RESEARCH REPORT
SWINE HEALTH

Title: Development and Optimization of a Blocking ELISA for Type 1 and Type 2 Strains of Porcine Reproductive and Respiratory Syndrome Virus – **NPB #05-168**

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Abstract:

The PRRS virus (PRRSV) continues to be a significant economic concern for swine producers in the U.S. and throughout the world. Recently, the presence of an emerging European-like strain that is genetically and antigenically distinct from the original European Type 1 and U.S. Type 2 strains has impacted the sensitivity of current diagnostic techniques and consequently has complicated the detection of PRRSV in swine herds. The IDEXX HerdChek® PRRS assay, a commercially available enzyme-linked immunosorbant assay (ELISA) has become the industry standard for the detection of antibodies against PRRSV. The need to accurately determine the PRRSV serostatus of herds and individual animals has prompted the development of confirmatory tests that enable differentiation of true positive samples from presumed false positives. A highly specific and repeatable blocking ELISA (bELISA) was developed with the use of both U.S. Type 1 & 2 nucleocapsid (N) proteins as the antigen and two competitive monoclonal antibodies specific for highly conserved regions within the N protein. Validation of the bELISA was performed by using 537 serum samples from 42 individual animals that were experimentally infected with either U.S. Type 1 or U.S. Type 2 PRRSV. Receiver operating characteristic analysis determined a diagnostic sensitivity and specificity of 99.3% & 99.1%, respectively. Further analysis of the data enabled us to establish a definitive cutoff point of 39.7 and to identify times of seroconversion for both PRRSV genotypes. Furthermore, the bELISA was able to resolve 72% of unexpected positive IDEXX ELISA results obtained from a collection of 196 diagnostic field samples. Our results show that the bELISA may be useful as a confirmatory test to evaluate suspect results obtained with the IDEXX ELISA, due to its increased sensitivity and detection capabilities.

Introduction:

The PRRS virus continues to be a major economic concern for swine producers in the U.S. and throughout the world. Control of the PRRS virus remains problematic, due to its asymptomatic persistent infections and ability to rapidly evolve. PRRSV can be divided into two genotypes (Type 1 or Type 2) based upon antigenic diversity and genetic heterogeneity between strains. European-Like and North American strains show an overall nucleotide identity of only 60-63% at the genomic level (Nelsen et al., 1999, Allende et al., 1999). Recently, the presence of an emerging European-like strain that is genetically distinct from both the original European Type 1 and U.S. Type 2 strains has complicated the detection of PRRS virus in swine herds. The presence of this new strain of PRRS has impacted the sensitivity of current diagnostic techniques, due to the variation in antigenicity. Diagnostic techniques for the detection of PRRS virus such as serum virus neutralization (SVN), virus isolation (VI) or indirect fluorescent antibody assays (IFA) can be used to monitor the status of a herd; however, they have varying sensitivity and specificity for different PRRSV strains and may only be able to detect the virus at certain time points during infection. A commercially available indirect ELISA (IDEXX HerdChek® 2XR PRRS ELISA) is currently used as the industry standard for monitoring the PRRS status of swine herds. This IDEXX ELISA shows high sensitivity and specificity for detecting PRRS, has good reproducibility, and is efficient and cost-effective; however, the presence of false-positive results from otherwise seronegative herds is a concern for veterinarians and producers. We have developed a blocking PRRS ELISA (bELISA) to address the concerns of false-positives in seronegative herds. This bELISA has been shown to be a highly specific, highly repeatable ELISA able to detect newly emerging of strains of PRRSV. Ultimately, the objective of our research was to develop, optimize and validate a bELISA for use in a commercial kit and extend its capabilities to detect EU-like and EU-PRRS virus strains.

Objectives:

Overall Objective – To develop and optimize an accurate and specific blocking ELISA (bELISA) that will detect an antibody response against both North American Type 2 and European Type 1 strains of PRRSV. This work builds on previous studies by investigators at South Dakota State University and represents a major step in moving this assay toward widespread availability as a commercial kit.

Objective 1: Development of preservative methods for bELISA reagents. The current blocking ELISA developed by researchers at SDSU has been tested and validated in a research laboratory using freshly prepared reagents. The components of this assay must now be stabilized using preservatives and/or dehydration to enable long-term storage with no loss of activity. Once viable preservative methods have been determined and optimized for all components, the assay must then be re-validated with known PRRSV negative and positive serum samples.

Objective 2. Broaden detection capabilities of the bELISA assay. Because the EU-like Type 1 PRRS virus is now found in North American swine herds, it has become important to detect these new strains as well. Developing both a capture antigen and an antibody for use in the detection of the new EU-like strain and the European strains is currently a goal of the Center for Infectious Disease Research and Vaccinology at SDSU. The final goals of the bELISA are to broaden the detection capabilities of the assay by the implementation of a dual set of monoclonal antibodies and capture antigen against EU-PRRSV and EU-like PRRSV. All reagents will be combined with components from the previously described blocking ELISA so that one assay would detect all strains of the PRRS virus.

Materials and Methods:

Expression of PRRSV nucleocapsid antigen:

The PRRSV bELISA utilized the recombinant nucleocapsid antigen from both VR2332 (North American Type 2 PRRSV) and 01-08 (European-Like Type 1 PRRSV) isolates. PRRSV VR2332, ORF-7 was cloned into the plasmid pET 24b (Novagen) and expressed in *E. coli* BL21 cells (Stratagene) as fusion proteins containing an amino terminal myc-tag and a carboxyl terminal 6x histidine tag. The construct was kindly provided by Dr. Mike Murtaugh at the University of Minnesota. The transformed cells were plated onto Luria-Bertamo agar plates containing 50 ug/ml Kanamycin. Next, a 20 ml culture of 2xYT containing 50ul/ml Kanamycin was inoculated with a single colony and grown overnight at 37C. The next morning, 1L of 2xYT was inoculated with the overnight culture (1:50 dilution) and grown for 2 h at 37C. The expression of nucleocapsid protein was induced by the addition of 1mm IPTG in 1L of 2xYT medium containing 50ug/ml Kanamycin with shaking at 37C for 5 hours.

Plasmid pBAD/TOPO Thiofusion (Invitrogen) was used for the cloning and expression of PRRSV 01-08 ORF-7 within TOP10 cells grown in LB medium and supplemented with 50 μg/ml of Ampicillin. A 20ml overnight culture was prepared as stated previously and expanded to a 1 liter culture the following day. Expression of the European-like nucleocapsid fusion protein was induced with 0.02% arabinose for 5 hours. The translated product contained a carboxyl terminal 6x histidine tag and an amino terminal HP-thioredoxin tag designed to increase solubility of the antigen. Expressed proteins were purified twice by pH dependent, nickel column chromatography using Ni-NTA agarose (Qiagen) and quantitated by the Lowry assay.

Monoclonal antibody preparation.

