Development of a highly reliable Linear-After-The-
Exponential PCR assay for the detection of African Swine
Fever virus

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Abstract
African Swine Fever Virus (ASFV) is a highly pathogenic hemorrhagic DNA virus that infects domestic pigs with mortality rates approaching 100%. The virus is endemic to Sub-Saharan Africa and Sardinia, with outbreaks recently occurring in Russia and Uganda. There is no vaccine or cure for infection and all infected animals, as well as suspected cases of infection, are culled. Currently, the most rapid methods of ASFV detection use symmetric PCR to amplify a small section of the highly conserved B646L (VP72) gene. These assays are specific for ASFV detection, but due to the limitations of symmetric PCR, they do not provide reliable detection of low levels of viral DNA (Pierce et al. 2007). Linear-After-The-Exponential-PCR (LATE-PCR) is an advanced form of asymmetric PCR which overcomes these technical problems, and using this method we have constructed an assay for ASFV that is capable of detecting both very low and very high viral titres. The assay is designed to be used in either a laboratory setting or in the field using the Bio-SeeqII, a portable PCR machine built by Smiths Detection, Inc. (Watford, UK).
1. Introduction

Virus Morphology and Life Cycle

African Swine Fever (ASF) is an economically important, highly lethal disease of domestic pigs that is listed as notifiable to the OIE (World Organization for Animal Health). It is caused by African Swine Fever Virus (ASFV); previously classified as an iridovirus based on its morphology, it is now classified as the sole member within the family Astroviridae (genus Astivirus) [ICTvdB, 2006]. ASFV is similar to other viruses, including iridoviruses and poxviruses [Yañez et al., 1995], but it is the only DNA virus transmitted by bloodsucking arthropods [Dixon et al., 2000]. ASFV virions are composed of a nucleoprotein complex with a double-stranded DNA genome, a matrix domain, a core, an icosahedral capsid and an envelope. They are approximately 200-300 nm in diameter and spherical [ICTvdB 2006].

Most of the replication of the ASFV virion occurs in specialized virus factories located in the cytoplasm [Nufies et al., 1975] and ASFV is therefore classified as a cytoplasmic virus. But, DNA synthesis begins in the nucleus [Garcia-Beato et al., 1992]. It is then transported to the cytoplasmic virus factories where construction of a mature virion continues. Virion production uses energy supplied by host mitochondria that migrate en masse to the viral replication centers in response to early viral signals [Rojo et al., 1998]. Higher virus titres are produced as pathogenicity increases. Thus, the total amount of energy consumed increases with pathogenicity.

The clinical symptoms of ASFV infection are similar to those caused by Classical Swine Fever Virus (CSF), an RNA virus. The two viruses are virtually indistinguishable symptomatically, and diagnosis therefore heavily relies on laboratory tests [Agüero et al., 2004]. Availability of a field-based molecular diagnostic assay for ASFV would significantly shorten the response time to an outbreak and improve the chances of containing the infection.
Epidemiology of ASFV

The first documented outbreak of ASFV occurred in Kenya in 1921 [Montgomery et al., 1921]. The virus has since become endemic to Sub-Saharan Africa and Sardinia with outbreaks periodically occurring throughout Europe [Nix et al., 2006]. The most recent outbreaks occurred in March, 2009 in the Apanesenkovsky district of Stravropol Krai and the Salsky district of the Rostov region of Russia where 45 pigs died and another 34 were culled [Pig Progress, pigprogress.net, 06 Apr 2009]. In areas where the virus remains endemic, ASFV stays active in a sylvatic cycle between species of soft-tick from the genus Ornithodoros and wild suids (wild boars, bushpigs, and warthogs) [Anderson et al., 1998, Boinas et al., 2004, Kleiboeker, et al., 2001, Oura et al., 1998a]. These animals act as reservoirs and are capable of maintaining transmittable virus particles up to 80 days after infection [Anderson et al., 1998].

Transmission of ASFV occurs via three cycles: wild suid to wild suid, soft ticks to wild suids/domestic pigs, and/or wild suids to domestic pigs via direct transmission. Soft ticks of the Ornithodoros species, which are natural, bloodsucking parasites of warthogs, wild boars, and bushpigs, maintain, amplify and transmit the virus when feeding on wild suids and domestic pigs [Anderson et al., 1998]. Transmission may also occur via direct or indirect contact with secretions or excretions from infected pigs or from ingestion of infected pig flesh [Anderson et al., 1998, Boinas et al., 2004, Kleiboeker et al., 2001, Oura et al., 1998a]. ASFV is very stable in the environment and capable of remaining active in excretions, pork products and tissue samples for long periods of time, making it difficult to eradicate [McKercher et al., 1978, Plowright et al., 1987].

Pathogenesis of ASFV

Different strains of ASFV exhibit varying levels of pathogenicity, ranging from highly pathogenic (infected individuals acquire peracute and acute infections) to low pathogenic (infected individuals acquire subacute and chronic infections or remain
asymptomatic) [Boinas et al., 2004]. Infection with peracute or acute strains of ASFV may cause death before symptoms appear or antibodies can be made [Dixon et al., 2004]. Symptoms of infection include lethargy, weakness, fever, vomiting, hemorrhage of multiple organs (liver, kidneys, spleen), skin necroses, and death.

There is an extreme difference in the infectious dose and lethal dose required of the different strains. For highly pathogenic strains, the amount of virus needed to cause infection (infectious dose) is ≤10 virus units and all infected animals die (the infectious dose is equal to the lethal dose) [Pan et al., 1984]. Viruses that are mildly pathogenic are equally infectious (≤10 virus units required), but the lethal dose is 2-50x greater (20-562 virus units required) than highly pathogenic strains. Strains with low pathogenicity require an infectious dose of 56-10,000 virus units and a lethal dose of 56,200 – 3,120,000 virus units [Pan et al., 1984]. These strains may cause infection, but death occurs much later, if at all. These differences in infectious and lethal dose in part explain the differences in morbidity and mortality caused by the various strains. Host response, however, has proven to be extremely complicated and variable. Different pigs infected with the same low pathogenic strain can either remain asymptomatic or show signs of chronic infection [Leitão, et al., 2001]. It is still unclear what host factors are responsible for this difference in response.

