RESEARCH REPORT
SWINE HEALTH

Title: Detection and differentiation of field strains and commonly used vaccine strains of Type 2 PRRS virus in the US, **NPB #16-196**

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Industry Summary:

PRRS remains the most economically devastating swine disease with an estimated annual loss of $664 million in the US. The PRRS genome is constantly changing, making accurate diagnosis difficult. Many pairs of primers have been used in some commercial PRRS PCR kits to cover the genetic diversity of the PRRS genomes. Hypothetically, using more primers can bring potential issues such as non-specific amplifications. On the vaccine side, there are four PRRS vaccines that have been used in the US, namely PrimePac; Ingelvac MLV, Ingelvac ATP and Fostera. Due to their close relationships to some field strains, differentiation between vaccine strains and field strains are challenging using common real-time PCR methods. Currently the differentiation is mostly done by PRRS ORF5 sequencing, which is expensive and time consuming. Luminex technology allows us to detect more molecular targets in a single reaction with the cost similar to that of one real-time PCR reaction. Beads of different colors are coupled with target-specific, i.e., pathogen-specific, capture oligos in the Luminex platform. A short piece of pathogen genome is amplified by target-specific primers. A region in the PCR product is complementary to the capture oligo on the beads, thus is hybridized to the beads for specific target identifications. Designing a Luminex PCR assay without a probe that is required in most real-time PCR assays, can simplify the designing process, and potentially resolve the multiplexing problem encountered in real-time PCR assays. By analyzing all available full- and near full-genome sequences of PRRS, we were able to design two pairs of primers that hypothetically can detect 98% of all PRRS sequences, thus the assay is predicted to detect 98% of filed strains. We were also able to design a pair of primers for each vaccine strains, which can differentiate PRRS strains that are 98% or less identical to the vaccine strains. Analytical sensitivity of this Luminex PRRS assay is lower than, but close to the sensitivity level of a typical real-time PCR assay. Testing on the vaccine strains and 489 PRRS field strains indicated that the Luminex PRRS assay we have developed can detect 95.2% of the field strains, and can differentiate majority of the vaccine strains especially for MLV-like strains. Although Luminex assays do not provide quantification data like Ct values generated by real-time PCR assays, it provides a cost-effective way of comprehensive detection of rather divergent PRRS strains, at the same time to differentiate the PRRS vaccine strains without using the more expensive sequencing procedure.

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Keywords: PRRS, detection, diagnostics, Luminex, vaccine differentiation

Scientific Abstract:

PRRS remains the most economically devastating swine disease with an estimated annual loss of $664 million in the US. The PRRS genome is constantly changing, making accurate diagnosis difficult. Many pairs of primers have been used in some commercial PRRS PCR kits to cover the genetic diversity of the PRRS genomes. Hypothetically, using more primers can bring potential issues such as non-specific amplifications. On the vaccine side, four PRRS vaccines have been used in the US, namely PrimePac; Ingelvac MLV, Ingelvac ATP and Fostera. Due to their close relationships to some field strains, differentiation between vaccine strains and field strains are challenging using common real-time PCR methods. Currently the differentiation is mostly done by PRRS ORF5 sequencing, which is expensive and time consuming. Luminex technology allows us to detect more molecular targets in a single reaction with the cost similar to that of one real-time PCR reaction. Beads of different colors are coupled with target-specific, i.e., pathogen-specific, capture oligos in the Luminex platform. A short piece of pathogen genome is amplified by target-specific primers. A region in the PCR product is complementary to the capture oligo on the beads, thus is hybridized to the beads for specific target identifications. Designing a Luminex PCR assay without a probe that is required in most real-time PCR assays, can simplify the designing process, and can potentially resolve the multiplexing problem encountered in real-time PCR assays. By analyzing all available full- and near full-genome sequences of PRRS, we were able to design two pairs of primers that are predicted to detect 98% of filed PRRS strains. We were also able to design a pair of primers for each vaccine strains, which can differentiate PRRS strains that are 98% or less identical to the vaccine strains. Analytical sensitivity of this Luminex PRRS assay is one half log to one log lower than that of a typical real-time PCR assay. Testing on the vaccine strains and 489 PRRS field strains indicated that the Luminex PRRS assay we have developed can detect 95.2% of the field strains, and can differentiate majority of the vaccine-like strains especially for MLV-like strains. Although Luminex assays do not provide quantification data as these Ct values generated by real-time PCR assays, it provides a cost-effective way of comprehensive detection of rather divergent PRRS strains; at the same time, differentiates the PRRS vaccine strains without suing the more expensive sequencing procedure.