Monoclonal antibodies (MAbs) SDOW-17 & AT-13 recognize highly conserved epitopes on the PRRSV N protein (Nelson et al., 1993) and were used to increase sensitivity of the assay. Antibodies in raw ascites fluid were partially purified by ammonium sulfate precipitation. Next, a 10mg/ml solution of biotin-N-hydroxysuccinimide (biotin NHS) was prepared in DMSO and added to purified MAbs to a final concentration of 15% and incubated for 4h at room temperature with gentle stirring. The biotin NHS was neutralized with the addition of 80ul of 1 M NH_4Cl per mg of biotin NHS. The solution was dialyzed twice using Spectra/Por dialysis tubing (MWCO 14 kda; Spectrum Laboratories) in 4 L of PBS for more than 8 hours. A solution of bovine serum albumin (BSA) was added to a final concentration of 1% for stability then the biotinylated MAbs were aliquoted and stored at -80C.

Antibody measurement by blocking ELISA format:

A "strip-well" ELISA plate format utilizing a mixture of expressed North American and European PRRSV nucleocapsid proteins was selected. We worked with a commercial company (Chemicon) to optimize methods for drying, blocking and stabilizing antigen on ELISA plates. Specifically, the blocking ELISA was performed by coating flat-bottomed, Immulon 2HB, 96 well microtiter plates (Thermo Labsystems, Franklin, Mass) with 500 ng of *E. coli* expressed, recombinant, European and North American nucleocapsid protein. The antigen was diluted appropriately in ACB (15mM sodium carbonate and 35 mM sodium bicarbonate and buffered to pH 8.8) then added to alternating wells. Optimum concentration of recombinant fusion protein was determined by checkerboard titration so that antibody concentrations generated an optical density of approximately 2.0. Uncoated wells were treated with 100ul of ACB to serve as a background control. Once coated, the plates were incubated for 1 h at 37 C in a humidified incubator then held overnight at 4C. The following day, the plates were washed 6 times with PBS–T (1x PBS+0.05% Tween-20 vol/vol) using an ImmunoWash eight channel manifold (Nalgene Nunc International). Plates were immediately blocked with the addition of 200ul of PBS containing 2% BSA (wt/vol) and allowed to incubate 1h at 37C. After washing 6 times, test and control porcine sera were diluted 1:3 in SMD (1x PBS containing 0.05% tween-20 and BSA 0.1%, wt/vol) then 100ul was added in duplicate to each of the antigen-coated and background wells. Plates were then incubated at 37C for 1hour. Following sample incubation, 100ul of a 1:999 dilution of both AT-13 and SDOW-17 was added to all wells. Plates were gently swirled to mix the serum/antibody cocktail then incubated for an additional 30 minutes. Next, plates were washed 6 times with 300ul PBS-T, then Streptavidin-horseradish peroxidase

conjugate (Zymed) was diluted to a final concentration of 125 ug/ml in SMD and 100ul was added to each well. Plates were incubated for 1 hour at 37C. Following another washing step, 100ul of the chromagenic substrate ABTS (KPL) was added then subsequently stopped after 15 minutes with the addition of 100 ul ABTS stop solution to all wells. A_{405} absorbance values were read spectrophotometrically on a multichannel ELISA reader (ELx 808, Bio Tex, Winooski, VT). Data for each test sera were expressed as percent inhibition (PI) calculated by the following formula:

$PI = 1- [(A_{405}$ sample - A_{405} buffer control)/ (A_{405} neg control - A_{405} buffer control)] X 100

Measurement of repeatability:

The measurement of repeatability of the bELISA was assessed by running the same lot of internal quality control sera 20 times on one plate, once per plate on 10 plates in a single run and at least once in 10 different runs. The values for mean, standard deviation, and percent coefficient of variation have been previously reported (Ferrin et. al 2004). The same lot of internal-quality control serum has been used with the IDEXX ELISA, IFA and bELISA at the South Dakota Animal Disease Research and Diagnostic Laboratory (SD, ADRDL) since 2001.

Preservative method evaluation:

A variety of methods were evaluated to stabilize other assay components, including the biotinylated primary antibody, secondary antibodies and wash solutions to be included as kit components. Four different chemicals were tested, first as a preservative for the competitive/detection MAbs AT-13 and SDOW-17 and second as a preservative for the secondary-conjugated antibody and wash solutions. The preservatives that were tested include Gentamicin (50 ug/ml, Atlanta Biologicals); 2-chloroacetamide (0.02% Sigma-Aldrich, St. Louis, MO); 2-methyl-4-isothiazolin-3-one hydrochloride (MITZ, 0.1% Sigma-Aldrich, St. Louis, MO); and sodium azide (0.01% Eastman Kodak, Rochester, NY). The aforementioned chemicals and their respective concentrations have been previously reported as effective stabilizers in a variety of other ELISA based applications (Schafer et al., 1972). Individual "kit" components including the detection MAbs, the secondary-conjugated antibodies, and a 10X wash solution were tested with each preservative at its respective concentration.

Statistical cutoff determination:

In order to accurately assess the diagnostic sensitivity and specificity of the bELISA, a total of 537 confirmed PRRSV positive and negative serum samples from individual animals were used. The established PRRSV positive test serum used was obtained from two separate animal challenge studies. The first study involved four groups of eight, six week-old pigs in which each group was inoculated with one of four different plaque purified European-Like Type 1 strains of PRRSV. The strains used were based upon a distinct phylogenic profile and geographic location from which each strain was isolated. All pigs within each of the four groups were bled on regular weekly intervals and the serostatus of individuals were monitored between 21 and 85 days post inoculation (Nelson et al., 2006). The second study consisted of 10 pigs inoculated with the well characterized North American Type 2 PRRSV strain VR2332 and the serostatus of pigs was evaluated between 28 and 154 days post inoculation. As with both studies great care was taken to ensure that all PRRSV inoculated individuals had seroconverted before inclusion into the validation study. Negative testing was performed with serum samples from individuals from the same challenge studies and considered to be negative (uninfected) when collected prior to inoculation or when collected from uninfected control pigs. No negative (uninfected) serum samples from commercial herds were included in the validation study due the prevalence of PRRSV in field isolates. A positive/negative cutoff level was assessed using Receiver Operating Characteristics (ROC) software (GraphROC, version 2.0) which is a statistical program for the non-parametric estimation of sensitivity, specificity and corresponding confidence limits.

Detection of seroconversion:

A comparison of seroconversion was made between the IDEXX ELISA and the bELISA using 300 serum samples taken from 42 positive (infected) individual pigs. Determination of seroconversion was determined separately for North American Type 2 PRRSV and European-like PRRSV isolates. For the determination of seroconversion for the North American infected pigs, a total of 130 serum samples were analyzed from 10 pigs infected with North American PRRSV VR-2332 between 0 and 154 days post inoculation (dpi). The remaining 170 serum samples were from four groups of eight, six week-old pigs in which each group was inoculated with one of four phylogenetically distinct, plaque purified European-Like Type 1 strains of PRRSV (01-08, 01-07, 02-11 and 03-15) as previously described (Nelson et al., 2006). All pigs within each of the four groups were bled on regular weekly intervals between 0 and 85 dpi during which time seroconversion of all virus strains were assessed.