Currently there is no vaccine, treatment or cure for African Swine Fever and animals infected or suspected of infection are slaughtered. The ease and rapidity with which the virus spreads and kills make early, accurate detection necessary.

**Viral Genome and Key Players**

The first full ASFV genome was published by Yañez et al. in 1995 and was based on the non-pathogenic strain, Ba71V [Yañez et al., 1995]. Since then, nine more complete genomes have been sequenced [Chapman et al., 2008, Kutish et al., 2004 unpublished data] The ASFV genome is linear, non-segmented, 170kb to 190kb in length [Blasco et al.,
1989b] and contains 151 to 165 open reading frames (ORF), depending on the strain [Blasco et al., 1989a, Kleibocker et al., 2001]. The genome is relatively conserved from strain to strain, but contains three main variable regions: a central variable region (CVR), which shows the highest variability [Nix et al., 2006] and two terminal variable regions [Sumption et al., 1990, Blasco et al., 1989a,b]. The terminal variable regions make up the left 35kb and right 15kb ends of the genome and contain genes responsible for the virus host range and virulence [Blasco et al., 1989a,b, Burrage et al., 2004, Neilan et al., 2002, Sumption et al., 1990, Zsak et al., 1996, Zsak, et al., 2001]. There are many key genes located in these regions, including members of the multigene families (MGF) 360 and 530, as well as the UK and NL genes [Zsak et al., 2001, Zsak et al., 1996, Zsak et al., 1998], which play a role in determining virulence. Removal of the UK and NL genes is insufficient to attenuate all pathogenic ASFV strains, but removal of an 8kb region including several MGF 360 and 530 genes, has been shown to reduce the pathogenicity of some strains [Neilan et al., 2002].

One gene in particular is very highly conserved across all strains. The B646L gene (aka VP72), encodes the major capsid protein p72 [Bastos et al., 2003, Bastos et al., 2004]. This gene is almost 100% conserved across strains, with a C-terminal variable region used for genotyping. So far, 16 major genotypes have been determined using p72 characterization [Lubisi et al., 2005]. Genotyping of the B602L gene, located in the CVR, has further been used to identify subtypes of closely related ASFV strains in a combined p72-CVR approach [Lubisi et al., 2007, Bastos et al., 2004]. Current detection assays for ASFV target the VP72 gene based on its high level of conservation across strains.

**Immune Response and Immune Evasion**

The ASFV virus can infect multiple cell types, but cells of the monocyte-macrophage lineage are the first, and most severely affected [Wardley et al., 1977]. Early infection of the macrophage is characterized by up-regulation of pro-inflammatory
cytokines, including IL-6, TNF-α, and IFN-β, as well as chemokines of the CC and CXC groups [Zhang et al., 2006]. This initial up-regulation triggers the recruitment and activation of other inflammatory cells. Early transcriptional activation of pro-inflammatory genes is suspected to play a role in cell to cell virus transmission [Wardley et al., 1977]. The initial burst of cytokine and chemokine activity, however, is short-lived. During the later stages of infection, ASFV down regulates macrophage cytokine and chemokine transcription (and therefore inflammatory responses) [Powell et al., 1996]. The genes involved include the A238L gene, which inhibits pro-inflammatory NFκB-dependent gene transcription, and many members of MGF 530 and 360 [Powell et al., 1996]. The down-regulation of pro-inflammatory pathways, along with other mechanisms, helps prevent apoptosis of infected cells [Dixon et al., 2004]. ASFV is also capable of inducing apoptosis in nearby cells [Oura et al., 1998b, Salguero et al., 2002, Zhang et al., 2006]. The ASFV immune evasion techniques lead to a general decrease in B and T-lymphocyte responses, NK activity and the phagocytic activity of macrophages. ASFV is also a hemorrhagic virus. Transcription of the 8-DR gene produces a protein similar in structure and function to the CD2 molecule located on the surface of T lymphocytes [Borca et al., 1998]. This gene product causes adhesion of erythrocytes to infected cells, aiding in virus transmission and later leading to destruction of the red blood cells [Borca et al., 1998].

The immune response to ASFV infection varies from host to host. A study by Leitão et al. (2001) showed that a general increase in Natural Killer cell (NK) activity occurs in response to infection with low pathogenic virus [Leitão et al., 2001]. However, the immune response is not consistent. Domestic pigs infected with the same titre of the same low pathogenic virus often experience different clinical symptoms. One group in the Leitão study developed chronic infection, showing an increase in plasma Ig concentrations and anti-ASFV specific antibodies, but a decrease in NK activity. The other group (asymptomatic) had an increase in NK activity but normal Ig levels and low levels of anti-ASFV specific antibodies. Only the latter group developed immunity to a high-pathogenic
strain [Leitão et al., 2001]. Other studies show that an ASFV specific cytotoxic CD8+ T-lymphocyte response to infection with a low pathogenic strain can lead to immunity against a highly pathogenic strain [Martins et al., 1993, Oura et al., 2005]. Primary infection with a highly pathogenic strain often causes death before antibodies can be produced or an effective immune response can be generated.

ASFV is extremely difficult to eradicate due to its capability to survive outside of the body for long periods of time. This presents a danger even after removal of infected pigs. Individuals who have contracted and survived mildly pathogenic strains of ASFV often have persistent infection but acquire immunity to other pathogenic strains. Those who contract and survive a non-pathogenic strain also gain protective immunity to pathogenic strains [Leitao et al., 2001, Onisk et al., 1994, Zsak et al., 1993].

