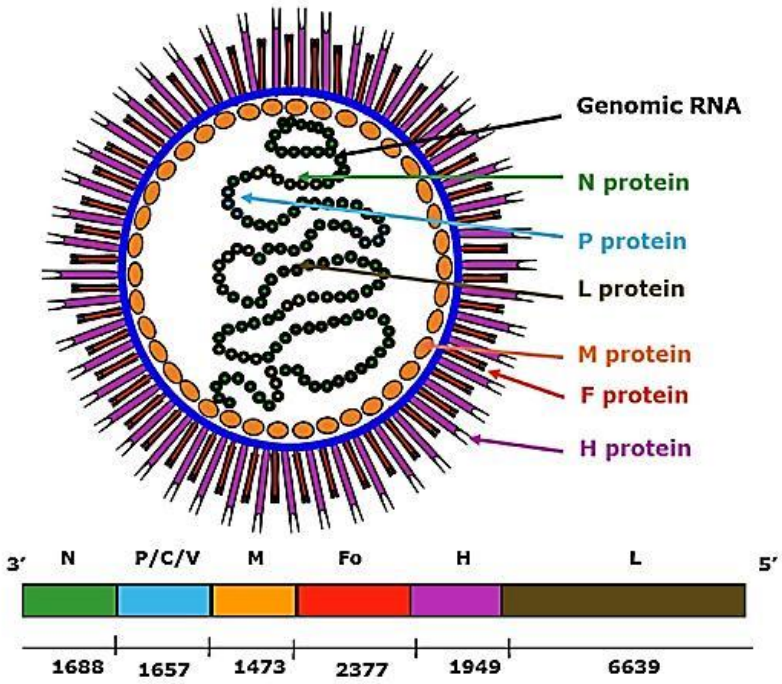


**Figure 5**: Morphological representation of the PPR morbillivirus (courtesy of Djeneba Keita, Source: Albina et al; 2013 [60]).

#### 2.1.2.4 Bluetongue virus (BTV)

BT is a non-contagious viral disease of domestic and wild ruminants, induced by the BTV [15]. Owing to its socioeconomic effect, BT is a WOAH-OIE classified multispecies disease [61, 62]. BTV infection results in significant economic losses because of the associated morbidity, mortality, abortion, and lethal defects. Indirect expenses are incurred as a result of trade limitations placed on natural ruminant circulation and animal product exports, diagnostics, immunization, vector control, and treatment of diseased animals [62-64]. BTV is a double-stranded RNA virus (dsRNA) of the family *Reoviridae* and the genus *Orbivirus* [65]. Based on 9 to 12 linear double stranded (ds) RNA segments, the currently 15 genera were designed [66]. Overall, 10 segments that are coding for seven structural (VP1-VP2) and six non-structural proteins (NS1-NS5, NS3a) [67-69]. The morphology of BTV is depicted in Figure 6.

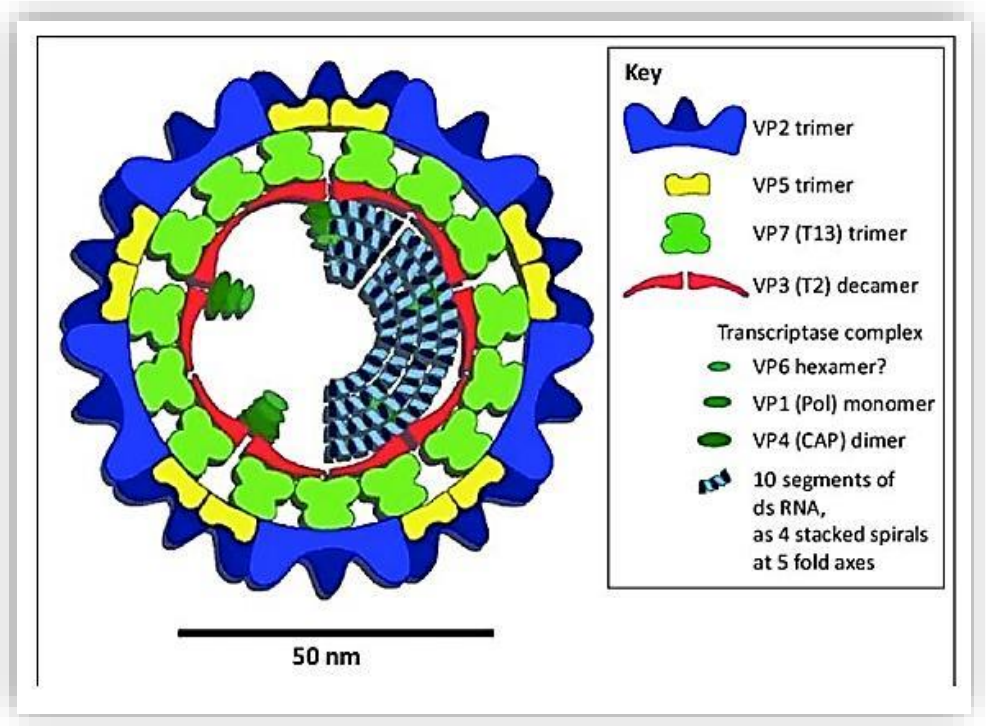


**Figure 6**: Overview of the BTV virus structure (Source: Mertens et al., 2004 [70]).

## 2.2. Laboratory molecular diagnostic methods

Laboratory diagnostics have been crucial for the rapid control and/or elimination of disease. Because of the accompanying socioeconomic and health effects, it is vital to respond rapidly and efficiently, not just during but between epidemics (i.e., increased preparedness). The usual surveillance strategy for emerging diseases is based on a combination of passive (clinical) detection and active sampling. However, conventional passive monitoring based on a small number of samples is restricted by diagnostic sensitivity, accuracy, visibility, and specificity of clinical symptoms. Active surveillance based on sampling is expensive and time demanding [65]. Hence, differential diagnosis based on laboratory analysis for a variety of diseases might increase pathogen identification, control, and extermination [66]. Well-trained laboratories with appropriate capability and high-quality analytical tools and resources are crucial for quick disease diagnosis, control, and/or elimination [67, 68]. However, also the WOAH observed that rapid diagnosis of novel diseases could be enhanced in the underdeveloped countries and even in some

industrialized ones due to a lack of infrastructure, laboratory capacity, and veterinary expertise [69].

National and international organizations, animal health authorities, scientific institutions, diagnostic laboratories, and field personnel are intensively working toward preventing and controlling TADs. Early warning systems (with rapid, sensitive and highly specific methods of detection) are critical to prevent the spread of disease among animal populations over wide regional areas. Therefore, the development of innovative robust diagnostic tests ("fit-for-purpose") is an important topic in modern veterinary research and animal healthcare. Especially molecular virology provides a variety of innovative approaches to improve the detection of viral diseases. Novel tests enable very rapid, sensitive and specific diagnosis within hours or even minutes. Virus detection can be conducted directly or indirectly. For the direct detection of ASFV, LSDV, IAV, PPRV, and BTV, various molecular techniques can be used, including conventional gel-based PCR, qPCR, multiplex PCR, and enhanced in situ hybridization in addition to classical methods including virus isolation (VI), and antigen detection by enzyme-linked immunosorbent assays (ELISAs). The most common methods for indirect detection are approaches for antibody detection, such as ELISA systems [71] [72] [73].

### 2.2.1 Identification of ASFV

For ASFV identification, there is a variety of laboratory diagnostic methods available [74], including VI, ELISA, immunofluorescence-based assays, PCR, qPCR and isothermal assays. VI can be performed in primary leukocyte cultures or bone marrow cells. First passage takes 7 days (in the clearly positive case ~2 d), and the second passage needs another 7 days. Virus replication in these cells can be identified by the haemadsorption test (HAD) [74]. Positive HAD results are definitive for ASF diagnosis, while negative HAD samples should be tested by PCR to avoid false negative results. Antigen detection can be performed using a fluorescent antibody test (FAT). A positive FAT result with clinical symptoms, including lesions, provides a preliminary ASF diagnosis [75]. Furthermore, ELISA can detect viral antigens but is only useful for acute forms of the disease and is not considered to be as sensitive as qPCR [76].

Two indirect sandwich ELISAs have been developed, one using a polyclonal serum and the second one combines monoclonal and polyclonal sera. Both techniques identified antigens from a variety of field isolates, although the polyclonal antiserum-based assay is marginally more sensitive than the monoclonal antibody-based ELISA [77]. The indirect fluorescent antibody test (IFAT) can be conducted to verify ASFV diagnosis [78, 79]. Immunoblotting tests can be applied as an alternative to the IFA test to corroborate the results with individualized sera [80, 81], along with the classical antibody detection ELISA, based on different purified or recombinant antigens [82, 83]. qPCR is considered the 'gold standard' in the direct detection of ASFV, and is established in all laboratories performing ASFV diagnostics [84-89]. qPCR provides sensitive, specific, and rapid detection and amplification of the ASFV genome and is recommended, especially in a major outbreak of ASF [90]. qPCR can also be applied when the material provided is inappropriate for virus isolation and antigen detection due to decomposition. Using qPCR, ASFV can be detected in tissues, blood, and, to a lesser extent, serum samples from an early stage of infection [91]. Nucleic acid purification or isolation is important for the identification of ASFV using qPCR. Depending on the infrastructure available at a laboratory, different extraction technologies can be used and applied in different platforms. So far, there are two major methods for DNA extraction using either silica membrane-based or magnetic bead-based extraction techniques [92] [93].

### 2.2.2 Identification of LSDV

The LSDV genome can be directly detected by PCR, which is comparatively inexpensive and considered the quickest method for detection. Various PCR methods were validated for the simultaneous detection of all three capripox virus species [94]. Virus isolation in cell culture or embryonated chicken eggs can be complemented with PCR to identify the virus strain [94-96]. Different antigen capture ELISA kits are suitable for LSDV diagnosis, but with limited sensitivity [97, 98]. LSDV can be also identified by electron microscopy [99-101], though without differentiating the genus or species. Another useful time-saving method in preliminary field investigation is the use of immunofluorescence or immunoperoxidase tests [102]. The virus neutralization test (VNT) is the main standard method for antibody detection against poxviruses (LSDV) [103, 104]. It is recommended by WOAH and is also known as "serum neutralization test" (SNT) (OIE, 2017). ELISA-based assays can be

performed with various surface coating antigens [105, 106] and the IFAT [107]. Western blotting is another antibody diagnostic test, which is highly sensitive but expensive and more difficult to implement [108, 109].

### 2.2.3 Identification of IAV

Virus isolation in embryonated chicken eggs or in a variety of cell lines e.g., DF1, MDCK, A549 is the gold standard for the diagnosis of IAV in poultry, pigs and humans. VI is important to obtain viable viruses for further pathotyping and in vitro characterisation. Nevertheless, VI in eggs or cell culture is tedious and time consuming. Moreover, isolation of HPAIV requires biosafety level 3 (BSL3) facilities which are available in fewer laboratories only. Pathotyping of AIV is usually done by intravenous injection of chickens to determine the intravenous pathogenicity index (IVPI) during a 10-day-observation period. LPAIV have an IVPI of less than 1.2 while HPAIV show an IVPI > 1.2. RT-qPCR is more sensitive and faster than VI. Genome detection by RT-qPCR can also distinguish subtypes by using HA- and NA-specific primers or by sequence analysis of HA and NA genes [110-112]. Furthermore, RT-qPCR assays have been developed to simultaneously detect and differentiate between LPAIV and HPAIV within a few hours [113] [114]. There is a monobasic $HA_0$ cleavage site of LPAIV which permits cleavage into the subunits $HA_1$ and $HA_2$ by trypsin-like proteases located in the respiratory and intestinal tract. On the contrary, HPAIV carry a polybasic $HA_0$ cleavage site cleaved by ubiquitously cellular proteases [115]. Thus, the differentiation can be achieved depending on the pathogenicity and the HA endoproteolytic cleavage site (HACS) [116]. Moreover, Agar gel immunodiffusion (AGID) is a diagnostic test to detect the matrix antigens of the influenza A virus in amnioallantoic fluid as well as to detect antibodies [117], which could also be detected by ELISA assays [118]. Due to the antigenic similarities between Influenza A viruses (nucleoprotein and matrix antigens), AGID tests can be utilized to determine antibodies to these antigens [117, 119]. ELISA-based assays have been deployed to detect IAV-specific and subtype-specific antibodies to Influenza A virus type-specific antigens in either species-dependent or -independent competitive tests [120-124]. Haemagglutinin (HA) inhibition tests have been conducted in serological diagnosis, however the HA inhibition assay is subtype specific [125-127].

### 2.2.4 Identification of PPRV

PPRV genome detection can be performed by several molecular techniques, such as RT-qPCR, RT loop-mediated isothermal amplification (RT-LAMP), and RT recombinase polymerase amplification (RT-RPA) [128, 129]. However, RT-qPCR is preferred due to its high sensitivity and specificity and because it can detect all four lineages of the virus; numerous assays have been established for genome detection [130-134]. Indeed, PCR is 1,000 times more sensitive than classical virus isolation methods (OIE, 2009). For the indirect detection of the PPRV, one can use a VNT, which is sensitive but time-consuming [135, 136], or competitive ELISA-based assays, which are based on PPRV-specific monoclonal antibodies (MAbs) [137, 138].

### 2.2.5 Identification of BTV

For BTV detection, virus isolation can be performed in embryonated chicken eggs and cell cultures. For virus isolation in cell cultures, different mammalian and insect derived cell lines have been established (OIE, Terrestial Manual. P. Chapter 3.1.3 Bluetongue) [139]. For genome detection, TaqMan-based RT-qPCR has become the most popular technique [140]. several RT-qPCR protocols have been validated targeting various BTV genome segments (e.g. 1, 5, or 10). Partial or whole genome sequencing are widely applied for detecting BTV serotype/strain [141]. For the indirect detection of BTV, different ELISA systems (Blocking or double Ag ELISA) were developed to detect the BTV antibodies as a standard method. The AGID test can be used, which is simple to carry out and the antigen is relatively simple to generate, however it lacks specificity for other Orbiviruses, however the Virus Neutralisation Test (VNT) is recommended for antibody detection for serotyping purposes [142-144].

In general, some of the presented diagnostic methods show limitations in terms of sensitivity, specificity and/or throughput. Classical virological methods are only conditionally suitable for the very simple and rapid detection of specific pathogens in a resource-limited laboratory or in the field. Due to the high sensitivity and specificity of molecular detection methods on the one hand and the universal application of real-time PCR technology on the other hand, the main focus of the presented work was on the simplification and acceleration

of nucleic acid extraction and subsequent genome detection by real-time PCR. Of course, the simplifications presented here, especially in the rapid extraction of viral nucleic acid, are also applicable to alternative molecular procedures such as isothermal amplification or next-generation sequencing.

## 2.3 Viral nucleic acid detection using qPCR

### 2.3.1 Nucleic acid extraction and release

To optimize PCR amplification, DNA/RNA extraction is a crucial first step in the diagnostic process [92]. Nucleic acid isolation requires a high labour input, technical facilities, and trained personnel to perform the extraction and avoid the most common causes of contamination, while the procedure delivers limited throughput depending on the particular method and shows variability in the efficiency of extraction [145]. DNA can be extracted from clinical (e.g., fine-needle aspirates of body secretions and tissue specimens) and analytical samples (e.g., dried bloodstains, swabs, fingerprints, soil, and tissues). Furthermore, nucleic acids can be extracted from cell cultures, insects, protozoa, bacteria, and yeast [146]. Successful nucleic acid purification requires effective distribution of cells after its lysis, inactivation of nucleases (DNase for DNA extraction and RNase for RNA extraction), removal of inhibitory substances and sterile conditions [147]. The purity and sensitivity of the isolated nucleic acids will directly influence the results. There are different extraction systems e.g., viral nucleic acid isolation via Trizol buffer [148, 149], however the following two different nucleic acid extraction systems have become established as a standard in the diagnostic routine.

#### 2.3.1.1 Manual extraction methods

The manual silica membrane column-based extraction methods offer several commercial options, including complete kits containing most of the necessary components. It is laborious and requires repeated centrifugation followed by removal of the supernatant depending on the type of sample and additional mechanical treatment [92]. The column-based method is a

simple and safe method, and is readily available in kit form. In this approach, the binding of nucleic acids is optimized by specific buffer solutions and relatively regulated pH and salt concentrations [150]. Centrifugal force is used to pass the sample lysates through the silica membrane, where the RNA attaches to the silica membrane at the correct pH. The surface, which contains proteins and salt residues, is then washed to eliminate contaminants and the flow-through is removed. RNase-free water is then used to elute the nucleic acids (DNA/RNA) [92]. Column-based DNA/RNA extraction is one of the best available methods that offers a stable stationary phase for quick and accurate buffer exchange and thus nucleic acid isolation. The main drawback of this technique is that it requires a tiny centrifuge. Vacuum-based techniques can also be conducted instead of centrifugation to isolate impurities [150]. To achieve higher nucleic acid yields in less time, researchers can integrate an extraction with the spin column technique. This approach is appropriate for large-scale high-throughput processing, including automated systems. Incomplete lysis can also contribute to low viral RNA yields [151]. The principle of the silica membrane column-based extraction method is illustrated in Figure 7.

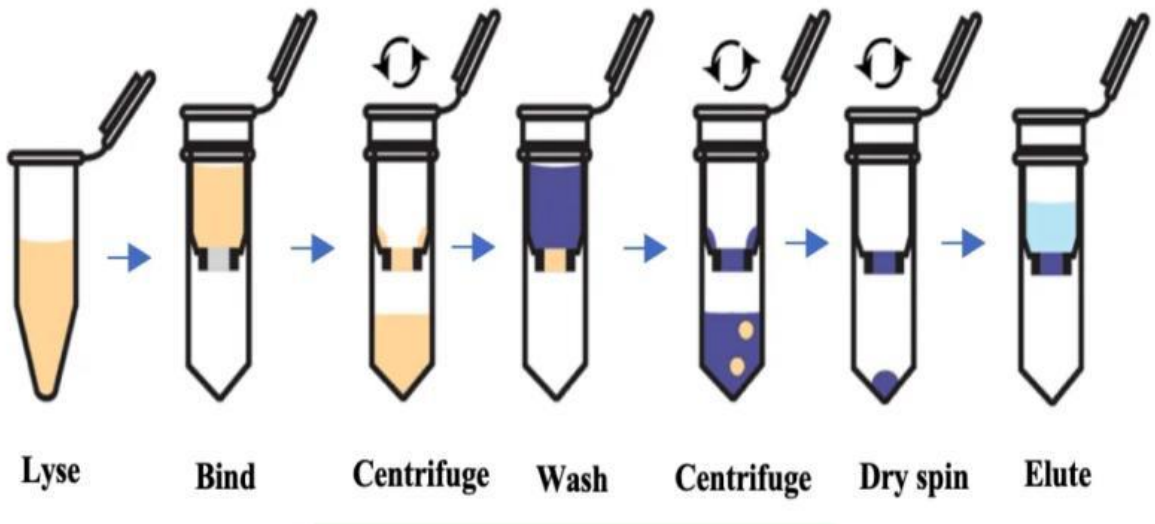


**Figure 7**: Overview of the silica membrane column-based extraction method (Source [and permission granted by]: Kbdna.com).

In our first study, we used a silica membrane-based extraction kit compared to alternative methods for DNA isolation of the ASFV genome by manual extraction from cell-free and cell-containing specimens [89, 152].

#### 2.3.1.2 Automated extraction methods

Automated tools for medium to large laboratories have become increasingly popular in recent years as they provide an alternative to time-consuming manual procedures. These technologies allow high sample throughput while limiting cross-contaminations, which would maximize nucleic acid yield, purity, repeatability, and scalability as well as assay speed, precision, and reliability [92, 153-156]. There are a growing number of automated extraction systems on the market, and their use in various pathogen detection tests has been described [93, 157-161]. Most of the automated extraction systems are based on the magnetic bead-based system. For nucleic acid binding, the beads have a paramagnetic base that is typically coated with silica. The sample is homogenized in a buffer containing RNase inhibitors before being handled with the magnetic beads, which enable the particles to attach to RNA molecules. When the magnetic beads are placed near an external magnetic field, they can be quickly collected. The supernatant is gathered, and the beads are rinsed in a suitable buffer while the magnetic field is removed. This procedure is simply replicated for several washes. The DNA/RNA is eluted from the magnetic beads into solution using DNase/RNase-free water, and the supernatant (containing the purified nucleic acid) is then transferred separately to (sterile) tubes [150, 162]. The methods for collecting magnetic beads are simple and fast. Since no membrane is involved, there is less risk of clogging. This approach is well suited for scaling, high throughput separation, and automation. Because of the movement of the beads, purification is effective. However, thick samples may restrict bead movement, and the final sample may occasionally be contaminated with magnetic beads [163]. The procedure of the magnetic bead-based extraction system is detailed in Figure 8.

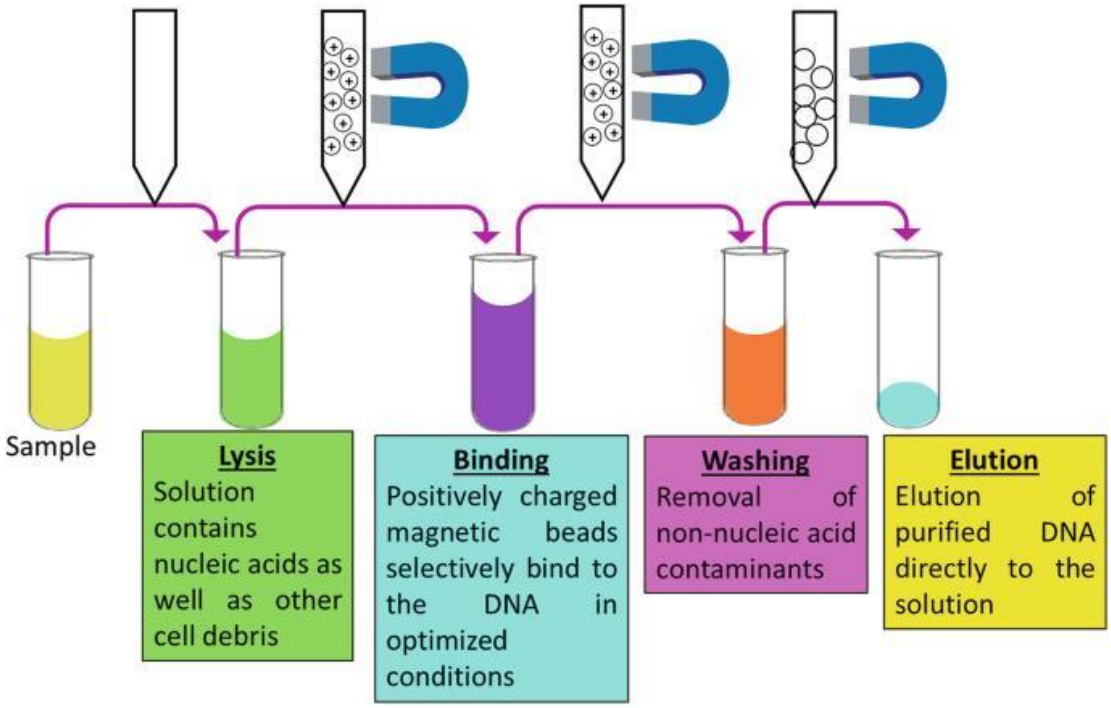


**Figure 8**: Overview of the magnetic bead-based extraction system (Source [and permission granted by]: Kbdna.com).

Different magnetic bead-based extraction kits and automated extraction instruments were validated for ASFV genome detection in our first study. The availability of ready-to-use or prefilled reagents for nucleic acid isolation could facilitate the extraction procedure in the diagnostic process, save time, and avoid all possible forms of contamination [152]. A main concern in the application of automated instrumentation to isolate nucleic acids for use in amplification assays is the potential for cross contamination or malfunctioning robotics. Other important practical considerations include volume, throughput, flexibility, and costs.

For further improvement of the diagnostic method for the field application, a rapid and sensitive magnetic bead-based hand extraction method is required that can compete with standard automated laboratory-based extraction methods. The so-called “Easy Express Extraction system ‘Triple*E*’” - a rapid, reliable, portable, and cost-effective nucleic acid extraction technique, that does not require advanced technical skills or electricity, is such a method. This portable device is reliable and can be used in the field as part of rapid molecular diagnostics. Because it is easily adaptable to a wide range of downstream

molecular targets, it can be used both inside and outside the laboratory. It is a simple device for nucleic acid extraction that does not require centrifugation or electricity, which is a significant advantage over existing commercial extraction methods [164]. It has the ability to isolate manually up to eight sample matrices in one extraction cycle within 10 min. This method was validated for the extraction of both viral DNA and RNA from different pathogens in our third study [165] and combined with direct qPCR amplification using thee different portable systems for the detection of ASFV in our fourth study [166].

Direct qPCR amplification without nucleic acid extraction was conducted by the dilution of the original ASFV sample (1:40) in RNase-free water and subsequent PCR testing. Several qPCR assays have been described for the detection of ASFV [84, 85, 87, 167, 168]. Moreover, various studies have validated the application of sensitive, high-speed, and portable real-time PCR systems, which can be operated in the field for a rapid detection of ASFV [169-171]. Battery-powered portable PCR instruments can be playing an important role in field diagnostics, especially in remote areas where electricity is not available. They can also provide well-defined data analysis to various users.

#### 2.3.1.3 Release of nucleic acid using different buffers

Release of nucleic acids via specific buffers could simplify DNA/RNA isolation. Different buffers have been used for improving the release of nucleic acids, such as Chelex® Resin 100, Tris-EDTA (TE) buffer, and virotype tissue lysis reagent (TLR). These buffers were validated for the rapid preparation of various sample types without the necessity for an extraction kit or any complicated nucleic acid isolation procedures. Moreover, these buffers can be utilized with Flinders Technology Associates (FTA) cards or filter papers [172], which can be used for the transport and storage of biological sample materials [173]. These buffers were validated for the detection of different infectious diseases, e.g. TE buffer for different diseases (e.g. avian Influenza [174], Newcastle disease [175], FMD [176], and rabies virus [177]). Chelex® Resin 100 was e.g. used for improving the diagnostics of COVID-19 genome detection [178]. In our first and second study, TLR was used and validated for ASFV genome detection [152], which followed a successful application of TLR for the release of the viral RNA of bovine viral diarrhoea virus (BVDV) [179]. TE, TLR, and Chelex® Resin 100 could be implemented in the detection of ASFV and IAV [180]. This validation of releasing buffers has been conducted in

combination with direct qPCR amplification, which we propose as a suitable alternative for nucleic acid extraction.

FTA cards are an important technology for field applications. Whatman FTA® filter paper cards consist of a chemically treated cellulose membrane that lyses cells, nuclei, and organelles from a variety of sources (e.g., blood, saliva, and plant tissue). They are commercially available in a variety of configurations to meet application requirements and custom specifications. FTA cards are impregnated with chaotropic chemicals that also inactivate infectious agents and limit the biological hazard potential of the sample during processing. In this way, biological material on the FTA cards can be stored at room temperature for long durations. Since no refrigerators or freezers are required, storage costs are significantly reduced [181]. Specimens stored on FTA cards could be delivered by regular mail without any special handling restrictions, making them a convenient tool for collecting and preserving field specimens [173]. FTA cards are used in the veterinary field as an alternative method for collection, transport, and temporary storage of specimens for molecular diagnostics and have been used for many diseases, including avian influenza [174], Newcastle disease [175], porcine reproductive and respiratory syndrome [182], foot-and-mouth disease [176], rabies [177], and ASFV [183]. The release of DNA and RNA via FTA cards using different releasing buffers has allowed direct qPCR amplification without extraction procedures, which we also validated in our second study [180].

### 2.3.2 Real-time PCR (qPCR)

qPCR is based on Kary Mullis' innovative PCR method, which permits researchers to amplify specific DNA segments by more than a billion-fold [184, 185]. Quantitative real-time PCR is the accurate detection and quantification of the products created during each cycle of PCR, which are directly related to the amount of template utilized. The thermostable *Thermus aquaticus* (Taq) DNA polymerase has been exhibited to have 5'–3' exonuclease activity. Splitting of a target probe during PCR by the 5'-nuclease function of Taq polymerase can be performed to detect amplification of the target-specific product [186]. PCR-based technologies have advanced molecular biology by making it easier for researchers to alter

DNA, facilitating both simple processes, such as cloning, and large-scale analyses, such as the Human Genome Project [187, 188]. This is in part because of the tremendous sensitivity of PCR being paired with the accuracy provided by real-time monitoring of PCR products as they are produced [189]. A Roche molecular system was developed to conduct the first real-time PCR by Higuchi et al. [190, 191]. They were able to observe and record the assembly of DNA with a video camera by incorporating the typical fluorescent dye ethidium bromide (EtBr) into the PCR and performing the reaction under UV light. Since 1966, EtBr has been known to increase its fluorescence when binding nucleic acids [192], but qPCR was not developed until the early 1990s by integrating this fluorescent chemistry with PCR and real-time data. As a result, this technology rapidly developed into a competitive alternative and gained both diagnostic and scientific importance [189]. This technique has allowed the automation of molecular diagnostics with high throughput and shorter turnaround times [193]. Probe-based qPCR assays provide revolutionary methods for rapid detection of viruses in diagnostic facilities. Today, several qPCR methods are in use, including TaqMan, Molecular Beacons (MB) and dual probe systems, such as LightCycler®, dye-labelled oligonucleotide ligation (DOL), and the primer–probe energy transfer system (PriProET).