Evaluation of IDEXX ELISA field negative and unexpected positive results:

The use of a bELISA was employed to confirm the status of negative field samples and to resolve the status of serum samples demonstrating unexpected false positive IDEXX ELISA results that arise in routinely sampled negative herds. A total of 752 serum samples from 37 cases of expected negative herds were first analyzed from SDSU ADRDL using the IDEXX ELISA. The same samples were subsequently retested by the bELISA to verify the seronegative status of individual samples and cases. In addition, 195 serum samples from 127 different cases resulting in suspect false positive IDEXX ELISA and IFA values were revaluated using the bELISA. The true PRRSV status was determined retrospectively on the basis of monthly serological testing of the herd according to the established herd management practices of the producer.

Results:

Objective I. Development of preservative methods for the bELISA

Procedures to stabilize antigen, antibody and other test components were optimized in regard to ratios, combined with appropriate preservatives and incorporated into the new assay. First, the bELISA incorporated a "strip-well" plate format utilizing a mixture of expressed recombinant North American and European PRRSV nucleocapsid proteins. Before use, the antigen was extracted and purified twice via nickel-column chromatography (Figure 1). Next, optimization of antigen coating for the assay was performed by coating plates with various dilutions and ratios of the two expressed proteins to determine the optimum sensitivity and specificity for known PRRSV positive and negative serum samples. In addition, a dual set of biotinylated monoclonal antibodies were optimized for the assay. A European-like (Type1) PRRSV monoclonal antibody (AT-13) was mixed with a well characterized North American Type 2 mAb (SDOW-17) to broaden the sensitivity of the bELISA. Ratios of the MAbs were optimized utilizing a checkerboard titration format to determine appropriate levels of binding in direct competition to control swine serum containing antibodies against Type 1 and Type2 PRRSV. Once antigen coating and antibody blocking was optimized, control sera of known serostatus was tested in preparation for validation. Figure 2 shows a sample bELISA plate with 6 PRRSV negative serum samples in quadruplicate (left side) and 6 PRRSV positive serum samples in quadruplicate (right side utilizing equal numbers of Type 1 and Type 2 PRRSV positive sera).

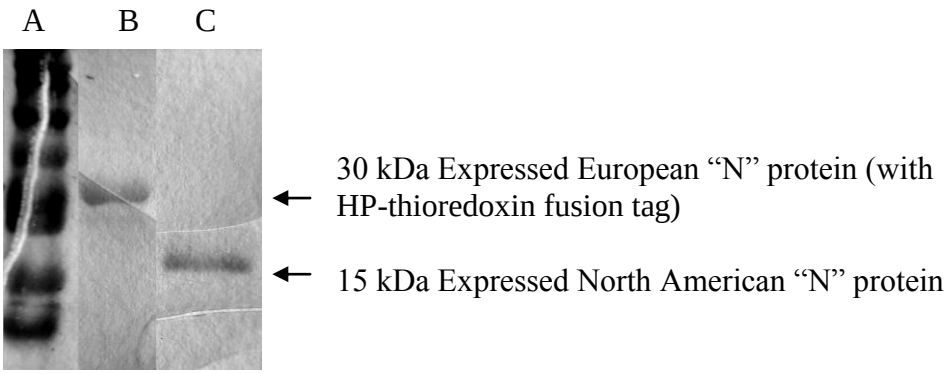
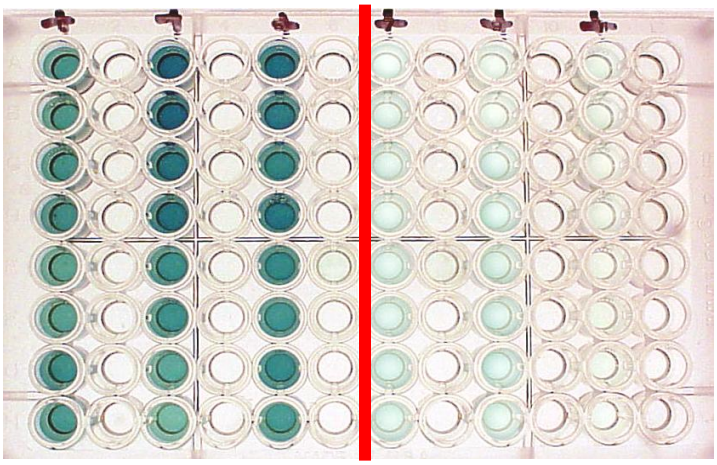


Figure 1. Coomassie Blue staining of purified recombinant nucleocapsid (N) proteins. Lane A shows molecular mass markers. Lane B shows European-like PRRSV N protein expressed as an HP-Thioredoxin fusion protein to improve solubility. Lane C shows expressed North American PRRSV N protein.

Columns of wells alternately coated with PRRSV antigen (+) or mock antigen (blank)



Negative Serum **Positive Serum**

Figure 2. ELISA plate showing PRRSV antibody positive and negative samples. PRRSV antibody negative samples show strong color development due to antigen binding of competing labeled monoclonal antibodies. PRRSV antibody positive samples show minimal color development due to specific sample antibody blocking the binding of labeled monoclonal antibody.

Stability Evaluation: Comparison of commercial "dry" and SDSU coated "wet" plates

Once optimized, antigen was prepared for commercial coating onto Immulon 2HB ELISA plates in a "strip-well" format and subsequent "wet-dry" analyses were performed to determine if there were any differences in sensitivity between the commercially prepared plates and the custom "wet" plates prepared by SDSU for the bELISA. The comparisons were made using well-characterized known PRRSV positive and PRRSV negative serum samples obtained from the SD-ADRDL. Results show no statistical difference

between commercially prepared bELISA plates and those previously prepared by SDSU (Figure 3). However, slight improvements in performance and consistency were noted for the commercially dried plates.

Also, when comparing commercially coated plates using two different blocking conditions, it was determined that there was a 14% decrease in signal development (P = 0.009 by two tailed Student's t-Test assuming equal variance) between commercially coated plates blocked at 37C versus those that were blocked at room temperature (23C).

In another controlled stability test, comparisons were made between commercially coated plates blocked at 37C and refrigerated until use, versus those that were held at room temperature for six months (Figure 4). These data indicate that there was no difference between the two blocking conditions (P >0.05) even though the plates that were immediately refrigerated after coating show less background noise when using PRRSV negative sera than those held at room temperature for half a year.