Introduction:

PRRS remains the most economically devastating swine disease with high morbidity and mortality since its emergence in the US (Gauger et al., 2012; Kappes and Faaberg, 2015). The estimated annual loss in the US reaches $664 million (Holtkamp et al., 2013). The fact that emerging and re-emerging PRRS strains have been causing outbreaks for more than two decades, and the increasing knowledge of PRRS sequencing data indicating that the PRRS genome is constantly changing for infection and adaptation (Goldberg et al., 2000; Lunney, Benfield, and Rowland, 2010; Murtaugh et al., 2010; Shi et al., 2010). Type 2 PRRSV is the major genotype causing problem in the US. A significant variation in the Type 2 PRRSV strains have been observed in the US (Meng et al., 1995), Canada (Larochelle, D'Allaire, and Magar, 2003), Denmark (Madsen et al., 1998), Asian countries (An et al., 2007; Cha et al., 2006; Key et al., 2001; Ni et al., 2013), and other regions. A phylogenetic analysis on a recent collection of 694 full- and near full-Type 2 PRRS genomes illustrated an extensive variation in the PRRS genome. The divergent genomes of PRRSV make identification difficult.

The commonly used molecular diagnostic method for PRRSV is real-time RT-PCR (qRT-PCR)-based assays. qRT-PCR is a very sensitive method for the detection of PRRS viruses, yet the variation of the PRRSV genome made it difficult to detect all variants. With current technology,

most real-time RT-PCR (qRT-PCR) machines can only detect up to 5 molecular targets. Practically, most published qPCR assays describe 2-3 targets (Huang et al., 2011; Mackay, 2004). Multiple qPCR reactions would be required for a disease panel testing, which are accompanied with higher cost and longer turnaround time. This largely limits detection capabilities (Hamza, Jurzik, and Wilhelm, 2014), especially for the detection of polymicrobial syndromes, or a pathogen with very divergent and constantly changing genome like PRRSV. Differentiating PRRS vaccine strains is even more challenging because of the subtle differences between the vaccine strains and some field strains. There is no published protocol, to our knowledge, that can simultaneously detect and differentiate the four US vaccine strains from the field strains. Currently vaccine identification is done by DNA sequencing, which is expensive and time-consuming.

Luminex technology has greatly enhanced the multiplexing capability of serological detections (Christopher-Hennings et al., 2013; Langenhorst et al., 2012). In recent years, nucleic acid-based multiplex Luminex detection assays have also been established for human and animal pathogen detections.

In this study, a Luminex assay was built to detect the majority of field PRRS strains by using different pairs of primers, at the same time to differentiate the four PRRS vaccine strains used in the US by using vaccine-specific primers.

Objectives: From your research proposal.

1. Development of a comprehensive, multiplex assay for the detection of Type 2 PRRSV field strains, and differentiation of vaccine stains through thorough bioinformatics analysis of most current sequences
2. Optimization and validation of the PRRS assay using a panel of field swine samples.
3. Sequencing verification of the vaccine strains, and selected vaccine-like and wild type strains.

Materials & Methods:

Assay design

There are 694 type 2 PRRS full- and near full-genome sequences were available at the time of design. All 694 sequences were downloaded and aligned in CLC Genomic Workbench. The aligned sequences were used to identify the conserved regions for the detection primer designs, and used to identify regions that are specific to each vaccine strains for vaccine differentiation designs. Two pairs of detection primers were identified that can detect 85.4% and 91.2% of the 694 full genomes, respectively, with combined detection coverage of 98.1%. A pair of primer for each vaccine strains were identified. Magnetic beads (MagPlex-TAG microspheres) that have unique capture sequence attached to each bead were purchased from Luminex. Two-oligo design illustrated in Figure 1 was used to reduce the assay complexity. Each forward primer was designed to attach a probe sequence (in maroon in Figure 1) that is complementary to the capture oligo coupled on the beads. After the PCR reaction, the PCR amplicon was captured on the beads by the hybridization between the probe and the capture oligo. The reverse primer for each designed primer pair was synthesized with a biotin attached to generate signal for positive detection. The primers were then pooled and used for amplification. Amplified PCR products were purified and hybridized to the beads. Not hybridized PCR products were washed away, and the remaining that hybridized to the beads were run through a BioRad Bio-Plex 200 system to generate detection results.