The qPCR assay contains an oligonucleotide probe intended to hybridize inside the target sequence. This probe is labelled at the 5’ end and is not extendable at the 3’ end to prevent it from acting as a primer. During amplification, annealing of the probe to one of the PCR product strands produces a target appropriate for exonuclease activity. In addition, the 5’-to-3’ exonuclease activity of Taq DNA polymerase (when the enzyme extends from an upstream primer into the region of the probe) disassembles the probe into smaller pieces that could be distinguished from the undegraded probe. The development of double-labelled fluorogenic oligonucleotide probes has suppressed the need for post-PCR processing for probe degradation analysis [194]. A reporter fluorescent dye is bound to the 5’ end of the probe and a quencher dye is attached to the 3’ end. The close proximity of the quencher dye to the probe significantly reduces the fluorescence generated by the reporter dye while the probe is intact [195]. Several real-time fluorescent PCR chemistries are available, but the most common used are 5´-nuclease (TaqMan®) and SYBR® Green dye-based assays.

The 5´-nuclease assay is termed for the 5´-nuclease activity of the TaqMan DNA polymerase. The 5´-nuclease domain can cleave DNA bound to the template after it has been synthesized. Fluorescence resonance energy transfer (FRET) is a second essential component of the 5´-nuclease assay. The TaqMan® probe has a gene-specific sequence and is intended to attach to the target gene between the two PCR primers. Bound to the 5´-end of the TaqMan® probe is the 'reporter', a fluorescent dye that reports amplification of the target. At the 3´ end of the probe is a quencher, which quenches the fluorescence of the reporter in intact probes. The principles of real-time PCR using TaqMan assay are illustrated in (Figure 9).

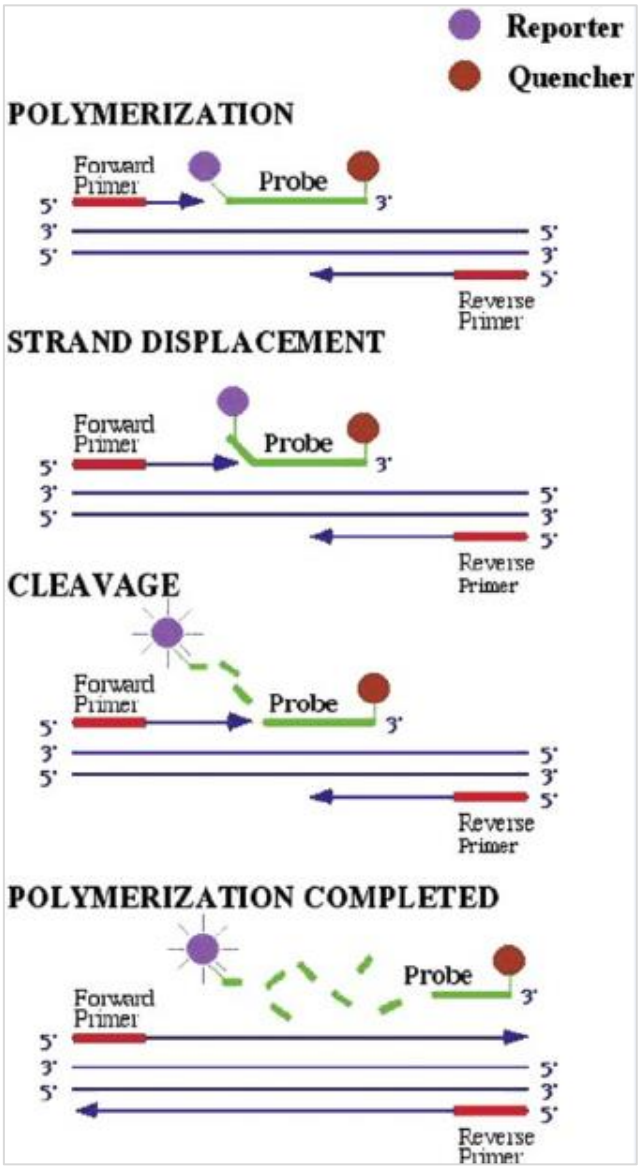


**Figure 9:** The principles of real-time PCR using TaqMan chemistry, (Source: Schaad et al., 2003 [196]).

The observed enhancement of fluorescence during qPCR is linearly related to the rise in the concentration of the amplifying product and thus relies on the initial amount of the target sequence [191]. For quantification, the amplification cycle in which the fluorescent signal initially crosses the background signal is referred to as the quantification cycle (Cq) [197]. The Cq-value is used to calculate the initial DNA copy number, because the Cq-value is related to the starting amount of the target [198]. After analysing a serial dilution of a known sample, e.g., a plasmid carrying the sequence of the complete amplicon or a synthetic single-stranded positive sensor oligonucleotide, a standard curve is generated by analysing the Cq-value against the logarithm of the original copy number. The copy number of the unknown sample of the sequence of interest is determined by linear regression of the standard curves [193, 199]. For time-course studies, for example, untreated samples or samples from the initial time point are utilized as reference controls. To generate valid results, the amplification efficiencies of the housekeeping gene and the target sequences must be relatively equivalent [193, 200].

Duplex PCR and qPCR have a wide range of applications. Co-amplification of an internal control (IC) and pathogen-specific target is a common application of duplex PCR, i.e. amplification of two targets. The possibility of amplifying different targets (i.e. multiplex qPCR) with modern real-time PCR machines using different fluorophores could be also realised. The use of ICs is crucial for verifying effective nucleic acid extraction and ensuring the absence of PCR-inhibitors, especially when studying potentially problematic biological matrices [201, 202].

Compared to ‘classical’ single or nested PCR methods, the diagnostic application of qPCR assays has several advantages, including: (1) faster and higher throughput; (2) no post-treatment of PCR products; (3) a non-nested qPCR design provides sensitivity comparable to conventional nested PCR; (4) amplified products are detected by fluorescence in the reaction tube without the need to open the system, reducing the risk of contamination and allowing a higher specificity; (5) qPCR assays provide a quantitative estimate, not only a qualitative result; (6) qPCR is more accurate and less labour intensive than alternative quantitative PCR

techniques; (7) compared to classical detection in agarose gels followed by EtBr staining, the hands-on time is much shorter; (8) qPCR enables automation and can be mechanized by using robots for DNA/RNA extraction and pipetting; and (9) qPCR probes can be labelled with a variety of fluorophores that serve as separate reporter dyes for different primer sets, thereby enabling multiplex PCR analysis (10) and reducing diagnostic costs. The advantages of real-time diagnostic PCR assays have been summarized in various reviews [201, 203-208].

## 2.4. Point-of-care (POC) testing and field application

Accurate and timely detection of infectious diseases remains a challenge in the field, especially during epidemics and in developing countries. Long travel distances and unreliable logistics for specimen transport (e.g., poorly maintained roads and vehicles, fuel shortages, inadequate courier networks, seasonally inaccessible roads), difficulties in maintaining the cold chain, inadequately equipped laboratories, lack of qualified personnel, and excessively high operating costs all contribute to long turnaround times between specimen collection, laboratory diagnosis, and further medical treatment. Point-of-care tests (POCTs) are "*a fully or partially automated benchtop, portable, or disposable device that can be operated in a nonlaboratory setting by nontechnical personnel to provide a clinically relevant diagnostic test result on the same day in the field*" [209]. POCTs, also referred to as 'rapid diagnostic tests', 'point-of-need tests', 'near-patient tests', or "pen-site tests" are available in a variety of forms and are used in clinical, veterinary, and botanical industries worldwide. They are designed to be portable and user-friendly with a minimal turnaround time from sample to result, allowing diagnosis and management decisions to be made during the same visit. POCTs could also be efficiently deployed at border crossings, airports, and other border entry points where rapid detection of diseased animals or animal products is critical. Although centralized clinical laboratories offer sensitive and specific tests, such as blood cultures, high-throughput immunoassays, PCR, and mass spectrometry (MS), they are often time and labour intensive, expensive, and dependent on sophisticated instrumentation and well-trained personnel. POC diagnostics, on the other hand, provide immediate results at resource-limited settings, enabling rapid and accurate treatment [210]**.** According to the WHO, POC testing for infectious disease control, especially in developing countries, should

meet the following 'ASSURED' criteria: (1) affordability, (2) sensitivity, (3) specificity, (4) ease of use, (5) speed and robustness, (6) device-free application, and (7) handover to the end user [211]. Numerous POCT platforms and formats exist, ranging from paper-based lateral flow assays (LFAs) and portable nucleic acid detection systems (e.g., loop-mediated isothermal assays, recombinase polymerase assays, portable and/or isothermal PCR devices) to portable nanopore sequencers, wearable electronic sensors, and 'smart' textiles [212-216]. Some POCTs detect a single analyte or pathogen, while others allow multiplexing to test two or more targets; some are single-use disposable cartridges or cassettes, while others provide a portable multipurpose platform. POCTs can be used for a variety of clinical applications, including screening, diagnosis, monitoring, prognosis, and surveillance (WHO, 2019a). Many POCTs use a cellular network, Wi-Fi, and/or Bluetooth to transfer data between remote field sites and central databases [213, 217].

There are several POC assays available for different viral pathogens, including LFAs, recombinant polymerase amplification (RPA), and loop-mediated isothermal amplification (LAMP). There are various types of isothermal nucleic acid amplification methods, such as LAMP of DNA, transcription-mediated amplification (TMA), single-mediated amplification of RNA technology (SMART), nucleic acid sequence-based amplification (NASBA), strand displacement amplification (SDA), isothermal multiple displacement amplification (IMDA), and helicase dependent amplification (HDA). These are the leading methods in detecting and analysing a small quantity of nucleic acids [218]. Isothermal technologies are useful in the laboratory diagnosis of ASF and complement existing molecular approaches to provide rapid differential diagnosis of suspected swine fever. Unlike other molecular formats, such as PCR, isothermal experiments can be performed, eliminating the need for expensive thermocycling [219]. However, isothermal amplification technologies have some limitations: Some methods are inefficient at amplifying long target sequences and other methods, such as LAMP, need four to six specific primers, which complicate the experimental procedure and require more sensitivity [220]. Therefore, it would be advantageous to use the qPCR technology combined with a rapid manual extraction method, which can offer high-speed amplification and simultaneous detection of multiple target sequences within a single reaction.

## 2.5 Advantages and disadvantages of different diagnostic systems for field application

POC technologies offer many advantages: they are portable, self-contained, and either instrument-free or battery-powered. The thermostable and lyophilized reagents do not require cold chains or reconstitution. Overall diagnostic process time is reduced compared to laboratory-based methods. They require minimal training and only brief protocols for preparation or extraction prior to sample testing [221, 222]. However, there are also some limitations, such as cost-effectiveness, which could be an important deterrent to adopting POC testing in resource-limited or low/middle-income populations. Low demand for POC technologies could result in unreasonable market prices, not even considering the additional costs of personnel, equipment, storage, and transportation of reagents to the field site [223]. Due to the short analysis time, POCT usually have a lower sensitivity but often a high specificity. Thus, a positive result can be considered very safe, a negative result should be interpreted with caution and may need to be confirmed again by further laboratory testing. Therefore, it should be kept in mind that POC testing is rarely intended to completely replace standard laboratory testing, as samples usually still require laboratory testing for confirmation and genetic characterization of pathogens, especially in outbreaks of notifiable diseases.

All diagnostic methods used in this dissertation are illustrated with the dis/advantages in Table 2.

**Table 2** An overview about the diagnostic methods used in this work.

| Nucleic acid extraction/release | Viruses | Sample no./time (minutes) per run | Advantages | Disadvantages |
|---|---|---|---|---|
| Silica membrane column-based extraction system (e.g., QIAamp Viral RNA | *ASFV* | *12/30* | Reliable and suitable method in most regional | Long time in high-throughputs with possible manual |

| | | | | |
|---|---|---|---|---|
| Mini Kit) | | | laboratories | contamination risks |
| Magnetic bead-based automated extraction system (e.g., IndiMag Pathogen Kit on the IndiMag 48 instrument) | *ASFV, IAV, LSDV, PPRV and BTV* | *48/31* | Reliable, sensitive method, kit availability in prefilled form and use in high-throughputs | Might be expensive for some labs with limited resources |
| Magnetic bead-based automated extraction system (e.g., NucleoMagVet Kit on the KingFhisher Flex instrument) | *ASFV, IAV, LSDV, PPRV and BTV* | *96/20* | Reliable, sensitive method, kit availability in prefilled form and use in high-throughput | Might be expensive for some labs with limited resources |
| POC manual hand extraction (e.g., Triple*E*) | *ASFV, LSDV, PPRV and BTV* | *8/10* | Rapid, sensitive, electricity-free method, availability in prefilled form and application in the lab and field | Sample no. limitation and unavailability as a commercial kit |
| Release via TLR buffer | *ASFV* | *Up to 16/65* | Reliable, suitable method in most regional laboratories and no need for extraction kit | Long incubation time with high number of samples and samples heating is required for inactivation |
| Release via FTA cards using different buffers (e.g., Chelex 100, TE) | *ASFV and IAV* | *4/30-60* | Suitable for samples shipped from the field, transport and storage of biological materials | Long incubation time with high number of samples and not suitable for high-throughput analysis |
| | | | | |
| **Real-time (qPCR) amplification** | | | | |
| Lab-based systems | | | | |
| Standard laboratory-based PCR system (e.g., Bio-Rad | *ASFV, IAV,* | *96/76* | Reliable and sensitive standard | Cannot be applied in |

| | | | | |
|---|---|---|---|---|
| CFX 96) | *LSDV, PPRV and BTV* | | PCR system | the field |
| Direct qPCR amplification in the lab | *ASFV and IAV* | *96/76* | No need for nucleic acid extraction kit | Less sensitivity |
| POC-based systems | | | | |
| IndiField | *ASFV* | *9/54* | Rapid, sensitive PCR system, availability in lyophilised form and application in the field | Might be expensive in some remote areas |
| Liberty 16 | *ASFV* | *16/39* | Rapid, sensitive PCR system and application in the field | Might be expensive in some remote areas |
| UF-300 Genechecker | *ASFV* | *10/20* | Ultra-rapid, sensitive PCR system and application in the field | Might be expensive in some remote areas |
| Direct qPCR amplification in the field using different POC PCR machines | *ASFV* | *9-16/20-54* | No need for nucleic acid extraction kit and time-saving | Reduced sensitivity |

# 3. Objectives

## 3.1. Simplifying the molecular diagnostic tools for a sensitive detection of ASFV

PCR and qPCR are the standard methods recommended by the WOAH for direct detection of ASFV DNA. In this dissertation, we aimed at the development and validation of qPCR assays and the evaluation of sample matrices and the feasibility of different nucleic acid extraction methods to increase the sensitivity of detection of ASFV.

## 3.2. Optimization of released nucleic acids from FTA cards for the detection of ASFV and IAV

The Whatman FTA® filter paper cards facilitate the collection, transport, inactivation and temporary storage of biological samples. During animal disease outbreaks, safe and reliable transportation options are required between the field site and regional laboratory. Here, we analysed the efficacy of viral DNA (ASFV in EDTA blood) release versus RNA (IAV in allantoic fluid) release from seven manufacturers of FTA cards using seven different techniques and release methods.

## 3.3. Development of innovative molecular diagnostics in the lab and field for the detection of ASFV and other transboundary animal diseases

The complexity of current nucleic acid extraction methods limits their application outside modern laboratories. Therefore, a rapid and cost-effective approach (i.e., Triple*E*) for the purification of nucleic acids was developed that does not require a high level of technical expertise or effort. Validation data were obtained by testing two DNA (ASFV and LSDV) and two RNA viruses (PPRV and BTV). The Triple*E* system was validated and compared to standard extraction by an automated system (IndiMag 48) and subsequently compared to direct qPCR amplification as a rapid detection method in the field by the dilution of the original ASFV sample (1:40) in RNase-free water. Furthermore, a validation of the high-speed real-time PCR system was performed. In this study, four different qPCR systems and three portable PCR thermal cyclers were used to optimize the performance and sensitivity of the diagnostic tool in the field.

## 4. Results

The manuscripts are presented in the form they were accepted for publication. Each manuscript has its own reference section formatted in the style of the respective journal; references and abbreviations from the manuscripts are not included at the end of this document. Figures and tables are numbered individually within each of the manuscripts.

Results

4.1. Swift and reliable “easy lab” methods for the sensitive molecular detection of African swine fever virus

Publication I

**Swift and reliable “easy lab” methods for the sensitive molecular detection of African swine fever virus**

Ahmed Elnagar, Jutta Pikalo, Martin Beer, Sandra Blome and Bernd Hoffmann

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# Swift and Reliable "Easy Lab" Methods for the Sensitive Molecular Detection of African Swine Fever Virus

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**Abstract:** African swine fever (ASF) is a contagious viral hemorrhagic disease of domestic pigs and wild boars. The disease is notifiable to the World Organisation for Animal Health (OIE) and is responsible for high mortality and serious economic losses. PCR and real-time PCR (qPCR) are the OIE-recommended standard methods for the direct detection of African swine fever virus (ASFV) DNA. The aim of our work was the simplification and standardization of the molecular diagnostic workflow in the lab. For validation of this "easy lab" workflow, different sample materials from animal trials were collected and analyzed (EDTA blood, serum, oral swabs, chewing ropes, and tissue samples) to identify the optimal sample material for diagnostics in live animals. Based on our data, the EDTA blood samples or bloody tissue samples represent the best specimens for ASFV detection in the early and late phases of infection. The application of prefilled ready-to-use reagents for nucleic acid extraction or the use of a Tissue Lysis Reagent (TLR) delivers simple and reliable alternatives for the release of the ASFV nucleic acids. For the qPCR detection of ASFV, different published and commercial kits were compared. Here, a lyophilized commercial kit shows the best results mainly based on the increased template input. The good results of the "easy lab" strategy could be confirmed by the ASFV detection in field samples from wild boars collected from the 2020 ASFV outbreak in Germany. Appropriate internal control systems for extraction and PCR are key features of the "easy lab" concept and reduce the risk of false-negative and false-positive results. In addition, the use of easy-to-handle machines and software reduces training efforts and the misinterpretation of results. The PCR diagnostics based on the "easy lab" strategy can realize a high sensitivity and specificity comparable to the standard PCR methods and should be especially usable for labs with limited experiences and resources.

**Keywords:** African swine fever virus; DNA extraction; real-time PCR; easy lab



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## 1. Introduction

African swine fever (ASF) is an OIE (World Organisation for Animal Health)-listed and devastating disease of domestic pigs and wild boars caused by a complex DNA virus of the genus *Asfivirus* in the *Asfarviridae* family [1]. The length of the African swine fever virus (ASFV) genome varies from 170 to 190 kbp among different isolates, and the number of open reading frames (ORFs) ranges from 151 to 167 [2]. In Africa, argasid ticks of the genus *Ornithodoros* can transmit the virus [3], while outside Africa, transmission via direct contact is more prevalent. ASFV can deliver very high lethality (up to 100%) in susceptible Suidae and causes significant economic losses to the pig industry [4].

ASFV is currently endemic in large parts of sub-Saharan Africa and Sardinia [5]. In 2007, the virus emerged in Georgia, and then it spread to several countries in Europe and Asia. Here, the outbreak of ASF causes a large number of deaths among domestic pigs and wild boars [6]. The typical clinical signs of ASF are high fever, rapidly deteriorating general health, respiratory distress, and hemorrhage [7]. Currently, no vaccine is available, and surveillance strategies, strict outbreak response policies, and eradication programs are the only tools to prevent the further emergence and spread of ASFV.

The common laboratory diagnostic methods for the direct detection of ASFV include virus isolation (VI), hemadsorption test (HAD), and different molecular genetic techniques, such as loop-mediated isothermal amplification (LAMP), recombinase polymerase amplification (RPA), and polymerase chain reaction (PCR). Furthermore, antigen detection can be performed by the enzyme-linked immunosorbent assay (ELISA) or fluorescent antibody tests (IFTs). However, some methods are very laborious (virus isolation) or not sensitive enough for animals with low virus levels. Antigen detection can be impaired in the presence of antibodies [8].

Therefore, conventional and real-time PCR have been considered to be reliable methods for ASFV detection [7,9] and are recommended by the OIE. In addition, PCR has been shown to be an excellent and rapid technique that can be used as a routine diagnostic tool for ASFV in either surveillance, control, or eradication program [7,9–13].

The objective of this study was to evaluate and validate reliable and easy molecular diagnostic methods for the so-called "easy lab" concept. Therefore, prefilled and easy-to-handle DNA extraction/releasing procedures were combined with established standard PCR procedures for the detection of ASFV. Easy lab can be defined as the simplification and standardization of the molecular diagnostic workflow in the lab aimed at realizing a high sensitivity and specificity with maximal repeatability, reproducibility, and robustness. It should be applicable for users and labs with limited facilities and resources in molecular diagnostics.

Three key points were investigated in this study. First, we identified the best sample material for accurate diagnosis of ASFV in the "easy lab" setting based on different specimens originating from different animal experiments and field samples from wild boars during the 2020 ASFV outbreak in Germany. Second, we evaluated several extraction methods for DNA isolation by comparing standard methods with different manual and automated extraction systems and other alternatives for nucleic acid release without the need to use a commercial extraction kit. Third, we tested different commercial real-time PCR kits, assays, and thermocyclers for improving the speed, sensitivity, and specificity of ASFV detection. Based on the generated data, the identification of the optimal workflow for ASFV nucleic acid detection in differently equipped and experienced labs should be supported.

## 2. Results

### *2.1. Identification of the Best Sample Matrix for ASFV Detection*

Based on three different animal experiments (1, 6, and 7), a comparison of samples was undertaken to select the best sample with regard to different matrices (EDTA blood, serum, oral swabs, and chewing ropes) and different time phases after inoculation (initial and late). The data showed that EDTA blood could detect ASFV DNA in both phases of the infection. ASFV DNA could be detected also in other matrices, but with restrictions (Figure 1A,B). Serum samples delivered comparable results to EDTA blood, but only in the later stage of infection (Figure 1B and Table S1). In contrast, oral swab and chewing rope samples showed lower viral genome loads at all sampling dates; some even yielded negative results. Therefore, oral swabs and chewing ropes could be defined as inappropriate specimens (Tables S1 and S2). Of the bloody tissue samples, spleen showed the most reliable results with a comparative sensitivity to EDTA blood samples (Tables S1).

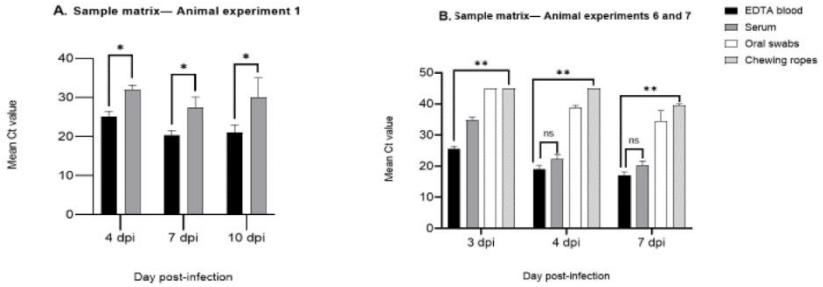


**Figure 1.** (**A**) Sample matrix comparison (EDTA blood and serum) from animal experiment 1. The mean Ct values based on five live domestic pigs (animal numbers 30, 31, 32, 35, and 37) inoculated with African swine fever virus (ASFV) Estonia 2014 at different time points, 4, 7, and 10 dpi (number of replicates = 7, Table S1) are shown. Standard deviation (SD) for EDTA blood (2.68) and serum (1.44). An unpaired *t*-test was performed for statistical analysis, and EDTA blood showed significantly lower Ct values among the different time points, 4, 7, and 10 dpi (* *p*-value < 0.01, number of replicates = 7, Table S1). (**B**) Sample matrix comparison (EDTA blood, serum, and oral swabs) and two animal pens (chewing ropes) from animal experiments 6 and 7, mean Ct values based on four live domestic pigs (animal numbers 48, 51, 53, and 58) inoculated with two different ASFV strains (KAB 6/2 and SUM 14/11) at different time points, 3, 4, 7, and 8 dpi. SD values for EDTA blood (4.45), serum (7.90), oral swabs (5.27), and chewing ropes (3.20). Comparing the overall genome loads, an unpaired *t*-test was performed to test the significance of each matrix. EDTA blood showed highly significant Ct values compared with other matrix samples at 3 dpi (** *p*-value = 0.002). A similar significance level could be identified for oral swabs (** *p*-value = 0.009) and for chewing ropes (** *p*-value = 0.002). However, at 4 and 7 dpi, the ASFV genome load in serum was not significantly different from the genome load in EDTA blood (ns *p*-value = 0.3).

### 2.2. *DNA Extraction Methods*

To obtain a wide applicable range for viral DNA isolation, a comparison was performed between seven extraction methods (Figure 2, Table 1 and Table S1). All methods were analyzed by the qPCR assay published by Haines et al. [13]. It could be demonstrated that all tested methods were quite sensitive, efficient, and convenient for DNA isolation from all sample materials, depicted in Figure 2. The qPCR results for sample DNA obtained by the tested extraction kits were found to be very similar in terms of Ct values. No differences could be observed between the silica membrane- and magnetic bead-based kits, the 100 and 200 µL sample starting volumes, and the non-prefilled and prefilled extraction plates. No performance differences could be observed between using the IndiMag® Pathogen Kit (non-prefilled) and the IndiMag® Pathogen Cartridge formats (prefilled). Furthermore, the IndiMag® Pathogen IM48 Cartridge and the IndiMag® Pathogen KF96 Cartridge performed equally well irrespective of the magnetic bead processing platform (KingFisher Flex and IndiMag48) used. A slightly lower sensitivity was obtained from the genome release method by virotype Tissue Lysis Reagent (TLR), whereby false-negative results were only observed for a few samples with a very low genome load (Ct value > 33, Table S1).

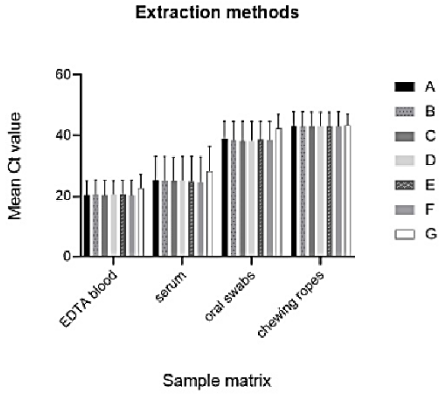


**Figure 2.** Extraction method comparison, mean Ct values obtained from 30 animals (EDTA blood), 25 animals (serum), 20 animals (oral swabs), and 6 animal pens (chewing ropes). (**A**) QIAamp Viral RNA Mini Kit (70 µL sample volume). (**B**) NucleoMagVet Kit (100 µL sample volume). (**C**) NucleoMagVet kit (200 µL sample volume). (**D**) IndiMag® Pathogen Kit. (**E**) IndiMag® Pathogen IM48 Cartridge. (**F**) IndiMag® Pathogen KF96 Cartridge. (**G**) Nucleic acid release by virotype TLR. (Sample volume for all IndiMag® extraction formats was 200 µL). SD analysis was carried out (number of replicates = 30); for mean Ct values, see Table 1. SD value for **A**, 10.79; **B**, 10.68; **C**, 10.75; **D**, 10.68; **E**, 10.76; **F**, 10.86; and **G** (10.41). Standard error of the mean value for **A** is 5.39; **B**, 5.34; **C**, 5.37; **D**, 5.34; **E**, 5.37; **F**, 5.42; and **G**, 5.20. A one-way ANOVA was performed to test the significance between the different extraction methods based on the same matrix samples with a resulting $p$-value > 0.99 for the taken samples, which is not statistically significant.

**Table 1.** Mean Ct values of different DNA extraction methods using different sample materials.

| Sample Matrix | Extraction Methods * | | | | | | |
|---|---|---|---|---|---|---|---|
| | **A** | **B** | **C** | **D** | **E** | **F** | **G** |
| EDTA blood | 20.44 | 20.61 | 20.04 | 20.64 | 20.71 | 20.38 | 22.67 |
| Serum | 25.22 | 24.98 | 24.96 | 25.09 | 24.88 | 24.63 | 28.22 |
| Oral swabs | 38.92 | 38.47 | 38.26 | 38.43 | 38.68 | 38.55 | 42.49 |
| Chewing ropes | 43.05 | 43.03 | 43.11 | 43.15 | 43.15 | 43.03 | 43.58 |

* For description of the extraction methods, use the legend of Figure 2.

### *2.3. Rapid Amplification and ASFV Detection Using Different qPCR Assays*

Using the extracted eluates of method E (IndiMag®Pathogen IM48 Cartridge), a comparison of four different qPCR assays was carried out (Figure 3 and Table S2). The in-house Haines qPCR (Haines assay), the modified Universal Probe Library (UPL) qPCR method from the EU reference laboratory (EURL assay), and the commercial virotype ASFV 2.0 qPCR (virotype assay) were conducted on the Bio-Rad CFX96 real-time PCR cycler, whereby the commercial lyophilized IndiField ASFV PCR (IndiField assay) was applied on the IndiField thermocycler based on the matching PCR tubes. To avoid false-negative results due to PCR inhibitors or improper nucleic acid extraction, external and internal controls were co-amplified for all samples. The corresponding results of the internal controls are presented in Table S2.

Regarding the target detection, all tested samples amplified on the Bio-Rad CFX96 cycler produced identical qualitative positive and negative ASFV results. Furthermore, the variability of the Ct value between the three assays was very low, while the template volumes of the three assays with 2.5, 2.0, and 5.0 µL were slightly different. In comparison

with the OIE-recommended modified UPL PCR assay (EURL assay), the ΔCt value was calculated for the tested samples. For the EDTA blood samples, the mean Ct values of the virotype assay and the in-house Haines assay were 1.2 and 1.1 Cts lower in comparison with the EURL method. This difference was further confirmed with the serum and oral swab samples (Table 2).