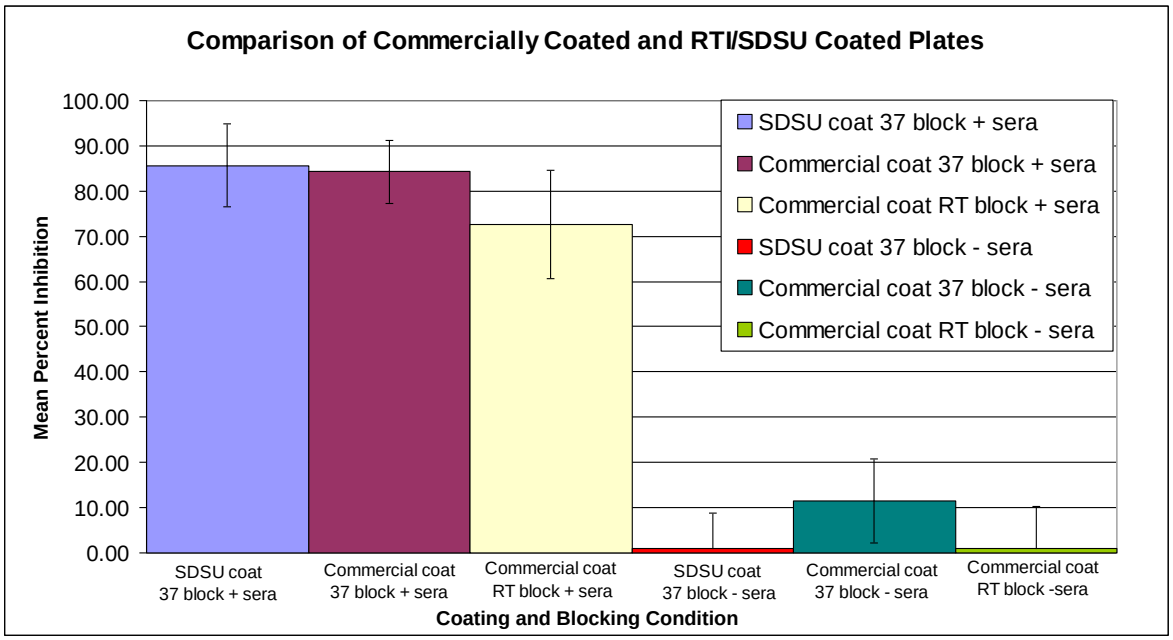


Figure 3. Results showing comparison of commercially prepared, stabilized and blocked "ready-to-use" bELISA plates under two different blocking conditions and those prepared by SDSU using "wet" reagents and a separate blocking step at 37C.

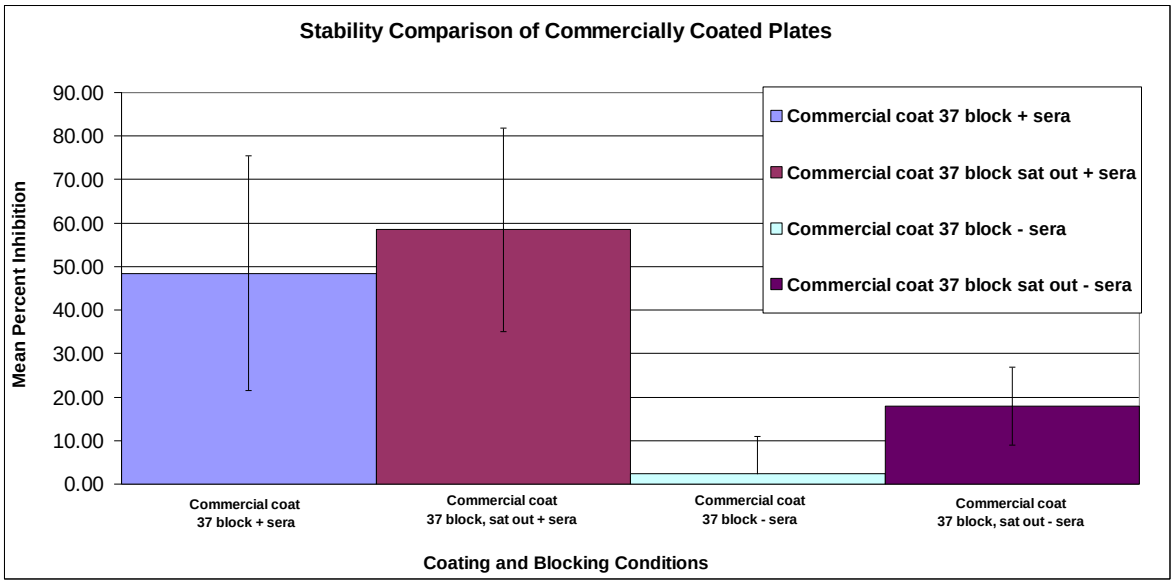


Figure 4. Results showing a longevity/stability comparison of commercially coated, stabilized and blocked plates tested under two separate conditions: Plates immediately refrigerated after blocking versus plates allowed to remain at room temperature (23C) for six months.

Preservative method evaluation:

A variety of methods were evaluated to stabilize other assay components, including the biotinylated primary antibody, secondary antibodies and wash solutions to be included as kit components. Four different chemicals were tested, first as a preservative for the competitive/detection monoclonal antibodies (AT-13 & SDOW-17) and second, as a preservative for the secondary-conjugated antibody and wash solutions. The preservatives that were tested include Gentamicin (50 ug/ml, Atlanta Biologicals); 2-chloroacetamide (0.02% Sigma-Aldrich); 2-methyl-4-isothiazolin-3-one hydrochloride (MITZ, 0.1% Sigma-Aldrich); and sodium azide (0.01% Eastman Kodak). Individual "kit" components including the detection monoclonal antibodies, the secondary-conjugated antibodies and a 10X wash solution were tested with each preservative and its respective concentration. Our results showed that when all preservatives were tested individually with the biotinylated-monoclonal antibodies there was no inhibition of signal development (data not shown). However, when the preservatives were added to the secondary-HRP conjugated antibodies and the wash solution, it was found that MITZ and sodium azide dramatically inhibited signal development when substrate was applied (Figure 5). Conversely, gentamicin and 2-chloroacetamide demonstrated no signal inhibition at the indicated concentrations or even at ten-times higher concentration.

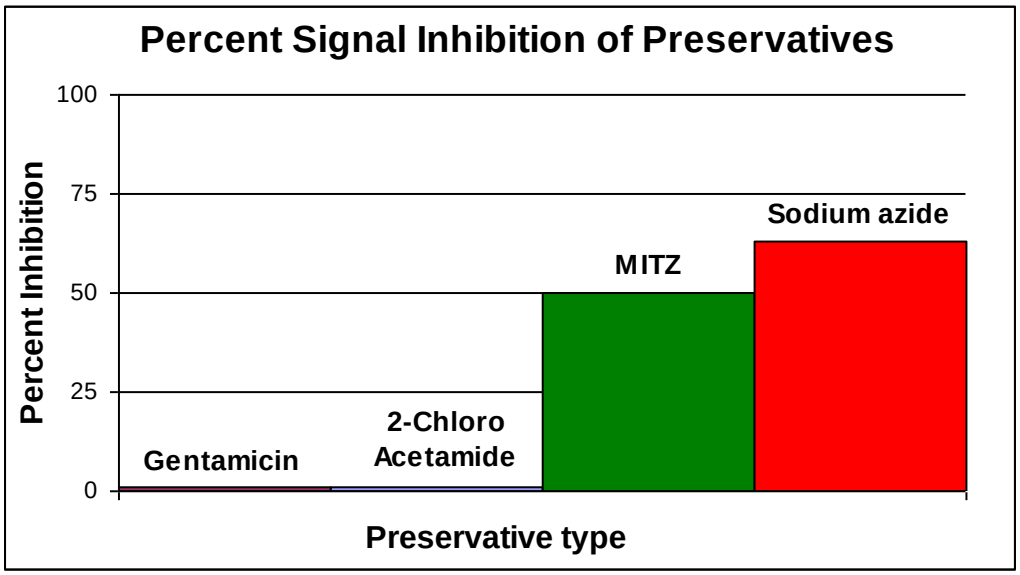


Figure 5. Testing of preservative methods: The following four chemicals were tested as preservatives: Gentamicin (50 ug/ml); 2-chloroacetamide (0.02%); 2-methyl-4-isothiazolin-3-one hydrochloride (MITZ, 0.1%); Sodium azide (0.01%).

