Supplementary Report S1:

Comparative testing of commercial extraction kits for swine fever diagnostics

Prior to the reported study, the German NRL for CSF and ASF at the Friedrich-Loeffler-Institute (FLI) performed a small comparative study on automated nucleic acid extractions. The background was to replace the former routine method, the MagAttract Virus M48 kit (Qiagen), that was no longer available. We include the data here to give a background for interested readers that may also want to detect both swine fever viruses, African swine fever virus (ASFV) and classical swine fever virus (CSFV).

Study design and methodology

Nucleic acid extraction using three magnetic bead-based nucleic acid extraction kits on automated workstations

I.1 Samples and matrices

Extractions were performed on samples from experimental infections that had been carried out at the high containment facilities of the Friedrich-Loeffler-Institute (FLI), Greifswald – Insel Riems, Germany. The study comprised three sample subsets that were aggregated as follows: subset I compromised 80 ASFV positive samples. These samples originated from twenty different animals, in detail five domestic pigs infected with the genotype I isolate "Netherlands'86", five wild boar infected with genotype II isolate "Estonia" and ten domestic pigs inoculated with another genotype II isolate "Armenia08". From each animal, four sample types were tested (EDTA blood, serum, lung and spleen), reflecting routine diagnostic samples. Subset II comprised CSFV positive samples from twenty different domestic pigs. In detail, eight animals were infected with a Peruvian CSFV strain (genotype 1.1), six pigs with the isolate "Lithuania" belonging to genotype 2.1 and another six animals with strain "Roesrath" (genotype 2.3). Here, tonsil samples were tested instead of spleen samples. To assess specificity, negative blood, serum, lung, spleen, and tonsil samples were added from sows and piglets that were part of animal trials with porcine epidemic diarrhea virus (subset III). Positive samples were selected to cover a wide range of viral loads.

I.2 Nucleic acid extraction

The following kits were included: NucleoMag® VET (Macherey - Nagel), MagAttract Virus M48 (Qiagen), and MagMAX™ CORE (Thermo Fisher Scientific, workflow C). While the NucleoMag® VET and MagMAX™ CORE kits are set up for simultaneous detection of both RNA and DNA, the MagAttract Virus M48 was designed for the extraction of viral RNA. However, the latter was validated at the German NRL for ASF and was routinely used to extract also ASF samples from both experimental trials and diagnostic investigations. Tissue samples were processed for extraction as follows: A small piece (size of a small pea) was cut from all tissue samples and homogenized in serum free medium for 3 min at 30 Hz with a steal bead in a Tissue Lyser II (Qiagen). After a short centrifugation step, the liquid supernatant was used along with the other liquid samples for the different extraction methods. All samples were extracted in triplicate on the KingFisher extraction platform (Thermo Fisher Scientific) according to the manufacturer's instructions. To all extractions, a heterologous internal control RNA or DNA was added (Hoffmann et al., 2006). The extraction procedures for the different kits are briefly described below.

I.3 Detection of viral genome and internal controls

For the detection of ASFV, the qPCR protocol published by King et al. (2003) was used as duplex assay with the detection of an internal control (see above). This assay is accredited at the NRL as "ASF-System 1" (LAM03ASP). The qPCR was performed using the QuantiTect Multiplex PCR Kit no ROX (Qiagen) on a BioRad CFX96 Real-time detection system (version 3.1) with the following temperature profile: 15 min activation at 95 °C followed by 45 cycles of 60 sec 95°C and 60 sec 60°C. For the detection of CSFV, the RT-qPCR developed by Hoffmann et al. (2005) was used. Including detection of an internal control, this system is accredited as CSF-System 1 (LAM03KSP). The RT-qPCR was carried out using the Quantitect Probe RT-PCR Kit (Qiagen) on a BioRad CFX96 Real-time detection system (version 3.1) with the following temperature profile: reverse transcription for 30 min at 50°C, 15 min activation at 95 °C followed by 42 cycles of 15 sec 95°C, 30 sec 57°C and 30 sec 68°C.