**Table 2.** Mean Ct values of ASFV qPCR assays using different sample materials.

| Sample Matrix | Total Sample Number | Haines Assay Mean Ct Value (pos. Sample No.) | EURL Assay Mean Ct Value (pos. Sample No.) | Virotype Assay Mean Ct Value (pos. Sample No.) | IndiField Assay Mean Ct Value (pos. Sample No.) |
|---|---|---|---|---|---|
| EDTA blood | 36 | 20.7 (34) | 21.8 (34) | 20.6 (34) | 18.1 (35) |
| Serum | 25 | 24.8 (23) | 25.8 (23) | 24.5 (23) | 21.2 (25) |
| Oral swabs | 20 | 38.6 (11) | 39.1 (11) | 38.4 (11) | 34.5 (16) |
| Chewing ropes | 6 | 43.0 (1) | 43.2 (1) | 43.0 (1) | 38.7 (3) |

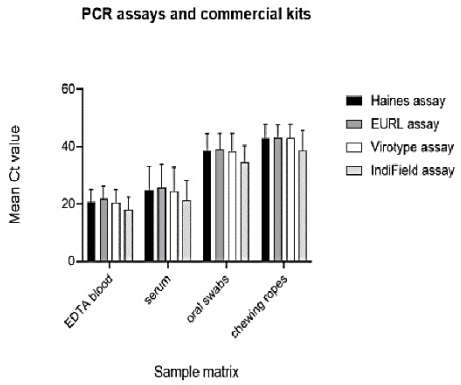


**Figure 3.** Comparison of PCR assays and commercial kits, mean Ct values obtained from 30 animals (EDTA blood), 25 animals (serum), 20 animals (oral swabs), and 6 animal pens (chewing ropes). (1) PerfeCTa qPCR ToughMix Kit (Haines assay). (2) LightCycler 480 Probes Master Kit (EURL assay). (3) Virotype ASFV 2.0 PCR Kit (virotype assay). (4) IndiField ASFV PCR (IndiField assay). SD analysis was carried out (number of replicates = 30); for mean Ct values, see Table 2. SD values for Haines assay, 10.07; EURL assay, 10.25; virotype assay, 10.77; and IndiField assay, 10.02. Standard error of the mean value for Haines assay is 5.34; EURL assay, 5.12; virotype assay, 5.38; and IndiField assay, 5.00. A one-way ANOVA was performed to test the significance between the different PCR assays based on the same matrix samples with a resulting $p$-value = 0.93 for the taken samples, which is not statistically significant.

Overall, the IndiField ASFV PCR showed the lowest Ct values and the highest sensitivity. In comparison with the EURL method, the mean Ct value for the EDTA blood samples was 3.7 cycles earlier with the IndiField PCR. Similar Ct values could be identified for the other tested matrices (Table 2). Furthermore, 10 samples with a very low viral load scored negative with the three methods performed on the Bio-Rad CFX96 cycler, but positive on the IndiField thermocycler using the IndiField ASFV PCR. Here, positive results with Ct values between 33.0 and 40.4 could be ascertained for these 10 samples (Table S2). The improved sensitivity of the IndiField ASFV PCR is probably based on the higher template input of 20 µL.

*2.4. Analysis of Field Samples from ASFV Outbreak in Germany 2020*

A comparison was carried out between three different extraction methods (B, E, and G) and four different qPCR assays (1, 2, 3, and 4). For the amplification of the TLR-released blood samples with the IndiField ASFV PCR on the IndiField thermocycler using 20 µL template, some inhibition effects could be observed. Therefore, we diluted the template in RNase-free water with a 1:1 dilution factor (10 µL template added to 10 µL water) to reduce the concentration of PCR inhibitors. All samples extracted by the NucleoMagVet Kit and the IndiMag®Pathogen Kit were detected positive. Only 12 out of 14 samples extracted with the virotype TLR method were detected positive (2 out of 14 samples were detected negative by this method). This result was independent of the used qPCR system. The Ct values from the three extraction procedures amplified with the four different qPCR assays are presented in Table 3 (mean Ct values) and Supplementary Table S3. In general, differences could be shown for the extraction methods only. All qPCR assays, regardless of whether lyophilized or not, delivered very similar results. While showing the lowest Ct values, the lyophilized IndiField ASFV PCR was also not able to detect the two borderline samples, 3 and 11 (Supplementary Table S3), extracted with the TLR method that were also not detected by the nonlyophilized qPCR assays.

**Table 3.** Testing of ASFV-positive field samples from the outbreak 2020 in Germany. The Ct values of three different extraction methods and four different ASFV qPCR assays are shown.

| Animal | Sample Matrix | (1) Haines Assay | | | (2) EURL Assay | | | (3) Virotype Assay | | | (4) IndiField Assay | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | N | I | T | N | I | T | N | I | T | N | I | T |
| | | Ct Value | | | Ct Value | | | Ct Value | | | Ct Value | | |
| 1 | SwS | 30.7 | 30.4 | 35.9 | 31.3 | 31.2 | 36.4 | 29.3 | 28.9 | 35.1 | 25.8 | 25.8 | 38.6 |
| 2 | SwS | 27.6 | 28.1 | 31.4 | 28.1 | 28.8 | 32.4 | 26.0 | 26.6 | 29.8 | 22.7 | 23.1 | 28.2 |
| 3 | SwS | 31.9 | 31.7 | - | 32.7 | 32.7 | - | 30.3 | 30.6 | - | 27.1 | 26.8 | - |
| 4 | SwS | 25.4 | 25.3 | 30.9 | 26.0 | 26.0 | 32.1 | 23.7 | 23.7 | 29.1 | 19.8 | 20.2 | 26.8 |
| 5 | Serum | 29.9 | 30.2 | 31.1 | 29.5 | 30.0 | 31.3 | 29.0 | 28.3 | 29.3 | 25.2 | 25.0 | 27.3 |
| 6 | SwS | 28.1 | 28.0 | 30.9 | 28.3 | 28.7 | 31.9 | 26.3 | 26.3 | 29.2 | 23.0 | 23.0 | 27.7 |
| 7 | SwS | 29.6 | 30.4 | 35.6 | 30.1 | 30.7 | 36.7 | 27.9 | 28.7 | 36.5 | 24.8 | 24.9 | 32.1 |
| 8 | SwS | 20.5 | 20.6 | 23.6 | 21.1 | 21.5 | 24.6 | 19.2 | 19.3 | 22.5 | 16.0 | 16.0 | 25.2 |
| 9 | BM | 21.9 | 21.4 | 26.7 | 22.1 | 22.1 | 28.0 | 20.5 | 19.8 | 27.8 | 17.0 | 17.0 | 25.8 |
| 10 | BM | 18.8 | 18.5 | 21.5 | 19.2 | 19.1 | 22.6 | 17.3 | 17.2 | 20.4 | 14.1 | 15.1 | 22.6 |
| 11 | BM | 34.5 | 35.0 | - | 36.3 | 36.9 | - | 33.1 | 34.5 | - | 30.7 | 31.6 | - |
| 12 | SwS | 26.1 | 25.0 | 29.1 | 26.1 | 26.0 | 29.9 | 24.1 | 23.9 | 27.4 | 20.0 | 19.8 | 25.9 |
| 13 | SwS | 22.7 | 22.1 | 25.4 | 23.0 | 22.6 | 26.8 | 21.1 | 20.3 | 24.1 | 17.0 | 16.9 | 23.9 |
| 14 | SwS | 27.8 | 27.6 | 30.1 | 27.9 | 28.0 | 31.3 | 26.0 | 25.8 | 28.8 | 22.1 | 22.1 | 26.9 |
| 15 | DIC | - | - | - | - | - | - | - | - | - | - | - | - |
| 16 | DIC | - | - | - | - | - | - | - | - | - | - | - | - |

(**1**) PCR assay based on the protocol published by Haines [13]. (2) EURL PCR assay, which is an OIE-recommended method [9]. (**3**) Virotype ASFV 2.0 PCR Kit [14]. (**4**) IndiField ASFV PCR. Abbreviations: N = NucleoMagVet kit (Macherey-Nagel), I = IndiMag® Pathogen Kit (Indical Bioscience), T = Tissue Lysis Reagent (Indical Bioscience), SwS = swab suspension, BM = bone marrow, DIC = DNA isolation control (ASFV negative serum), - = no Ct.

## 3. Discussion

African swine fever has triggered global concerns; highly significant economic impact and mortality rates have led to a major threat to the pig industry. Without ASF-specific treatment or an effective vaccine, rapid and accurate laboratory diagnosis is an important tool for timely intervention and thus ASF control. The actual lab diagnosis focuses on viral nucleic acid isolation and PCR from available specimens and antibody detection from liquid samples [15]. Molecular diagnostic techniques in the EU reference laboratories are mainly based on OIE-recommended methods (i.e., conventional [10] and real-time PCR systems [3,7,9,12,13,16] and several commercial ASFV real-time PCR kits).

In this study, seven nucleic acid extraction methods and four different real-time PCR assays for ASFV detection were compared. Different sample materials were used and collected from several animal experiments with strains of different genotypes. The results

showed that a simplification of this kind of assays and workflows can be achieved with no relevant loss of sensitivity or specificity. This should encourage the use of its broad application in different labs.

The data analyses for matrix selection confirmed that EDTA blood is the most suitable choice for ASFV genome detection in both initial and late phases of infection of live animals. This result correlates with the work of other groups [9,13,17]. Serum samples could be also detected in the early stage of infection, but with a considerably reduced viral genome load in comparison with EDTA blood. Alternative specimens, like oral swabs or chewing ropes, could detect ASFV to a certain extent in the late phase of infection based on the increased viremia with significantly lower genome loads. For postmortem analyses, we could confirm that spleen is the most appropriate material for ASFV detection. This result was consistent with similar investigations [14]. In general, EDTA blood or bloody tissue materials are recommended for ASFV detection from both experimentally infected animals and dead carcasses in the field.

All tested silica membrane- or magnetic bead-based extraction methods were comparatively sensitive for DNA isolation. The manual QIAamp Viral RNA Mini Kit (Qiagen), based on the silica membrane system, could successfully isolate the viral DNA of the ASFV genome. Similar results could be ascertained in a study of Haines et al. [13]. The authors could also demonstrate that this kit is convenient for viral DNA extraction from ASFV. Additionally, our study showed that this kit could deliver almost identical results in regard to Ct values compared with automated magnetic bead-based extraction methods. For the automated magnetic bead-based systems, no differences between the usage of different input sample volumes or prefilled or non-prefilled reagents and different instruments (KingFisher Flex System or IndiMag48) could be observed. However, prefilled reagents have the ability of being conducted on both automated systems. The advantage of the IndiMag48 instrument is the possibility to extract nearly all exact sample numbers between 1 and 48 based on the individual composition of plasticware for 1, 8, and 24 samples. On the other hand, the KingFisher system has a wide range of extraction of up to 96 samples simultaneously, which could be perfectly practical in case of high-throughput scenarios as free testing of swine populations in ASFV restriction zones.

The virotype Tissue Lysis Reagent (TLR) was developed for the fast preparation of various sample types without the need for an extraction kit or any complicated nucleic acid isolation procedures and has been successfully used for Bovine viral diarrhea virus (BVDV) diagnosis from ear notch samples [18]. The viral ASFV genome release by TLR showed a slightly lower sensitivity compared with the standard silica membrane- and magnetic bead-based systems. However, the TLR could have the advantage of a successful application in a wide range of diagnostic laboratories in case of limited or unavailable commercial extraction kits or reagents. Especially, the COVID-19 pandemic situation has generated a huge consumption of extraction kits, and thus, the TLR method could be an effective alternative for the continuation of molecular ASFV diagnostics. For high-throughput scenarios, up to 96 samples can be processed with the TLR in appropriate PCR plates. The incubation can be performed in a conventional PCR thermocycler, followed by centrifugation in a plate centrifuge (e.g., 5804 R centrifuge, Eppendorf, Hamburg, Germany).

The four tested real-time PCR assays could detect the ASFV genome with similar efficiency. The most sensitive PCR was obtained from the IndiField ASFV PCR, which was amplified on the IndiField thermocycler. The study of Daigle et al. (2020) has ascertained the functionality of the IndiField thermocycler [19]. The slightly increased analytical sensitivity of the IndiField ASFV PCR compared with the other tested PCR assays can be most likely explained by the high template volume, possibly due to the lyophilized format of the kit. Interestingly, the IndiField ASFV PCR delivered excellent PCR result in a short time using a temperature profile of less than 60 min. The other three PCR assays with their liquid chemistry could achieve comparable results with high sensitivity and efficiency, which were conducted on a standard real-time thermocycler (Bio-Rad CFX96). The liquid master mixes can be used on different real-time PCR thermocyclers. However, it

was not suitable to analyze the complete test panel on the IndiField thermocycler due to its limitation of up to nine samples per run. Nevertheless, the use of lyophilized ready-to-use reagents and the related higher template input, as well as master mix stability, may in the future also be available for standard real-time PCR platforms if appropriate plastic is used. A previous study successfully demonstrated that ASFV could be detected by the use of lyophilized reagents for qPCR amplification [20]. Here, prefilled single tubes, 8-well strips, 24-well blocks, and complete 96-well plates can be used for the individual application of cycler-specific PCR kits. In general, the application of prefilled (lyophilized) pathogen-specific PCR kits would be an excellent extension of the use of prefilled reagents for nucleic acid extraction and would further reduce the risk of contaminations and the working time in the molecular diagnostic procedures. This was correlated to the works of other groups, which were performed with different pathogens, such as the influenza A virus [21] and bluetongue virus [22].

The standard and "easy lab" methods were successfully applied for ASFV detection in field specimens collected from dead wild boars during the 2020 ASFV outbreak in Germany. The data showed that all methods not only are convenient for samples from live animals but also can be successfully applied for different sample materials from carcasses of wild boars.

## 4. Materials and Methods

### *4.1. Sample Collection from Experimentally Infected Animals*

A panel consisted of 90 samples from domestic pigs and wild boar that had been obtained in seven different animal experiments with ASFV strains of different genotypes (Table 4). The animal trials were approved by a competent authority (Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei (LALLF) Mecklenburg-Vorpommern, Rostock, Germany) under reference number 7221.3-2.011/19. Different samples of these animal trials were used for the validation study (EDTA blood, serum, oral swabs, tissue homogenate spleen samples, and chewing ropes collected at different time points post-infection). In summary, 36 EDTA blood samples, 25 serum samples, 20 oral swabs, 6 chewing ropes, and 3 tissue homogenate spleen samples were used in this study (details shown in Table S1). The animals were housed in groups in the high containment facility of the Friedrich-Loeffler-Institut (FLI) ($L3^+$). The animals were fed a commercial pig food with corn and hay cob supplement and had access to water ad libitum.

**Table 4.** African swine fever virus isolates used in this study. Abbreviations: o.-n. = oro-nasally; i.m. = intramuscularly; HAD = hemadsorbing doses.

| Animal Experiment | Genotype | Isolate | Country of Origin | Year | Infection Route | Infection Dose (HAD50 /mL) |
|---|---|---|---|---|---|---|
| 1 | II | Estonia 2014 | Estonia | 2014 | o.-n. | $10^{5.25}$ |
| 2 | IV | RSA W1/99 | South Africa | 1999 | i.m. | $10^{0.83}$ |
| 3 | XII | MFUE 6/1 | Zambia | 1982 | i.m. | $10^{1.16}$ |
| 4 | XIX | CHZT 90/1 | Zimbabwe | 1990 | i.m. | $10^{1.0}$ |
| 5 | II | Belgium 2018/1 | Belgium | 2018 | o.-n. | $10^{4.6}$ |
| 6 | XI | KAB 6/2 | Zambia | 1983 | i.m. | $10^{3.25}$ |
| 7 | XIII | SUM 14/11 | Zambia | 1983 | i.m. | $10^{3.3}$ |

EDTA blood and serum samples were collected by using the KABEVETTE®G system (KABE Labortechnik, Nümbrecht, Germany). Afterwards, blood samples were prepared for long-term storage at +4 °C by adding penicillin/streptomycin, 100× (Thermo Fisher, Darmstadt, Germany) and gentamicin/amphotericin B solution, 500× (Thermo Fisher, Darmstadt, Germany), while serum samples were centrifuged at 4000 rpm for 10 min. Finally, both sample types were stored at +4 °C until the DNA extraction step. An amount of 0.5 g of organ tissue samples was homogenized by grinding with a 5 mm steel ball

within 1 mL cell culture medium in 2 mL bolted tubes using the TissueLyser II (Qiagen, Hilden, Germany).

Additionally, oral swabs (Copan Diagnostics Inc., Brescia, Italy) from individual pigs and chewing ropes from each stable were used for noninvasive sample collection. Oral swabs and pieces of chewing rope samples were enriched in 2 mL standard cell culture medium including antibiotics (see above) and incubated at room temperature on a thermoshaker (VWR International GmbH, Darmstadt, Germany) for 30 min (oral swabs) or 24 h (chewing ropes). The supernatant was used for the DNA extraction procedures.

*4.2. Field Samples from ASFV Outbreak in Germany*

Different specimens with sufficient sample volume collected from the first ASFV outbreaks in September 2020 in Germany were used for the evaluation. The samples delivered from the State Laboratory Berlin-Brandenburg were collected from carcasses found in the border region to Poland. A total of 14 samples (serum, bone marrow, and bloody swab suspensions) from 14 different wild boars were selected for the investigations. This panel consisted of 10 swab suspensions, 1 serum sample, and 3 bone marrow homogenates (gathered in Table S3).

Swab suspension was generated in 1.5 mL cell culture medium; the serum samples were centrifuged at 8000 rpm for 1 min before use. Bone marrow samples were homogenized by grinding 0.5 g of organ tissue with a 5 mm steel ball within 1 mL phosphate-buffered saline in 2 mL bolted tubes.

*4.3. DNA Extraction*

Seven different extraction and releasing methods were applied for the ASFV DNA isolation.

A. QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany): This silica membrane-based extraction kit is well established and is widely used for the manual extraction of both DNA and RNA from cell-free and cell-containing specimens. Briefly, a reduced sample volume of 70 µL to avoid the overload of the silica membrane was mixed with 560 µL AVL lysis buffer of the kit. An amount of 5 µL of internal control DNA (IC2-DNA) [23] was added to the sample–lysis buffer mixture, vortexed, and incubated at room temperature for 10 min. The following steps of the extraction procedure are based on the manufacturer's instructions. Finally, the nucleic acid was eluted in 50 µL elution buffer and stored at −20 °C. Using this kit, DNA/RNA for up to 12 samples can be extracted in approximately 30 min.

B. NucleoMagVet Kit (Macherey-Nagel, Düren, Germany): This magnetic bead-based extraction kit was conducted on the KingFisher Flex System (Thermo Fisher Scientific, Darmstadt, Germany). Briefly, 100 µL sample volume was added to 100 µL VL1 lysis buffer and processed according to the instructions of the manufacturer. For internal control, 10 µL IC-DNA was mixed with 350 µL VEB binding buffer per sample and was added to the sample–lysis buffer mixture. After three washing steps, the extracted nucleic acid was eluted in 100 µL elution buffer. The extraction protocol on the KingFisher Flex System needs approximately 20 min for up to 96 samples. Details of the KingFisher protocol can be provided on request.

C. NucleoMagVet- Kit (Macherey-Nagel) on the KingFisher Flex System, which was performed identically with the same protocol as described above in B, however, it was used with a different sample input volume of 200 µL.

D. IndiMag® Pathogen Kit: This magnetic bead-based extraction kit was applied on the IndiMag48 instrument (both kit and machine from Indical Bioscience, Leipzig, Germany). An interesting highlight of the IndiMag48 instrument is the variability of the number of extraction samples, which can be performed per run. Plastic blocks for 1, 8, or 24 samples can be combined to cover nearly all numbers between 1 and 48 samples. For each sample, four wells were used for the extraction procedure. Briefly, in the first well, 20 µL proteinase K was mixed with 200 µL sample and

500 µL VXL mixture (100 µL VXL lysis buffer, 400 µL ACB binding buffer, 25 µL magnetic beads, and 10 µL IC-DNA). In the second and third wells, the AW1 buffer (wash 1) and the AW2 buffer (wash 2) were housed, respectively. Finally, the nucleic acid was eluted in 100 µL elution buffer. The extraction procedure was realized according to the manufacturer's instructions, and the extraction time for up to 48 samples on the IndiMag48 platform was 31 min.

E. IndiMag® Pathogen IM48 Cartridge (IndiMag® Pathogen Kit prefilled for the IndiMag48 instrument): Here, the different buffers were prefilled into the four wells used per sample for the extraction. In the first well, the 20 µL proteinase K and, in the second well, the AW1 buffer mixed with magnetic beads were present. The AW2 buffer and the elution buffer were prefilled in wells 3 and 4, respectively. The prefilled and sealed plates were produced by Indical Bioscience and used according to the manufacturer's instructions. An amount of 200 µL sample volume, 500 µL VXL/ACB mixture without magnetic beads, and 10 µL IC-DNA (supplied with the virotype ASFV 2.0 PCR Kit) were added in the first well and then conducted directly on the IndiMag48 instrument with the same protocol used as for the non-prefilled extractions.

F. IndiMag® Pathogen KF96 Cartridge (IndiMag® Pathogen Kit prefilled for the KingFisher Flex System): Here, five prefilled 96 deep-well plates were provided by Indical Bioscience (plate 1 = proteinase K, plate 2 = AW1 buffer mixed with magnetic beads, plate 3 = AW2 buffer, plate 4 = AW3 buffer (supplementary wash step), and plate 5 = elution buffer). For the extraction, 200 µL sample, 500 µL VXL/ACB mixture without magnetic beads, and 10 µL IC-DNA (supplied with the virotype ASFV 2.0 PCR Kit) were added into the wells of the first plate. Extraction time was 32 min.

G. Nucleic acid release method of the ASFV genome by virotype Tissue Lysis Reagent (TLR) from Indical Bioscience: Here in this study, 10 µL ASFV sample was added to 90 µL TLR buffer in a standard 1.5 mL Eppendorf tube and mixed very well by pipetting up and down. The sample–TLR buffer mixture was incubated at 65 °C for 30 min and at 98 °C for 15 min, followed by cooling to room temperature. Afterwards, the sample–TLR buffer mix was centrifuged at 10 000 $\times g$ for 10 min. Finally, the cleared supernatant was transferred directly into the PCR reaction tube as template.

In all the extraction procedures, two exogenous extraction control DNAs were added to all lysis buffers of each extraction method (enhanced green fluorescent protein gene mix [23] and IC-DNA from the virotype ASFV 2.0 PCR Kit) according the references. The extracted template nucleic acids were stored at −20 °C until use.

*4.4. Real-Time PCR Kits and Assays for ASFV Detection*

Four different qPCR assays for ASFV genome detection were comparatively tested:

1. Haines PCR: The PCR assay described by Haines et al. [13] was modified by using a lab-specific amplification mix and the integration of a lab-specific internal control system utilizing the PerfeCTa®qPCR ToughMix®Kit from Quanta BioSciences (Gaithersburg, MD, USA). A FAM-labelled ASFV primer–probe mixture consisted of 800 nM ASFV-p72IVI-F, 800 nM ASFV-p72IVI, and 200 nM ASFV-p72IVI probe in 0.1 × TE buffer (pH 8.0). For the control of extraction and qPCR amplification, a heterologous control system, published by Hoffmann et al. [23], was integrated. Here, a HEX-labelled primer–probe mixture consisted of 200 nM EGFP1-F, 200 nM EGFP2-R, and 200 nM EGFP probe 1 in 0.1 × TE buffer (pH 8.0). The 12.5 µL total reaction mix was established by 1.75 µL RNase-free water, 6.25 µL 2× PerfeCTa qPCR ToughMix, 1.0 µL ASFV primer–probe mix (ASFV-P72-IVI-Mix-FAM), 1.0 µL internal control primer–probe mix (EGFP-Mix1-HEX), and 2.5 µL DNA template. The following thermoprofile was used for amplification: 3 min at 95 °C, 45 cycles at 95 °C for 15 s, 60 °C for 20 s, and 75 °C for 20 s. The fluorescence data in the FAM and HEX channel were collected during the annealing step, and the total run time on the Bio-Rad CFX96

Real-Time Detection System (Bio-Rad, Hercules, CA, USA) was 1 h and 16 min. For the data analyses, the Bio-Rad Maestro software (version 4. 1.2433. 1219) was used.

2. EURL PCR: This method is recommended by the EU reference lab for ASF and based on the publication of Fernández-Pinero et al. [9]. The qPCR is listed as the official method by the OIE. Because the original UPL probe is not commercially available anymore, an alternative TaqMan probe was introduced by the EURL-ASF. In our tests, the LightCycler 480 Probes Master Kit (Roche Applied Science, Mannheim, Germany) was used for the amplification according the standard operating procedure on the website of the EURL-ASF (https://asf-referencelab.info/asf/en/procedures-diagnosis/sops, accessed on 25 January 2021). Briefly, FAM-labelled ASF-VP72 primer–probe mixtures consisted of 600 nM ASF-VP72-F, 600 nM ASF-VP72-R, and 200 nM ASF-VP72P1-FAM in 0.1 × TE buffer (pH 8.0). For the internal control amplification, the EGFP-Mix1-HEX, as described above, was used. A total reaction PCR mix of 20 µL volume containing 6.0 µL RNase-free water, 10.0 µL of 2× LC480 Probes Master PCR Mix, 1.0 µL ASF-VP72-Mix-FAM, 1.0 µL EGFP-Mix1-HEX, and 2.0 µL template DNA was prepared. The PCR conditions were 5 min at 95 °C, followed by 45 cycles at 95 °C for 10 s and 60 °C for 30 s. The fluorescence data in the FAM and HEX channel were collected during the annealing step, and the total run time on the CFX96 Real-Time Detection System was 1 h and 13 min.
3. Virotype ASFV 2.0 PCR Kit (Indical Bioscience, Leipzig, Germany): This qPCR assay is a commercial kit for the detection of ASFV and is licensed for the German market. An amount of 20 µL of the ready-to-use master mix was filled in the PCR reaction well, and 5 µL of the template DNA was added to give a final reaction volume of 25 µL. Besides the ASFV target amplification, the master mix features two independent control systems. The homologous (endogenous) extraction and amplification control is detected in the HEX/JOE channel, whereas an additional heterologous (exogenous) extraction control is detected in the Cy5 channel. The exogenous control (IC-DNA) is supplied with the virotype ASFV 2.0 PCR Kit and is added to the lysis buffer during extraction. These controls serve to control extraction from the animal sample and to identify samples showing full and partial inhibition, thus excluding false-negative ASFV samples. According the supplier's instructions, a run time of 59 min on the CFX96 Real-Time Detection System with the following temperature profile was conducted: 2 min at 95 °C, 40 cycles at 95 °C for 5 s, and 60 °C for 30 s [14].
4. IndiField ASFV PCR (Indical Bioscience, Leipzig, Germany): This commercial real-time PCR amplifies the ASFV genome in the FAM channel and a homologous internal extraction control in the Amber/Texas Red channel. Interestingly, the PCR reactions were prepared as ready-to-use lyophilized reagents in the individual PCR tubes of the ultraportable IndiField thermocycler. The reaction mix was prepared by adding 20 µL DNA template directly to the lyophilized master mix. The cycler is fully controlled by a smartphone, and up to nine samples in one run can be analyzed in parallel. The PCR data can be uploaded to a cloud-based storage and analysis system. A PCR thermoprofile of 1 min at 95 °C, followed by 45 cycles at 95 °C for 1 s and 60 °C for 20 sec, will be introduced by scanning the specific QR code on the package of the lyophilized IndiField ASFV PCR. The total run time for this system on the IndiField thermocycler is 56 min.

Dilution series of an ASFV DNA standard (ASFV Estonia 2014) were applied in each PCR run to confirm the sensitivity and reproducibility of the performed analyses (Tables S1 and S2).

*4.5. Statistical Analysis*

Initial data recording and analyses (comparison of mean values and transformation of values) were done using Microsoft Excel 2010 (Microsoft Germany GmbH, Munich, Germany). GraphPad Prism 8 (GraphPad Software Inc., San Diego, CA, USA) was used for further statistical analyses and graph creation. Statistically significant differences were

investigated by two statistical tests (unpaired *t*-test and one-way ANOVA) to test the significance of the results. Statistical significance was defined as $p < 0.05$ and indicated with an asterisk (*); $p < 0.01$ was indicated with two asterisks (**).

## 5. Conclusions

EDTA blood and bloody materials are the sample matrices of choice for a sensitive ASFV genome detection, independent of the course and phase of the disease. Serum samples also work fine in general, but here, the sensitivity of the DNA detection in the early phase of infection can be reduced. Noninvasive sample materials (oral swabs and chewing ropes) are clearly less suitable for the detection based on the minimal virus excretion.

If the optimal specimens are used for the molecular detection of ASFV, several extraction and qPCR methods are "fit for purpose." The selection of ideal systems for a specific lab depends on various factors. To name a few, the number of analyses per day, the available lab equipment, the budget, the personal and technical resources, and the necessity to use certified kits are of relevance. Depending on the specific situation in the lab, the different methods for extraction and qPCR presented here can be combined in a modular regime. In addition, viral DNA release via the TLR procedure can be an option in the molecular diagnostics of ASFV, especially if standard extraction kits are expensive or not available.

In our study, we could show that simplification of DNA extraction and qPCR does not result in reduced diagnostic sensitivity per se. Based on the minimization of manual handling and working time, the use of commercially available and prefilled reagents for extraction and qPCR can reduce the risk of false-negative and false-positive results especially in high-throughput scenarios. The implementation of state-of-the-art internal control systems and easy-to-handle software in the used machines, combined with improved storage stability by using lyophilized PCR kits, will further improve the diagnostic safety and robustness of molecular diagnostics.