Objective II. Broaden detection capabilities of the bELISA assay:

Cutoff determination, diagnostic sensitivity and specificity.

PRRSV positive serum samples were obtained from a population of 290 positive testing clinical serum samples from North American Type 2 (n = 10) and European-like Type1 (n = 32) infected pigs as described in materials and methods. As with both studies, great care was taken to ensure that all PRRSV inoculated individuals had seroconverted before inclusion into the validation study. All North American and European-Like sera used were from pigs bled between 28-154 DPI and 21-85 dpi respectively. Negative testing was performed with serum samples from individuals from the same challenge studies and considered to be negative (uninfected) when collected prior to inoculation or when collected from uninfected control pigs. GRAPHROC software was used for ROC analysis of both PRRSV genotypes to compare histograms of statistical results obtained with the seronegative and seronegative populations to determine an optimized cutoff that maximized both the diagnostic sensitivity and specificity of the assay (Table 1). A two-graph ROC plot for the bELISA was constructed for both Type 1 and Type 2 PRRSV genotypes (Figure 6A-6C). An optimized cutoff level that maximized the efficiency of the assay for Type 1 and Type 2 PRRSV was calculated at 45.5 and 32.4 PI respectively. While ROC analysis showed levels of 99.0% diagnostic sensitivity and 99.3% specificity for Type 2 PRRSV, the assay was even more responsive towards Type1 PRRSV showing levels of 100% diagnostic sensitivity and specificity. Furthermore, there were no diagnostic false positive results seen with the European-Like PPRSV samples in contrast to the North American PRRSV samples where there was one. Figure 6C shows the ROC merger of Type 1 & Type 2 PRRSV PI values where there is a compromise in sensitivity and specificity of the two genotypes. The values given in Table 3 show that the combined results of the bELISA have a diagnostic sensitivity of 99.3% with a 95% confidence interval of 97.5 to 99.9% and a specificity of 99.2% with a 95% confidence interval of 97.1 to 99.9%. Finally, the overall cutoff value for the optimization of the assay using serum from both genotypes was recalculated to a statistical value of 39.7 PI within a 95% confidence interval level.