Detection Methods

The severity of the ASFV infection and lack of a cure or vaccination makes early detection necessary to prevent further spread and devastation. Current detection methods require the use of expensive, non-portable laboratory equipment and are often time consuming. This thesis describes the construction of a rapid, reliable, and sensitive assay for ASFV detection that can be used either in a laboratory setting or in the field on the Bio-SeeqII, a portable, battery operated instrument from Smiths Detection, Inc. (http://www.smithsdetection.com/eng/1025_4312.php), that has been designed for use by veterinarians with a minimum of training.

Several methods have been described for the detection of ASFV [Agüero et al., 2004, Alcaraz et al., 1990, Giammarioli et al., 2008, Hutchings et al., 2006, King et al., 2003, Malmquist et al., 1960, McKillen et al., 2007, Yves et al., 1992, Zsak et al., 2005]. Most of these assays produce accurate results within twenty-four hours, including sample preparation and virus detection. One method currently being used detects ASFV antigens via enzyme-linked immunosorbent (ELISA) assays [Alcaraz et al., 1990, Hutchings et al.,
2006]. These assays use an enzyme-linked antibody to detect specific antigens on the ASFV virion, especially viral proteins p243, p172, p73, p25.5, p15, and p12 and the infection proteins p30 and p23.5, p25, and p21.5 [Alcaraz et al., 1990]. Though ELISA assays are still used for laboratory testing, quicker, more reliable methods involve symmetric polymerase chain reaction (PCR).

PCR-based assays developed to detect ASFV target the highly conserved p72 region of the genome [Agüero et al., 2004, Bastos et al., 2003, Boshoff et al., 2007, Giammarioli et al., 2008, King et al., 2003, Lubisi et al., 2005, Lubisi et al., 2007, McKillen et al., 2007, Wambura et al., 2006, Yves et al., 1992]. The assay currently used by most reference laboratories (National Veterinary Institute, Uppsala, Sweden) was developed by King et al. (2003). It is a closed-tube, TaqMan® PCR assay designed to amplify a short region of the VP72 gene. Two TaqMan fluorescent probes are used to detect the ASFV VP72 sequence and an internal control. This assay provides detection of ASFV DNA.

![Figure 1: King. et. al. (2003) Comparison of the results obtained by the TaqMan® PCR and the OIE PCR method. Note: In both cases the yield of specific products diminishes significantly as the stock of viral DNA is diluted.](image-url)
within 24 hours of sample receipt [King et al., 2003]. While the assay provides a quick method of detection relative to antibody tests, the use of a TaqMan® probe system dependent on 5’ nuclease activity requires probes to be of relatively high temperature, meaning probe binding potentially competes with primer annealing, reducing the efficiency of the assay [Pierce et al., 2007]. This would be most noticeable at low concentrations of target and could account for the drop in fluorescence seen in the current reference assay (Figure 1) [Figure 4 in King et al. 2003]. As amplification goes on, large amounts of double-stranded DNA are produced, which can also act as competition for the TaqMan® probes, meaning the signal achieved may only reflect partial probe binding [Pierce et al., 2007]. This thesis provides a new, more fruitful method of detecting ASFV DNA using fluorescent probes in an asymmetric PCR assay.

Asymmetric PCR is the use of two primers of unequal concentration. The primer with the lower concentration, the Limiting Primer (LP), is used up in the reaction, thereby limiting the amount of double stranded DNA produced. The excess primer continues to amplify, producing large amounts of single-stranded DNA, which can then be detected using fluorescent probes. Previous attempts at asymmetric PCR were inefficient and unsuccessful at production of clean, single-stranded product. The drop in efficiency of most asymmetric assays is caused by the drop in concentration of the LP, which lowers the melting temperature (Tm) and prevents full participation in the reaction at the annealing temperature. This often generates non-specific product, which then inhibits the proper action of the DNA polymerase.

Recently, Pierce et al. (2005) described a new method of efficient asymmetric PCR. This technology, called Linear-After-The-Exponential Polymerase Chain Reaction (LATE-PCR) uses primers of unequal concentration and unequal temperature to produce high yields of single-stranded DNA [Sanchez et al., 2004, Pierce et al., 2005]. LATE-PCR solves the problem of primer inefficiency by raising the $T_m$ of the limiting primer ($T_m^L$) above the excess primer $T_m$ ($T_m^X$) so that $T_m^L - T_m^X > 0$. This provides the opportunity for
both primers to efficiently participate in the reaction. LATE-PCR therefore produces clean, single-stranded product that can then be probed. Another advantage of LATE-PCR is the separation of the annealing and detection steps. Symmetric PCR assays with fluorescent probes typically read at the annealing step. LATE-PCR, on the other hand, allows for an "endpoint" analysis, meaning the read step does not occur until after all cycles of amplification have been performed. After amplification, there are many copies of single-stranded DNA to which a probe can bind without competition with a complement strand. This allows for a larger range of potential temperatures at which the probe can bind. More probes can be designed and therefore complex assays (multiplexes) become reasonable.

**Designing a LATE-PCR Assay for ASFV**

The production of single-stranded DNA is a helpful diagnostic tool, because it provides a perfect target for probe hybridization, meaning detection of samples can occur in a closed-tube manner without risk of contamination. LATE-PCR allows for the production of specific, single-stranded DNA from as little as a single target copy [Pierce et al., 2007]. This proves especially helpful for detection of viruses present in low titres early in the infection cycle. For ASFV, which does not have a vaccine or cure, detection of low virus titres in samples from asymptomatic, but exposed pigs would be extremely helpful. It would allow for a quicker response time, which could reduce the spread of virus. The similarity of symptoms between ASFV and CSFV can often delay response time due to the wait for laboratory confirmation. Consequently, the virus has more time to spread. The most effective ASFV assay would therefore be one that can specifically identify ASFV in the field, reducing the amount of processing time.