**Supplementary Materials:** Supplementary materials can be found at https://www.mdpi.com/1422-0067/22/5/2307/s1.

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**Data Availability Statement:** The data set used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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4.2. Optimizing release of nucleic acids of African swine fever virus and Influenza A virus from FTA cards

Publication II

**Optimizing release of nucleic acids of African swine fever virus and Influenza A virus from FTA cards**

Ahmed Elnagar, Timm C. Harder, Sandra Blome, Martin Beer and Bernd Hoffmann

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# Optimizing Release of Nucleic Acids of *African Swine Fever Virus* and *Influenza A Virus* from FTA Cards

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**Abstract:** FTA cards and related products simplify the collection, transport, and transient storage of biological sample fluids. Here, we have compared the yield and quality of DNA and RNA released from seven different FTA cards using seven releasing/extraction methods with eleven experimental eluates. For the validation, dilution series of *African swine fever virus* (ASFV) positive EDTA blood and *Influenza A virus* (IAV) positive allantoic fluid were used. Based on our data, we conclude that direct PCR amplification without the need for additional nucleic acid extraction and purification could be suitable and more convenient for ASFV DNA release from FTA cards. In contrast, IAV RNA loads can be amplified from FTA card punches if a standard extraction procedure including a lysis step is applied. These differences between the amplifiable viral DNA and RNA after releasing and extraction are not influenced by the type of commercial FTA card or the eleven different nucleic acid releasing procedures used for the comparative analyses. In general, different commercial FTA cards were successfully used for the storage and recovery of the ASFV and IAV genetic material suitable for PCR. Nevertheless, the usage of optimized nucleic acid releasing protocols could improve the recovery of the viral genome of both viruses. Here, the application of Chelex® Resin 100 buffer mixed with 1 × Tris EDTA buffer (TE, pH 8.0) or with TED 10 (TE buffer and Dimethylsulfoxid) delivered the best results and can be used as a universal method for releasing viral DNA and RNA from FTA cards.

**Keywords:** *African swine fever virus*; *Influenza A virus*; nucleic acid release; DNA/RNA isolation; direct PCR amplification; FTA cards



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## 1. Introduction

The Flinders Technology Associates (FTA®) Whatman filter paper cards are based on a chemically-treated cellulose membrane, which lyses cells, their nuclei, and organelles from a variety of sources (e.g., blood, saliva, plant tissue). Upon immediate cell lysis, the released nucleic acid is bound within the supporting material, the card fiber. The matrix protects the nucleic acids from damaging agents (e.g., nucleases, oxidative agents, and bacterial growth) which serves to reduce degradation [1]. They are commercially available in a variety of configurations to meet application requirements and custom configurations. FTA cards are impregnated with chaotropic agents that inactivate infectious agents and reduce the biohazard potential of the sample, thereby minimizing risks of exposure to the technical staff during sample processing. This enables the storage of biological material on FTA cards at room temperature for extended periods. No refrigerators or freezers are required, which significantly reduces storage costs [2]. Samples stored on FTA cards can be shipped through regular postal service with no special handling restrictions, making them a very useful tool for the field collection of biological samples [3]. FTA cards are used in the veterinary field as an alternative method for collecting, transporting, and transiently storing samples for molecular diagnostics and have been applied for many viruses, including avian Influenza [4], *Newcastle disease* [5], *porcine reproductive and respiratory syndrome* [6],

*infectious bursal disease* [7], *foot and mouth disease* [8], *rabies* [9], and *African swine fever virus* [10]. However, it should be noted that the risk of cross-contamination of samples on FTA cards is higher compared with liquid samples collected in separate tubes. A further disadvantage of the FTA cards is the less efficient nucleic acid extraction based on the reduced recovery of the DNA and RNA from the filter paper matrix [11]. Although the probability of detecting the pathogens on FTA cards is lower than in fresh samples, the cards offer unique advantages for the collection, transportation, and storage of samples. Different procedures for the release of the nucleic acids from the FTA cards were described by the commercial suppliers or in context with specific viruses and cards [12].

In case of animal epidemics, we need safe and stable transport possibilities. It has been shown that swabs, filter papers, and also FTA cards can be helpful; however, the market and the possibilities of reprocessing are large. The objective of this study was to systematically evaluate the efficacy of release of viral DNA (ASFV in EDTA blood) and viral RNA (IAV in allantoic fluid) from seven brands of FTA cards on the market with seven different methods (eleven eluates). Released nucleic acid was measured via direct qPCR amplification as well as by qPCR after DNA/RNA extraction on a standard automated extraction system.

## 2. Results

For the study, four dilutions ($10^{-1}$ to $10^{-4}$) of ASFV-positive EDTA blood and IAV-positive allantois fluid in the same sample matrix of healthy donors were used as surrogates for DNA and RNA viruses, respectively. The ASFV EDTA blood sample has been diluted with ASFV-negative EDTA blood, and the IAV-positive sample has been diluted with negative allantoid fluid. For the dilutions of ASFV-positive EDTA blood, Ct values of 21.9, 24.1, 27.3, and 30.4 could be ascertained after standard extraction and qPCR. For the IAV dilution series in allantois fluid, Ct values of 17.1, 21.6, 24.5, and 28.4 were defined. In general, all generated raw data of the study are presented in the Supplemental Materials file (Supplementary Tables S1 and S2), including the data of the internal, positive, and negative controls. For better understanding and easier comparison of the generated data, qualitative and quantitative evaluations were performed, and these are summarized in the table and figures of the main text.

### 2.1. Comparison of Nucleic Acid Releasing/Extraction Methods

The comparison was performed with eleven different eluates derived from seven different releasing methods using seven different FTA cards for the releasing of both viral DNA and RNA. All tested methods were analyzed by the direct qPCR amplification and simultaneously by qPCR amplification after nucleic acid extraction on the KingFisher Flex System.

The ASFV genome was positively detected via direct qPCR amplification and amplification after DNA extraction by all methods up to the dilution $10^{-2}$. For the dilutions $10^{-3}$ and $10^{-4}$, direct qPCR compared to amplification after additional DNA isolation gave slightly higher numbers of positives (Table 1). As a tendency, the qualitative results were best when using method M2-E2 for direct amplification of the ASFV genome (Table 1). Very similar results could be obtained by the methods M3-E2, M6-E2, and M7-E2, respectively. Thus, all four releasing methods were suitable for the detection of ASFV genome via direct PCR (Table 1 and Supplementary Table S1). The qualitative data (positive vs. negative) were confirmed by the quantitative analysis of the Ct values after direct qPCR and qPCR after extraction. The results of all four dilution steps were combined for the quantitative analyses. Taking qualitative and quantitative data into consideration, direct qPCR outperformed qPCR following DNA extraction (Figure 1A, Supplementary Table S1).

**Table 1.** Qualitative PCR results for 11 DNA/RNA releasing methods using seven different FTA cards and four dilution steps of ASFV DNA and IAV RNA (the positive cards based on each isolation method and dilution series are presented, number of PCR positives/number of FTA brands used).

| Method Type | ASFV-qPCR Amplification | | | | | | | | | | IAV-RT-qPCR Amplification | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Direct qPCR | | | | | qPCR after DNA Extraction | | | | | Direct RT-qPCR | | | | | RT-qPCR after RNA Extraction | | | | |
| | Dilution Series | | | | | Dilution Series | | | | | Dilution Series | | | | | Dilution Series | | | | |
| | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | $10^{-4}$ | Sum | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | $10^{-4}$ | Sum | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | $10^{-4}$ | Sum | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | $10^{-4}$ | Sum |
| M1 | 7/7 | 7/7 | 7/7 | 2/7 | 23 | 7/7 | 7/7 | 7/7 | 1/7 | 22 | 7/7 | 7/7 | 0/7 | 0/7 | 14 | 7/7 | 7/7 | 7/7 | 1/7 | 22 |
| M2-E1 | 7/7 | 7/7 | 7/7 | 3/7 | 24 | 7/7 | 7/7 | 7/7 | 1/7 | 22 | 7/7 | 7/7 | 0/7 | 0/7 | 14 | 7/7 | 7/7 | 5/7 | 1/7 | 20 |
| M2-E2 | 7/7 | 7/7 | 7/7 | 7/7 | 28 | 7/7 | 7/7 | 7/7 | 3/7 | 24 | 7/7 | 7/7 | 3/7 | 0/7 | 17 | 7/7 | 7/7 | 7/7 | 2/7 | 23 |
| M3-E1 | 7/7 | 7/7 | 7/7 | 0/7 | 21 | 7/7 | 7/7 | 5/7 | 0/7 | 19 | 7/7 | 3/7 | 0/7 | 0/7 | 10 | 7/7 | 7/7 | 4/7 | 0/7 | 18 |
| M3-E2 | 7/7 | 7/7 | 7/7 | 4/7 | 25 | 7/7 | 7/7 | 7/7 | 4/7 | 25 | 7/7 | 7/7 | 3/7 | 0/7 | 17 | 7/7 | 7/7 | 7/7 | 1/7 | 22 |
| M4 | 7/7 | 7/7 | 7/7 | 1/7 | 22 | 7/7 | 7/7 | 5/7 | 1/7 | 20 | 7/7 | 7/7 | 0/7 | 0/7 | 14 | 7/7 | 7/7 | 7/7 | 2/7 | 23 |
| M5 | 7/7 | 7/7 | 7/7 | 0/7 | 21 | 7/7 | 7/7 | 5/7 | 0/7 | 19 | 7/7 | 7/7 | 0/7 | 0/7 | 14 | 7/7 | 7/7 | 7/7 | 1/7 | 22 |
| M6-E1 | 7/7 | 7/7 | 6/7 | 0/7 | 20 | 7/7 | 7/7 | 3/7 | 0/7 | 17 | 7/7 | 5/7 | 0/7 | 0/7 | 12 | 7/7 | 7/7 | 5/7 | 0/7 | 19 |
| M6-E2 | 7/7 | 7/7 | 7/7 | 4/7 | 25 | 7/7 | 7/7 | 7/7 | 5/7 | 26 | 7/7 | 7/7 | 7/7 | 2/7 | 23 | 7/7 | 7/7 | 7/7 | 4/7 | 25 |
| M7-E1 | 7/7 | 7/7 | 5/7 | 1/7 | 20 | 7/7 | 7/7 | 5/7 | 0/7 | 19 | 7/7 | 6/7 | 0/7 | 0/7 | 13 | 7/7 | 7/7 | 5/7 | 0/7 | 19 |
| M7-E2 | 7/7 | 7/7 | 7/7 | 5/7 | 26 | 7/7 | 7/7 | 7/7 | 4/7 | 25 | 7/7 | 7/7 | 7/7 | 2/7 | 23 | 7/7 | 7/7 | 7/7 | 4/7 | 25 |

ASFV (*African swine fever virus*), IAV (*Influenza A virus*), (M1) Method 1 (FTA Purification Reagent + Proteinase K), (M2-E1) Method 2–Eluate 1 (TE buffer + Proteinase K + FTA Elute buffer), (M2-E2) Method 2–Eluate 2, (M3-E1) Method 3–Eluate 1 (TE buffer + TLR buffer), (M3-E2) Method 3–Eluate 2, (M4) Method 4 (TE buffer), (M5) Method 5 (M-lysis Reagent), (M6-E1) Method 6–Eluate 1 (TE buffer + Chelex® 100 Resin), (M6-E2) Method 6–Eluate2, (M7-E1) Method 7–Eluate 1 (TED10 + Chelex® 100 Resin), (M7-E2) Method 7–Eluate 2.

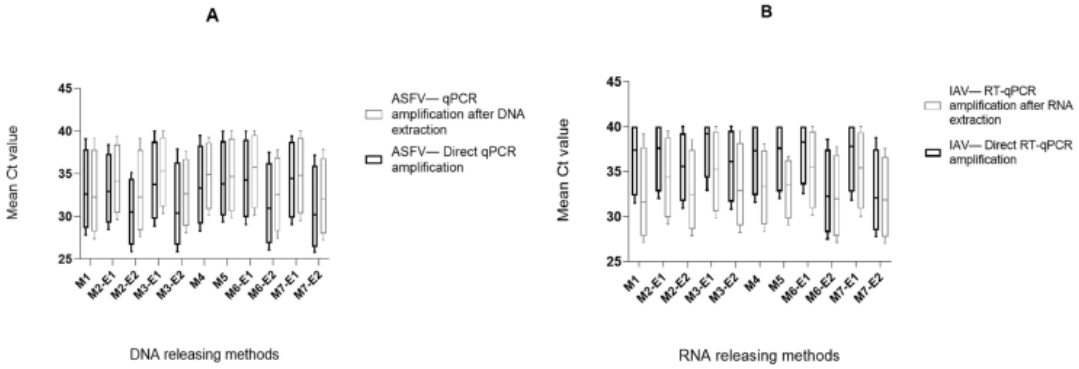


**Figure 1.** (**A**) Comparison of 11 different releasing methods for the ASFV genome detection with and without nucleic acid extraction. The mean Ct values based on the PCR results of all four dilution steps tested with seven FTA cards are shown. The used methods are described in the legend of Table 1. SD analysis was carried out (number of replicates = 11, Supplementary Table S1) are shown. The standard deviation (SD) value for all methods by the direct qPCR amplification is 1.96 and for qPCR amplification with extraction is 1.72. The standard error of the mean value for all methods by direct amplification is 0.59 and for the amplification with extraction is 0.51. An unpaired multiple *t*-test was performed to test the significance between the different RNA releasing methods based on the both qPCR amplification direct and with extraction with a resulting *p*-value > 0.99 for the taken values, which is not statistically significant. (**B**) Comparison of 11 different releasing methods for the IAV genome detection with and without nucleic acid extraction. The mean Ct values based on RNA-releasing methods of all four dilution steps. SD analysis was carried out (number of replicates = 11, Supplementary Table S1) are shown. Standard deviation (SD) value for all methods by the direct RT-qPCR amplification is 2.43 and for RT-qPCR amplification with extraction is 1.58. The standard error of the mean value for all methods by direct amplification is 0.73 and for the amplification with extraction is 0.47. An unpaired multiple *t*-test was performed to test the significance between the different RNA releasing methods based on the both RT-qPCR amplification direct and with extraction with a resulting *p*-value > 0.99 for the taken values, which is not statistically significant.

In general, the IAV genome detection via direct RT-qPCR showed more restrictions compared to the amplification results after RNA extraction of the released card eluates. Using the direct RT-qPCR, only the $10^{-1}$ dilution from all cards gave positive results with all releasing methods. In contrast, the dilution $10^{-2}$ could only be amplified from all cards if the RNA extraction step for the released eluates was added. The RT-qPCR amplification after RNA extraction demonstrated a higher number of positive results and lower Ct

values than the direct amplification, as became clear also for the dilutions $10^{-3}$ and $10^{-4}$. The data demonstrated that the methods M6-E2 and M7-E2 had slightly more sensitive results compared to other methods, regarding qualitative and quantitative values (Table 1, Supplementary Table S1 and Figure 1B). Interestingly, these two methods delivered good qualitative and quantitative results also in the direct RT-qPCR. Nevertheless, the positive number of cards and the Ct values were slightly better for the RT-qPCR after extraction compared to the direct RT-qPCR.

To confirm the efficiency and accuracy of the qualitative and quantitative results, a ΔCt was calculated among the four-dilution series between the values of the released eluates from the FTA cards compared to the same values of the extracted original samples on the KingFisher Flex system, (see Supplementary Table S3). For ASFV, based on the best releasing method, M2-E2 showed tendentially the lowest Δ Ct value (mean value of the four-dilution series) with a difference of 4.58 after direct qPCR. Two further methods (M3-E2 and M7-E2) showed good results with Δ Ct value of 4.83 and 4.92, respectively. Good results were defined based on the represented values in the study apart from possession of no statistically significant differences in the results. For IAV-RNA, M6-E2 and M7-E2 demonstrated the most sensitive results for the RT-qPCR amplification of extracted RNA with Δ Ct differences of 8.96 and 9.15 compared to the original sample fluids (Supplementary Table S3).

### 2.2. Comparison of Different FTA Cards

In general, all of the FTA cards were comparable to each other and delivered similar results. The ASFV genome could be detected from all card types with the direct qPCR and the qPCR after DNA extraction up to the $10^{-2}$ dilution. Not all cards spiked with the ASFV dilutions $10^{-3}$ and $10^{-4}$ delivered positive amplification results after genome releasing with the 11 methods. This result was independent from the use of direct qPCR or the amplification after additional ASFV-DNA extraction. Nevertheless, the direct qPCR delivered slightly more positive cards and generated the lower Ct values in general (Table 2, Supplementary Table S2 and Figure 2A).

**Table 2.** Qualitative data analysis of the values of the different DNA/RNA eleven releasing methods based on the seven tested FTA cards (the positive methods based on each card type and dilution series are shown, number of PCR positives/number of releasing methods used).

| Card Type | ASFV-qPCR Amplification | | | | | | | | | | IAV-RT-qPCR Amplification | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Direct qPCR | | | | | qPCR after DNA Extraction | | | | | Direct RT-qPCR | | | | | RT-qPCR after RNA Extraction | | | | |
| | Dilution Series | | | | | Dilution Series | | | | | Dilution Series | | | | | Dilution Series | | | | |
| | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | $10^{-4}$ | Sum | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | $10^{-4}$ | Sum | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | $10^{-4}$ | Sum | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | $10^{-4}$ | Sum |
| 1 | 11/11 | 11/11 | 11/11 | 4/11 | 37 | 11/11 | 11/11 | 10/11 | 0/11 | 32 | 11/11 | 11/11 | 2/11 | 0/11 | 24 | 11/11 | 11/11 | 11/11 | 5/11 | 38 |
| 2 | 11/11 | 11/11 | 11/11 | 2/11 | 35 | 11/11 | 11/11 | 6/11 | 1/11 | 29 | 11/11 | 11/11 | 2/11 | 0/11 | 24 | 11/11 | 11/11 | 11/11 | 3/11 | 36 |
| 3 | 11/11 | 11/11 | 10/11 | 3/11 | 35 | 11/11 | 11/11 | 9/11 | 3/11 | 34 | 11/11 | 11/11 | 4/11 | 2/11 | 28 | 11/11 | 11/11 | 10/11 | 2/11 | 34 |
| 4 | 11/11 | 11/11 | 11/11 | 7/11 | 40 | 11/11 | 11/11 | 11/11 | 5/11 | 38 | 11/11 | 8/11 | 4/11 | 0/11 | 23 | 11/11 | 11/11 | 11/11 | 5/11 | 38 |
| 5 | 11/11 | 11/11 | 9/11 | 4/11 | 35 | 11/11 | 11/11 | 8/11 | 5/11 | 35 | 11/11 | 10/11 | 4/11 | 2/11 | 27 | 11/11 | 11/11 | 11/11 | 0/11 | 33 |
| 6 | 11/11 | 11/11 | 11/11 | 4/11 | 37 | 11/11 | 11/11 | 11/11 | 1/11 | 34 | 11/11 | 9/11 | 2/11 | 4/11 | 26 | 11/11 | 11/11 | 7/11 | 0/11 | 29 |
| 7 | 11/11 | 11/11 | 11/11 | 3/11 | 36 | 11/11 | 11/11 | 10/11 | 4/11 | 36 | 11/11 | 10/11 | 2/11 | 3/11 | 26 | 11/11 | 11/11 | 7/11 | 1/11 | 30 |

1 FTA classic card, 2 Indicating FTA Elute card, 3 GenSaver, 4 GenSaver 2.0, 5 Human ID Bloodstain card, 6 COPAN Nucleic card, 7 Nucleo card/Blood sample storage card.

The direct RT-qPCR could detect the IAV genome from all cards using all methods only for the $10^{-1}$ dilution. However, the RT-qPCR after extraction of the viral RNA was also positive for the dilution step $10^{-2}$ for all cards and methods. The increased sensitivity of the RT-qPCR including extraction was further supported by analyses of the $10^{-3}$ and $10^{-4}$ dilutions. The direct RT-qPCR amplification for IAV showed more negative results and higher Ct values compared to the RT-PCR amplification after RNA isolation (Table 2, Supplementary Table S2 and Figure 2B). The FTA classic card and the GenSaver 2.0 card delivered the best qualitative and quantitative results for the IAV-RNA detection.

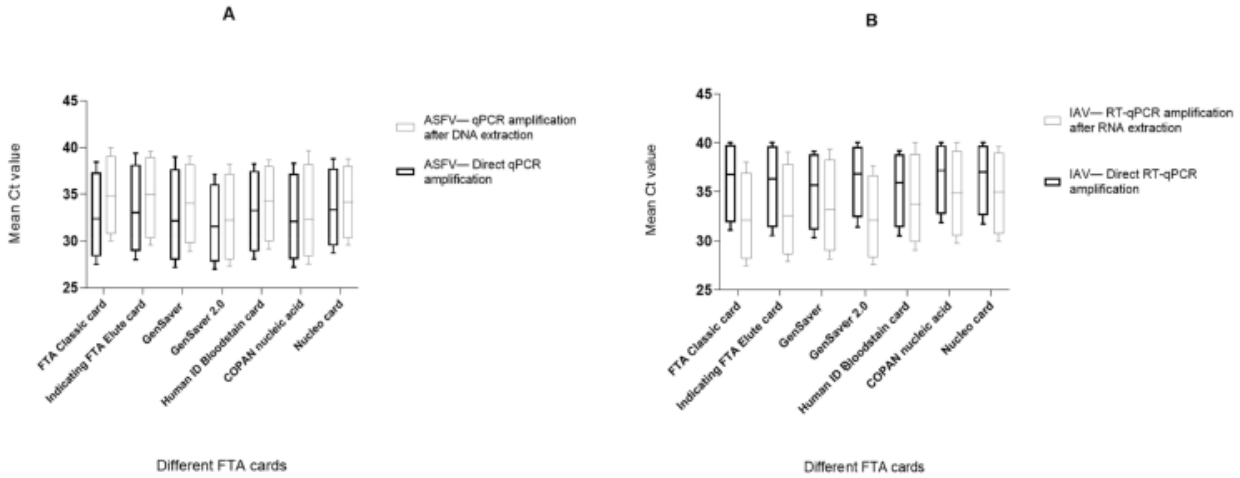


**Figure 2.** (**A**) Direct qPCR amplification with and without nucleic acid extraction for ASFV detection. The mean Ct values based on different FTA cards. SD analysis was carried out (number of replicates = 7, Supplementary Table S2) are shown. Standard deviation (SD) value for all cards by the direct qPCR amplification is 0.78 and for qPCR amplification with extraction is 1.14. The standard error of the mean value for all methods by direct amplification is 0.29 and for the amplification with extraction is 0.43. An unpaired multiple *t*-test was performed to test the significance between the different FTA cards based on both the qPCR amplification direct and with extraction with a resulting *p*-value > 0.99 for the taken values, which is not statistically significant. (**B**) Direct RT-qPCR amplification with and without nucleic acid extraction for IAV detection. The mean Ct values are based on different FTA cards. SD analysis was carried out (number of replicates = 7, Supplementary Table S2) are shown. Standard deviation (SD) value for all cards by the direct RT-qPCR amplification is 0.76 and for RT-qPCR amplification with extraction is 1.74. The standard error of the mean value for all methods by direct amplification is 0.28 and for the amplification with extraction is 0.55. An unpaired multiple *t*-test was performed to test the significance between the different FTA cards based on the both RT-qPCR amplification direct and with extraction with a resulting *p*-value > 0.99 for the taken values, which is not statistically significant.

Finally, the Ct values defined from the different FTA cards were compared to the Ct values from the extracted original samples on the KingFisher system (Supplementary Table S3). Here, the GenSaver 2.0 card demonstrated the smallest ΔCt value of 5.87 after the direct qPCR amplification of ASFV-DNA. In addition, the GenSaver 2.0 card and the FTA classic cards delivered the lowest ΔCt value of 9.43 and 9.48 for RNA amplification after the RNA extraction procedure (Supplementary Table S3).

## 3. Discussion

FTA cards have the advantage of inactivating pathogens and preventing degradation, thus allowing safe transport of the samples and its ability to be mailed as any other document [13]. The feasibility of performing molecular analysis of samples collected on FTA cards has been demonstrated previously [14]. The quality of nucleic acid stored on the cards, the low budget needed for storage and handling, the ease in transporting, and the simple extraction method makes FTA cards a compelling, convenient alternative to traditional methods for the storage and transport of samples [15]. In this study, eleven different methods for the releasing/isolation of DNA/RNA and seven different FTA cards were used and compared for the viral genome detection of ASFV and IAV. There are a variety of purposes for the use of the different FTA cards according to each manufacturer. Some FTA cards are designed for the isolation and purification of nucleic acids, while other cards are consisting of filter papers that are specialized for the collection, transport, and storage of biological samples.

Independent of the different purposes of the cards, our results showed that all types of cards could be used for the isolation of viral DNA and RNA. All tested isolation methods showed comparable yields of DNA via FTA cards for ASFV detection. Among all methods, M2-Eluate 2 (TE buffer + PK + FTA Elute buffer), M3-Eluate 2 (TE buffer + TLR buffer), and

M7-Eluate 2 (TED10 + Chelex® 100 Resin) have represented the best values and sensitivity for the detection of ASFV via direct qPCR. Whereas M1 (FTA purification Reagent + PK), M2-Eluate 2 (TE + PK + FTA Elute buffer), M6-Eluate 2, and M7-Eluate 2 showed the best results for IAV detection. Here, the additional extraction of the viral RNA from the released material will improve the qualitative and quantitative detection of the genome. In general, our work correlates with the work of other groups, which used very often TE buffer for the nucleic acid releasing from FTA cards [12,16,17]. Our study showed that the addition of the virotype Tissue Lysis Reagent (TLR) to TE buffer can deliver sensitive results, especially for DNA releasing. The TLR was originally developed for the fast preparation of various sample types without the need for an extraction kit or any complicated nucleic acid isolation procedures [18]. The study of Rodiño et al. (2016) has ascertained the functionality of using FTA purification Reagent for DNA isolation [13]. Surprisingly, this releasing method and the decreases in Ct values were more notable for the RNA and showed only average results for the ASFV-DNA releasing. Although no significant differences for all the tested releasing methods can be ascertained, the Chelex® 100 Resin methods (M6-E2 and M7-E2) provided excellent results for viral DNA and RNA releasing. The good results were independent of the used amplification method PCR with or without additional extraction. This result was consistent with similar investigations using Chelex® 100 Resin for the development of a direct qRT-PCR for SARS-CoV-2 [19].

Our data support that direct PCR amplification could be suitable for ASFV detection by using FTA cards, which is probably based on the stability of the viral DNA genome of ASFV and the robustness of the used PCR master mix. The direct PCR is a fast, sensitive, and cost-effective method for the detection of ASFV. In contrast, the released viral RNA is less stable than DNA [12] and needs an additional extraction procedure for the inhibition-free RNA isolation.

All used FTA cards could store and release the viral genome of both viruses. After releasing, extraction, and amplification, all cards showed more or less comparable Ct values. The most appropriate cards for ASFV-DNA isolation were the GenSaver 2.0, which was followed by COPAN nucleic acid, GenSaver, and the FTA classic cards, while GenSaver 2.0 and FTA classic cards were more suitable for the IAV detection. Similar results could be ascertained by using FTA classic cards for the detection of both RNA and DNA [12,20]. The feature of using indicating FTA cards could be also demonstrated by the study of other groups [21,22].

It must be noted that the loss on analytical sensitivity by using FTA cards is higher for RNA than DNA approaches. Compared with the liquid original sample material, nearly 5 to 7 Ct values will be lost for the ASFV-DNA amplification via direct PCR after FTA storage and release. In contrast, the best IAV releasing and extraction procedures lost approximately 9 to 11 Ct values compared to the original material. One FTA card spot with a diameter of 2.5 cm and approximately 4.9 $cm^2$ will be spiked with 120 µL of the sample material. Only a small part of the spiked card will be used for further analyses. In our study, we used 3 punches with a diameter of 0.3 cm (each 0.07 $cm^2$) reflecting approximately 0.21 $cm^2$. Thus, the releasing of the nucleic acid from the FTA card represents less than 5% of the original sample. Based on this reflection and the knowledge that the testing of less than 5% of the original sample volume, which would result in an estimated Ct value increase of ≈4.3, it can be concluded that substantial amounts of DNA will be released from the FTA card. The releasing of RNA is markedly decreased compared to the releasing of DNA (at least by a factor of 10), and the reduced recovery will be most likely caused by clogging of the single-stranded RNA in the fiber matrix of the cards based on the complex secondary and tertiary structure. The partial destroying of the viral RNA by the chemicals on the membrane are not likely, because here, substantial differences between the cards and the recovery over the time would be expected.

Based on the data in our study, different nucleic acid releasing methods and commercial FTA cards were successfully applied for the detection of ASFV and IAV. Thus, the FTA card was considered as a reliable diagnostic tool for the storing and extracting of DNA

and RNA viruses. This could be applicable in different labs based on the time and costs saving, while in the field, it also fits the purpose of molecular epidemiology research due to its easy transportation and sampling. The data presented here are based on standardized, experimentally generated samples and are therefore not necessarily the same as samples from the field. Nevertheless, it can be assumed that even if the sample quality is poor, the results of the methods used here should be very comparable. The prerequisite for this is that the FTA cards are used according to the manufacturer's specifications and are not overloaded.

## 4. Materials and Methods

### *4.1. Sample Collection/Viruses*

For the generation of the 10-fold dilution series of ASFV positive sample material, an EDTA blood specimen collected from a domestic pig inoculated with ASFV strain Belgium/2018 was used. The trial was performed for strain characterization and reference material acquisition (approved by the competent authority, Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei (LALLF) Mecklenburg-Vorpommern, Rostock, Germany, under reference number 7221.3-2.011/19). For the 10-fold IAV dilution series, allantoid fluid from eggs infected with *Influenza A virus* (A/Mallard/Germany/2009/H5/N3) was applied. Dilution series have been performed from $10^{-1}$ to $10^{-4}$ for both viruses. Based on the different FTA card types and different DNA/RNA releasing methods, we tested 28 EDTA blood samples for the ASFV detection and 28 allantoid fluid samples for the IAV detection.