I.4 Data analysis

Inter-assay reproducibility

To assess method robustness, the spiked internal control RNA for CSFV and DNA for ASFV was monitored (i.e. detection of the internal control). The assay to assay reproducibility was assessed by repeating the (RT-) qPCRs three times (technical replicates). The cycle quantification (Cq)-value means were calculated and then used to calculate the overall mean, standard deviation and coefficient of variability [CV, %]. The average of the CVs is reported as inter-assay CV.

Intra-assay reproducibility

To monitor the variation within one run, the spiked internal control in negative samples was used. The Cq value means were calculated and then used to calculate the standard deviation and coefficient of variability. The average of the individual CVs was reported as the intra-assay CV.

Concordance analysis

To compare the results of the different extraction methods with each other, the Cq values obtained after the different extractions were depicted in a Bland-Altman plot. This statistical method displays the difference between a pair of measurements made with the two methods on the y-axis in relation to the mean of this Cq-value results on the x-axis. The 95% limits of agreement were given by the mean difference plus or minus 1.96 multiplied with the standard deviation of the difference.

Results

Robustness

The reproducibility of the three magnetic-bead based extraction kits for automatization was assessed by calculating the coefficient of variation [%]. The variation within one PCR run lay between 0.54 and 2.16%, between three different PCR runs with the same samples between 1.00 and 3.71%. The NucleoMag VET kits had the highest reproducibility whereas the highest variations were observed with the MagAttract Virus M48 kit. Details are depicted in table 1.

Diagnostic sensitivity and specificity, concordance

No false-positive reactions were observed. However, some false-negative samples were detected (see table 2). For ASFV, eight samples were negative after MagAttract Virus M48 kit extraction whereas with the other two methods only one or two samples could not be detected. For CSFV, all samples were correctly positive after the extraction with NuceloMag VET. One or two samples were not correctly scored after extraction with the other kits.
To tests whether the failure of detection depends on the sample type, all Cq values obtained after the different extractions were depicted sorted by sample type (see figure 1, A-D for ASFV and figure 2 for CSFV). Apart from a certain variation among kits, false-negative results were mostly found in EDTA blood samples. No problems occurred with serum samples. For CSFV positive samples, the variation of the Cq-values was general lower (see figure 2). Here, all false negative samples, were lung samples. This matrix showed also the highest variation among kits.

Concordance analysis depicted by Bland-Altman plots (see figure 3, A-C) showed a good concordance between the NucleoMag VET kit and the MagMAX CORE kit independent from the sample type. The mean average lies near zero and the limits of agreement indicating nearly no differences between the two methods. The scattering of the points shows no bias and only three CSFV samples and five ASFV samples are falling out of range (figure 3B). A direct comparison of the NucleoMag VET and the MagAttract Virus M48 kit shows likewise accordance between the two kits, but with a higher range and a shifted mean of the difference. It can be seen, that CSFV samples show a lower Cq-value extracted with MagAttract Virus M48 kit while it is the other way round for ASFV samples (figure 3A). The Bland-Altman plot for the comparison of the MagMAX CORE kit with the MagAttract kit (figure 3C) looks similar. The scattering of the points is a bit wider resulting in a higher limit of agreement range. This diagram shows again the lower Cq-values for the CSFV samples and has the same high ASFV Cq-values which fall out of the range.

Practicability

Regarding the need of equipment and further reagents, all three extraction kits are quite similar. The NucleoMag VET is the only one with no need of a thermo-incubator and the MagMAX CORE kit scores with the saving of plastic material (see table 3). The NucleoMagVet kit has a shorter preparation time, mainly due to a shorter lysis time, but has a longer processing time of the standard KingFisher flex protocol resulting in the same overall nucleic acid extraction time for all three kits.

Conclusions

All bead-based extraction kits showed high reproducibility, repeatability and robustness with variations below 5%. Despite slight differences, concordance analyses showed a good correlation between the three extraction methods and all three kits are regarded as suitable for the simultaneously extraction of viral RNA and DNA.

The NRL chose to use the NucleoMag VET kit for further diagnostic work.

References

Hoffmann, B., M. Beer, C. Schelp, H. Schirrmeier and K. Depner, 2005: Validation of a real-time RT-PCR assay for sensitive and specific detection of classical swine fever. *J Virol Methods,* 130, 36-44.

Hoffmann, B., K. Depner, H. Schirrmeier and M. Beer, 2006: A universal heterologous internal control system for duplex real-time RT-PCR assays used in a detection system for pestiviruses. *J Virol Methods,* 136, 200-209.