Figure 6A
ROC analysis of Type 1 PRRSV

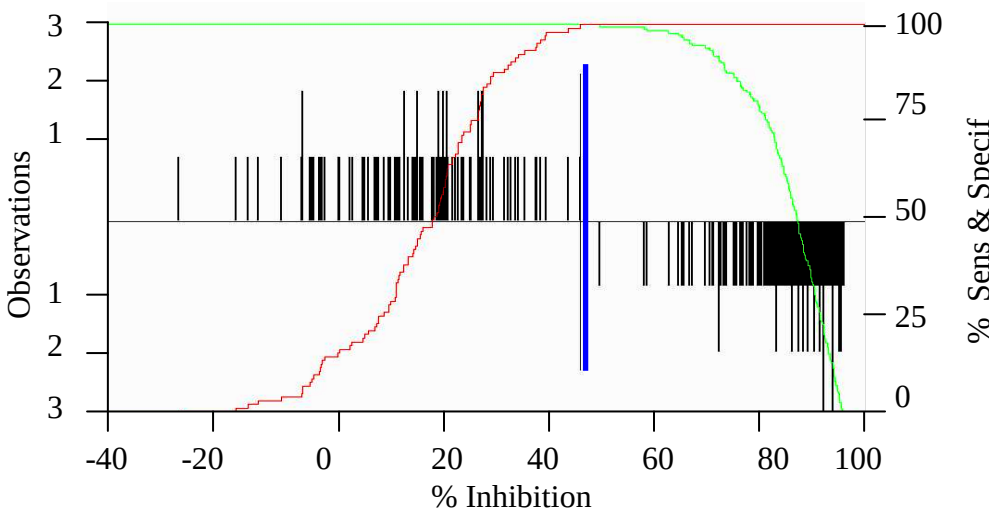


Figure 6B
ROC analysis of Type 2 PRRSV

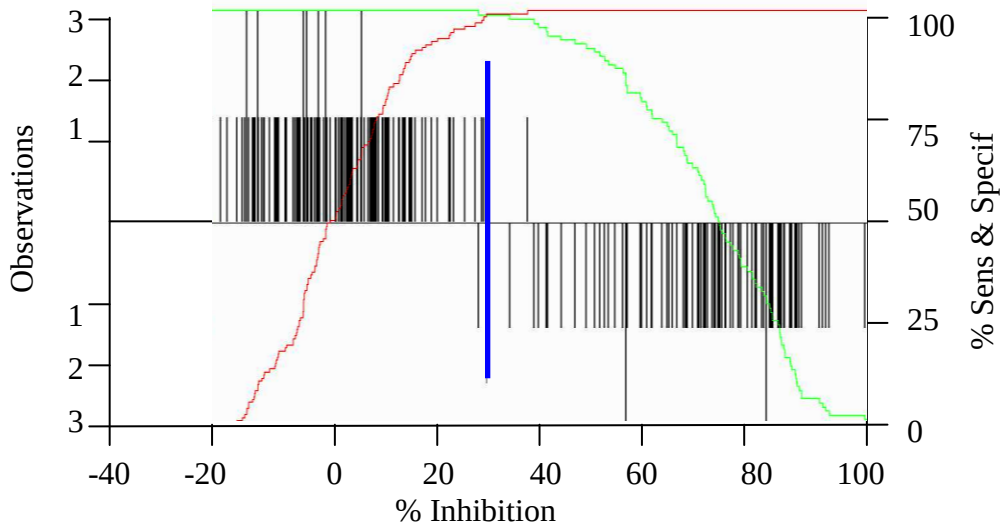


Figure 6C
ROC analysis of Type 1 & Type 2 PRRSV

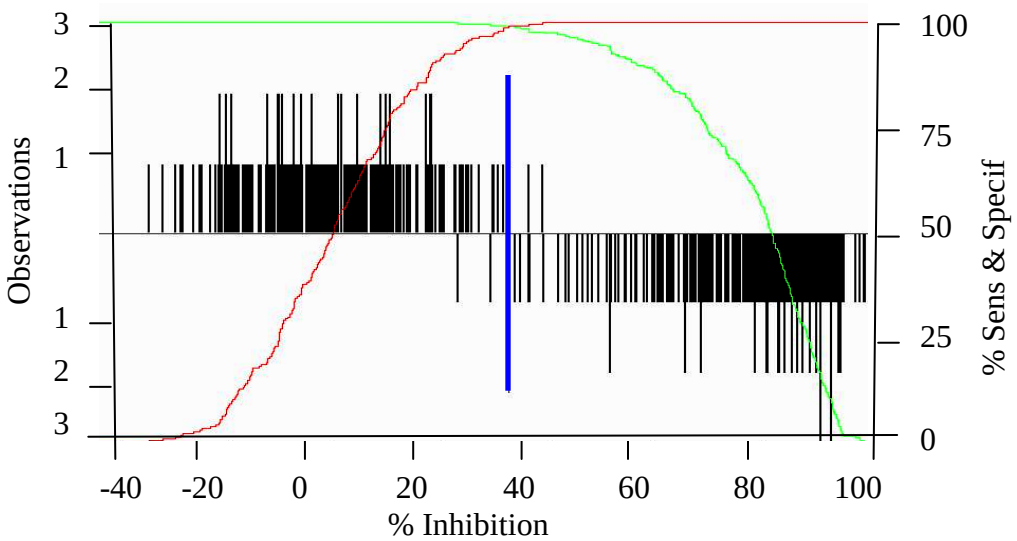


Figure 6. Two Graph ROC plots of the final validated PRRSV blocking ELISA. (A) ROC plot for European Type 1 PRRSV, sera n=294. (B) ROC plot for North American Type 2 PRRSV sera, n=243. (C) ROC plot for combined analysis for both Type 1 and Type 2 PRRSV, n=537. The upward-pointing gray histogram on the left side of the figure represents known uninfected animals. The downward-pointing black histogram on the right side of the figure represents known PRRSV-infected animals. The solid green line represents the diagnostic sensitivity of the assay as the cutoff is moved from 1 to 100 percent inhibition (PI). The red line represents the changes in diagnostic specificity as the cutoff is moved from 1 to 100 PI. The blue vertical line represents the optimized cutoff value corresponding to the maximum diagnostic sensitivity and diagnostic specificity.

Table 1.

Statistical ROC analysis

	bELISA ROC Values		
ROC Characteristic	European- Like Type 1 PRRSV	North American Type 2 PRRSV	Combined Type 1 & Type 2 PRRSV
No. Serum samples	243	294	537
Optimized Cutoff	45.5 PI	32.4 PI	39.7 PI
Mean (+) samples	86.2	72.4	81.3
(-) samples	13.3	6.04	9.19
Std Dev (+) samples	8.85	15.0	13.2
(-) samples	17.4	11.1	14.6
Diagnostic sensitivity (%)	100	99.0	99.3
95% confidence level	98.1-100	94.6- 100	97.5-99.9
Diagnostic specificity (%)	100	99.3	99.2
95% confidence level	96.6 - 100	95.9- 100	97.1-99.9

Table 1: Two graph ROC validation data from U.S. Type 1 and Type 2 PRRSV infected pigs collected from two separate clinical studies.

Figure 7A

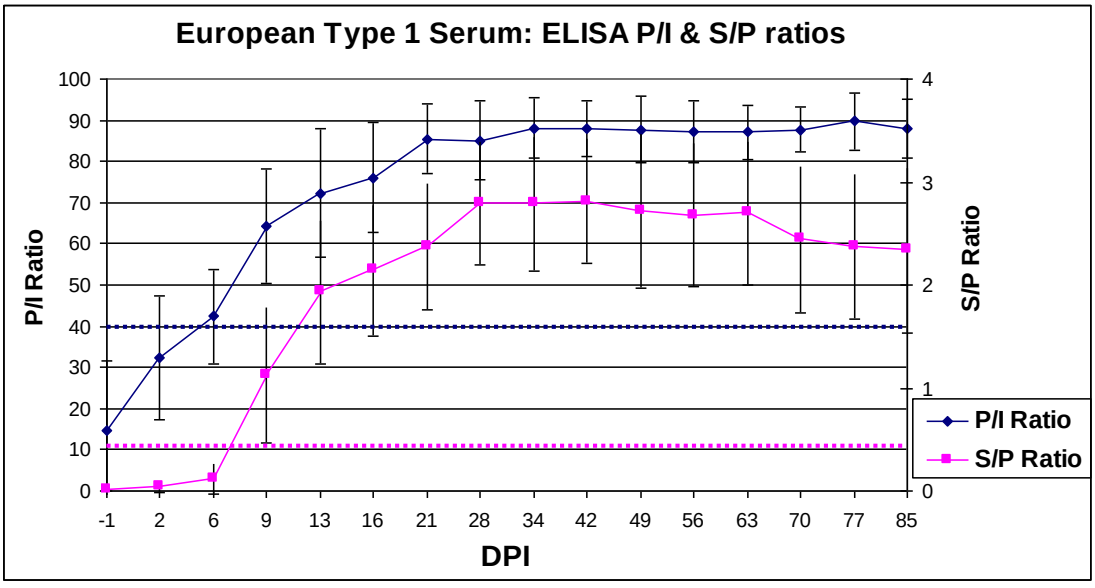


Figure 7B

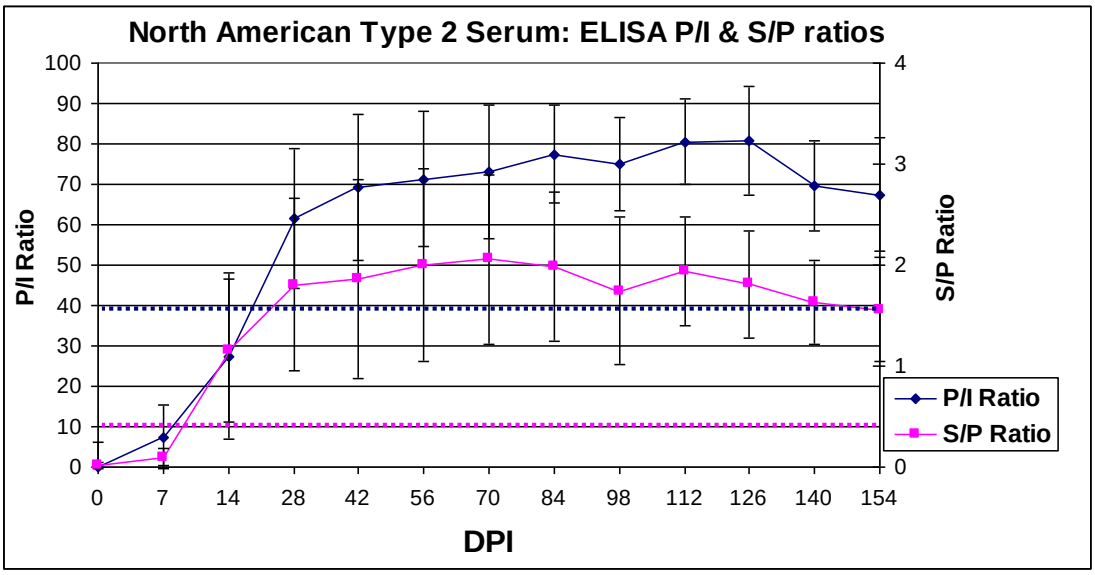


Figure 7. Times of seroconversion of European Type 1 PRRSV (**A**) and North American Type2 PRRSV (**B**) as determined by IDEXX ELISA S/P ratios (0.4 cutoff value) and bELISA PI (39.7 cutoff value).