### *4.2. FTA Cards*

The following FTA cards were used in the study:

1. FTA classic card (GE Healthcare Life Science-Whatman, Buckinghamshire, UK), an FTA card that is suitable for the isolation, purification, and storage of nucleic acids.
2. Indicating FTA Elute micro card (GE Healthcare Life Science-Whatman, Buckinghamshire, UK), an FTA card that is designed to simplify the handling, processing, and isolation of nucleic acids.
3. GenSaver (Ahlstrom-Munksjö Germany GmbH, Bärenstein, Germany) is a collection card that is suitable for direct amplification from a paper punch/disc, thus eliminating the extraction step.
4. GenSaver 2.0 (Ahlstrom-Munksjö Germany GmbH, Bärenstein, Germany) is a collection card that is designed for the collection, transport, and storage at ambient temperature of DNA from biological fluids. The fiber-based material of these cards is made of pure absorbent fibers impregnated with a property chemical formulation intended to prevent environmentally-induced degradation of long-term ambient preservation of DNA.
5. Human ID Bloodstain card (GE Healthcare Life Science-Whatman, Buckinghamshire, UK) is a card that is made from absorbent filter paper and designed for the collection and transport of blood and bodily fluids. It is appropriate for short-term handling of specimens.
6. Copan nucleic card (Copan Flock Technologies Srl, Brescia, Italy) is designed to collect, transport and store human DNA from buccal cells, saliva, blood, etc. The lysis treatment on the nucleic card allows a direct PCR short-tandem repeats (STR) analysis on a small punch of the card, without the need for the extraction step.
7. Nucleocard is a blood sample storage card (Macherey-Nagel, Düren, Germany) and FTA card that contains an impregnated specialized filter paper and designed for blood storage for subsequent DNA extraction.

First, 120 µL of each sample were spotted on each FTA card type and left for 48 h to be dried. After spotting and drying, the cards were stored at −20 °C to reduce any damage of nucleic acid under ambient conditions [12]. All steps for the nucleic acid releasing from the cards were conducted at room temperature.

*4.3. Nucleic Acid-Releasing Methods*

By using seven different nucleic acid-releasing methods, eleven eluates were created. Four extra eluates (eluate 1) were created from methods 2, 3, 5, and 6. The cause of generating eluate 1 was trying to reduce the time of releasing process and to show if there are variations between eluate 1 and 2. The selected releasing methods were based on the publications of the supplier, published protocols, and our own experiences. In general, 3 punches of 3 mm size were punched out from each FTA card with a Rayher punch pliers 3.0 mm (Rayher Hobby GmbH, Laupheim, Germany) and then transferred into a 2 mL Eppendorf tube. All eleven supernatants were tested directly in the ASFV and AIV real-time PCR. In addition, 100 µL of the releasing supernatant were extracted with the NucleoMagVet kit (Machery-Nagel, Düren, Germany) on the KingFisher Flex extraction system (ThermoFisher, Darmstadt, Germany), followed by qPCR and RT-qPCR. All card types were processed using the different following procedures:

1. Method 1 (M1), Nucleic acids (DNA and RNA) isolation using FTA purification reagent (GE Healthcare Life Science-Whatman, Buckinghamshire, UK) and proteinase K (Indical Bioscience, Leipzig, Germany): Here, 200 µL of FTA purification reagent and 20 µL of proteinase K were added to the FTA card punches. Afterwards, they were vortexed for 15 s, incubated at 1400 rpm in a thermal shaker at 56 °C for 60 min, and then left to be cooled at room temperature for 5 min. After centrifugation at 7000× *g* for 30 s, the supernatant was transferred in a new reaction tube. The output from FTA card pieces was used as a PCR template for the direct qPCR amplification and as an input sample material for the further nucleic acid extraction.
2. Method 2 (M2-E1), Nucleic acid isolation using FTA Elute buffer (Qiagen, Hilden, Germany), Tris EDTA (TE buffer) (Sigma-Aldrich, St. Louis, MO, USA) and Proteinase K (Indical Bioscience): 500 µL of 1× TE buffer (pH 8.0) were added to the FTA punches, vortexed for 5 s, and then, the supernatant was taken and stored as eluate 1 to be used for the further extraction and direct PCR amplification.
3. Method 2 (M2-E2), Following the last step from M2-E1, FTA card punches were washed 2 times with TE buffer, and afterwards, the supernatants were discarded. Then, 400 µL of FTA Elute buffer (Qiagen GmbH, Hilden, Germany) and 14 µL of Proteinase K were added, which was followed by incubation at 1000 rpm, 60 °C for 25 min, and then incubation at 1000 rpm, 90 °C for 5 min in a thermal shaker. After centrifugation at 7000× *g* for 30 s, the supernatant was transferred to a new Eppendorf tube and stored as eluate 2 and used for both nucleic acid extraction and for the direct PCR amplification.
4. Method 3 (M3-E1), Nucleic acid isolation using Tissue Lysis Reagent (TLR) (Indical Bioscience) and TE buffer (Sigma-Aldrich): TLR buffer has been successfully used for the direct RT-qPCR of Bovine viral diarrhea virus genome from ear notch samples [23]. The punches were taken as described before. First, 500 µL of TE buffer were added, vortexed for 5 s, and then, the supernatant was taken and stored as eluate 1 to be used for further extraction and direct PCR amplification.
5. Method 3 (M3-E2), Subsequently, FTA card punches were washed 2 times with TE buffer, and afterwards, the supernatants were discarded. Then, 400 µL of TLR were added, followed by incubation at 1000 rpm, 60 °C for 25 min, and then incubation at 1000 rpm, 90 °C for 5 min in a thermal shaker. After centrifugation at 7000× *g* for 30 s, the supernatant was transferred to a new Eppendorf tube and used as eluate 2 for both nucleic acid extraction and direct PCR amplification.
6. Method 4 (M4), Nucleic acid isolation using TE buffer (pH 8.0, Sigma-Aldrich): 500 µL of TE buffer were added to the FTA punches and then incubated at 1000 rpm, 26 °C for 30 min in a thermal shaker, which was followed by centrifugation at 7000× *g* for 30 s. The supernatant was transferred to a new Eppendorf tube and used for both nucleic acid extraction and for the direct PCR amplification. TE has been successfully used for nucleic acid releasing [12,16,17].

7. Method 5 (M5), Nucleic acid isolation using complete lysis-M reagent (Roche Diagnostics GmbH, Mannheim, Germany): 400 µL of M-lysis reagent were added to the three punches and then incubated at 1000 rpm, 26 °C for 30 min in a thermal shaker, which was followed by centrifugation at 7000× *g* for 30 s. The supernatant was transferred to a new Eppendorf tube and used for both nucleic acid extraction and for the direct PCR amplification. This buffer was still successfully used for the viral RNA releasing from FTA cards [24].
8. Method 6 (M6-E1), Nucleic acid isolation using Chelex® 100 Resin (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and TE buffer (pH8.0, Sigma-Aldrich): 500 µL of TE buffer were added to the punches followed by the incubation at 1000 rpm, 26 °C for 30 min in a thermal shaker, followed by centrifugation at 7000× *g* for 30 s. The supernatant was transferred to a new Eppendorf tube and stored as eluate 1 [19].
9. Method 6 (M6-E2): First, 500 µL of a 5% *w/v* suspension of Chelex® 100 Resin in sterile water were added to the punches, which was followed by incubation at 1000 rpm at 60 °C for 25 min and at 90 °C for 5 min in a thermal shaker. After centrifugation at 20,000× *g* for 3 min, the supernatant was transferred to new Eppendorf tube and then used as eluate 2 for both the nucleic acid extraction and for the direct PCR amplification.
10. Method 7 (M7-E1), Nucleic acid isolation using Chelex® 100 Resin (Bio-Rad Laboratories, Inc, Hercules, CA, USA) and TED10, which consisted of TE buffer including 10% of dimethylsulfoxid (Carl Roth GmbH, Karlsruhe, Germany): This TED10 solution has been used successfully for the effective viral RNA releasing and direct amplification of SARS-CoV-2 [19]. First, 500 µL of TED10 (90% TE buffer + 10% DMSO) were added to the 3 taken punches from each card and then incubated at 1000 rpm, 26 °C for 15 min in a thermal shaker, which was followed by centrifugation at 7000× *g* for 30 s. The supernatant was transferred to a new Eppendorf tube and stored as eluate 1.
11. Method 7 (M7-E2), 500 µL of 5% *w/v* suspension of Chelex® 100 Resin in sterile water were added, which was followed by incubation at 60 °C for 25 min and then at 90 °C for 5 min in a thermal shaker at 1000 rpm. After centrifugation at 20,000× *g* for 3 min, the supernatant was transferred to a new Eppendorf tube and was used as eluate 2 for both nucleic acid extraction and for the direct PCR amplification.

### 4.4. DNA/RNA Extraction

For the magnetic bead-based extraction, the NucleoMagVet Kit (Macherey-Nagel, Düren, Germany) was conducted on the KingFisher Flex System (Thermo Fisher Scientific, Darmstadt, Germany). Briefly, 100 µL of sample volume were added to 100 µL VL1 lysis buffer and processed according to the instructions of the manufacturer. For internal control, 10 µL IC2-DNA/RNA [25] were mixed with 350 µL VEB binding buffer per sample and added to the sample–lysis buffer mixture. After three washing steps, the extracted nucleic acid was eluted in 100 µL elution buffer. The extraction protocol on the KingFisher Flex System needs approximately 20 min for up to 96 samples. Details of the KingFisher run protocol can be provided on request. The extracted template nucleic acids were stored at −20 °C until use.

### 4.5. Real-Time PCR

*African swine fever virus* (ASFV) detection: The ASFV qPCR assay described by Haines et al. [26] was modified by using a lab-specific amplification mix and the integration of a lab-specific internal control system [18]. Very concretely, for the amplification, the PerfeCTa® qPCR ToughMix® Kit from Quanta BioSciences (Gaithersburg, MD, USA) was applied. A FAM-labeled ASFV primer–probe mixtures consisting of 800 nM primer ASFV-p72IVI-F (5′-GAT GAT GAT TAC CTT YGC TTT GAA-3′), 800 nM primer ASFV-p72IVI-R (5′-TCT CTT GCT CTR GAT ACR TTA ATA TGA-3′), and 200 nM probe ASFV-p72IVI-FAM (5′-FAM-CCA CGG GAG GAA TAC CAA CCC AGT G-BHQ1-3′) in 0.1 × TE buffer (pH 8.0) was realized. For the control of extraction and qPCR, a heterologous control system, published

by Hoffmann et al. (2006) [25], was integrated. Here, a HEX-labeled primer-probe-mixture consisting of 200 nM primer EGFP1-F (5′-GAC CAC TAC CAG CAG AAC AC-3′), 200 nM primer EGFP2-R (5′-GAA CTC CAG CAG GAC CAT G-3′), and 200 nM EGFP-probe 1 (5′-HEX-AGC ACC CAG TCC GCC CTG AGC A-BHQ1-3′) in 0.1 × TE buffer (pH 8.0) was prepared. The 12.5 µL total reaction mix was established by 1.75 µL of RNase free water, 6.25 µL of 2 × PerfeCTa qPCR ToughMix, 1.0 µL of ASFV primer probe mix (ASFV-P72-IVI-Mix-FAM), 1.0 µL of the internal control primer probe mix (EGFP-Mix1-HEX), and 2.5 µL DNA template. The following thermo-profile was used for the amplification: 3 min at 95 °C, 45 cycles at 95 °C for 15 s, 60 °C for 20 s, and 75 °C for 20 s. The fluorescence data in the FAM and HEX channel were collected during the annealing step, and the total run time on the CFX96 real-time detection system (Bio-Rad, Hercules, CA, USA) could be ascertained with 1 h and 16 min. For data analyses, the Bio-Rad Maestro software (Version: 4. 1.2433. 1219) was used.

*Influenza A virus* (IAV) detection: Real-time RT-qPCR was performed using the Ag-PathID One-Step RT-qPCR kit (Applied Biosystems, Foster City, CA, USA). The composition of a single reaction of 12.5 µL was as follows: 1.25 µL of RNase-free water, 6.25 µL of 2 × RT-PCR buffer, 0.5 µL of RT-PCR Enzyme Mix, 1 µL of primer–probe mix for the internal control (EGFP-Mix1-HEX) and 1 µL of IAV specific primer–probe mix. Finally, 2.5 µL of RNA template was added. Cycling conditions were 50 °C for 30′, 94 °C for 2′ min, and 45 cycles of 15′′ at 94 °C and 30′′ at 56 °C and 30′′ at 68 °C. Fluorescence was measured during the 56 °C annealing/extension step. Nuclease-free water served as negative control in all experiments. Briefly, the FAM-labeled IAV-NP2 primer–probe mixtures consisted of 600 nM primer NP-1448-F (5′-GGG AGT CTT CGA GCT CTC-3′), 600 nM primer NP-1543-R (5′-GCA TTG TCT CCG AAG AAA TAA GA-3′), and 200 nM probe IAV-NP-1473-FAM (5′-FAM- AAG GCA VCG ARC CCG ATC GTGC-TAMRA-3′), in 0.1 × TE buffer (pH 8.0). For the internal control amplification, the EGFP-Mix1-HEX, as described above, was used.

Dilution series of an ASFV DNA (ASFV Estonia 2014) and IAV RNA (IAV H5N3) standard were applied in each PCR run to confirm the sensitivity and reproducibility of the performed analyses (Supplementary Tables S1 and S2).

### 4.6. Direct qPCR Amplification

The supernatants of the card's punches including the released nucleic acid were added directly to the master mix for PCR analysis without prior processing. For the detection of ASFV and IAV, the same PCR assays and kits were used as mentioned above. For internal process control, the internal control assay was changed. Here, 1.0 µL internal control primer–probe mix (Beta-Actin-Mix2-HEX) was used instead of the EGFP–Mix1–HEX described before. Briefly, HEX-labeled Beta-Actin-Mix2 primer–probe mixtures consisting of 600 nM primer ACT-1005-F (5′-CAGCACAATGAAGATCAAGATCATC-3′), 600 nM primer ACT-1135-R (5′-CGGACTCATCGTACTCCTGCTT- 3′), and 200 nM probe ACT-1081-HEX (5′-HEX-TCGCTGTCCACCTTCCAGCAGATGT-BHQ1-3′), in 0.1 × TE buffer (pH 8.0).

### 4.7. Statistical Analysis

Initial data recording and analyses comparison of mean values and transformation of values (comparison of the mean Ct values for each serial dilution based on the 11 different releasing methods, comparison of the mean Ct values for each serial dilution based on the 7 different FTA cards and estimating the delta Ct values between the released output of the FTA card and the extracted original sample based on the different releasing methods and different FTA cards) were done using Microsoft Excel 2010 (Microsoft Germany GmbH, Munich, Germany). GraphPad Prism 8 (GraphPad software Inc., San Diego, CA, USA) was used for further statistical analyses and graph creation. Statistically significant differences were investigated by the statistical test (unpaired multiple *t*-tests) to test the significance of the results (comparison between direct PCR and extraction and then PCR among the different assays and FTA cards; see Supplementary Table S4). Statistical significance would be defined as $p < 0.05$ with an asterisk (*).

## 5. Conclusions

The use of FTA cards seems to be a feasible and easy way for the storage and transport of biological samples for molecular testing. Although all tested FTA cards and releasing methods tested in this study can be applied successfully for the recovery of ASFV-DNA and IAV-RNA, slight differences exist in the analytical sensitivity of the used cards and releasing methods. Interestingly, the direct PCR of ASFV genome delivered moderately (not statistically significant) lower Ct values than samples that underwent a separate extraction. Given the lack of improvement with a second step, the more efficient lab process of direct PCR is sufficient, and additional steps not warranted for general use. In contrast, only a reduced amount of IAV RNA could be amplified from the FTA card by direct RT-qPCR and an increased sensitivity (although not statistically significant) was noted after performing an additional nucleic acid extraction step. For this reason, depending on the downstream analyses, a secondary extraction step may be required and recommended for the RNA detection. Nevertheless, the molecular analyses of strong positive samples via FTA cards can be a helpful option in the diagnostics of pathogens circulating worldwide.

**Supplementary Materials:** The following are available online at www.mdpi.com/article/10.3390/ijms222312915/s1.

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**Institutional Review Board Statement:** The ASFV animal trial was performed for strain characterization and reference material acquisition (approved by the competent authority, Landesamt für Landwirtschaft, Lebensmittel-sicherheit und Fischerei (LALLF) Mecklenburg-Vorpommern, Rostock, Germany, under reference number 7221.3-2.011/19).

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** The data set used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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## 4.3. Easy Express Extraction (Triple*E*)-A universal, electricity-free nucleic acid extraction system for the lab and the pen

Publication III

**Easy Express Extraction (Triple*E*)-A universal, electricity-free nucleic acid extraction system for the lab and the pen**

Christian Korthase, Ahmed Elnagar, Martin Beer and Bernd Hoffmann

Microorganisms

2022

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*Article*

# Easy Express Extraction (Triple*E*)—A Universal, Electricity-Free Nucleic Acid Extraction System for the Lab and the Pen

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**Abstract:** The complexity of the current nucleic acid isolation methods limits their use outside of the modern laboratory environment. Here, we describe a fast and affordable method (easy express extraction, called Triple*E*) as a centrifugation-free and electricity-free nucleic acid isolation method. The procedure is based on the well-established magnetic-bead extraction technology using an in-house self-made magnetic 8-channel and a rod cover. With this extraction system, nucleic acids can be isolated with two simple and universal protocols. One method was designed for the extraction of the nucleic acid in resource-limited "easy labs", and the other method can be used for RNA/DNA extraction in the field for so-called molecular "pen-side tests". In both scenarios, users can extract up to 8 samples in 6 to 10 min, without the need for any electricity, centrifuges or robotic systems. In order to evaluate and compare both methods, clinical samples from various viruses (African swine fever virus; lumpy skin disease virus; peste des petits ruminants virus; bluetongue virus), matrices and animals were tested and compared with standard magnetic-bead nucleic acid extraction technology based on the KingFisher platform. Hence, validation data were generated by evaluating two DNA viruses as well as one single-stranded and one double-stranded RNA virus. The results showed that the fast, easy, portable and electricity-free extraction protocols allowed rapid and reliable nucleic acid extraction for a variety of viruses and most likely also for other pathogens, without a substantial loss of sensitivity compared to standard procedures. The speed and simplicity of the methods make them ideally suited for molecular applications, both within and outside the laboratory, including limited-resource settings.

**Keywords:** nucleic acid extraction; field application; African swine fever virus; lumpy skin disease virus; peste des petits ruminants virus; bluetongue virus



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## 1. Introduction

Transboundary animal diseases, such as African swine fever (ASF), bluetongue disease, lumpy skin disease and peste des petits ruminants (PPR), result in serious socio-economic consequences for affected countries [1–8]. Thus, early diagnosis and reaction to disease outbreaks are essential to carry out control activities. Rapid and reliable diagnostic tools are of paramount importance for the confirmation of clinical cases and the early implementation of control measures, which is crucial to prevent further spread of the disease [9].

The diagnosis of the above-mentioned infectious diseases can be performed by direct and/or indirect detection of the infectious agents. The molecular diagnostics by polymerase chain reaction (PCR), isothermal amplification, nucleic acid sequence-based amplification or loop-mediated isothermal amplification are widely used as direct detection methods, as listed in the official World Organisation for Animal Health (OIE) manual of diagnostics [10–12]. They are all based on the amplification and detection of viral nucleic acids, so that no pathogens need to be cultivated, and, at the same time, they allow for the relatively rapid confirmation of the disease [13–21].

Nonetheless, all of these methods require reliable and safe nucleic acid purification. These can be either manually or automatically performed. The advantages of automated nucleic acid extraction are the streamlined and efficient extraction process, the possibility of high and scalable throughput, the reduced manual handling with a lower risk of contaminations and a less time-consuming extraction work flow. Therefore, although most laboratories rely on manual DNA or RNA extraction methods, automated nucleic acid extraction has become an attractive alternative to labor-intensive manual methods [22–24]. For this reason, a growing number of automated extraction platforms are available, and there are multiple reports of their use in assays for pathogen detection [25]. In addition to silica-membrane-based column systems, which realize the flow of the sample to be extracted via centrifugation or the application of vacuum, magnetic-bead-based extraction methods have become more and more widely used. In general, all these systems are designed for the extraction of RNA and DNA, and which system to use in a laboratory depends on many factors (number of samples, sample continuity, matrix, human resources, technical equipment, etc.). A trend towards the use of ready-to-use extraction kits and prefilled systems has been observed in recent years. This further harmonizes and standardizes molecular diagnostics. In addition, such standardized kits will speed up the extraction procedure and will reduce the risk of sample contaminations. The efficiency of these kits has already been showed by Schlottau et al. [26], wherein it was described that, in approximately 20 min, up to 96 samples can be easily extracted without cross contamination between samples [27].

Nevertheless, automated extraction systems are often expensive and not available for all laboratories worldwide. In addition, automatic, as well as manual, nucleic acid extraction needs electricity, at least for repeated centrifugation steps. Thus, the development of an affordable, nonelectric device would cover a large diagnostic market area, since underdeveloped countries and low-budget diagnostic laboratories would profit from low-cost but high-quality extraction methods. Furthermore, electricity-free and rapid nucleic acid extraction is a prerequisite for the realization of so-called "molecular pen-side tests", which will combine the sensitivity and specificity of molecular diagnostic tests with the simplicity and speed of antigen-based point-of-care test systems.

In our study, we aimed to establish and validate a rapid, reliable, portable and affordable nucleic acid extraction method, called Triple*E*, which does not require extensive technical skills. Therefore, all the listed advantages make this small and portable device a piece of reliable equipment that can be used in the field as a part of a fast-molecular diagnostic tool. Therefore, it can be used both inside and outside the laboratory, as it can be easily adapted to a wide range of downstream molecular assays. It is considered a simple instrument for nucleic acid extraction without the need for centrifugation steps, which is a significant advantage of this technique over other commercial extraction systems. [28]. For an initial validation, samples of different matrices infected with four viruses of emerging diseases were used. Nucleic acid of two DNA viruses (African swine fever virus (ASFV), lumpy skin disease virus (LSDV)) and two RNA viruses (peste des petits ruminants virus (PPRV) and bluetongue virus (BTV)) were extracted with the novel Triple*E* procedure in comparison to a standard magnetic bead extraction method on an semi-automated KingFisher platform and analyzed in well-established follow-up real-time PCR systems.

## 2. Materials and Methods

### *2.1. Sample Collection and Viruses*

A panel composed of 64 samples was used. (i) ASFV-positive samples ($n$ = 16) consisted of field-collected specimens ($n$ = 12; EDTA wild boar blood samples) that had been submitted to the Friedrich-Loeffler-Institut for disease confirmation and samples ($n$ = 4; EDTA blood samples) obtained from experimentally infected domestic pigs. (ii) LSDV-positive samples ($n$ = 16) were composed of EDTA blood, serum, oral/nasal swabs and crusted skin specimens collected from experimentally infected ruminants. (iii) PPRV-positive samples ($n$ = 16) were composed of oronasal and conjunctival swabs and spleens from experimentally infected goats. (iv) BTV-positive samples ($n$ = 16) were composed of

EDTA blood samples from experimentally infected ruminants. The sample composition is listed in detail in Table S1.

All experimental protocols were reviewed by a state ethics commission and approved by the competent authority (Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei (LALLF) Mecklenburg-Vorpommern, Rostock, Germany). Six different animal trials were conducted at the FLI with the following reference numbers: ASFV Estonia 2014 (M-V/TSD/7221.3-2.011/19), LSDV Macedonia 2016 (M-V/TSD/7221.3-2.1-022/10), LSDV-Nigeria-V281 (M-V/TSD/7221.3-2-004/18), PPRV- Kurdistan 2011 (M-V/TSD/7221.3-1-018/14), BTV-27 and BTV-4 (M-V/TSD/7221.3-1.1-058/10), as well as BTV-33 and BTV-8 (M-V/TSD/7221.3-1-048/19).

### *2.2. Nucleic Acid Extraction*

All samples were extracted twice by each of the magnetic bead-based extraction system. A preliminary step was carried out for all swab-collected specimens. In detail, swabs were collected using FLOQSwabs (Copan, Brescia, Italy), submerged into 2 mL of non-supplemented MEM medium, shaken for 30 min at RT and transferred to 2 mL tubes (Eppendorf, Hamburg, Germany). Samples were stored at 4 °C until extraction. A summary of all four validated extraction methods can be found in the Supplemental Material in Table S1.

#### 2.2.1. KingFisher Flex Extraction System

As standard reference method, the NucleoMagVet kit (Macherey-Nagel, Düren, Germany) on the semi-automated KingFisher Flex platform (Thermo-Fisher-Scientific, Waltham, MA, USA) was applied. Extraction was performed following the manufacturer's instructions. Briefly, 100 µL sample volume was added to the KingFisher 96 deep-well plate, followed by the addition of 20 µL Proteinase K and 100 µL lysis buffer VL1. Subsequently, 350 µL binding buffer VEB and 20 µL NucleoMag B-Beads were added to the sample-lysis buffer mix. After three washing steps, the extracted nucleic acids were eluted in 100 µL elution buffer VEL.

#### 2.2.2. IndiMag 48 Extraction System

A second method was used for comparative purposes, the IndiMag Pathogen Kit on the IndiMag 48 platform was used (Indical Bioscience, Leipzig, Germany), following the instructions described by Elnagar et al. [29].

#### 2.2.3. Easy Express Extraction (Triple*E*) System

Finally, the Triple*E* method was established and validated. This extraction system was composed by a nonelectric extraction procedure using the IndiMag Pathogen Kit (Indical Bioscience).

#### Extraction Instrument

As shown in Figure 1, the Triple*E* procedure was performed by using three hardware components. The first one was the in-house self-made magnetic 8-channel (Figure 1A) which originated from an IndiMag 48 extraction machine and was subsequently modified for manual handling. The second was the IndiMag 48 PW Rod cover as shown in Figure 1B, which was used in combination with the magnetic 8-channel (Figure 1C); finally, the third was an IndiMag 48 PW 24-Sample Block (Figure 1D), wherein the buffers were placed. All three listed components were obtained from Indical Biosciences (Leipzig, Germany).

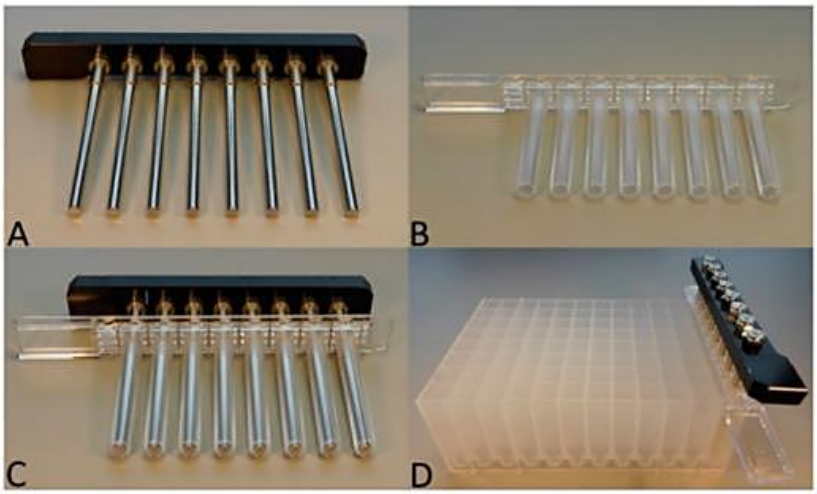


**Figure 1.** Materials used for the Triple*E* system. (**A**) In-house-self-made magnetic 8-channel. (**B**) IndiMag 48 PW Rod cover. (**C**) Magnetic channel combined with rod cover. (**D**) Magnetic-tip comp inserted into the park position in column 12 of the IndiMag 48 PW 24- Sample Block.

Extraction Plate and Buffers

Before the extraction could be started, plates were prepared as follows. IndiMag Pathogen Kit buffers were placed in the IndiMag 48 PW 24-Sample Block (96-deep well plate) as described in Figure 2. Briefly, in column 1, the proteinase K (20 µL/well) was placed. In column 2, the first washing buffer AW1 (500 µL/well) mixed with the magnetic beads (25 µL MagAttract suspension G/well) were located. In columns 3, 4 and 5, three further washing buffers (AW1, AW2 and ethanol (80%)) were placed (500 µL/well). In column 7, the AVE elution buffer (100 µL/well) was filled. The separate location of the elution buffer should minimize the risk of contamination during the extraction procedure. Subsequently, the prefilled 96-deep well plate was covered with a Thermo-Bond Heat Seal foil (Biozym Scientific, Oldendorf, Germany) and heat-sealed for 4 s at 175 °C using the Sally Heat Sealer (Biozym Scientific). Prefilled plates were stored at room temperature (RT) until further use.

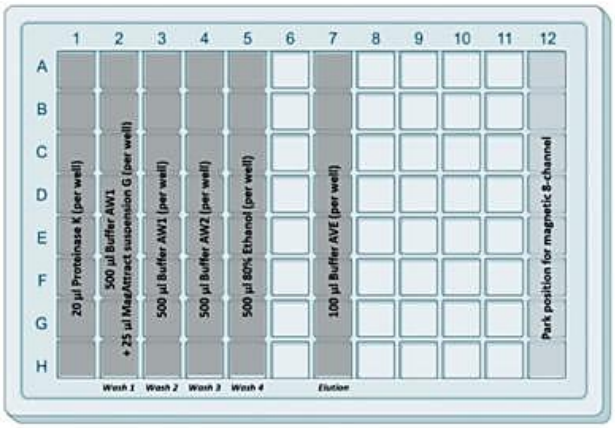


**Figure 2.** Design of the 96-deep well plate with the prefilled reagents. In the first column, 20 µL proteinase K solution was added, and the second column was filled with 25 µL of magnetic beads (Mag-Attract Suspension G) per well. Washing buffers AW1, AW2 and 80% Ethanol were filled in columns 2 to 5 (500 µL/well) as illustrated. Finally, in column 7, 100 µL/well of elution buffer AVE was added. Columns 6 and 8–12 remained empty. (The figure was created with a 96-well square well plate template from BioRender.com with modifications).