King, D. P., S. M. Reid, G. H. Hutchings, S. S. Grierson, P. J. Wilkinson, L. K. Dixon, A. D. Bastos and T. W. Drew, 2003: Development of a TaqMan PCR assay with internal amplification control for the detection of African swine fever virus. *J Virol Methods,* 107, 53-61.

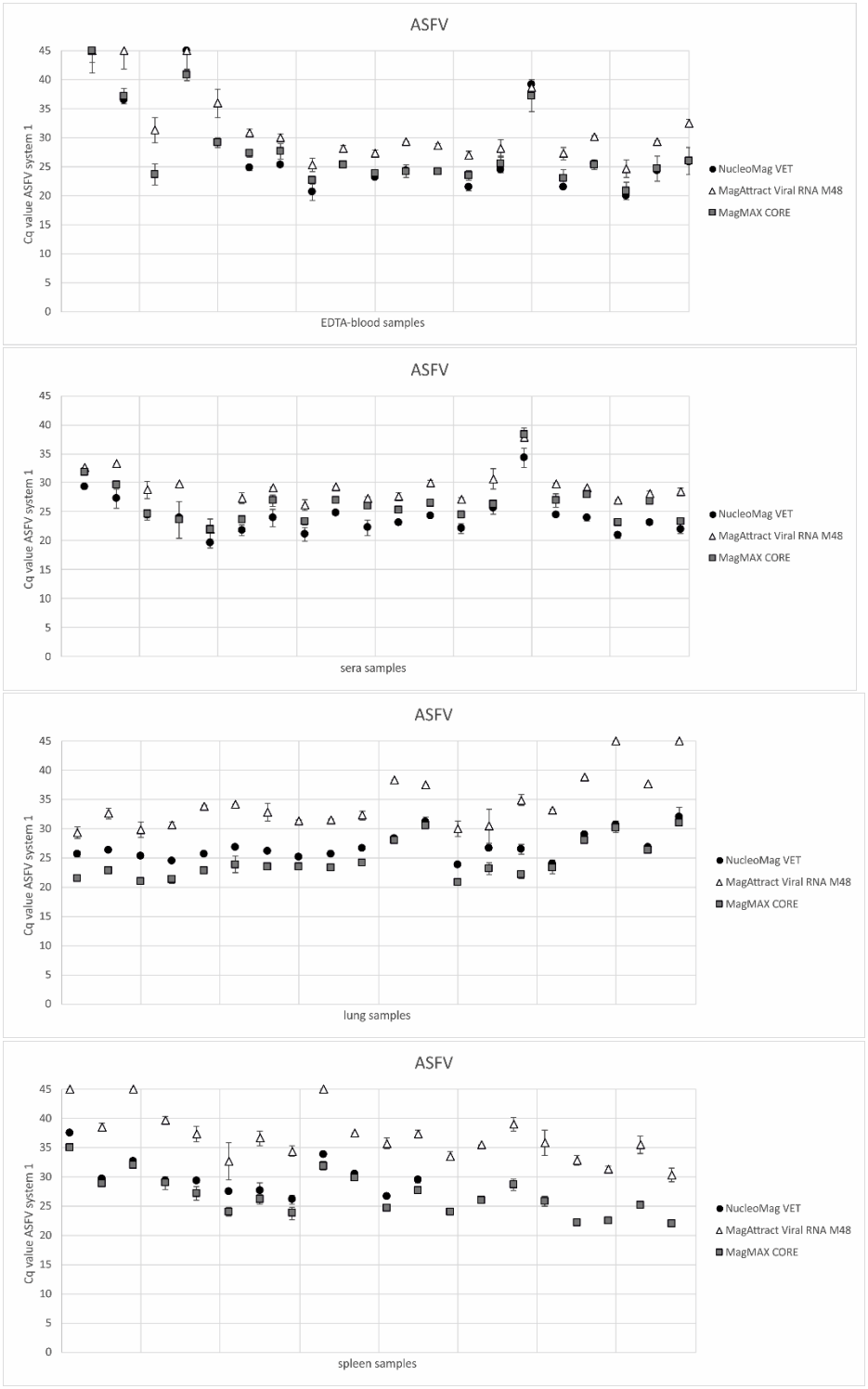

Figure 1: Cq-values of ASFV positive A) EDTA blood, B) serum, C) lung and D) spleen samples extracted with the three different kits (see figure legend).