Detection of seroconversion:

A comparison of seroconversion was made between the IDEXX ELISA and the bELISA with 300 serum samples from 42 positive (infected) pigs. Determination of seroconversion was determined separately for North American Type 2 PRRSV and European-like PRRSV isolates. For the determination of seroconversion of European-Like Type 1 PRRSV, 170 serum samples were analyzed from four groups of eight pigs in which each group was inoculated with a phylogenetically distinct, plaque purified strain of PRRSV (01-08, 01-07, 02-11 and 03-15) as previously described (Nelson et al., 2006). All pigs within each of the four groups were bled on regular weekly intervals between 0 and 85 dpi during which time seroconversion of all virus strains were assessed. These data show that that the bELISA was able to establish an initial time of seroconversion as early as 6 dpi, similarly, the IDEXX ELISA established an initial seroconversion time of 9 dpi (Figure 7A). Both tests are in agreement showing that 100% of the pigs seroconverted roughly in a little more than a week and remained seropositive for the duration of the 85 day study. For the determination of seroconversion of North American infected pigs (Figure 7B), a total of 130 serum samples were analyzed from 10 pigs infected with North American PRRSV VR-2332 between 0 and 154 dpi. Results show that the bELISA and IDEXX ELISA both demonstrated initial times of seroconversion from 14 and 28 dpi. However, no samples were collected from this group of pigs between 14 and 28 dpi to allow finer discrimination of exact times of seroconversion. Both test show that all pigs remained seropositive throughout the 154 day study.

Assessment of negative field sera and evaluation of unexpected positive results:

The use of a blocking ELISA was employed to confirm the status of negative field samples and to resolve the status of serum samples demonstrating unexpected false positive IDEXX ELISA results that arise in routinely sampled negative herds. A total of 752 serum samples from 37 cases of expected negative herds were first analyzed from SDSU ADRDL using the IDEXX ELISA. Of the total number of presumed negative samples, the bELISA confirmed
739 (98.3%) of them to be negative in agreement with the IDEXX results (Table 2).

In addition, 196 serum samples collected from 127 different cases resulting in suspect IDEXX ELISA and IFA unexpected positive values were revaluated using the bELISA. The average IDEXX S/P ratio among the group was 0.685. A comparison of the percentages of the unexpected positive results between the two ELISA formats was made (Table 2). The bELISA format yielded a 72% reduction in the number of unexpected false

positive samples. A reduction from 196 unexpected positives using the IDEXX ELISA down to 55 unexpected positive samples assessed by the bELISA.

Table 2.

Negative testing of serum samples

Assay Type	Analysis of neg field samples from presumably neg herds	Analysis of unexpected pos samples in presumably neg herds
IDEXX ELISA and IFA	752 neg samples	196 unexpected positive samples
bELISA	739 (98.3%) Presumed neg in agreement with IDEXX	55 unexpected positive samples (72% reduction)

Table 2. Testing of negative and unexpected positive samples was compared using the IDEXX ELISA, IFA and the bELISA.

Discussion:

PRRSV can be divided into two distinct genotypes, the European and North American types. These types show significant genetic and antigenic differences with an overall nucleotide identity of 63% (Nelsen et al., 1999; Meng et al., 1995). The antigenic heterogeneity within new emerging European-like strains presents new diagnostic challenges for the industry. In addition, monitoring the serostatus of PRRSV negative or low-prevalence herds also presents an issue of high importance to producers. The IDEXX HerdChek® PRRS ELISA has been used for years as the "gold-standard" for the detection and screening of PRRSV antibodies. Other methods such as IFA and immunoperoxidase monolayer assays have been developed, but differ in sensitivity with disadvantages such as the need for actual virus replication and the influence of antigenic differences between genotypes. (Yoon et al., 1992; Ropp et al., 2004).

The N protein of North American and European strains is comprised of 123 or 128 amino acid residues, respectively, and is expressed as the most abundant protein with a virion content of between 20-40% (Bautista et al., 1996). It also represents the most immunodominant protein among all PRRSV structural proteins, and is encoded by a relatively well-conserved region of the viral genome with a high degree of amino acid sequence identity among stains. The kinetics of the humoral response has been well documented and several reports have described the use of anti-N MAbs that recognize epitopes shared by North American or European strains of PRRSV (Nelson et al., 1993; Dea et al., 1996). Therefore, a bELISA was developed using a full length, recombinant expressed nucleocapsid protein as capture antigen to coat 96 well microtiter plates. In order to increase the specificity of the assay, purified nucleocapsid antigen from both European-like and North American strains was utilized.

The choice of detection used in an ELISA assay can affect the overall specificity and influence the performance of the assay. A dual set of biotinylated monoclonal, blocking antibodies and a streptavidin conjugate was chosen in order to maximize the sensitivity at subsequent steps. Further analytical specificity and sensitivity is imparted by the use of the MAbs SDOW-17 and AT-13. Both are directed against highly conserved epitopes on the nucleocapsid protein and have been shown to cross react with nearly all isolates of PRRSV- including both North American and European isolates (Nelson et al., 1993; Wooton et al., 1998). Ratios of these biotinylated monoclonal antibodies were then optimized in experiments utilizing multiple sets of varying dilutions to provide optimum binding to recombinant antigen and appropriate levels of competition with control swine serum containing antibodies against Type 1 and Type 2 PRRSV.

This test format proved ideal for the intended application of this assay for follow-up testing of unexpected positive IDEXX ELISA results. The internal control sera used for the bELISA has been routinely used for the IDEXX ELISA by the SD ADRDL and has been well characterized over several years. The overall

performance of this assay builds upon work previously reported by Ferrin et al., 1994. Optimization of antigen coating methods has resulted in stabilized commercially coated, "dry" ELISA plates that eliminate the separate blocking steps previously required with "wet" reagents and improve throughput of the assay. Extensive comparisons between commercially prepared "dry" plates and traditional freshly prepared ELISA plates demonstrated equal performance to the "ready-to-use" "dry" plates. Long term stability assessment of the stabilized plates was performed by demonstrating that commercially coated sealed plates allowed to sit at room temperature for 6 months showed no significant decrease in signal development compared to those immediately refrigerated once coated. However, commercially coated plates blocked at 37C showed a significant increase in signal compared to plates blocked at room temperature. The effect of different blocking temperatures on the performance of the bELISA has also previously been noted when analyzing serum samples on freshly coated "wet" plates (data not shown).

Testing of potential preservatives was performed using the following preservatives: Gentamicin (50 ug/ml); 2-chloroacetamide (0.02%); 2-methyl-4-isothiazolin-3-one hydrochloride (MITZ, 0.1%); Sodium azide (0.01%). Only gentamicin and 2-chloroacetamide were determined to be effective preservatives in all kit components.