Extraction Workflow

Next, the Triple*E* easy-lab workflow was carried out. To this end, the in-house self-made magnetic 8-channel and the rod cover were used either separated or combined during the extraction, depending on the requirements of each step, as illustrated in Figure 3. The following extraction protocol was carried out:

1. *Lysis-binding steps*: A 100 µL sample was placed on 1.5 mL tubes (Eppendorf) prefilled with 100 µL VXL lysis buffer and 400 µL ACB binding buffer (IndiMag Pathogen Kit, Indical Bioscience). Then the 600 µL sample-lysis-binding mix was thoroughly mixed by repeated pipetting and added to the first column (including the Proteinase K) of the prefilled 96-deep well plate. Then, magnetic beads were collected from column 2 with the magnetic channel inserted into the rod cover. This was carried out by dipping up and down the magnetic channel-rod cover up to 10 times (Figure 3—1). Subsequently, the magnetic channel-rod cover with the attached magnetic beads was transferred into column 1 (Figure 3—2), then the magnetic channel was removed and placed the parking position in column 12. Now, the separate rod cover in column 1 was dipped up and down 30 times and was incubated for 3 min at RT (Figure 3—3). Next, the magnetic channel, picked up from the park position, was inserted into the rod cover (Figure 3—4), and the combo was dipped slowly 10 times up and down to collect the magnetic beads again.
2. *Washing steps*: The magnetic channel-rod cover with the attached magnetic beads was inserted into column 2 (Figure 3—5). The washing step was performed by dipping up and down 30 times with the combined magnetic channel-rod cover without the complete releasing of the magnetic beads. Detached beads were recollected by dipping with slower movements 10 times. This latter step was used to catch the maximum number of magnetic beads free in solution. Subsequently, the described washing procedure was applied to the next three washing steps using columns 3, 4 and 5 (Figure 3—6–8).
3. *Elution step*: Finally, the magnetic channel-rod cover with the attached magnetic beads was inserted into column 7 (Figure 3—9) and was dipped up and down 30 times, again followed by a dipping step consisting of 10 slower movements for catching the maximum number of magnetic beads. Thereafter, the rod cover and the attached

magnetic beads were discarded. The ready-to-use nucleic acids remained in column 7 for subsequent real-time PCR amplification or other molecular analyses.

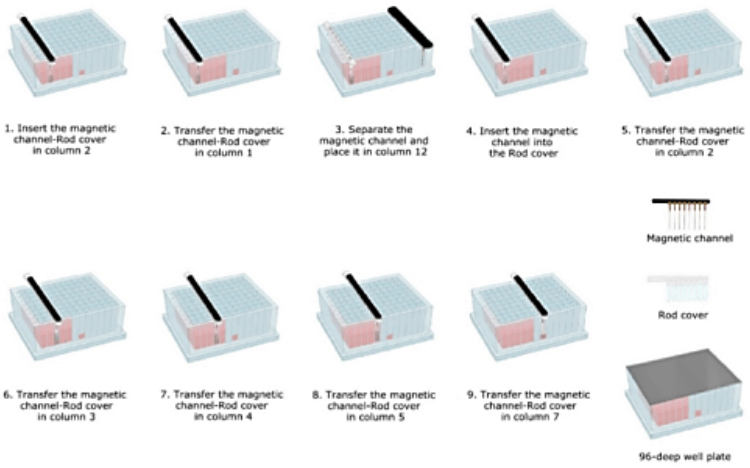


**Figure 3.** Workflow performed for the Triple*E* easy-lab and point-of-care (POC).

The Triple*E* point-of-care (POC) protocol was performed with modified incubation and dipping steps as follows: (i) the incubation time for the binding step was reduced from 3 min to 1 min, (ii) the fast up and down dippings during the washing steps were reduced from 30 to 10 and (iii) the up and down dippings for magnetic bead collection with slower movements were also reduced from 10 to 5 times. All reagents and volumes were applied as described in the Triple*E* easy-lab protocol.

### 2.3. *Real-Time PCR*

All primers and probes used in this study are listed in Table 1. The FAM mix consisted of: 10 µL of each primer (100 pmol/µL), 2.5 µL probe (100 pmol/µL) and 77.5 µL 0.1 × TE buffer (pH 8.0). The HEX ß-Actin mix was composed of 2.5 µL of each primer (100 pmol/µL), 2.5 µL probe (100 pmol/µL) and 77.5 µL 0.1× TE buffer (pH 8.0).

PerfeCTa qPCR ToughMix (Quanta BioSciences, Gaithersburg, MD, USA) was used for ASFV and LSDV nucleic acid amplification. The reaction mix consisted 1.75 µL nuclease-free water, 6.25 µL PerfeCTa qPCR ToughMix, 1 µL FAM mix, 1 µL HEX ß Actin mix and 2.5 µL DNA template. The temperature profile was 3 min activation of Taq polymerase at 95 °C, followed by 45 cycles of 15 s at 95 °C denaturation, 15 s at 60 °C annealing and 15 s at 72 °C elongation. [30–32]. Additionally, RT-qPCR for BTV and PPRV was run with qScript XLT One-Step RT-qPCR ToughMix (Quanta BioSciences, Gaithersburg, MD, USA). Before each RT-qPCR was performed, denaturation of the double-stranded extracted RNA was carried out as previously described [33]. The reaction mix was composed of 1.75 µL nuclease-free water, 6.25 µL qScript XLT One-Step RT-qPCR ToughMix, 1 µL FAM mix, 1 µL HEX ß Actin mix and 2.5 µL RNA template. The temperature profile was 10 min reverse transcription at 50 °C, 1 min activation of Taq polymerase at 95 °C and 45 cycles 15 s at 95 °C denaturation, 20 s at 57 °C annealing and 30 s at 72 °C elongation. All analyses were measured with the CFX96 Real-Time System (BIO-RAD, Hercules, USA). Fluorescence data were collected during the annealing phase, and results were considered positive when Ct values were <45.

**Table 1.** Sequences of primers and probes.

| PCR Assay | Genome Detection of | Primer/Probe | Sequence 5′-3′ | Amplicon (Base Pair) | Reference |
|---|---|---|---|---|---|
| ASFV-P72-IVI-mix | ASFV | ASFV-p72IVI-F<br>ASFV-p72IVI-R<br>ASFV-p72IVI-FAM | GAT GAT GAT TAC CTT YGC TTT GAA<br>TCT CTT GCT CTR GAT ACR TTA ATA TGA<br>FAM-CCA CGG GAG GAA TAC CAA CCC AGT G-BHQ1 | 78 | Haines et al., 2013 [30] |
| Capri-p32-mix | Capripoxvirus | Capri-p32for<br>Capri-p32rev<br>Capri-p32-FAM | AAA ACG GTA TAT GGA ATA GAG TTG GAA<br>AAA TGA AAC CAA TGG ATG GGA TA<br>FAM-ATG GAT GGC TCA TAG ATT TCC TGA T-BHQ1 | 89 | Bowden et al., 2008 [31] modified; Dietze et al., 2018 [32] |
| Pan BTV-IVI-mix | BTV | Orru_BTV_IVI_F2<br>Orru_BTV_IVI_R2<br>Orru_BTV_IVI_FAM | TGG AYA AAG CRA TGT CAA A<br>ACR TCA TCA CGA AAC GCT TC<br>FAM-ARG CTG CAT TCG CAT CGT ACG C-BHQ1 | 97 | OIE terrestrial manual (version May 2021) |
| PPRV-Batten-mix | PPRV | PPRV-N-483F<br>PPRV-N-624R<br>PPRV-N-551FAM | AGA GTT CAA TAT GTT RTT AGC CTC CAT<br>TTC CCC ART CAC TCT YCT TTG T<br>FAM-CAC CGG AYA CKG CAG CTG ACT CAG AA-BHQ1 | 142 | Batten et al., 2011 [34] |
| ß-Actin-DNA-mix 2 | beta-actin mRNA | ACT-1030-F<br>ACT-1135-R<br>ACT-1081-HEX | AGC GCA AGT ACT CCG TGT G<br>CGG ACT CAT CGT ACT CCT GCT T<br>HEX-TCG CTG TCC ACC TTC CAG CAG ATG T-BHQ1 | 106 | Toussaint et al., 2007 [35] modified; Wernike et al., 2011 [36] |

### 2.4. Data Analyses and Statistics

Data were recorded and evaluated using Microsoft Excel 2019 (Microsoft Deutschland GmbH, Munich, Germany). The analytical performance of each extraction method was carried out by comparing the average differences of Ct values using the Bland–Altman test [37]. To this end, this test considers the two extraction systems to be in agreement, if their results fall within the so-called limit of agreement (LoA) interval. This interval was calculated using the mean difference ± 1.96 standard deviation (SD) of the Ct values obtained using both extraction systems.

To test the analytical sensitivity of each method, ten-fold dilution series of virus-positive samples were prepared. For each extraction method, the samples were tested in duplicates and analyzed with the standard (RT)-qPCR assay as described. PCR efficiencies were calculated based on the resulting standard curves. Next, regression analysis and the Pearson's correlation coefficient were calculated to compare the extraction.

GraphPad Prism 9 (Graphpad Software Inc., San Diego, CA, USA) was used for statistical analyses and graph creation.

## 3. Results

### 3.1. Reproducibility of the Extraction Methods

A panel of field- and laboratory-collected samples was used to assess the diagnostic sensitivity of the rapid extraction protocols for ASFV, PPRV, BTV and LSDV. As presented in Table 2, mean Ct values, as well as the intra-run variability of the different extraction systems, showed that all studied methods maintained high reproducibility. The automated Triple*E* extraction instrument delivered comparable results as the commercially available alternatives for the DNA viruses. However, after the extraction and amplification of BTV and PPRV, a difference of approximately 2 Ct values was observed between the automated and manual Triple*E* systems.

**Table 2.** Reproducibility of the standard protocol compared with four extraction protocols. Intra-run variability.

| Virus | Extraction | Mean Ct | SD | CV (%) |
|---|---|---|---|---|
| ASFV | KingFisher Flex | 21.03 | 0.20 | 0.92 |
| | IndiMag 48 | 21.77 | 0.26 | 1.18 |
| | Triple*E* POC | 21.63 | 0.18 | 0.93 |
| | Triple*E* easy-lab | 21.47 | 0.17 | 0.81 |
| LSDV | KingFisher Flex | 24.87 | 0.16 | 0.63 |
| | IndiMag 48 | 24.72 | 0.27 | 0.98 |
| | Triple*E* POC | 25.82 | 0.18 | 0.01 |
| | Triple*E* easy-lab | 25.99 | 0.20 | 0.72 |
| PPRV | KingFisher Flex | 23.87 | 0.14 | 0.59 |
| | IndiMag 48 | 23.44 | 0.18 | 0.77 |
| | Triple*E* POC | 25.23 | 0.30 | 1.13 |
| | Triple*E* easy-lab | 25.28 | 0.15 | 0.56 |
| BTV | KingFisher Flex | 29.14 | 0.33 | 1.12 |
| | IndiMag 48 | 29.07 | 0.50 | 1.72 |
| | Triple*E* POC | 31.55 | 0.41 | 1.29 |
| | Triple*E* easy-lab | 31.51 | 0.51 | 1.59 |

SD = standard deviation; CV% = coefficient of variation.

### 3.2. *Analytical Performance of the Extraction Methods*

The agreement between the KingFisher Flex automated extraction and each of the other used methods was evaluated using Bland–Altman plots (see Figure 4). All recorded row data are summarized in Table S2 of the Supplemental Material.

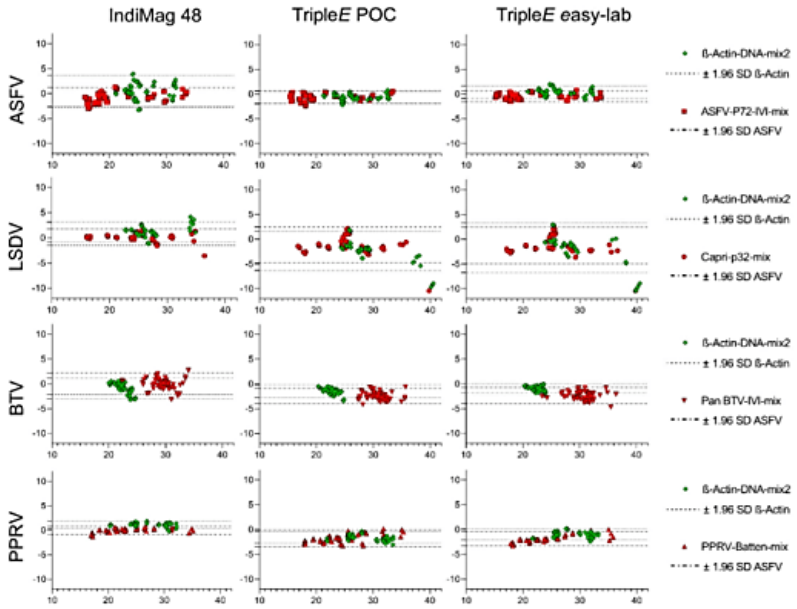


**Figure 4. Evaluation of the analytical performance of the extraction methods.** Bland-Altman plots comparing the KingFisher Flex automated method to the commercially available IndiMag 48 and the hand-made Triple*E* method. The dotted lines represent the limits (upper and lower) of agreement, for both the virus target (red) and the ß-actin (green). The plots show at the Y axis the differences between the Ct values obtained after real-time amplification for each of the evaluated viruses after KingFisher Flex extraction and at the X axis the tested systems against the average of the Ct values detected.

When comparing the ASFV results, a point-by-point comparison showed a low degree of variability with a trend for strong detection in all samples for all devices. Nonetheless, for β-actin detection the bias, i.e., the average discrepancy that could indicate a systematic difference, was highest for the IndiMag 48 extraction method. This observation coincided with wide limits of agreement and several samples outside the limits (see Figure 4).

The LSDV detection was in general successful for all samples using all four methods. Only one sample was borderline detected using the Triple*E* methods, because only three of four results were positive in the qPCR. Furthermore, a wider limit of agreement and some samples outside the limits for Triple*E* systems could be ascertained (see Figure 4). A manual in-detail comparison revealed that samples obtained from swabs were detected with a distinct shift in Ct values for the extraction of the virus. Similarly, the detection of β-actin in these samples showed a wider LoA for Triple*E* methods when compared with IndiMag 48.

Subsequently, BTV-positive blood displayed a very similar outcome when comparing all extraction systems. Samples above a Ct value of 30 exceed the LoA, suggesting that samples up to these values are in agreement and that the results obtained by any of these

extraction methods are comparable. In addition, the β-actin results always lay between the LoA in all systems with almost no discrepancies.

Finally, for PPRV, the Bland–Altman test showed a perfect match, with no LoA exceeded for either the target or the endogenous internal control. Overall, all systems and viruses revealed that, for all sample matrices, with the exception of LSDV-infected swabs, all methods are in agreement with the KingFisher Flex automated method. Hence, no under- or over-estimation of Ct values was detected in this study for any of the hereby tested methods.

### *3.3. Linearity and Analytical Sensitivity of Extraction Methods*

Finally, the analytical sensitivity of the four extraction methods was tested for each virus by performing a ten-fold dilution series of virus-positive samples. Overall, all extraction systems allowed viral detection up to a $10^{-4}$ dilution. Linearity for ASFV and PPRV indicated a 0.99 correlation coefficient, while LSDV and BTV ranged from 0.83 to 0.97 for the Triple*E* system (see Figure 5).

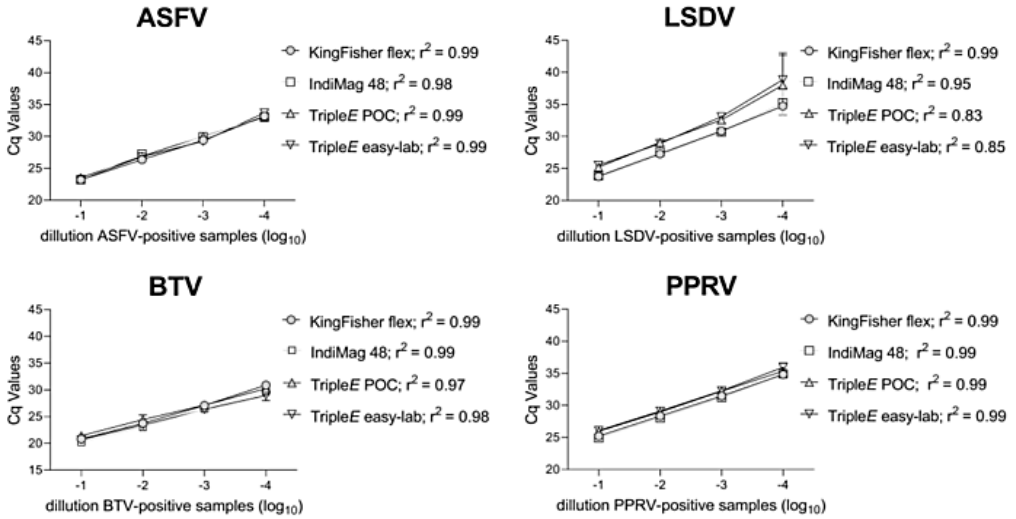


**Figure 5. Linearity and analytical sensitivity of the four different extraction methods.** The ASFV-, LSDV-, PPRV- and BTV-positive samples were diluted ten-fold. Extracted RNA or DNA was quantified as previously described. Regression lines are illustrated and the correlation coefficient given in the legend for each method. POC: Point-of-care.

## 4. Discussion

Animal disease outbreaks of ASF, LSD, bluetongue and PPR have caused suffering, death and economic losses worldwide [1,3,5–7,38,39]. These viral pathogens can threaten global health and food security. Nucleic-acid-based diagnosis has allowed authorities to rapidly react and control the outbreaks; thus, (RT)-qPCR has become the standard molecular-diagnostic tool in many countries [40]. However, nucleic acid extraction remains one of the most important steps leading to a successful diagnosis. Over the past few years, significant progress has been made to simplify and speed up the viral nucleic acid isolation process of different sample matrices [9,26,29,41] Therefore, in this study, three extraction systems were compared with our well-established reference method, the NucleoMagVet kit (Macherey-Nagel) on the KingFisher Flex platform. Beside an alternative automated extraction method, the commercially available IndiMag Pathogen Kit on the IndiMag

48 platform (both from Indical Bioscience, Leipzig, Germany), and two manual extraction systems based on magnetic bead technology, were evaluated for the simple, universal and electricity-free extraction of nucleic acid (called "Triple*E*" (easy express extraction).

Both manual extraction methods use a hand-made, magnetic channel for processing eight samples in parallel. This 8-well magnetic channel, as well as the rod cover and the deep well plate, originate from the IndiMag 48 system. In addition, the reagents of the IndiMag Pathogen Kit were implemented in the manual systems. Differences exist regarding the manual processing of the magnetic beads and two additional washing steps added in the universal, electricity-free extraction procedure. Both of these additional washing steps are not possible for the automated IndiMag 48 systems based on technical limitations. The processing of the magnetic beads by hand overcome this technical limitation and allow the integration of several additional washing steps in the extraction process. Here, we included an additional washing step using the AW1 buffer and a second additional washing step using 80% ethanol for the further reduction of inhibitory factors in the eluate. For the simplification of sample processing, the magnetic channel was not removed from the rod cover during the washing steps. The limited washing effect by the non-released magnetic beads was widely compensated for by the two additional washing steps.

The described both Triple*E* systems (easy-lab and POC) can be also defined as two variants of one extraction method. For easier understanding, both variants/methods were described and analyzed separately in the presented study. The incubation time for lysis and binding, as well as the number of movements for bead washing and bead collection, are different between both methods/variants. The "easy lab" system is recommended for resource-limited labs without the possibility of using a robotic system. The "pen-side" system is further speed-optimized, and the proposed application is molecular testing in the field (pen-side, point-of-care). The 96-well plate can be prepared and stored for a longer time at room temperature. Accordingly, prefilled extraction plates for IndiMag 48 or the KingFisher platform based on the IndiMag Pathogen kit will be offered by Indical Biosciences. Thus, it can be concluded that prefilled Triple*E* plates will be functional for long-term storage and applications in the field.

Samples from a wide range of matrices, hosts and viruses were used for the comparative validation approach, and the NucleoMagVet kit on the KingFisher platform acted as reference extraction system. The functionality and power of the alternative automated system (IndiMag Pathogen kit on the IndiMag 48 robotic platform) for ASFV could be shown previously [29]. Here, we present similar validation data for additional viral pathogens, namely LSDV, BTV and PPRV. The chosen viruses reflect a broad range of types of viral nucleic acid (dsDNA, dsRNA and ssRNA), and, based on the analyzed data, the suitability of the IndiMag system for the genome extraction of different viral genomes could be confirmed. Thus, the IndiMag system represents a useful and practicable alternative automated extraction system, especially if 48 or fewer samples are processed in parallel.

Nevertheless, both automated systems need the robotic platforms and electricity for extraction. For some labs with limited resources, unstable supply of electricity or for molecular analyses in the field, the application of robotic systems can be problematic or impossible. In particular, the further development of molecular pen-side tests requires techniques that are mobile and independent from public electricity. For PCR and isothermal amplification, devices with an integrated battery are still available. An example for such techniques are the Franklin cycler from Biomeme, the Liberty16 from Ubiquitome or the Genie III from Optigene. The simple and mobile, electricity-free or battery-based extraction of nucleic acid is still a bottleneck for the further improvement and acceptance of a molecular pen-side test. Here, the M1 sample preparation cartridge from Biomeme represents a commercially available electricity-free nucleic acid extraction systems, which has been successful applied for molecular pen-side tests of SARS-CoV-2 and other pathogens [42,43]. Nevertheless, the M1 sample preparation cartridge needs 5–10 min for one sample and showed reduced sensitivity for protein-rich samples like serum or blood [43]. Nucleic acid extraction with alternative, but electricity-based, field methods are also described [44,45].

Nevertheless, our Triple*E* systems allow the extraction of up to 8 liquid samples in 5–10 min, independent from the sample matrix, host and most likely the pathogen. The chemical basis is the well-established and FDA-approved IndiMag Pathogen kit, which was developed for the viral RNA/DNA and bacterial DNA extraction from a broad range of veterinary sample matrices, e.g., whole blood, serum, swabs and other body fluids. On the other side, the flexible Triple*E* hardware is universal for magnetic bead processing. Thus, the use of alternative extraction kits (e.g., optimized for food or environmental samples) is in general possible, but the incubation times and magnetic bead dipping movements should be validated accordingly for maximum sensitivity. Ready-to-use reagents and buffers minimize human error and lead to further harmonized and standardized nucleic acid extraction. The kits used in this study ensured reliable and robust DNA and RNA extraction, which is advantageous for laboratories with an adequate budget. Nevertheless, the extraction procedures presented here are open and flexible, and thus the use of inexpensive, homemade nucleic acid extraction buffers is also possible [46].

The presented data showed that the Triple*E* systems, easy-lab and POC could successfully isolate viral DNA and RNA from the four targeted viruses. Qualitatively, all tested samples were successfully detected by PCR, and a good agreement of Ct values between the automated and the manual systems was observed.

NucleoMagVet kit as highlighted by the Bland–Altmann test [37]. However, according to the data presented in this study, one matrix seemed to influence the LSDV positivity of the qPCR results. Swabs obtained from experimentally infected ruminants showed a shift in Ct values for sample detection that exceeded the LoA. The reason for the shift of approximately 2 Ct values for the extraction with the Triple*E* system of LSDV-positive swab samples remains unclear. Nevertheless, for the molecular testing of clinically affected cattle/animals, the reduced sensitivity of 2 Ct values seems acceptable based on the expected high viral genome load in oral and nasal swabs [47].

In terms of reproducibility, the Triple*E* systems showed low intra-run variations when compared with the two automated extraction methods. This fact will be confirmed by a comparison of the data from the internal control assay. Here, the housekeeping gene β-actin was used to ensure the quality of the host genetic material [36]. All automated extraction methods studied here allowed the successful detection of the endogenous internal control in all samples examined. Furthermore, the data of the internal β-actin control of the Triple*E* system show only very slight deviations from the results of the automated extractions. Thus, the functionality of the internal process control is successfully confirmed in the manual procedures, which is of particular importance for virus-negative samples [40,48].

## 5. Conclusions

The automated IndiMag system and the manual Triple*E* provide very comparable results to the NucleoMag/KingFisher system for extraction of viral nucleic acid. The Triple*E* system represents an easy-to-handle manual method for the electricity-free extraction of DNA and RNA in diagnostic laboratories with limited resources. Furthermore, it can easily be implemented in the field for the extraction of nucleic acid using molecular pen-side tests. Our study offers solid data supporting this nonelectric, sensitive and robust extraction method for up to 8 samples in less than 10 min. Direct detection and characterization of pathogens by nucleic-acid-based detection and sequencing techniques will continue to gain importance. The rapid and cost-effective identification of pathogens in endemic, often low-resource countries or directly in the field will play a crucial role for the surveillance and control of animal health worldwide. The Triple*E* system we have developed and evaluated could make a valuable contribution to this.

**Supplementary Materials:** Supplementary materials can be found at https://www.mdpi.com/article/10.3390/microorganisms10051074/s1, Table S1: Summary of all four extraction methods, Table S2: Raw data of RT-qPCR analyses.

**Author Contributions:** Conceptualization, B.H., C.K. and A.E.; methodology, C.K.; validation, B.H., A.E. and C.K.; formal analysis, A.E.; investigation, B.H. and A.E.; resources, M.B. and B.H.; writing—original draft preparation, C.K. and A.E.; writing—review and editing, C.K., A.E., M.B. and B.H.; visualization, B.H.; supervision, B.H.; project administration, M.B. and B.H.; funding acquisition, B.H. All authors have read and agreed to the published version of the manuscript.

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## 4.4. Point-of-care testing for sensitive detection of the African swine fever virus genome

Publication IV

**Point-of-care testing for sensitive detection of the African swine fever virus genome**

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# Point-of-Care Testing for Sensitive Detection of the African Swine Fever Virus Genome

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**Abstract:** African swine fever (ASF) is a contagious viral hemorrhagic disease that affects domestic pigs and wild boar. The disease is notifiable to the World Organization of Animal Health (WOAH), and causes significant deaths and economic losses. There is currently no fully licensed vaccine available. As a result, early identification of the causative agent, ASF virus (ASFV), is crucial for the implementation of control measures. PCR and real-time PCR are the WOAH-recommended standard methods for the direct detection of ASFV. However, under special field conditions or in simple or remote field laboratories, there may be no sophisticated equipment or even stable electricity available. Under these circumstances, point-of-care systems can be put in place. Along these lines, a previously published, rapid, reliable, and electricity-free extraction method (Triple*E*) was used to isolate viral nucleic acid from diagnostic specimens. With this tool, nucleic acid extraction from up to eight diagnostic samples can be realized in one run in less than 10 min. In addition, the possibility of completely omitting viral DNA extraction was analyzed with so-called direct real-time PCR protocols using ASFV original samples diluted to 1:40 in RNase-free water. Furthermore, three real-time PCR cyclers, developed for use under field conditions (IndiField, Liberty16 and UF-300 Genechecker$^{TM}$), were comparatively applied for the sensitive high-speed detection of ASFV genomes, with overall PCR run times between 20 and 54 min. Depending on the viral DNA extraction/releasing method used and the point-of-care cycler applied, a total time for detection of 30 to 60 min for up to eight samples was feasible. As expected, the limitations in analytical sensitivity were positively correlated to the analysis time. These limitations are acceptable for ASFV diagnostics due to the expected high ASFV genome loads in diseased animals or carcasses.

**Keywords:** African swine fever virus; DNA isolation; portable real-time PCR; point-of-care (POC)



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## 1. Introduction

African swine fever virus (ASFV) is the only member of the *Asfarviridae* family and the genus *Asfivirus*. It is a complex double-stranded DNA virus with a size of 170–190 kbp, and around 151 to 167 open reading frames [1]. It is the causative agent of African swine fever (ASF), which only affects *Suidae*. ASF is a fatal disease that can cause death in up to 100% of infected domestic pigs and wild boar of the species *Sus Scrofa* [2]. It has generated enormous economic losses in the pig industry, especially since 2007 [3]. In Africa, argasid ticks of the genus *Ornithodoros* can spread the virus [4], although outside of Africa, transmission via direct contact with infected animals or carcasses is the most relevant way of transmission.

ASF outbreaks in Asia and Europe have killed millions of pigs, and the disease has recently been spreading throughout different countries in Europe (Bulgaria, Czech Republic, Hungary, Moldova and Romani, Belgium, Poland, and since 2020, Germany) [5]. There is currently no effective vaccination or therapy for ASF; hence, the early and swift detection of this virus is critical for any control measures.

Real-time polymerase chain reaction (PCR) is one of the fastest and most sensitive laboratory procedures for detecting pathogen nucleic acid material in clinical samples.

Therefore, conventional and real-time PCR are considered to be reliable methods for ASFV detection [6,7], and are recommended by the WOAH. In addition, PCR has been shown to be an excellent and rapid technique that can be used as a routine diagnostic tool for ASFV in surveillance, control, and eradication programs [6–11].

Point-of-care testing (POCT) has been developed to provide more efficient disease control and a reliable diagnostic tool under special field conditions, without the need to send samples to specialized or central diagnostic laboratories. POCT can be particularly useful for disease diagnosis in remote areas, where infrastructure and laboratory capacity are limited [9].