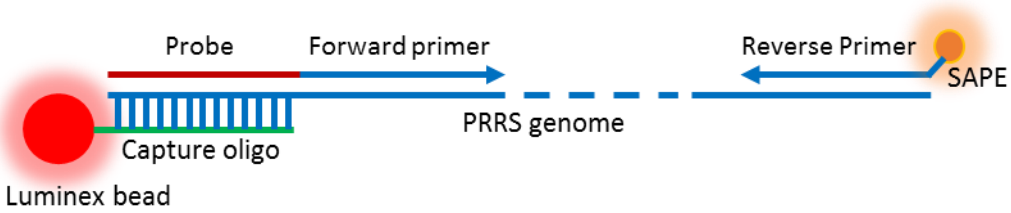


Fig. 1. Illustration of the Luminex PRRS assay design. Each Luminex bead is coupled with a capture oligo (Green). The forward primer is attached with a probe (Maroon) to its 5' end that is complementary to the capture oligo. The reverse primer is labeled with biotin that later generates signal when sample is positive.

The Luminex assay procedure

Standard laboratory procedure for Luminex assays was used. Briefly, multiplexed RT-PCR amplification using the forward primers that were attached with specific probes complementary to the capture oligo on the beads, and the biotinylated reverse primers were carried out. Optimized RT-PCR conditions was used with Qiagen One-step RT-PCR Kit (Qiagen, Valencia, CA). Each bottle of MagPlex-TAG microspheres was vortexed vigorously for 30s, then sonicated for 30 s. The working solution of microsphere mixture was prepared by diluting 2500 beads/well in 33uL 1.0X TMAC hybridization buffer. The bead mixture was vortexed again for 30 s, and sonicated for approximately 20 seconds then aliquoted in 33uL portions into 96-well skirted microplates. Two microliter of PCR product and 15 uL nuclease-free water were added to make total volume of 50uL/well. The plate was covered to prevent evaporation and hybridized in a thermal cycler with the following parameters: 95°C for 2 minutes (denaturation step) and 52°C for 20 minutes (hybridization step). During hybridization, fresh reporter mix was prepared by diluting SAPE to 3 µg/mL using 1X TMAC hybridization solution. After hybridization, reactions were shaken for 5 min, then washed three times with 75 uL of 1X TMAC Buffer and resuspended in 50uL SAPE, and shaken for 5 min. The plate was incubated at 52°C for 8 min, then washed three times in 50 uL of 1XTMAC Buffer, resuspended in 60 uL of 1XTMAC Buffer and was shaken for 5 min, and then analyzed by the Luminex analyzer according to manufacturer's instructions.

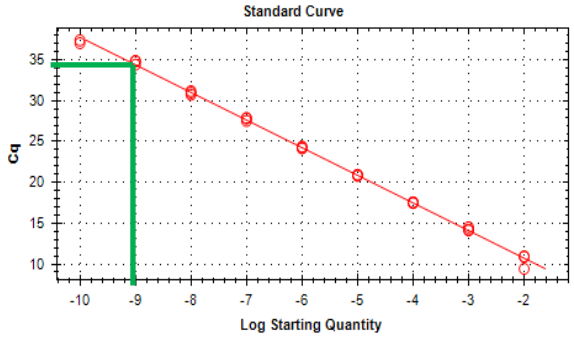
Samples

Prototype strains for vaccines Ingelvac MLV, Ingelvac ATP and Fostera were kindly provided by the Dr. Richard Hesse, Director of Diagnostic Virology Lab of KSVDL. Target region of vaccine strain PrimePac was synthesized based on the GenBank accession number DQ779791.1. Diagnostic sample RNAs were kindly provided by co-PI, Dr. Jianqiang Zhang of Iowa State University Veterinary Diagnostic Laboratory. A collection of 489 RNA samples that were used to generate PRRS ORF5 sequencing data were transported to KSVDL for evaluation of the PRRS Luminex assay.