This thesis describes the construction of a new, faster, and more reliable LATE-PCR assay for the detection of African Swine Fever Virus in the field. The assay is designed to be run as a duplex with an internal DNA control as a safeguard against a false negative. The primers and probe for ASFV were designed based on the most conserved
regions of the VP72 gene and follow the criteria for effective LATE-PCR design [Pierce et al., 2005]. Chosen sequences are specific to the African Swine Fever Virus strain E70, a pathogenic European strain, among others. The LATE-PCR assay is designed to amplify a 247 bp segment upstream of the current region used for the reference assay [King et al., 2003]. DNA control primers were designed based on the Xist gene expressed in female mouse embryos [Hartshorn et al., 2007]. This gene was chosen as a control because no homology was seen with the ASFV sequences. The probe for the DNA control was designed to follow proper LATE-PCR probe criteria and contains an arbitrarily chosen sequence to avoid non-specific interactions. The ASFV and DNA targets are distinguished by two probes of different T_m and different fluorescence. PCR testing in the field will be possible using the BioSeeqII portable laboratory from Smiths Detection, Inc.. This device is specifically engineered for use in the field with a minimum of user training. It includes an automated sample preparation unit that carries out sample preparation and LATE-PCR analysis on site in a matter of hours (http://www.smithsdetection.com/eng/1025_4312.php). ASFV can be detected in blood, feces, tissue or nasal epithelial cells, all of which can be processed by the BioSeeqII. The development of this assay provides a specific and more efficient method of ASFV detection on site.

The LATE-PCR ASFV assay described here is faster and more convenient than the current assays. Its specificity and sensitivity are further enhanced by inclusion of PrimeSafe™ II, an improve form of PrimeSafe™ that is a PCR additive that maintains the fidelity of amplification over a broad range of target concentrations by suppressing mispriming throughout the reaction (Rice et al. 2007; Wangh et al. personal communication).... The final optimized assay is capable of detecting viral titres down to approximately 1 genome copy/reaction and is specific for ASFV, even in the presence of a vast excess of porcine genomic DNA.
2. Materials and Methods

2.1 Primers and probe design

The ASFV primers (Limiting primer: LP, Excess primer: XP) and probe were designed to detect a 247bp region of pathogenic isolate E70 (GenBank Accession AY578692). The design for the DNA control primers was originally based on the Xist gene expressed in female mouse embryos [Hartshorn et al., 2007]. The primers were modified to match LATE-PCR primer criteria with melting temperatures close to the ASFV primer sequences. The DNA control probe is a synthetic sequence with no known origin designed to fit LATE-PCR probe criteria. All sequences are shown in Table 1. The ASFV probe was designed with a single G/T mismatch to the original target to reduce the effects of a hairpin in the probe structure. Nonspecific interactions were avoided based on Visual OMP (version 6.6.0) software (DNA Software, Inc., Ann Arbor, MI). This program was also used to calculate melting temperatures at the initial concentrations of the primers and probes.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5'→3')</th>
<th>Modification</th>
<th>(T_m) °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASFV LP</td>
<td>CTGATACTGTCACATTAAACCGAGAGTGAG</td>
<td>None</td>
<td>69.3°C</td>
</tr>
<tr>
<td>ASFV XP</td>
<td>CTGGGAAAGACCTGATCTCTCATCTCCTG</td>
<td>None</td>
<td>67.1°C</td>
</tr>
<tr>
<td>ASFV Probe</td>
<td>AACGAGATTTGGAAGTTTCCT</td>
<td>5' Quasar 670; 3' BHQ2*</td>
<td>55.5°C</td>
</tr>
<tr>
<td>DNA Control LP</td>
<td>CGTAACTTTGTGACGCGCTACGTTCACTCC</td>
<td>None</td>
<td>71.1°C</td>
</tr>
<tr>
<td>DNA Control XP</td>
<td>GAGCTGAACACCTACTCTCGATTCT</td>
<td>None</td>
<td>67.4°C</td>
</tr>
<tr>
<td>DNA Control Probe</td>
<td>AACGACTCTTAACACGCTT</td>
<td>5' Cal Orange 560; 3' BHQ1</td>
<td>57.2°C</td>
</tr>
</tbody>
</table>

The DNA control primer pair was designed to be within one degree of the respective ASFV primers (\(\Delta T_m^{XP} < 1^\circ C, \Delta T_m^{LP} < 1^\circ C\)). The ASFV probe was modified with a 5' Quasar 670 fluor (QSR670) and a 3' Black Hole Quencher 2 (BHQ2). The DNA control probe was modified with a Cal Orange fluor and a BHQ1. All melting temperatures were calculated by Visual OMP.

\* \(T_m\) = melting temperature at the starting concentration
2.2 Testing Materials

2.2.1 Synthesized oligonucleotide targets

The assay was initially tested against a truncated, synthetic ssDNA target designed to include the primer and probe regions for the ASF VP72 gene sequence (Sigma-Aldrich, MO). The duplex reaction included the synthetic ssDNA control target. All synthetic targets and primers were ordered from Sigma-Aldrich (St. Louis, MO, USA). Fluorescent probes were ordered from Biosearch Technologies (Novato, CA, USA). The sequences for the synthesized oligonucleotide targets are shown in Table 2.

Table 2: Sequences and Melting Temperatures of ASFV and DNA Control Synthetic Test Targets

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>$T_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASFV Target</td>
<td>CTGGAAAGAGCTGTATCTCTATCCTGGAAAGCTTACATGTCCGA</td>
<td>83.2°C</td>
</tr>
<tr>
<td></td>
<td>ACTTGCTGGAATCTCCGTTGAGGTGGGTGACCGTGCAGTTCAG</td>
<td></td>
</tr>
<tr>
<td>DNA Control Target</td>
<td>GAGCTGAACACTACTACTACTACTTTACTTTGGCTGTGATTAAGAGGC</td>
<td>79°C</td>
</tr>
<tr>
<td></td>
<td>GTCGAACATGGGAGTAAACACGTGGGCTCGACAAAGTTAAC</td>
<td></td>
</tr>
</tbody>
</table>

Synthetic test targets were manufactured as single-stranded molecules. The melting temperatures were calculated by Visual OMP. The real viral test target for ASFV is 247 bp.