Rapid antigen detection tests, such as lateral flow devices (LFDs), are easy to use under field conditions, but their current diagnostic performance has not yet been highly standardized, and especially their diagnostic sensitivity is reduced [12,13]. Various approaches have been described for POCT, but the most reliable solution still seems to be genome-based systems, especially in combination with simple extraction procedures. Loop-mediated isothermal amplification assays have the potential for field diagnosis of ASF, but concerns with either their diagnostic performance for clinical samples or their risk of contamination may have limited their wider application [14,15]. The problem of fast, efficient, and electroless nucleic acid extraction in the field must be clarified for all genome-based detection methods. In this context, Korthase et al. (2022) established a rapid and electricity-free extraction method applicable for all POCT that detects pathogen-specific RNA/DNA [16]. A recombinase polymerase amplification (RPA)-based method was reported as a simple, cost-effective, and fast diagnostic tool for rapid and specific detection of ASFV, as described by the study of Wang et al. (2017) [17]. Furthermore, Daigle et al. (2020) described the successful transfer of a highly sensitive and specific laboratory-validated real-time PCR assay to a portable pen-side thermocycler, which can be operated in the field for rapid detection of ASFV following quick manual nucleic acid extraction [18]. Other studies have developed highly sensitive and specific real-time PCR assays that have been validated and used in diagnostic laboratories around the world for the detection of genetic material in clinical samples [19–22]. Briefly, it has been demonstrated that the most reliable solution still seems to be PCR-based methods, especially in combination with simple extraction procedures.

All of these molecular tests could help in epidemiological investigations for diagnosing the disease in remote areas with sparse infrastructure and limited laboratory capacity. In addition, screening of wild boar carcasses directly at the site of discovery could save time and resources [13]. The transport of clinical samples to diagnostic laboratories in remote areas can take a longer time, prolonging the process of diagnosis and delaying the results needed for a rapid response.

The objective of our study was to evaluate molecular diagnostic tools for the so-called point-of-care (POC) concept. For this purpose, suitable methods for rapid and simple ASFV DNA extraction and release were tested, and different real-time POC PCR cyclers were analyzed comparatively. The recently described electricity-free hand extraction method (*Easy Express Extraction*, Triple*E* system) [16], which was able to isolate up to eight samples in less than 10 min, was validated in comparison to an automated routine extraction system that was also based on magnetic bead technology (IndiMag 48).

In addition, direct qPCR—based on the 1:40 dilution of ASFV-positive clinical specimens in water—was also performed. For specific genome detection, three portable real-time PCR thermal cyclers (IndiField from Indical Bioscience, Liberty16 from Ubiquitome, and UF-300 Genechecker$^{TM}$ from Genesystem) were compared with a standard real-time PCR cycler for the laboratory (CFX96 from Bio-Rad). The aim was to analyze the basic suitability of these POC cyclers for the sensitive detection of ASFV genomes under strongly reduced time conditions.

## 2. Materials and Methods

### *2.1. Sample Collection*

The panel consisted of 34 samples from domestic pigs and wild boar that had been collected in four different animal experiments, with ASFV strains of different genotypes. The animals were housed in groups at a high-containment facility in the Friedrich-Loeffler-Institut (FLI) (L3+). The animals were fed a commercial pig food with corn and a hay cob supplement, and had access to water ad libitum. The animal trials were approved by a competent authority (Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei (LALLF) Mecklenburg-Vorpommern, Rostock, Germany) under reference number 7221.3-2.011/19. For the analyses, sample matrices from 22 animals that were infected with several ASF virus strains were used (10 EDTA blood samples (Estonia 2014), one EDTA blood sample (CHZT 90/1), three EDTA blood samples (Belgium 2018), eight EDTA blood samples (SUM 14/11), two lung tissue samples, four spleen tissue samples, four liver tissue samples (all organ samples from a trial with Estonia in 2014), and eight bone marrow tissue field samples). The samples reflected different routine matrices and had been collected at different time points post-infection. Furthermore, eight bone marrow samples were collected from wild boar carcasses from actual outbreaks in Germany. The latter samples were delivered from the state laboratory by the local authority of the outbreak region. Overall, 42 specimens were used in this study (Table S1).

### *2.2. DNA Extraction/Releasing Methods*

The IndiMag 48 platform and an IndiMag® Pathogen Kit (both INDICAL BIOSCIENCE, Leipzig, Germany) were used as the standard automated extraction method for comparative purposes, as described in the study of Elnagar et al. (2021) [23].

Next, compared to the standard automated extraction system, we validated a recently described manual extraction method that does not need any electricity or centrifugation steps, which can therefore easily be performed under field conditions. This Easy Express Extraction (Triple*E*) system represents a fast and affordable magnetic bead-based extraction method that is also based on the IndiMag® Pathogen Kit (INDICAL BIOSCIENCE, Leipzig, Germany). We used this method, as described by Korthase et al. (2022) [16]. Compared to both extraction methods, we conducted direct qPCR amplification of the original materials by its dilution to 1:40 in RNase-free water, mixing it well by pipetting up and down, and subsequently used it directly without further treatment as a PCR template.

EDTA blood was carefully mixed several times before viral DNA extraction/releasing started. The tissue samples were homogenized by grinding approximately 0.5 g of organ tissue with a 5 mm steel ball within 1 mL of cell culture medium in 2 mL bolted tubes that were shaken up and down more than 30 times. The liquid supernatant of the homogenate was used for further processing.

### *2.3. Real-Time PCR Detection Systems*

#### 2.3.1. CFX 96 Standard System

The ASFV qPCR assay described by Haines et al. [11] was modified by the integration of a lab-specific internal control system [24]. For the amplification, a PerfeCTa® qPCR ToughMix® Kit from Quanta BioSciences (Gaithersburg, MD, USA) was applied. Briefly, a FAM-labeled ASFV primer–probe mixture consisting of 800 nM primer ASFVp72IVI-F (5′-GAT GAT GAT TAC CTT YGC TTT GAA-3′), 800 nM primer ASFV-p72IVI-R (5′-TCT CTT GCT CTR GAT ACR TTA ATA TGA-3′), and 200 nM probe ASFV-p72IVI-FAM (5′-FAM-CCA CGG GAG GAA TAC CAA CCC AGT G-BHQ1-3′) in 0.1 × TE buffer (pH 8.0) was realized. For control of the extraction and qPCR, a heterologous control system, published by Hoffmann et al. (2006) [24], was integrated. Here, a HEX-labeled primer–probe mixture consisting of 200 nM primer EGFP1-F (5′-GAC CAC TAC CAG CAG AAC AC-3′), 200 nM primer EGFP2-R (5′-GAA CTC CAG CAG GAC CAT G-3′), and 200 nM EGFP-probe 1 (5′-HEX-AGC ACC CAG TCC GCC CTG AGC A-BHQ1-3′) in 0.1 × TE buffer (pH 8.0) was prepared. Then, 12.5 µL of the total reaction mix was established with 1.75 µL of RNase-

free water, 6.25 µL of 2 × PerfeCTa qPCR ToughMix, 1.0 µL of the ASFV primer–probe mixture (ASFV-P72-IVI-Mix-FAM), 1.0 µL of the internal control primer–probe mixture (EGFP-Mix1-HEX), and 2.5 µL of the DNA template. The following thermoprofile was used for the amplification: 3 min at 95 °C, 45 cycles at 95 °C for 15 s, 60 °C for 20 s, and 72 °C for 20 s. The fluorescence data in the FAM and HEX channels were collected during the annealing step, and the total run time on the CFX96 real-time detection system (Bio-Rad, Hercules, CA, USA) could be ascertained as 1 h and 16 min. For data analyses, Bio-Rad Maestro software (Version: 4.1.2433.1219) was used.

#### 2.3.2. IndiField PCR System

The IndiField PCR system (INDICAL BIOSCIENCE, Leipzig, Germany) is an ultra-portable thermocycler that weighs around 1.4 kg, and has a rechargeable battery with a lifespan of approximately 8 h. It is fully controlled by a smartphone, holds up to nine samples, and has the ability to detect 27 analytes in parallel (three fluorescence channels per well). An IndiField ASFV PCR Kit (INDICAL BIOSCIENCE, Leipzig, Germany) was used, which was prepared as ready-to-use lyophilized reagents in the individual PCR tubes of the IndiField thermocycler. The reaction mix was prepared by adding 20 µL of the DNA template directly to the lyophilized master mix, including the ASFV target assay (FAM channel) and the internal control assay (ROX channel). The PCR data can be uploaded to a cloud-based storage and analysis system. A PCR thermoprofile of 1 min at 95 °C, followed by 45 cycles at 95 °C for 1 s and 60 °C for 20 s, was introduced by scanning the specific QR code on the package of the lyophilized IndiField ASFV PCR Kit [23]. The total run time for this system on the IndiField thermocycler was 56 min.

#### 2.3.3. Liberty16 PCR System

The Liberty16 PCR system (Ubiquitome, New Zealand) is an easy and fast thermocycler (FAM channel only) with an outside dimension of 3.2 kg; it is provided with an internal rechargeable lithium-ion battery. Here, a Biozym Blue Probe qPCR Mix Separate ROX (Biozym, Hessisch Oldedorf, Germany) was used for amplification. A total reaction of 12.5 µL consisted of 2.75 µL of water, 6.25 µL of 2 × Blue Probe qPCR ToughMix, 1.0 µL of the ASFV primer–probe mixture (ASFV-P72-IVI-Mix-FAM), and 2.5 µL of the DNA template. The PCR data do not require a laptop to be run; however, the Ubiquitome iPhone app does need to be downloaded from the App Store. This app allows for setting up the run, viewing the run-in progress, calling Cq dynamic graphing of the annotated real-time PCR amplification curves, and uploading data to share in the cloud. The PCR run was performed via a Bluetooth connection with a thermal profile of 1 min at 95 °C, followed by 40 cycles at 95 °C for 3 s and 60 °C for 3 s. The total run time for this Liberty16 PCR system was 37 min.

#### 2.3.4. Genechecker UF-300 PCR System

The UF-300 Genechecker™ dual channel real-time PCR system (Genesystem Co., Daejeon, Korea) is a compact and intuitive platform (3.3 kg) for point-of-care molecular diagnostics with available dual detection channels (FAM/ROX). For application in the field, the thermocycler can be operated via a vehicle cigarette lighter. The system has a touch panel interface (8 inches) so that users can intuitively set the parameters and instantly run tests. The screen consists of four simple menus, and test protocols can be pre-programmed for immediate startup. The system can finish a PCR run within 20 min. The high ramping rates for heating and cooling are based on a special microfluid PCR chip associated with a compact and sophisticated hardware mechanism. The microfluid chip has a capacity of 10 samples per PCR run. A Biozym Blue Probe qPCR Mix Separate ROX (Biozym, Hessisch Oldedorf, Germany) in a total reaction volume of 10 µL was also applied here for amplification. Finally, 5 µL of 2× Blue Probe qPCR ToughMix, 2.0 µL of the ASFV primer–probe mixture (ASFV-P72-IVI-Mix-FAM), and 3 µL of the DNA template were

mixed for one well. A PCR thermal profile of 1 min at 95 °C, followed by 40 cycles at 95 °C for 3 s and 60 °C for 3 s, was performed. The run time of the UF-300 was 19 min.

## 3. Results

A comparison of the three different nucleic acid extraction/releasing methods and four qPCR systems was performed to acquire a broad applicability range for ASFV DNA isolation and genome amplification in the field (Figures 1–3). All of the tested samples were first extracted with the IndiMag 48 system and amplified with the Bio-Rad CFX96 standard system using the in-house Haines qPCR (Haines assay), in order to generate qualitative and quantitative reference data (Tables S1 and S2). For the POCT, nucleic acid extraction/releasing, the Triple*E* system as well as direct qPCR amplification (samples diluted 1:40 with distilled water) were comparatively tested. In addition, the extracted/released ASFV DNA was tested by applying three different POCT thermocyclers (IndiField, Liberty16, and UF-300).

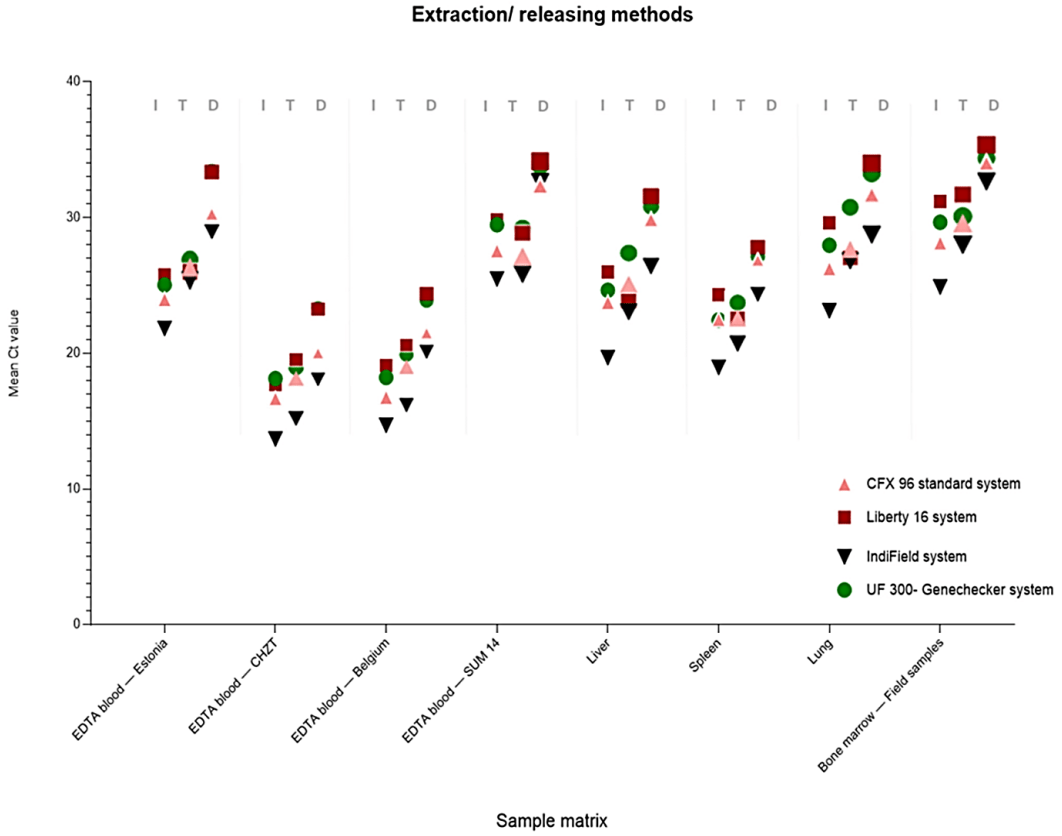


**Figure 1.** Comparison of the four different qPCR systems based on the three different extraction/releasing procedures. (I = standard automated IndiMag 48 extraction system; T = Triple*E* manual extraction system; D = released DNA amplified via direct qPCR). For the analyses, the qPCR results of different sample matrices from 22 animals, infected with several ASF virus strains, were used (see detailed information in the Methods and Materials section).

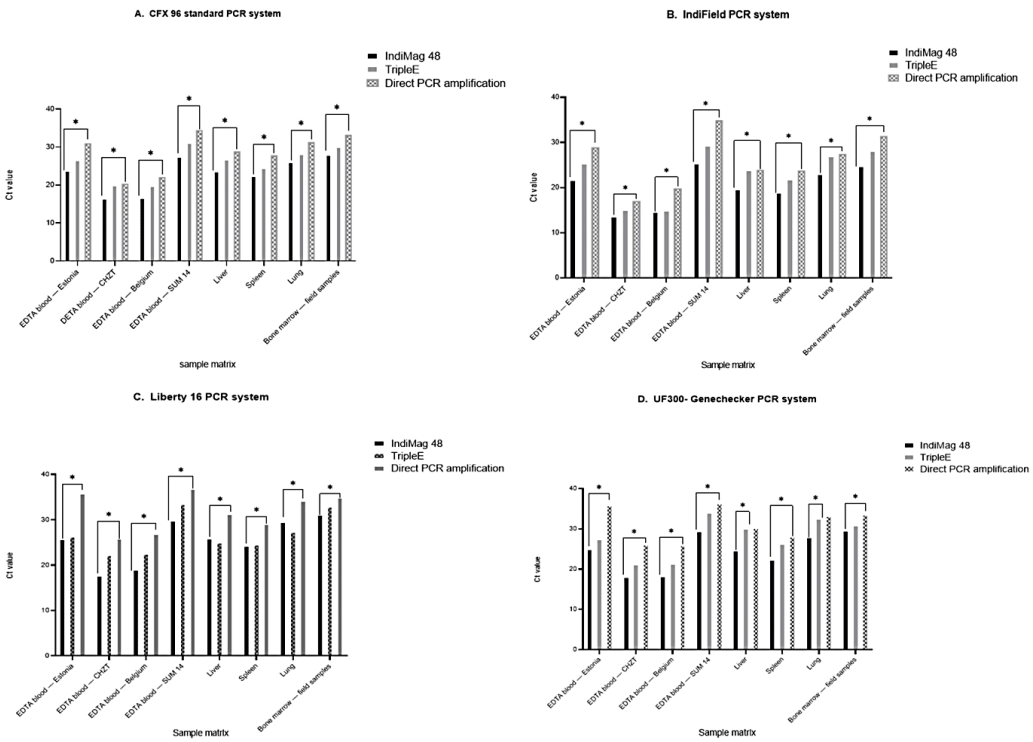


**Figure 2.** Statistical analyses of the different DNA extraction/releasing methods based on different real-time PCR systems. Based on the different sample matrices, an unpaired *t*-test was performed to test the significance of the different extraction/releasing methods. The IndiMag 48 and Triple*E* system showed highly significant Ct values compared to the direct qPCR amplification (* $p < 0.01$). However, there was no significant difference between the two extraction systems (IndiMag 48 and Triple*E*), and this was presented with a *p*-value > 0.99 (ns). (**A**) Standard deviation (SD) analysis was carried out for all DNA extraction/releasing methods, based on the standard CFX 96 PCR system. The SD value for IndiMag 48 was 4.44, 4.26 for Triple*E*, and 5.06 for direct PCR. (**B**) Based on the IndiField PCR system, the SD value for IndiMag 48 was 4.40, 5.55 for Triple*E*, and 5.92 for direct PCR amplification. (**C**) Based on the Liberty16 PCR system, the SD value for IndiMag 48 was 4.95, 4.31 for Triple*E*, and 4.18 for direct PCR amplification. (**D**) Based on the UF 300-Genechecker system, the SD value for IndiMag 48 was 4.59, 4.83 for Triple*E*, and 4.18 for direct PCR amplification.

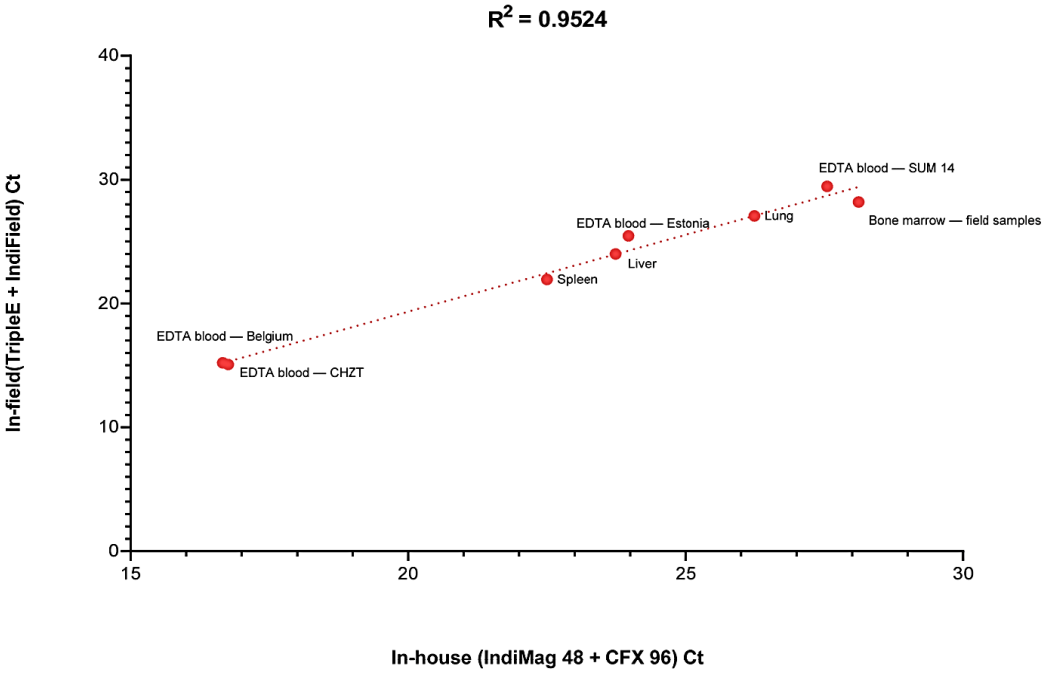


**Figure 3.** Comparison of the mean Ct values obtained from the same samples tested on the portable in-field system (Triple*E* + IndiField PCR system) versus the standard laboratory-based system (IndiMag 48 + CFX 96 PCR system). A Spearman correlation coefficient test was performed using GraphPad Prism 8 (GraphPad Software Inc., San Diego, CA, USA).

### *3.1. Qualitative Data Analysis*

In terms of the Ct values based on the standard PCR amplification on the Bio-Rad CFX 96, we divided the dataset of 42 samples overall into four groups, in order to determine and evaluate the efficacy and sensitivity of each portable PCR thermocycler based on the different extraction methods.

Group I comprised samples with Ct values between 15 and <20 (11 samples); Group II (Ct 20–<25) included 11 samples; Group III (Ct 25–<30) comprised 13 samples; and Group IV included 7 samples with Ct values higher than 30 (Table 1 and Table S1). Qualitative data evaluation based on the different extraction and qPCR methods showed very clearly that positive ASFV detection is dependent on the viral genome load in the different samples. All of the high-load Group I and II samples with Ct values between 15 and 25 could be successfully detected, regardless of the extraction method or the qPCR cycler used. The Group III samples (Ct 25–30) could always be detected when extracted with the IndiMag 48 or Triple*E* extraction system. Even when using direct qPCR, the samples of Group III could be successfully amplified in the vast majority of cases (all samples were detected positive). Only the combination of direct PCR and the Liberty16 cycler yielded a negative result for 4 of the 13 samples of the moderately loaded Group III. A similar result was obtained with the weak positive samples of Group IV (Ct > 30). Here, most of the samples could be successfully detected after extraction with the IndiMag 48 or Triple*E* method (24 and 19 of the 28 samples, respectively). In contrast, ASFV detection of these samples after direct qPCR was positive in only one of the 28 tests (Table 1). An overview about the estimated time of each DNA extraction and PCR amplification run was summarized in (Table 2).

**Table 1.** Qualitative data analysis of the PCR results representing the different extraction methods and qPCR thermocycler. In each column, the number of positive results related to the total number of tested ASFV-positive samples are presented.

| | IndiMag 48 | | | | | Triple*E* | | | | | Direct PCR | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **G I** | **G II** | **G III** | **G IV** | **Total** | **G I** | **G II** | **G III** | **G IV** | **Total** | **G I** | **G II** | **G III** | **G IV** | **Total** |
| CFX 96 | 11/11 | 11/11 | 13/13 | 5/7 | 40/42 | 11/11 | 11/11 | 13/13 | 5/7 | 40/42 | 11/11 | 11/11 | 13/13 | 1/7 | 36/42 |
| IndiField | 11/11 | 11/11 | 13/13 | 7/7 | 42/42 | 11/11 | 11/11 | 13/13 | 5/7 | 40/42 | 11/11 | 11/11 | 13/13 | 0/7 | 35/42 |
| Liberty16 | 11/11 | 11/11 | 13/13 | 6/7 | 41/42 | 11/11 | 11/11 | 13/13 | 5/7 | 40/42 | 11/11 | 11/11 | 9/13 | 0/7 | 31/42 |
| UF-300 | 11/11 | 11/11 | 13/13 | 6/7 | 41/42 | 11/11 | 11/11 | 13/13 | 4/7 | 39/42 | 11/11 | 11/11 | 13/13 | 0/7 | 35/42 |

G I = Group I represents samples with Ct values between 15 and <20; G II = Group II with Ct values between 20 and <25; G III = Group III with Ct values between 25 and <30; G IV = Group IV with Ct values >30 (for detailed raw data, see Table S1).

**Table 2.** Comparison of extraction/releasing time, qPCR run time, and total processing time for the tested extraction/releasing methods and real-time PCR cyclers (in min).

| | IndiMag 48 | | | Triple*E* | | | Direct PCR | | |
|---|---|---|---|---|---|---|---|---|---|
| | **Extraction Time** | **PCR Run Time** | **Total Processing Time** | **Extraction Time** | **PCR Run Time** | **Total Processing Time** | **Releasing Time** | **PCR Run Time** | **Total Processing Time** |
| CFX 96 | 31 | 76 | 107 | 10 | 76 | 86 | 5 | 76 | 81 |
| IndiField | 31 | 54 | 85 | 10 | 54 | 64 | 5 | 54 | 59 |
| Liberty16 | 31 | 37 | 68 | 10 | 37 | 47 | 5 | 37 | 42 |
| UF-300 | 31 | 19 | 50 | 10 | 19 | 29 | 5 | 19 | 24 |

### *3.2. Quantitative Data Analysis*

Based on the standard automated IndiMag 48 extraction system, all data obtained by the four qPCR systems showed comparable results in terms of Ct values, with a slightly higher sensitivity for the IndiField PCR system using the ASFV IndiField PCR Kit. This trend was confirmed with samples extracted with both POC extraction/releasing systems. Using the Triple*E* hand extraction system and the direct qPCR amplification, the quantitative results were similar among all tested samples. The ASFV IndiField PCR also showed the lowest Ct values and highest sensitivity.

Figure 1 shows the comparative mean Ct values for the different sample matrices as a function of the extraction method. The raw data for the analyses were compiled, and are presented in Tables S1 and S2. A one-way ANOVA was performed to test the significance of the different PCR systems being compared, with a resulting $p$-value of >0.99 for all samples taken, which is not statistically significant.

From the analyses, it can be seen that regardless of the ASFV strains or matrices tested, there was very good agreement between Ct values. The direct qPCR amplification presented less sensitivity than the data obtained using extracted DNA for qPCR.

Nevertheless, the highly simplified releasing procedure without any need for extraction was also able to detect the pathogen with acceptable Ct values, especially in samples with high viral loads.

The two different nucleic acid extraction methods delivered very similar results, demonstrating that the electricity-free hand extraction (Triple*E*) could be a very suitable component of the molecular POC testing procedure. Furthermore, the data showed that the two magnetic bead-based extraction methods are quite comparable; however, the automated IndiMag 48 platform had higher sensitivities. In addition, direct qPCR amplification presented a statistically significantly lower sensitivity compared to the IndiMag 48 extraction. Interestingly, the samples were classified correctly with all four different real-time PCR systems (Figure 2).

## 4. Discussion

African swine fever is one of the most serious viral infections of domestic pigs and wild boar, and has a tremendous impact on animal health and the pig industry. Due to the lack of vaccination or treatment options, early detection is of utmost importance to recognize outbreaks and apply control measures as soon as possible [25]. Domestic pigs and Eurasian wild boar show severe clinical manifestations after ASFV infection [26]. Since most clinical signs are very unspecific, laboratory testing is required to corroborate any clinical suspicions [19]. Here, two different DNA extraction systems were evaluated and compared to the performance of extraction-free direct qPCR amplification as a diagnostic tool in the field. Direct qPCR was performed as an alternative to DNA isolation methods using the output of the diluted original samples as a template for PCR amplification. This approach was validated to suit the field application against the various standard DNA extraction methods and PCR systems. It is clear that direct qPCR without prior nucleic acid extraction has limitations, as inhibitors present in the sample can influence the performance of the qPCR. Newly developed master mixes, such as the Biozym Blue Probe qPCR Mix, show improved tolerance to inhibitory substances. Nevertheless, genome amplification from blood is a particular challenge, as hemoglobin is considered to be a potent PCR inhibitor. It should also be noted that the inhibitor tolerance of DNA-dependent DNA polymerases is higher than that of RNA-dependent reverse transcriptases. Thus, the meaningful and successful use of direct qPCR is particularly dependent on the sample matrix, but also on whether (viral) RNA is to be detected in addition to DNA. The limitations of direct qPCR require qualified technical staff, which in turn can be a significant obstacle in the field [27].

These difficulties may be overcome by employing an on-site hand nucleic acid extraction tool. Manual processing of the magnetic beads bypasses this technological barrier, allowing the extraction process to include numerous additional washing steps. The performance of the so-called Triple*E* system has been shown previously [16]. Moreover, other studies have shown that on-site sample preparation extraction systems/kits could be a good option for the diagnostic process in the field [18,28–30], offering the advantage of conducting a fast nucleic acid extraction process that helps in the rapid detection of pathogens. Interestingly, direct qPCR amplification of diluted sample materials using the standard CFX 96 PCR system delivered comparable qualitative results for the tested samples. ASFV genomes were detected via direct PCR amplification in all samples with high to moderate viral loads (Ct < 30). Only samples with low viral genome loads (CT > 30) scored mostly negative results (one positive out of seven samples). Nevertheless, 36 samples (out of 42) were detected as positive overall with direct PCR.

Furthermore, the performance of the standard real-time CFX 96 PCR system was compared with three portable qPCR systems (IndiField, Liberty16, and UF-300 Genechecker) based on the isolated DNA samples. For the IndiField cycler, the commercially available lyophilized PCR reagents and the recommended temperature profiles were used. The lyophilized master mix allows the addition of 20 µL of a DNA template, and the kit-based protocol has a total run time of 56 min. This relatively long PCR run time compared to the other POC cyclers, and the 8-fold amount of template, most likely account for the high analytical sensitivity of this workflow among the other thermocyclers. For the other two POCTs on the Liberty16 and the Genechecker, respectively, the ASF p72 gene real-time PCR assay of Haines et al. (2013) was used [11]. These two portable cyclers were tested with a maximum time-reduced temperature profile (37 min for Liberty16 and 19 min for UF-300). For both workflows, the extracted ASFV DNA was amplified and detected with high sensitivity (at least 39 of the 42 samples were positive) despite the short overall PCR run times. Only direct qPCR of the weakly positive samples showed negative results. Surprisingly, the tube-based Liberty16 system with a 37 min runtime performed slightly worse (31 of the 42 samples were positive) than the extremely fast microchip-based UF-300 system with a 19 min runtime (35 of the 42 samples were positive).