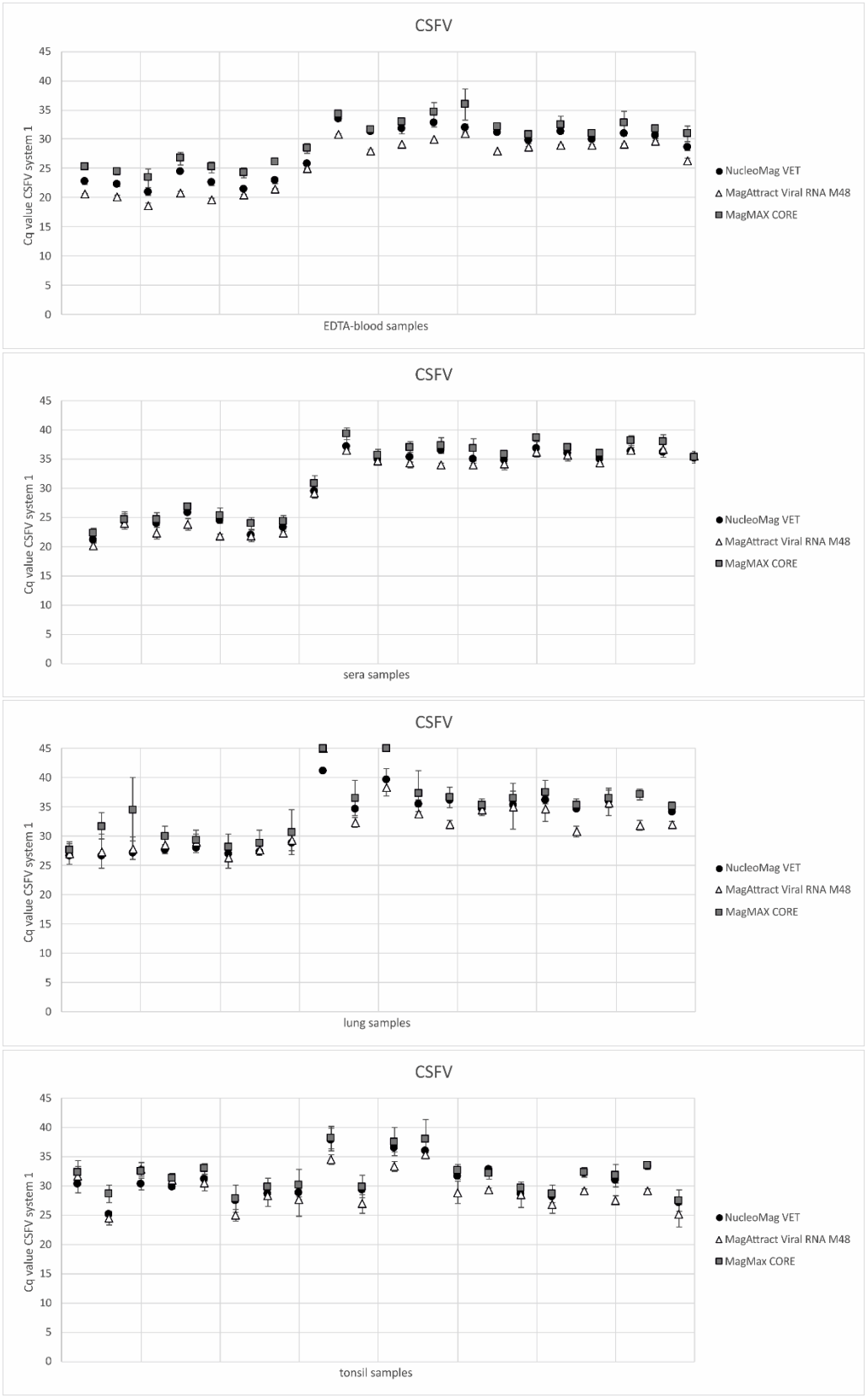

Figure 2: Cq-values of CSFV positive A) EDTA blood, B) serum, C) lung and D) tonsil samples extracted with the three different kits (see figure legend).