2.2.2 Samples from CISA-INIA

Fourteen ASFV DNA reference controls were kindly provided by Dr. Carmin Gallardo of the ASF EU Reference Laboratory (CISA-INIA). DNA was extracted directly from primary cell cultures (leukocytes and/or alveolar macrophages) using a nucleic acid extraction kit (Nucleospin/Machery-Nagel–Cultek) following the manufacturer’s procedures. The DNA was then concentrated by ethanol precipitation: 1/10 volume of 3M NaOAC and 3 volumes ethanol were added to the DNA solution then left overnight at -70 °C. The solution was spun in a microcentrifuge for 10 minutes to pellet the DNA, then washed with 70% ethanol and spun for another 10 minutes. The DNA was air-dried and
resuspended in a final volume of 100 µl of distillate RNase-free water. Testing on the viral DNA samples was performed by Mikhayil Hakhverdyan of the National Veterinary Institute (SVA) in Uppsala, Sweden.

2.3 LATE PCR assay

This endpoint LATE-PCR ASFV assay was designed for the amplification of the VP72 gene of African Swine Fever Virus based on an alignment of 32 sequences from GenBank using ClustalW alignment software (http://www.ebi.ac.uk/Tools/clustalw2/index.html). It is a duplex assay that includes an internal DNA control, which is a synthetic target of no known function. Primer and probe design for both ASF and the DNA control followed the criteria of LATE-PCR outlined by Sanchez et al. (2004), and Pierce et. al (2005). Fluorescent reads are acquired using endpoint analysis after PCR amplification. Amplification of the correct product was verified via melt analysis.

2.3.1 Assay Composition

Each reaction was run in a final volume of 25 µl and contained the following reagents: 1x PCR buffer (Invitrogen, Cat. No: 60684-050), 3 mM MgCl₂, 250µM dNTPs, 50 nM ASFV Limiting Primer, 1 µM ASFV Excess Primer, 50 nM DNA Control Limiting Primer, 1µM DNA Control Excess Primer, 100 nM ASFV Probe with a 5' QSR670 fluor and a 3' Black Hole Quencher 2, 100 nM DNA Control probe with a 5' Cal Orange 560 fluor and a 3' Black Hole Quencher 1, 300 nM Primesafe™II (a mis-priming-preventing compound) (Rice et al. 2007) and 2 units of antibody-complexed Platinum® TFi exo (-) DNA polymerase (Invitrogen, Carlsbad, CA).

2.3.2 Conditions using Stratagene Mx3005P Sequence Detector

PCR of synthetic targets was carried out in a Stratagene Mx3005P Sequence Detector (Stratagene, La Jolla, CA) with the following thermal profile: 1 cycle at 95°C for 3 minutes; 50 cycles of 95°C for 10 sec, 58°C for 15 sec, and 72°C for 30 sec; and 1 cycle
at 70°C for 3 minutes, 50°C for 3 minutes, and 35°C for 3 minutes with fluorescence acquisition during the last cycle at 70°C, 50°C, and 35°C in the Quasar 670 and Cal Orange channels. Experiments are run using endpoint analysis rather than real time to reduce nonspecific product. Data analysis was carried out using Microsoft Excel Software.

2.3.3 Conditions using Corbett Rotorgene 3000

PCR of real viral targets was carried out in a Rotorgene 3000 (Qiagen/Corbett Life Science, Valencia, CA) with the following thermal profile: 1 cycle at 95°C for 3 minutes; 50 cycles of 95°C for 10 sec, 58°C for 15 sec, and 72°C for 30 sec; and 1 cycle at 70°C for 3 minutes, 50°C for 3 minutes, 40°C for 3 minutes, with fluorescence acquisition during the last cycle at 70°C, 50°C, and 40°C in the Cy5 Channel (Source 625 nm, Detector 660 high pass filter nm) and JOE channel (Source 530 nm, Detector 555 nm). The lowest detection temperature is 40°C due to the temperature limitations of the Rotorgene thermocycler.

2.4 Sensitivity determination and PCR efficiency

To determine sensitivity, a series of dilutions of known concentration of the synthetic ASFV target were tested. Dilutions ranged from $1.5 \times 10^7$ target copies/µl to approximately 1 copy/reaction. Dilutions were tested using both SYBR Green, reading real time at 72°C to test primer efficiency, and at end point and real time using probes.

2.5 Specificity tests

To determine the specificity of the assay, 1:10 dilutions of 14 real viral samples were tested (Table 6). The samples were extracted from porcine DNA and were estimated to contain <1% viral DNA (total DNA in samples estimated to ~90ng/µlitr).
3. Results

3.1 Assay optimization

This LATE-PCR assay was initially optimized using synthetic targets for ASFV and the DNA control in separate monoplex reactions (Figure 2 and Figure 4 respectively). Serial dilutions were tested using SYBR Green detection to determine the efficiency and specificity of each pair of primers. Amplification of both targets was carried out for 60 cycles reading in real time at 72°C with no PrimeSafe™II. All dilutions were detected (see Table 3 and Table 4). Figure 2 and 3 show the ASFV monoplex using SYBR detection.

![Graph showing fluorescence vs cycle for SYBR Green detection](image1)

Figure 2: SYBR Green detection of ASFV monoplex serial dilution. Real time analysis shows the appearance of dilutions in decreasing order from $10^7$ copies/reaction. The lowest concentration is estimated at 1 copy/reaction. The clustering of concentrations $10^2$ and $10^3$ is a function of starting amplification with single-stranded targets. Average Ct values are listed in Table 3.

(baseline subtracted). Figure 3 presents the melt derivative of the ASFV monoplex, which peaks at approximately 83°C. Figures 4 and 5 show the serial dilution of the DNA control synthetic target monoplex (baseline subtracted). Figure 5 shows the melt derivative of the
DNA control, which peaks at approximately 81°C. The optimization and testing of synthetic targets was carried out in a Stratagene Mx3005P thermocycler.