The different data suggested that portable molecular assays can be used to detect ASFV DNA in realistic sample materials almost as efficiently as laboratory-based methods.

It is clear that a maximum reduction in the PCR run time, as well as the lack of elimination of inhibitory factors when using direct qPCR, must lead to a reduced sensitivity of these methods. However, it is also clear that for diagnostics of clinically affected animals, a maximized sensitivity is in most cases not necessary. Here, according to the diagnostic requirements, a compromise between time and sensitivity must be found. Thus, a negative result in POCT must always be critically reviewed, and testing for freedom of disease in herds or individual animals can be generally limited. Nevertheless, the question of individual testing in the field can be answered positively with the best POC method presented here (Triple*E* + IndiField), as it can provide very comparable results compared to the standard laboratory method. These best POCT results were reached with the portable Triple*E* extraction system combined with the IndiField amplification. A high level of correlation ($R^2 > 0.95$) was observed between this POCT workflow and the laboratory-based reference method (IndiMag 48 extraction system followed by amplification on Bio-Rad CFX 96) (Figure 3). Moreover, pre-filled reagents of the Triple*E* extraction system could be stored at room temperature for months, and the qPCR reagents of the IndiField assay are lyophilized, which also eliminates the need for a cold chain here.

Some portable molecular assays for rapid on-site detection of African swine fever (ASF) have been described [12,31–35], and a few have been also evaluated in the field [36]. Other studies have demonstrated the importance of applying POCT as a molecular tool in the field, which may even reduce the workload for central laboratories [18,37]. In accordance with our study, the study of Daigle et al. (2020) showed and confirmed the applicability of a portable molecular assay in the field, which was successfully performed with clinical sample materials [18]. Our field molecular assay offers rapid and sensitive DNA/RNA extraction for eight samples in parallel within 10 min, and the possibility to detect ASFV genomes with different POC cyclers and assays. A portable assay may also be carried out in a small mobile laboratory or in a vehicle, avoiding the need to move instruments inside a possibly infected farm.

The limitations of molecular POCT should be presented with caution, and always in the context of the aims of investigations. The expected high diagnostic specificity of POCT can be used to define positive results with certainty. The reduced analytical sensitivity of POCT compared to routine laboratory-based methods may result in some difficulties for the free testing of samples. However, such weakly positive samples are unlikely to be found in diseased pigs, but may be an issue for wild boar carcasses in poor condition.

In summary, the presented data in this study showed that the universal Triple*E* electricity-free extraction system achieved a similar sensitivity to standard automated extraction such as the IndiMag 48, although the obtained Ct values were slightly lower with the standard method. In addition, ASFV genome detection was significantly less sensitive with direct qPCR amplification. This is evident from the analysis of both qualitative and quantitative PCR data. However, it appeared that direct qPCR amplification could be a sufficiently reliable POCT under certain circumstances (e.g., in clinically diseased animals). For molecular POCT, the portable PCR machines tested here using ultra-rapid temperature profiles are generally suitable, and provide comparable results to Bio-Rad's standard laboratory-based cycler.

## 5. Conclusions

The combination of a portable qPCR system and a manual extraction method resulted in a user-friendly, sensitive, and specific field-deployable diagnostic system. This would help the diagnosis process in remote areas, and could also reduce the amount of field samples that need to be shipped to central laboratories. The application of ASFV-direct qPCR in the field could be an alternative option for samples with high viral genome loads.

**Supplementary Materials:** The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/v14122827/s1: Table S1: Summary of all Ct values as a comparison between the extraction/releasing methods applied on the standard real-time PCR detection system; Table S2: Summary of all Ct values as a comparison between the extraction/releasing methods applied on the different portable real-time PCR detection systems.

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## 5. Discussion

The prevalence of transboundary animal diseases (TADs) has been on the rise in recent years in low-income and developing countries with high animal densities [224], which poses an increasingly global challenge. Globalisation, climate changes and mass production of animals contributed largely to the global spread and endemicity of several viral diseases in different animal production sectors. Some developing countries are affected by contagious animal diseases because of inadequate access to animal healthcare and veterinarians. These TADs caused tremendous socioeconomic losses worldwide, disturbed the international trade of animals and by-products, threatened the global food supply and some were also spread to several highly developed countries as a panzootic (e.g. ASFV or HPAIV H5). Monitoring and diagnosis of animal diseases are the first line of defence to early detect and prevent further spread of the TADS. Nevertheless, many of the current standard laboratory assays are time consuming (e.g., due to processing of samples before diagnosis, inactivation, transportation) or insensitive (e.g. due to interruption of cold-chain, low yield of nucleic acids, viral mutations). This diagnostic gap and resulting underreporting of disease can lead to the spread of new or re-emerging TADs with potentially severe impacts. Therefore, rapid diagnosis in and out of the laboratory has the potential to dramatically change the existing paradigm of animal health surveillance in many countries.

Outbreaks of ASFV, LSDV, BTV, HPAIV, and PPRV have caused suffering, death, and severe economic losses in different animals worldwide [29, 40, 225-229]. The appearance of ASF in the European Union has brought a previously exotic animal disease into the spotlight. Disease control is complicated by e.g., the lack of an effective vaccine. ASF has evolved from an exotic disease from sub-Saharan Africa to a significant threat to the central European swine sector. With the reintroduction of the disease into the European Union in 2014, it seems to have found an ideal breeding ground in the large wild pig population. However, the disease dynamics in north-eastern Europe were different, where long-term endemic cycles without the involvement of domestic pigs developed in all affected countries after the virus was introduced into the wild pig population. Knowledge of significant elements of disease transmission and dynamics is an important step in both risk assessment and developing of

control and contingency strategies. Despite the high virulence of viral strains and high mortality rates in feral pigs, viral life cycles have been self-sustaining for several years. This makes it impossible for competent authorities and veterinary services to make realistic risk assessments or to develop and implement appropriate control measures. Diagnostic tests are widely used in laboratories to determine the presence or absence of current and/or past diseases in various circumstances (i.e., early detection, characterization of disease spread, and follow-up to disease eradication). Due to the increasing prevalence of emerging diseases (with high zoonotic potential) in wild and domestic animals, establishing efficient laboratory services should be a priority before they pose a threat to animal and human health. The EU currently requires member states to be capable immediately responding to a few designated animal diseases (European Regulation (EU) 2016/429), but it is equally important to be prepared for emerging threats in different epidemiological scenarios (European Union, 2016). Therefore, the availability of rapid, specific and sensitive diagnostic methods is of a paramount importance to early detect and control different TADs [230].

### 5.1. Simplifying the molecular diagnostic tools for the sensitive detection of ASFV

In the absence of ASF-specific therapies or effective vaccines, rapid and accurate laboratory diagnosis is critical for rapid intervention and control of disease spread. Reliable diagnostic methods are based on the isolation of viral nucleic acids and PCR from different samples and antibody detection from fluid samples [170]. In EU reference laboratories, molecular diagnostic procedures are mostly based on WOAH-recommended methods (i.e. conventional [86] and real-time PCR systems [84, 85, 88, 89, 167, 231], as well as several commercial ASFV real-time PCR kits). In our study [152], we evaluated and optimized different steps of ASFV diagnosis using qPCR including sample types, DNA extraction methods and qPCR at early and late stage of infections of experimentally or naturally infected pigs. Several DNA isolation techniques (i.e., manual column-based and magnetic beads-based automated extraction systems) and qPCR assays (i.e., EU reference laboratory assay and other commercial real-time PCR systems) were validated and used to detect ASFV genomes. A variety of sample materials (i.e., EDTA blood, serum, oral swabs and chewing ropes) were used, derived from numerous strains (i.e., Estonia 2014, Belgium 2018/1 and other five ASFV isolates from South Africa) and genotypes (i.e., II, IV, XI, XII, XIII and XIX).

Firstly, the suitability of different sample types for detection of ASFV was evaluated. EDTA blood is the best option for ASFV genome detection in both early and late stages of infection in live animals [85, 89, 232]. Serum samples can also be used for detection at the early stages of disease, however with a much lower viral genomic load than EDTA blood. Because of an increasing viremia and spread to other organ systems, ASFV could be detected in alternative samples, such as oral swabs or chewing ropes, in the late stages of infection. Based on the higher genome loads, we found that the spleen is the most suitable material for ASFV detection in post-mortem studies, consistent with the findings of previous studies [233]. In general, EDTA blood or bloody tissue samples are recommended for ASFV detection in experimentally infected animals (**Publication I, Figure 1**).

We further evaluated the methods for extraction of ASFV DNA using column-based techniques, releasing the ASFV genome via Tissue Lysis Reagent buffer (TLR) without an extraction kit, and magnetic bead-based techniques using automated (IndiMag 48 and KingFisher Flex System extraction platforms) or manual approaches. All of the silica membrane manual or magnetic bead-based extraction systems were found to be efficient and sensitive for ASFV-DNA isolation. For the automated magnetic bead-based system, there was no difference in the results between different sample volumes, prefilled or non-prefilled reagents, or different equipment, consistent with the results of Haines et al. [89]. This diagnostic extraction system could therefore be applied to a variety of field sampling procedures and benefit various laboratories. Prefilled reagents, on the other hand, could be used on two different automated systems and offer the advantage of being applied to different ranges of sample materials. In one extraction run based on the availability of different forms of plastic cartridges, an isolation procedure could be performed for samples from 1 to 24 and, during the high-throughput scenarios, up to 96 samples could be processed, allowing flexible usage to suit different users. This promotes the applicability of prefilled reagents in high-throughput environments, such as complimentary or free testing of swine herds in ASFV restriction zones and for the screening purposes. The virotype TLR-mediated release of the viral ASFV genome is slightly less sensitive than conventional silica membrane and magnetic bead-based methods. However, in case of limited or no availability of commercial extraction kits or reagents, the TLR method could be successfully used in a range of diagnostic laboratories. For instance, the COVID-19 pandemic led to a scenarios of

limited extraction kits, in which case the TLR technique could be a suitable option. It has been successfully used for releasing the viral RNA of BVDV from ear notch samples (**Publication I, Figure 2**)[179].

Furthermore, we compared and validated different generic qPCRs for the detection of ASFV DNA using prefilled/lyophilised reagents in different thermocyclers (e.g., Bio-Rad CFX96 PCR system and IndiField thermal cycler). All qPCR assays/techniques, performed with different PCR thermal cyclers, detected the ASFV genome with comparable sensitivity and efficiency (**Publication I, Figure 3**). The IndiField POC thermal cycler combined with the lyophilized kit provided comparable results and showed a higher sensitivity than the standard Bio-Rad CFX96 PCR system. The increased analytical sensitivity of the IndiField PCR system can be most likely determined by the high template volume as recommended from the manufacturer, possibly due to the lyophilized format of the kit. The functionality of the IndiField PCR cycler was confirmed in another study for ASFV genome detection [169]. In general, the use of prefilled (lyophilized) pathogen-specific PCR kits could complement the use of ready-to-use reagents for nucleic acid extraction, further minimizing the risk of contamination, and reduce turnover time. The results showed that the simplification of standard laboratory assays and procedures is possible without significant loss of sensitivity or specificity. During the 2020 ASFV outbreak in Germany, these methods were not only suitable for samples from live animals, but could also be effectively used for various sample materials from wild boar carcasses. The state-of-the-art equipment and user-friendly software, as well as the improved storage and stability provided by lyophilized PCR kits, are not only ‘fit for purpose’ but also reduce training requirements and simplify interpretation of the results. The result is a highly sensitive and specific alternative to conventional PCR methods that is especially valuable for laboratories with limited personnel experience and resources.

## 5.2. Optimization of released nucleic acids from FTA cards for the detection of ASFV and IAV

FTA cards offer the advantage of inactivating microorganisms and preventing nucleic acid deterioration so that samples can be safely transported without affecting the possibility for amplification and sequencing of viral genome [234]. Some FTA cards are designed for separation and purification of nucleic acids, while others consist of filter sheets designed for

collection, transport, and storage of biological material. The practicality of molecular analysis of samples collected on FTA cards has already been demonstrated [235]. The high quality of the nucleic acids contained on the cards, minimal budget required for storage and handling, convenience of transport, and ease of extraction make FTA cards an attractive alternative to standard methods of sample storage and transport. In this work, eleven different techniques for the release/isolation of DNA/RNA and seven different FTA cards for detection of ASFV and IAV viral genomes were used and compared. Our results suggest that direct PCR amplification using FTA cards may be useful for ASFV detection, likely due to the stability of the viral ASFV DNA genome and robustness of the PCR master mix. Direct PCR is a rapid, sensitive, and inexpensive method for detecting ASFV. In contrast, released viral IAV RNA is less stable [242] and requires an additional extraction procedure for robust removal of inhibitors. Reverse transcriptase, necessary for the transcription of viral RNA into cDNA, is much more sensitive to inhibitory substances and these can be eliminated by an RNA extraction process.

We firstly compared the efficiency of different FTA cards from seven suppliers to yield highly sensitive DNA/RNA materials/eluates of ASFV and IAV. All FTA cards used can store and release the viral genomes of the two viruses. We further compared the variation of different FTA cards and found that after elution, extraction, and amplification, all cards showed comparable Cq values. Based on the results, the various nucleic acid release techniques and commercial FTA cards were successfully used to detect ASFV and IAV. Therefore, FTA cards are considered a reliable tool for DNA and RNA virus storage and extraction. This would promote their application in various laboratories due to the time and cost savings, while also meeting the demands for safe storage and transport for field samples (**Publication II, Figure 1 and 2**). The results reported here are based on standardized samples from spiked materials or experimentally-infected animals and may not be identical to field samples. Nevertheless, it is reasonable to assume that our results should be comparable, even with low sample quality. The FTA cards must be used according to the manufacturer's requirements and must not be overloaded. Although all FTA cards and release methods evaluated in this study were effective in recovering ASFV DNA and IAV RNA, there were minor differences in the analytical sensitivity of the cards and methods used. Surprisingly, direct PCR of the ASFV genome resulted in slightly lower Ct values than those extracted separately. Given the lack

of improvement by a second step, the more efficient laboratory procedure of direct PCR is sufficient for general use, and further steps are not required. In contrast, direct RT-qPCR was able to amplify only a small amount of IAV RNA from the FTA card, and higher sensitivity was observed after an additional nucleic acid extraction step. Consequently, depending on the downstream analysis, a separate extraction step for RNA detection may be necessary. Nevertheless, molecular analysis with qPCR of strongly positive samples obtained with FTA cards could be useful for diagnostic procedures in remote locations.

### 5.3. Development of innovative molecular diagnostics in the lab and field for the detection of ASFV and other transboundary animal diseases

One of the most important steps in disease diagnosis is the nucleic acid extraction. In recent years, significant progress has been made in simplifying and accelerating the isolation of viral nucleic acids from various sample matrices [149, 152, 236, 237]. However, the complexity of current nucleic acid isolation procedures limits their use outside the modern laboratory environment. As a centrifugation-free and electricity-free tool, the Triple*E* method provides rapid and consistent nucleic acid extraction for a wide range of viruses while maintaining sensitivity compared to conventional techniques (**Publication III, Figure 1**). Its speed and simplicity make the Triple*E* method ideal for the detection of viral nucleic acids both inside and outside the laboratory, especially those with limited resources.

We compared the Triple*E* system to two well-established methods, a NucleoMagVet kit (Macherey-Nagel) on the KingFisher Flex platform and a commercially available IndiMag Pathogen Kit on the IndiMag 48 platform. The Triple*E* method could be conducted in two manual extraction systems based on the magnetic bead technology (‘Triple*E* easy lab’ for use in various laboratories and ‘Triple*E* POC’ for use in the field). These systems have different incubation times for lysis and binding as well as a different number of steps for washing and collecting of the beads. The ‘easy lab’ solution is recommended for resource-limited laboratories that do not have access to a robotic system. The ‘pen-side/POC’ system is optimized to increase speed and enable application in the field. Four viral pathogens were used for validation: ASFV, LSDV, BTV, and PPRV—representing enveloped and unenveloped viruses and a variety of viral nucleic acid types (dsDNA, dsRNA, and ssRNA). For nucleic acid isolation, the two automated extraction technologies require robotic platforms and

electricity. The pen-side molecular assay requires diagnostic methods that are transportable and platforms with integrated batteries for PCR and isothermal amplification. An example of such a technology is the Franklin cycler from Biomeme or the Liberty 16 from Ubiquitome. Extraction of nucleic acids in a simple transportable battery-assisted manner remains a barrier to the advancement of a molecular pen-side assay. Other commercial rapid extraction techniques exist, such as the Biomeme M1 cartridge for sample preparation, which does not require electricity and has been successfully used as a molecular tool for the diagnosis of SARS-CoV-2 and other diseases [238, 239]. The M1 cartridge, on the other hand, requires 5–10 min for each sample and showed slightly lower sensitivity for serum and blood samples [169].

Nevertheless, our Triple*E* systems enabled the extraction of up to eight liquid samples in 5 to 10 min, regardless of sample matrix, host, and viral pathogen (DNA and RNA). The availability of ready-to-use reagents, which could reduce daily human errors in the laboratory, can further optimize nucleic acid extraction and sample throughput. The results reported in our study show that the two variants of the Triple*E* system provide equivalent results to conventional automated systems with highly comparable sensitivity (**Publication III, Figure 4**).

POCT were created to provide more effective disease control and a reliable diagnostic tool in the field without the need to transfer biological samples to specialized or central diagnostic laboratories. POCT can be very beneficial for diagnosis of viral pathogens in remote areas with limited infrastructure and facilities [87]. Various techniques have been developed for POCT, however, PCR-based systems appear to be the most reliable solution, especially when combined with simple nucleic acid extraction procedures. Triple*E* was optimised for application in the field as a rapid DNA extraction technique for ASFV detection. It was evaluated and compared with the performance of direct qPCR amplification and a standard extraction technique (IndiMag 48). All nucleic acids extracted using the IndiMag 48 magnetic extraction system, Triple*E* system, and direct qPCR amplification of the diluted original samples were first analysed using a Bio-Rad CFX96 system as a standard laboratory-based method and then using three portable field methods/PCR systems (IndiField, Liberty 16, and UF-300 Genechecker) to determine the accuracy and efficacy of ASFV genome detection.

This was demonstrated in the fourth study (under review). The capacity to efficiently extract nucleic acids in clinical samples while eliminating possible PCR inhibitors is crucial for sensitive detection of viral genome DNA in clinical samples; this has been a major difficulty in the field due to a lack of advanced equipment or competent technical staff [240]. Nucleic acids were successfully extracted from all sample types using automated and manual procedures, and Cq values were comparable between the Bio-Rad CFX96 and three portable qPCR systems. Interestingly, direct qPCR amplification produced more or less comparable results, allowing this approach to be used as an alternative molecular diagnostic tool (**Publication IV, Figure 1 and 2**). The qualitative results showed that the ASFV genomes could be detected with higher sensitivity by direct PCR amplification using the standard CFX96 PCR system, when compared to the IndiMag 48 automated extraction system. However, fewer positive samples were detected when amplified using a portable Liberty16 PCR instrument. In terms of nucleic acid extraction/release procedures, all thermocyclers showed relatively similar performance and sensitivity (**Publication IV, Table 1**). Manual processing of magnetic beads bypasses any technological requirements, allowing the extraction procedure to include additional washing steps, as we confirmed in the third study [165]. Several studies [169, 240-242] have found that on-site sample preparation extraction systems/kits could improve diagnostic procedures in the field. This has the advantage of allowing rapid DNA extraction, which is useful for pathogen identification and screening in different populations. However, the method has certain limitations given that only up to eight samples can be processed per extraction run. Compared to the standard system for PCR amplification, the portable PCR equipment showed comparable performance. Portable molecular tests for rapid on-site detection of ASF have been developed [243-246], and a few have been tested [247]. Other studies have shown the value of using pen-side tests in reducing the burden to transport samples to central laboratories [168, 169]. The results of the portable field-testing systems were comparable to the laboratory-based approaches, as measured by the correlation between methods (**Publication IV, Figure 3**). The study might show an improvement in the speed of using different portable molecular assays with a total process time that relies on the extraction technique and the employed thermocycler (**Publication IV, Table 2**). The field molecular assays have the benefit of incorporating a highly sensitive and specific molecular tool into a commercially accessible portable real-time PCR assay for the

quick detection of ASFV. The test sensitivity and specificity were equivalent to those of in-house laboratory-based procedures, indicating that the Triple*E* portable test could be effectively performed in the field without the requirement for advanced technical skills. Furthermore, it seems that direct qPCR amplification could be an appropriate reliable POCT under certain circumstances (e.g., in clinically infected animals). The portable PCR equipment evaluated here employing ultra-rapid thermal profiles are typically acceptable for molecular POCT and provide reliable comparable results to the Bio-Rad standard laboratory-based PCR system.

## 6. Summary

Molecular diagnostics is crucial for effective disease control and surveillance. Based on the socioeconomic importance of viral pathogens, especially after the global pandemic of COVID-19, efforts in the validation and development of molecular diagnostic methods should be more intensive. This is not only important for the detection of viral pathogens, but also bacterial, mycotic, and parasitic organisms. Simplification and standardization of methodology was the first and main objective of our analyses and validations. Hence, our innovative molecular methods have been validated for different emerging viral diseases (ASFV, LSDV, IAV, PPRV, and BTV).

The studies presented aimed to simplify and validate the molecular diagnostic workflow in various laboratory and field-based contexts, including the use of point-of-care testing systems. Point-of-care systems could be used in practice along with a molecular diagnostic tool with acceptable sensitivity and high specificity. Approaches should be user-friendly, with basic protocols, and easily interpretable results available in 30–60 min.

Simple diagnostic tools can be generated by reducing the number of manual steps to avoid contamination. Using ready-to-use reagents and prefilled extraction plates can save time compared to manual extraction procedures and would enhance high-throughput applications. Optimisation of the extraction process was done using an appropriate internal control system for nucleic acid extraction and PCR amplification. This has been developed for the detection of ASFV. Release of DNA/RNA could be performed using virotype tissue lysis reagent (TLR) without an extraction procedure or from FTA cards via different releasing buffers and it has been optimised for the detection of both RNA/DNA viruses (ASFV and IAV).

High-speed qPCR assays, ready-to-use lyophilised reagents, and battery-powered PCR systems combined with a validated hand extraction method (Triple*E*) were successfully applied in laboratory and field applications. In addition, a simple software was used for control, data analysis, and interpretation. After successful validation of the Triple*E* method for the detection of ASFV, LSDV, BTV and PPRV, it was further used as a point-of-care method and validated for the detection of ASFV in combination of different qPCR thermal

cyclers and the use of direct qPCR amplification in the field. Briefly, the combination of a portable qPCR system and a manual extraction approach resulted in a field-deployable diagnostic system for different transboundary animal diseases that is user-friendly, sensitive and specific. This might be beneficial in remote areas and may minimize the number of field samples that need to be sent to central laboratories. Reliable nucleic acid release methods have been verified for a wide range of viral diseases. A variety of diagnostic platforms was validated and a better understanding of how disease control could be improved, even in various resource-limited contexts, was provided. In the future, it would be more significant and beneficial to expand the use of the innovative molecular diagnostic methods for a wide range of different pathogens and more infectious diseases. This would be a powerful tool for fighting against emerging and re-emerging transboundary animal diseases.

## 7. Zusammenfassung

Die Molekulardiagnostik ist für eine wirksame Krankheitsbekämpfung und -überwachung von entscheidender Bedeutung. Angesichts der sozioökonomischen Bedeutung viraler Krankheitserreger, insbesondere nach der weltweiten COVID-19-Pandemie, sollten die Bemühungen um die Validierung und Entwicklung molekularer Diagnosemethoden intensiviert werden. Sie sind nicht nur für den Nachweis von viralen Erregern, sondern auch von bakteriellen, mykotischen und parasitären Organismen wichtig. Die Vereinfachung und Standardisierung der Methodik war das erste und wichtigste Ziel unserer Validierung. Daher wurden unsere innovativen molekularen Methoden für verschiedene neu auftretende Viruserkrankungen (ASFV, LSDV, IAV, PPRV und BTV) validiert.

Die vorgestellten Studien zielten darauf ab, die molekulardiagnostischen Arbeitsabläufe in verschiedenen labor- und feldbasierten Kontexten zu vereinfachen und zu standardisieren, einschließlich der Verwendung von Point-of-Care-Testsystemen. Point-of-Care-Systeme könnten in der Praxis zusammen mit einem molekularen Diagnoseinstrument mit akzeptabler Empfindlichkeit und hoher Spezifität eingesetzt werden. Die Verfahren sollten benutzerfreundlich sein, mit einfachen Protokollen und leicht interpretierbaren Ergebnissen, die innerhalb von 30-60 Minuten vorliegen. Einfache Diagnoseinstrumente können durch die Verringerung der Anzahl manueller Schritte erstellt werden, um Kontaminationen zu vermeiden. Die Verwendung gebrauchsfertiger Reagenzien und vorgefüllter Extraktionsplatten kann im Vergleich zu manuellen Extraktionsverfahren Zeit sparen und würde Anwendungen mit hohem Durchsatz verbessern. Die Optimierung des Extraktionsverfahrens erfolgte unter Verwendung eines geeigneten internen Kontrollsystems für die Nukleinsäure Extraktion und die PCR-Amplifikation. Dieses wurde für den Nachweis von ASFV entwickelt. Die Freisetzung von DNA/RNA konnte mit dem Virotype Tissue Lysis Reagent (TLR) ohne Extraktionsverfahren oder von FTA-Karten über verschiedene Freisetzungspuffer erfolgen und wurde für den Nachweis beider RNA/DNA-Viren (ASFV und IAV) optimiert.

Hochgeschwindigkeits-qPCR-Assays, gebrauchsfertige lyophilisierte Reagenzien und batteriebetriebene PCR-Systeme in Kombination mit einer validierten

Handextraktionsmethode (Triple*E*) konnten erfolgreich im Labor und im Feld eingesetzt werden. Darüber hinaus wird eine einfache Software für die Steuerung, Datenanalyse und -interpretation verwendet. Nach erfolgreicher Validierung der Triple*E*-Methode für den Nachweis von ASFV, LSDV, BTV und PPRV wurde sie als Point-of-Care-Methode weiterverwendet und für den Nachweis von ASFV in Kombination mit verschiedenen qPCR-Thermocyclern und dem Einsatz der direkten qPCR-Amplifikation im Feld validiert. Zusammengefasst führte die Kombination aus einem tragbaren qPCR-System und einem manuellen Extraktionsansatz zu einem vor Ort einsetzbaren Diagnosesystem, das benutzerfreundlich, empfindlich und spezifisch ist. Dies könnte in abgelegenen Gebieten von Vorteil sein und die Anzahl der Feldproben, die an zentrale Labors geschickt werden müssen, minimieren. Zuverlässige Methoden zur Freisetzung von Nukleinsäuren wurden im Rahmen dieser Arbeit für ein breites Spektrum von Viruskrankheiten verifiziert. Es wurden eine Vielzahl von Diagnoseplattformen validiert und ein besseres Verständnis dafür geschaffen, wie die Krankheitsbekämpfung selbst in verschiedenen ressourcenbeschränkten Kontexten verbessert werden kann. In Zukunft wäre es wichtiger und vorteilhafter, die Anwendung innovativer molekularer Diagnosemethoden auf ein breites Spektrum verschiedener Krankheitserreger und weiterer Infektionskrankheiten auszuweiten. Dies wäre ein wirksames Instrument zur Bekämpfung von neu auftretenden und wieder aufkommenden Tierseuchen.

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## 9. Abbreviations

| | |
|---|---|
| AI | Avian Influenza |
| ARBO | Arthropod-borne virus |
| ASFV | African swine fever virus |
| BTV | Bluetongue virus |
| BVDV | Bovine viral diarrhoea virus |
| CD | Cluster of differentiation |
| CHOP | CCAAT/enhancer binding protein homologous protein |
| COVID-19 | Corona virus disease-2019 |
| Cq | Quantification cycle |
| CSF | Classical swine fever |
| CTLs | Cytotoxic T cells |
| DNA | Deoxyribonucleic acid |
| DOL | Dye-labelled oligonucleotide ligation |
| dpi | Days post infection |
| dsDNA | Double-stranded deoxyribonucleic acid |
| dsRNA | Double-stranded ribonucleic acid |
| EC | European Commission |
| EDTA | Ethylenediaminetetraacetic acid |
| EFSA | European food safety authority |
| ELISA | Enzyme-linked immunosorbent assay |
| EU | European Union |

## Abbreviations

| | |
|---|---|
| FAO | Food and Agriculture Organisation of United Nations |
| FAT | Fluorescent antibody test |
| FMD | Foot-and-mouth disease |
| FTA | Flinders Technology Associates |
| GPS | Global positioning system |
| HAD | Haemadsorption test |
| HPAI | Highly pathogenic avian influenza |
| IAV | Influenza A virus |
| IFN | Interferon |
| (K)bp | (Kilo)base pair |
| LAMP | Loop mediated isothermal amplification |
| LFAs | Lateral flow assays |
| LFD | Lateral flow devices |
| LFIA | Lateral flow immuno-assays |
| LSDV | Lumpy skin disease virus |
| MB | Molecular beacons |
| MS | Mass spectrometry |
| NK | Natural killer |
| nm | Nanometre |
| OIE | World Organisation for Animal Health |
| ORFs | Open reading frames |
| PCR | Polymerase chain reaction |
| PERK | Protein kinase R-like endoplasmic reticulum kinase |

## Abbreviations

| | |
|---|---|
| POCT | Point-of-care testing |
| PPRV | Peste des petits ruminants virus |
| PRRS | Porcine reproductive and respiratory syndrome |
| qPCR | Quantitative real-time polymerase chain reaction |
| RNA | Ribonucleic acid |
| RPA | Recombinant polymerase amplification |
| ssRNA | Single-stranded ribonucleic acid |
| TADs | Transboundary animal diseases |
| TNF-a | Tumour necrosis factor-alpha |
| VI | Virus isolation |
| WHO | World Health Organization |

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