Results:

Analytical sensitivity

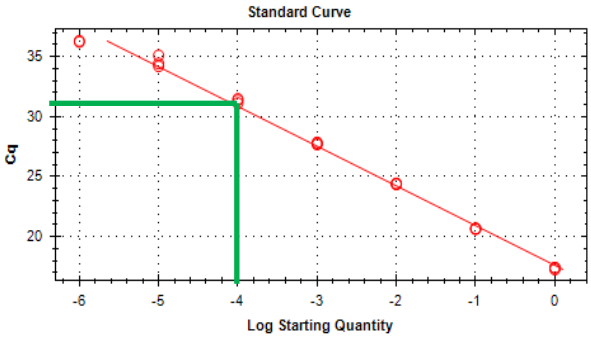
Analytical sensitivity was accessed using 10-fold dilutions of each vaccine strain and/or synthetic plasmid (PrimePac). Results were compared to a real-time PCR assay, as indicated in Figures 2 to Figure 5.



Sample	Detect1	Detect2	MLV	ATP	Primepac	Fostera
NTC	314	845	303.5	285	1235.5	332
10^-7	245	6131.5	8102	170.5	269	160.5
10^-8	147.5	4867	6238	104	296	153
10^-9	165	3476	3364.5	158	248	156.5
10^-10	213	1856	1077	227	423	243
10^-11	319	614	436	330	635	394

Fig. 2A.

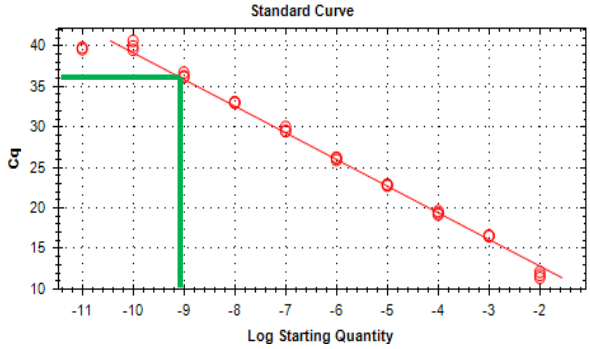
Detection limit for cloned Ingelvac MLV segment using the PRRS Lumines assay as compared with real-time PCR.



Sample	Detect1	Detect2	MLV	ATP	Primepac	Fostera
NTC	475	942	542.5	452	705.5	1361.5
10^0	9003	14773	16983	581	952.5	606
10^-1	4750	13200	16368	565	1165	628.5
10^-2	2652.5	11564	14208	629	1286.5	658.5
10^-3	749	6675	9604	470	1723.5	767
10^-4	506.5	2279	4891.5	496	2721	557
10^-5	555	802	1069.5	514	838.5	734

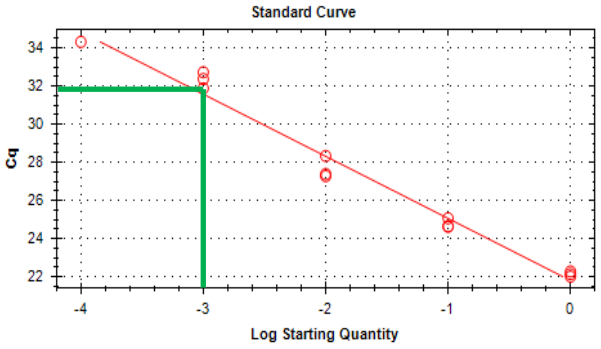
Fig. 2B.

Detection limit for the Ingelvac MLV strain using the PRRS Lumines assay as compared with real-time PCR.



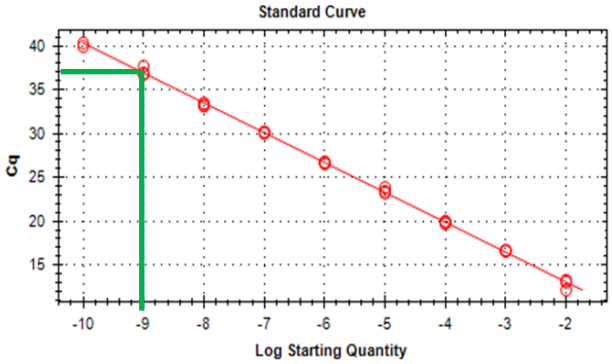
Sample	Detect1	Detect2	MLV	ATP	Primepac	Fostera
NTC	297	518.5	420.5	323	519	358
10^-7	235	5515	114	4787	167	127.5
10^-8	208	5414	218	3948.5	315.5	260.5
10^-9	454.5	5209	511	3125	757	424
10^10	471	2270.5	648	1045.5	1001	790

Fig. 3A. Detection limit for cloned Ingelvac ATP segment using the PRRS Lumines assay as compared with real-time PCR.