Table 3: Average Ct values for SYBR Green detection of ASFV monoplex serial dilution

<table>
<thead>
<tr>
<th>Sample</th>
<th>Threshold Value</th>
<th>Ct Value</th>
<th>Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>NTC</td>
<td>45.131</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1.5 x 10^7</td>
<td>45.131</td>
<td>14.70</td>
<td>0.017321</td>
</tr>
<tr>
<td>1.5 x 10^8</td>
<td>45.131</td>
<td>17.67</td>
<td>0.101489</td>
</tr>
<tr>
<td>1.5 x 10^9</td>
<td>45.131</td>
<td>21.72</td>
<td>0.070711</td>
</tr>
<tr>
<td>1.5 x 10^5</td>
<td>45.131</td>
<td>25.44</td>
<td>0.051962</td>
</tr>
<tr>
<td>1.5 x 10^4</td>
<td>45.131</td>
<td>29.74</td>
<td>0.250599</td>
</tr>
<tr>
<td>1.5 x 10^3</td>
<td>45.131</td>
<td>33.44</td>
<td>0.343123</td>
</tr>
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<td>1.5 x 10^2</td>
<td>45.131</td>
<td>33.02</td>
<td>0.341223</td>
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<tr>
<td>1.5 x 10^0</td>
<td>45.131</td>
<td>37.95</td>
<td>1.471371</td>
</tr>
</tbody>
</table>

The threshold value was calculated by the Stratagen Mx3005P algorithm. The Ct for each dilution was determined at the threshold value based on the amplification plot represented in Figure 2.

Figure 3: Melt derivative – SYBR Green detection of ASFV monoplex serial dilution. The $T_m$ for the synthetic ASFV target was calculated by Visual OMP to be 83.2°C. The observed $T_m$ was approximately 83°C.
Figure 4: SYBR Green detection of DNA control monoplex serial dilution. Real time analysis of the DNA control showed high amplification efficiency. The dilutions decreased from 10^6 copies/reaction to an estimated 1 copy/reaction. The average Ct values for the DNA control are listed in Table 4.

Table 4: Average Ct values for SYBR Green detection of DNA control monoplex serial dilution

<table>
<thead>
<tr>
<th>Sample</th>
<th>Threshold Value</th>
<th>Ct Value</th>
<th>Standard Deviation</th>
</tr>
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<tr>
<td>NTC</td>
<td>45.558</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1.5 x 10^7</td>
<td>45.558</td>
<td>10.03</td>
<td>0.060277</td>
</tr>
<tr>
<td>1.5 x 10^8</td>
<td>45.558</td>
<td>13.85</td>
<td>0.089629</td>
</tr>
<tr>
<td>1.5 x 10^5</td>
<td>45.558</td>
<td>17.08</td>
<td>0.037859</td>
</tr>
<tr>
<td>1.5 x 10^4</td>
<td>45.558</td>
<td>20.70</td>
<td>0.150997</td>
</tr>
<tr>
<td>1.5 x 10^3</td>
<td>45.558</td>
<td>24.23</td>
<td>0.09609</td>
</tr>
<tr>
<td>1.5 x 10^2</td>
<td>45.558</td>
<td>26.45</td>
<td>0.040415</td>
</tr>
<tr>
<td>1.5 x 10^1</td>
<td>45.558</td>
<td>30.07</td>
<td>0.06053</td>
</tr>
<tr>
<td>1.5 x 10^0</td>
<td>45.558</td>
<td>31.31</td>
<td>1.039423</td>
</tr>
</tbody>
</table>

The Ct was determined at the threshold value based on the amplification plot represented in Figure 4.

After optimization of the ASFV and DNA control monoplex reactions, the complete duplex reaction was tested (Figures 6, 7, 8, 9). This reaction was comprised of two ASFV
Figure 5: Melt Derivative – SYBR Green detection of DNA control monoplex serial dilution. The T
m of the DNA Control was calculated using Visual QMP to be 79°C. The melt derivative shows a T
m of approximately 81°C.

primers, two DNA control primers, one ASFV probe, one DNA control probe and 300 nM
PrimeSafe™II to prevent nonspecific interactions during amplification. A serial dilution of
the ASFV target was tested at endpoint after 50 cycles of amplification (Figure 6, 7),
reading in the QSR670 channel. The duplex assay was tested using both 50 copies/reaction and 150 copies/µl of the DNA control target. Each reaction reported here
contained 150 target copies/reaction of the DNA control. The data are presented as a
ratio of the fluorescence at 35°C to the fluorescence at 70°C with the baseline subtracted
(Figure 6).

A threshold value of 0.2 normalized fluorescent units above the negative control
(normalized to equal 0) was chosen to establish a positive signal. Fluorescent ratios
ranged from 0.6 (1 target/reaction) to 4.2 (10⁷ targets/reaction) normalized fluorescent
units. The samples containing only the DNA control produced a negative signal in the QSR670 channel. Figure 7 shows the melt derivative for the ASFV samples at approximately 53°C. The DNA control was read in the Cal Orange channel (Figures 8 and 9). The endpoint data is reported as a normalized value at 35°C. The threshold value for the DNA control was chosen as 0.2 for a positive signal. All of the DNA Control samples were detected in the Cal Orange channel with fluorescence ranging from 0.7 to 0.88 normalized fluorescent units. Figure 9 shows the melt derivative of the DNA control samples at approximately 56°C.