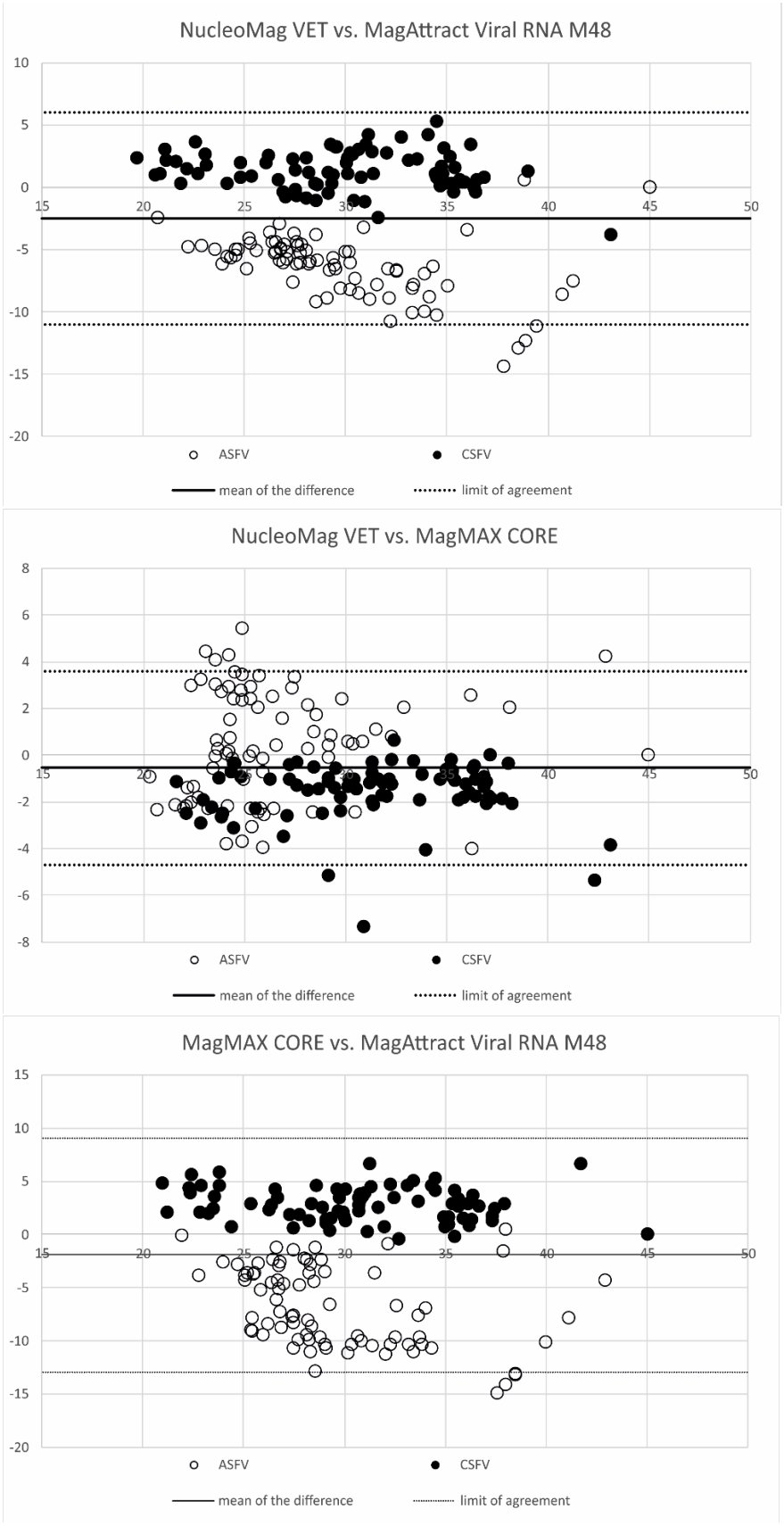

Figure 3: Bland-Altman plots of ASFV and CSFV Cq-values. Compared are NucleoMag VET and MagAttract Virus M48 kit (A), NucleoMag VET and MagMAX CORE kit (B) and MagMAX CORE kit and MagAttract Virus M48 kit (C).