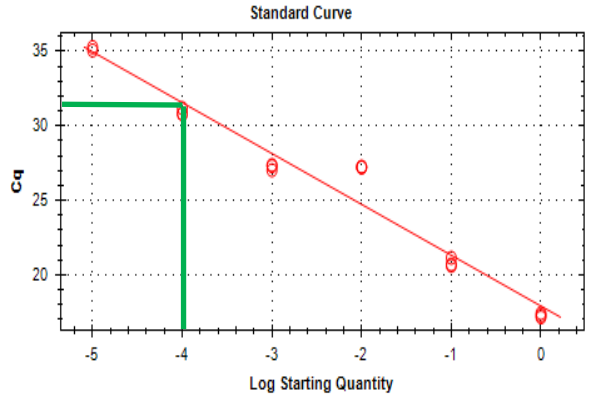
Sample	Detect1	Detect2	MLV	ATP	Primepac	Fostera
NTC	577	1342	484.5	406	2259	542.5
10^0	4093	13464	552.5	14457	870	535
10^-1	1484.5	13187	1278	12513	1138	542
10^-2	1068	12638	979	8343	1373.5	681
10^-3	693	7839	1467	2480.5	1719	750.5
10^-4	482	2594	604.5	528	745.5	592

Fig. 3B. Detection limit for the Ingelvac ATP strain using the PRRS Lumines assay as compared with real-time PCR.



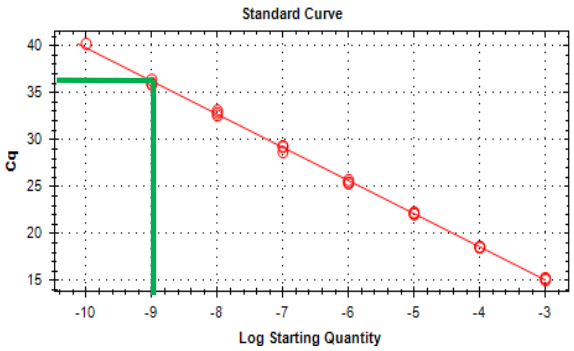
Sample	Detect1	Detect2	MLV	ATP	Primepac	Fostera
NTC	211.5	282.5	326	194	318	301
10^-7	351.5	7094	182	181	390	4341
10^-8	383	7979	338	351	621.5	3977
10^-9	634	6275.5	743	659	1258	2309

Fig. 4A. Detection limit for cloned Fostera segment using the PRRS Lumines assay as compared with real-time PCR.



Sample	Detect1	Detect2	MLV	ATP	Primepac	Fostera
NTC	347.5	720	545	417	2324	415
10^0	5944	10503	499	523.5	679	8367
10^-1	4964	12267	791.5	749.5	874	10479
10^-2	2234.5	12597	810.5	791	1950	10301
10^-3	918	10035	1075	661	2044	8373
10^-4	1046.5	6681.5	813.5	769	1837	5227
10^-5	811.5	1631	652	626	1364	1466

Fig. 4B. Detection limit for the Fostera strain using the PRRS Lumines assay as compared with real-time PCR.



Sample	Detect1	Detect2	MLV	ATP	Primepac	Fostera
NTC	708	680	547	405.5	884	602
10^-3	456	9613	663	501	5139	555
10^-4	375	8982.5	573	408	5257	561
10^-5	427	8908.5	539	403	5657	523.5
10^-6	593.5	10433	839	558	6155.5	752
10^-7	398	7095	380.5	270	5221	339
10^-8	762.5	8973	822	502	6123.5	620.5
10^-9	512	4875.5	775	491	4090	693

Fig. 5. Detection limit for cloned PrimePac segment using the PRRS Lumines assay as compared with real-time PCR.

A summary of detection limits of the Luminex PRRS assay for each vaccine clone and each vaccine strain defined by real-time PCR Ct values are summarized in Table 1 below.

Table 1. Summary of Luminex PRRS assay detection limits defined as Ct values generated by a real-time PCR assay

Vaccine strain	MLV		ATP		Fostera		PrimePac*
Target type	Clone	Strain	Clone	Strain	Clone	Strain	Clone
Real-time PCR Ct	34	31	36	32	37	32	36

*The PrimePac vaccine strain was not obtained, and only a synthesized clone was used.

Diagnostic sample evaluation.