3.2 Sensitivity and specificity using Viral DNA samples.

To determine the sensitivity and specificity of the ASFV LATE-PCR assay, both the monoplex and duplex were tested on a total of 14 real viral samples (Table 5) at the
National Veterinary Institute in Uppsala, Sweden. Samples were provided by Dr. Carmina Gallardo of the CISA-INIA in Madrid, Spain. Each sample contained purified DNA that was extracted from primary cell cultures (leukocytes and/or alveolar macrophages) from pigs, except MwLil 20/1, which was isolated from ticks at a pig pen, and Ba71, which is a verocell-adapted pig isolate. Each sample was diluted 1:10 in water and tested in a Corbett Rotorgene 3000 thermocycler. The 14 samples were tested with the ASFV monoplex (Figures 10 and 11) and one sample, E75, was tested with the duplex containing the DNA control (Figures 12 and 13). The concentration of viral DNA present in the samples was estimated to be <1% of the total DNA [Karl Stáhl, personal communication]. The remaining DNA was porcine.

Samples tested with the monoplex assay were amplified for 50 cycles using Taq Gold DNA polymerase (annealing temperature 60°C) without PrimeSafe™II and were detected with a Cal Orange probe. Data for the monoplex is presented at 50°C and has been normalized to 70°C with the baseline subtracted.
Figure 8: DNA control at 150 copies per reaction at 35°C (Cal Orange channel) plotted as each of the ASFV target concentrations. All values are normalized to 70°C with the baseline subtracted. Amplification was carried out for 50 cycles. All reactions contained the same copy number of DNA control target. Endpoint analysis shows all samples giving similar fluorescence. Samples labeled "control" contained only the DNA control (negative for ASFV target).

Figure 9: Melt derivative - DNA control at 150 copies per reaction at 35°C (Cal Orange channel) plotted as each of the ASFV target concentrations. The melting temperature of the DNA control probe was calculated using Visual OMP to be 57.2°C. The observed $T_m$ was 56.3°C.
The endpoint data show a strong positive signal above the threshold (0.2 normalized fluorescent units) for all of the samples tested (Figure 10). Fluorescent values ranged from 1.6 to 3.2 above the negative control (normalized to 0). Figure 11 shows the melt derivative for all 14 strains at approximately 55°C. Six strains (Ug03H, Ken06, Ken07, BF07, L60, and SS88) show a slight decrease in the melting temperature of the probe.

Table 5. ASFV strains used in the study.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Name</th>
<th>Origin/Source</th>
<th>Isolation Country</th>
<th>Institute Provider</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mozambique 1964</td>
<td>Moz64</td>
<td>Pig</td>
<td>Mozambique</td>
<td>CISA-INIA</td>
</tr>
<tr>
<td>Angola 1972</td>
<td>Ang72</td>
<td>Pig</td>
<td>Angola</td>
<td>CISA-INIA</td>
</tr>
<tr>
<td>Chalaswa 1983</td>
<td>MwLii 20/1</td>
<td>Tick, pig pen</td>
<td>Malawi</td>
<td>CISA-INIA</td>
</tr>
<tr>
<td>Cape Verde 1997</td>
<td>CV97</td>
<td>Pig</td>
<td>Cape Verde</td>
<td>CISA-INIA</td>
</tr>
<tr>
<td>Hoima 2003</td>
<td>Ug03H</td>
<td>Pig</td>
<td>Uganda</td>
<td>CISA-INIA</td>
</tr>
<tr>
<td>Kenya 2006</td>
<td>Ken06.B1</td>
<td>Pig</td>
<td>Kenya</td>
<td>CISA-INIA</td>
</tr>
<tr>
<td>Kenya 2007</td>
<td>Ken07.Eld1</td>
<td>Pig</td>
<td>Kenya</td>
<td>CISA-INIA</td>
</tr>
<tr>
<td>Burkina Faso 2007</td>
<td>BF07</td>
<td>Pig</td>
<td>Burkina Faso</td>
<td>CISA-INIA</td>
</tr>
<tr>
<td>Pontevedra 1970</td>
<td>E70</td>
<td>Pig</td>
<td>Spain</td>
<td>CISA-INIA</td>
</tr>
<tr>
<td>Badajoz 1971</td>
<td>Ba71V</td>
<td>Verocell adapted pig isolate</td>
<td>Spain</td>
<td>CISA-INIA</td>
</tr>
<tr>
<td>Lérida 1975</td>
<td>E75</td>
<td>Pig</td>
<td>Spain</td>
<td>CISA-INIA</td>
</tr>
<tr>
<td>Lisbon 60</td>
<td>L60</td>
<td>Pig</td>
<td>Portugal</td>
<td>CISA-INIA</td>
</tr>
<tr>
<td>Sardinia 1988</td>
<td>Ss88</td>
<td>Pig</td>
<td>Italy</td>
<td>CISA-INIA</td>
</tr>
<tr>
<td>Port-au-Prince 81</td>
<td>Haiti</td>
<td>Pig</td>
<td>Haiti</td>
<td>CISA-INIA</td>
</tr>
</tbody>
</table>

Abbreviations: CISA-INIA - Centro de Investigación en Sanidad Animal, del Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria. Samples were generously provided by Dr. Carmina Gallardo.
Figure 10: ASFV monoplex detection of multiple ASFV strains at endpoint (Cal Orange probe). Fourteen viral DNA strains were tested and all showed a positive signal at 50°C. Differences in fluorescence reflect differences in DNA concentration.

Figure 11: Melt Derivative - ASFV monoplex detection of multiple ASFV strains at endpoint (Cal Orange channel). The melt analysis shows a $T_m$ of 54.8°C for eight strains and 54.2°C for six strains.
Figure 12: ASFV duplex E75 strain test reading endpoint at 40°C in the QSR670 and Cal Orange Channels. The E75 strain was tested with the full duplex. Samples labeled as "E75" contain only E75 viral DNA. "E75 + Control" contain both the E75 strain and the DNA control. Samples labeled as "Control" contain the DNA control target only and were detected only in the Cal Orange channel.