Several steps were taken in order to validate and assess the accuracy of the bELISA assay. ROC analysis was performed using serum samples from 537 serum samples from 42 individual animals that were experimentally infected with either U.S. Type 1 or U.S. Type 2 PRRSV. In addition to using a large population of animals and serum samples, careful attention was taken to verify the PRRSV serostatus of each individual pig before inclusion into the validation population. Only those time periods whereby 100 percent of pigs had first seroconverted (most often by 21 dpi) was utilized.

Statistical analysis determined a diagnostic sensitivity and specificity of 99.3% and 99.1%, respectively for each PRRSV genotype. In addition, an optimized cutoff level that maximized the efficiency of the assay for Type 1 and Type 2 PRRSV was calculated at 45.5 and 32.4% PI respectively. Interestingly, there were no diagnostic false positive results seen with the European-Like PRRSV samples in contrast to the North American PRRSV samples where there was one. Finally a merger of both Type 1 and Type 2 PRRSV was performed which resulted in a change of the overall cutoff value for the optimized assay at a value of 39.7 PI. The high degree of sensitivity and specificity quantified within the assay was imparted by the use of control populations of animals to ensure that minimal overlap of ROC histograms occurred between positive and negative analytes. Our results also show that the high sensitivity of our assay indicates that the antibody response of the animals is able to compete effectively against both competing MAbs AT-13 and SDOW-17 for the binding of the capture nucleocapsid antigen.

The bELISA was developed primarily as a confirmatory test to the IDEXX ELISA when investigations of unexpected positive results in presumably negative herds come about. The detection of seroconversion was compared to results seen with the IDEXX ELISA by assaying samples obtained from two separate controlled clinical studies using both U.S. Type 1 and Type 2 sera. A total of 300 serum samples from 42 PRRSV positive (infected) animals were used, and a comparison of seroconversion was made between the IDEXX ELISA and the bELISA. Determination of seroconversion was determined separately for North American Type 2 PRRSV and European-like PRRSV isolates. The determination of seroconversion for the North American infected pigs, included 130 serum samples that were analyzed from 10 pigs infected with North American PRRSV VR-2332 between 0 and 154 dpi. The remaining 170 serum samples were from four groups of eight, six week-old pigs in which each group was inoculated with one of four phylogenetically distinct, plaque purified European-Like Type 1 strains of PRRSV. All pigs within each of the four groups were bled between 0 and 85 dpi and seroconversion was assessed. Overall, the agreement between the two methods in determining the times of seroconversion was very high. For the U.S. Type 1 strains, the times of seroconversion were nearly identical; however, these data show a delay of approximately a week for the bELISA. These data indicated that the bELISA has a window of detection that is quite similar to the IDEXX ELISA. There were no false-positive results with any samples within the seroconversion study, most likely because all animals were from one of two controlled studies; and hence, have had minimal prior exposure to other pathogens.

Lastly, the bELISA was employed to confirm the status of negative field samples and to resolve the status of serum samples demonstrating unexpected false positive IDEXX ELISA results that arise in routinely sampled

negative herds. A total of 752 serum samples from 37 cases of expected negative herds were first analyzed from SDSU ADRDL using the IDEXX ELISA. Then the same samples were reevaluated by the bELISA to verify the seronegative serostatus of the cases. Of the total number of presumed negative samples, the bELISA confirmed 739 (98.3%) of them to be negative in agreement with the IDEXX results. The 1.7% unaccountability may be due to variations in the individual immune responses of pigs or the sample quality. Reports have shown that there is a potentiation of PRRSV for other pathogens such as *M. hyopneumoniae* (Thacker et al., 1999) which may be reflective of herd management practices with the use of multiple vaccines or other therapeutic drugs.

Because the results of negative-testing demonstrated that the bELISA was highly repeatable, we proceeded with the assessment of 195 serum samples from 127 cases resulting in suspect IDEXX ELISA results. The bELISA format yielded a 72% reduction in the number of unexpected positive results from 196 unexpected positives using the IDEXX ELISA down to 55 unexpected positive using the bELISA. The average IDEXX S/P ratio among the group of 55 was 0.685 indicating a value close to the cutoff point of 0.4.

In conclusion, the IDEXX HerdChek® ELISA is a well-characterized and accepted assay; however, false positive samples continue to be a problem in herds expected to give negative results. This makes the serostatus determination of individual animals and herds unclear. The bELISA was designed with both Type 1 and Type 2 nucleocapsid antigens in mind, in addition to two biotinylated antibodies derived from the two different PRRSV genotypes (AT-13 and SDOW-17 MAb). These data, through negative testing, demonstrate that the bELISA is highly repeatable and shows a high degree of agreement with the IDEXX ELISA with respect to seroconversion. The bELISA also demonstrates a high level of resolving power when unexpected false positive results arise. The validation results of our optimized bELISA show that it will be useful as a confirmatory test when unexpected positive results arise in presumably seronegative herds.

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Lay Interpretation:

The NPB Initiative recognizes that the most effective way to control PRRS is to eliminate the virus from domestic swine herds. Control and elimination strategies should include diagnostic tools that can identify all PRRSV isolates so infected animals can be culled from the herd. This report demonstrates that our newly developed and optimized bELISA provides a more sensitive assay to confirm the true positive animals versus false positive in herds or when transient positive/negative results arise. It could also be used to validate IDEXX ELISA results when PRRSV antibodies are just above or below the level of detection (low S/P ratios). By adding components such as a dual set of monoclonal antibodies AT-13 and SDOW-17 and recombinant nucleocapsid protein derived from both North American and European-like PRRS virus strains, we demonstrate an increased specificity and sensitivity which ultimately leads to the assay's broadened detection capabilities.

The bELISA is a single assay taking just three and a half hours to perform and provides an accurate and convenient alternative method to assist with the evaluation of results obtained when using the IDEXX ELISA. Other candidate assays for a follow-up test have drawbacks. VI is sensitive within days of infection, but becomes less useful in the following weeks (Christopher-Hennings et al., 2001) Temperature of storage and

shipping of serum samples also may decrease the likelihood of virus recovery. SVN detects neutralizing antibodies that appear 30 to 60 days post infection, so it is a poor test for early infection. PCR provides a quick and sensitive method for the detection of viral antigen; however, it is very expensive to perform on a routine screening basis. Although the IFA detection window is similar to that of the IDEXX ELISA, it requires techniques and equipment not available in all laboratories. It is also very subjective and has less repeatability between diagnostic laboratories. The bELISA shows specificity and sensitivity over a wide time frame, and shows good repeatability. Therefore, the blocking ELISA assay is a better candidate for a confirmatory assay. The addition of antibody and antigen components to detect Type 1 PRRSV will expand the detection capabilities of the assay. We have developed the components of this assay into a stable "kit" format that will retain its activity after storage, making it a good candidate for commercial applications. This bELISA could supplement the current IDEXX ELISA and would directly benefit producers by allowing them to detect any PRRSV strains present in their herd quickly and accurately.

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