We have tested 489 PRRS RNA samples that were kindly provided by Dr. Jianqiang Zhang of ISU Veterinary Diagnostic Laboratory. Among these samples 412 were tested positive by the Luminex PRRS assay. Because the RNA samples were accumulated from the past sequencing cases, degradation of RNA is a possible cause of the negative results generated by the Luminex assay. To verify that this may be the case, we tested all 77 Luminex-negative samples by a PRRS real-time PCR assay. Only 21 of the 77 samples were positive, and 56 of the Luminex-negative samples were due to the degradation of RNA. Therefore, the diagnostic sensitivity of this Luminex PRRS assay should be 95.2% (412/(489-(77-21)), and not 84.3% (412/489).

Vaccine differentiations

Among the 412 Luminex positive samples, there were 84 MLV or MLV-like strains defined by a >99% identity to the ORF5 sequence of the vaccine strain. Among these 84 strains, 79 were identified MLV or MLV-like strains by the Luminex assay. Eighteen of the 23 Fostera-like strains identified by sequencing were identified by the Luminex assay as Fostera-like strains. Two of four ATP-like strains by sequencing were also identified by the Luminex assay as ATP-like strains. There was no PrimePac-like strain identified in the collection (Table 3).

Table 3. Number of vaccine strains identified by ORF5 sequencing and number of positives by the Luminex assay

	MLV	Fostera	ATP	PrimePac
ORF5 Sequencing	84	23	4	0
Luminex PRRS Assay	79	18	2	0

For better visualization, Luminex assay results for the four vaccine strains and selected positive and negative field strains are shown in Table 4 bellow.

Table 4. Luminex assay result on PRRS vaccine strains and selected field strains

Type	Detection1	Detection2	MLV	ATP	Prime Pac	Fostera
NTC	371	594	719	359.25	808.5	449.5
MLV	7685.5	13041	15112.5	350	652	536
Fostera	7240.5	15609	594.5	612.5	835	11397
ATP	2022.5	13542	506	13151	461	534
Prime Pac	225	7622	290.5	205.5	5953	192
Positive1	10257.5	4296.5	765	682	1966.5	655
Positive2	376.5	8149	8811	327	3232.5	414
Positive3	5773	11306	289	637	524	289
Positive4	7117	12501	538	886.5	1004	496
Negative1	454	1213.5	745	425	1859	609
Negative2	372.5	862	778	413.5	877.5	497.5
Negative3	570	1127	1101	635	1256	760.5
Negative4	399	1031.5	795	438	988.5	588

Discussion:

A multiplex Luminex assay was developed for the detection of field PRRS strains, at the same time for the differentiation of the four vaccine strains used in the US. The Luminex system have been well-documented for antibody and antigen detections. In recent years, the accumulated evidences indicated that it is also working well for nucleic acid detections. Our first-hands experience using a commercial Luminex *Salmonella* serotyping kit to identify serotypes of 200 *Salmonella* strains also indicated that it is working well for nucleic acid detections, and that encouraged us to pursue the development of this Luminex PRRS assay with the NPB support. With two pairs of primers, the assay is predicted to detect 98% of type 2 PRRS strains, analyzed by an *in silico* analysis of the coverages of the primers over a collection of 694 full-or near full-PRRS genome sequences. Based on the test results on 489 field strains, the assay detected 95.2% of the strains, which is lower than our prediction. Further improvement is possible through more detailed optimization of the assay.

Some field strains are very similar to one of the vaccine strains. The most commonly seen strains were MLV or MLV-like strains. There were 84 strains that have greater than 99% of the nucleic acid identity to the MLV vaccine strain identified by ORF5 sequencing. Among these 84 strains, 79 were also identified as MLV or MLV-like strains by the Luminex assay. Another commonly seen vaccine-like strain was Fostera-like strains. Eighteen of the 23 sequencing

confirmed strains were also identified as Fostera-like strains by the Luminex assay. Only four strains were ATP-like strains by sequencing, of which two were identified as ATP-like strains by the Luminex assay. There was no PrimePac-like strains identified. One factor that may contribute to vaccine identification differences between the ORF5 sequencing and the Luminex PRRS assay is that the sequencing prediction was based on the ORF5 gene, while primers in the Luminex PRRS assay were designed on other genes. Our analysis of full genome sequences of PRRS strains indicated that the ORF5 gene may not be the best region for vaccine differentiations.

The Luminex platform is capable of detecting many more targets than PCR-based technology. A comprehensive swine Luminex panel assay would be beneficial to the research community and industry, if major swine pathogens including PRRS, PCV2, PCV3, SIV, and others can be detected in a single reaction.

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