Figure 13: Melt Derivative - ASFV duplex E75 strain test - QSR670 and Cal Orange Channels. The graph on the left shows the melt derivative in the QSR670 channel. Only samples containing E75 viral DNA were amplified. The graph on the right shows the melt derivative in the Cal Orange channel. Only samples containing the DNA control were amplified. The observed T_m was 65°C for the DNA control and 52°C for the ASFV viral DNA.
The E75 strain was tested with the full duplex (Figures 12 and 13). Figure 12 shows the raw endpoint data at 40°C, provided by Mikhayil Hakhverdyan of the National Veterinary Institute in Uppsala, Sweden. Samples tested with the duplex were amplified for 50 cycles using Platinum® Tfi exo (-) DNA polymerase without PrimeSafe™II. Amplification was run for 50 cycles reading at 70°C, 50°C and 40°C. Each reaction contained ASFV viral DNA and either 10³ copies/4 μl of DNA control, or water. All of the samples containing ASFV viral DNA gave positive signals in the QSR670 channel and no signal in the Cal Orange channel. The DNA control gave a positive signal in the Cal Orange channel only. Figure 13 shows the melt derivative of the duplex test, peaking at approximately 55°C for the DNA control and 52°C for the ASFV viral DNA. Some nonspecific product can be seen in the melt derivative of the DNA target. Testing of the ASFV viral samples is now ongoing with added PrimeSafe™II.

4. Discussion
This thesis presents a novel assay for the detection of African Swine Fever Virus based on Linear-After-The-Exponential Polymerase Chain Reaction. Because of the properties of LATE-PCR, each reaction produces large amounts of specific, single-stranded DNA, which can then be probed with a sequence-specific probe. Against synthetic targets, the assay has proven to be specific and effective even at low target numbers. The typical sensitivity of symmetric PCR assays is approximately 10 targets/reaction. This assay is capable of detection down to approximately 1 molecule/reaction. After 50 cycles of amplification, the fluorescence at this concentration is relatively lower than samples with a higher concentration due to the presence of less single-stranded target to which the probe can bind. A simple increase in the number of amplification cycles will produce more targets and increase the fluorescence. The internal DNA control in this assay is also specific and sensitive at low copy number. Results show
a signal only in the Cal Orange channel. This indicates there are no nonspecific interactions or false positives produced by the assay.

The comparison of the LATE-PCR data to the real time, symmetric PCR data presented by King et al. 2003 revealed the inherent limitations of the symmetric PCR assay. The sensitivity of the symmetric PCR assay decreases dramatically with fewer targets. This decrease is mainly a result of inhibition of the DNA polymerase due to mis-priming. LATE-PCR produces large amounts of ssDNA that do not interfere with polymerase activity, and with the added effect of PrimeSafe™II, mis-priming is reduced. Therefore, smaller concentrations of target can be easily detected. LATE-PCR also allows for the use of probes with very low temperatures, increasing the range at which readings can be made. By reading these probes at endpoint, amplification and detection are separated, allowing amplification to be carried out under optimal conditions before the read step. The separation of the amplification and detection steps also reduces the total run time of the assay, which provides a quicker diagnosis.

The melt curve analysis of the assay on both synthetic and viral targets revealed a probe T$_m$ which is a few degrees below the theoretical temperature calculated by Visual OMP. This difference is most likely due to the Platinum® Tfi exo (-) buffer, which can reduce the predicted melting temperature 1-3°C.

Testing of real viral DNA from 14 ASFV strains extracted from pig tissue was performed at the SVA in Uppsala, Sweden. All 14 strains produced a positive signal when tested with the LATE-PCR monoplex assay. The melt analysis confirmed the correct product was amplified. The total amount of viral DNA present in each sample was estimated at less than 1% of the total DNA [Karl Ståhl, personal communication], indicating this assay is specific to ASFV viral DNA, even in a large background of porcine DNA. The melt analysis of the multiple strain test revealed a slight strain-specific difference in the melting temperature of the probe (Figure 11). This difference in melting temperature is most likely the result of sequence variation in or near the probe-binding
site. Variations can increase or decrease the efficiency of amplification and can also influence probe annealing. Sequence information is still unavailable for most of the strains tested; analysis of these targets by sequencing is ongoing in Sweden and may reveal the presence of mutations.

Testing of the duplex on one sample of viral DNA was also performed. The test of the E75 strain produced a strong positive signal with no false positives or negatives. The melt analysis of the ASFV samples showed generation of some nonspecific product. The samples were run without PrimeSafe™II, which reduces mis-priming. Inclusion of PrimeSafe™II in future tests should reduce these nonspecific interactions.

The major assays currently used for the detection of ASFV require 24 hours upon receipt of the sample before an answer is received and require the use of expensive, non-portable laboratory equipment. But, the devastation caused by an outbreak of African Swine Fever Virus makes early detection a necessity for the prevention of further loss. The LATE-PCR assay described in this thesis is a reliable, efficient asymmetric PCR assay that detects both low and high viral titres with high efficiency. The assay produces large amounts of single stranded DNA, which can then be detected at endpoint. This provides a more rapid method of detection than the current real-time assays. This assay is specific to African Swine Fever Virus and can detect viral targets even in a large background of porcine DNA. A further advantage of this assay is its ability to be used in the field on Smiths Detection, Inc.'s Bio-SeeqII portable sample preparation and PCR machine. The instrument includes five independent thermocyclers, each of which accommodates a Universal Sample Preparation Device (USPD) which processes a biopsy sample all the way through tissue homogenization, nucleic acid extraction and purification, PCR amplification, and results communication with a global command center. The required reagents are stored lyophilized in the reagent pack of the USPD which is labeled with a barcode that instructs the thermocycler what protocol of heating and cooling to use. The features of the BioSeeqII allow for on-site sample preparation and detection within hours.
5. Conclusions

In conclusion, the LATE-PCR assay described here for the detection of African Swine Fever Virus is an efficient, specific, and sensitive test. It provides reliable and reproducible results and can be used in either a laboratory setting or in the field. The use of LATE-PCR allows for endpoint analysis, which provides a quicker and more sensitive test than the current ASFV symmetric PCR assays.

Acknowledgements

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