Validation of a Milk Progesterone Fluorescence Polarization Assay - A Pilot Study -

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Ellie LLC has developed a fluorescence polarization assay (FPA) for the quantification of milk progesterone (P4) levels. To validate the analytical performance of this assay, we tested: 1) intra-assay, inter-assay, and inter-operator precision; 2) milk P4 storage stability after refrigerating or freezing samples for 24 h; 3) the effect of adding potassium dichromate as a preservative on the P4 concentration; and 4) the performance of the FPA versus a radioimmunoassay (RIA) in determining P4 concentration in milk and serum. In addition, we evaluated the performance of the milk P4 FPA as an "early non-pregnancy test" during at least one estrus cycle in 20 Holstein-Friesian and 14 Jersey cows. The milk P4 FPA demonstrated strong repeatability (total mean CV<5%) and reproducibility (total mean CV<9%). Sample storage stability was acceptable at both temperature conditions. The addition of potassium dichromate caused no significant variation in P4 FPA measurements. Milk P4 concentrations, when compared to RIA were biased, but the differences were not diagnostically significant. In all cases where "early non-pregnancy test" performance was evaluated, the P4 FPA yielded 100% specificity, when an arbitrary P4 cut-off value of 35 ΔmP was used. No animal was falsely classified as opened, and these results were later verified using classical methods. The same cut-off also yielded 60-100% sensitivity for Holstein-Friesian cows, depending on how the assay was used. In non-synchronized Jersey cows, the sensitivity was 28-57%. The tail paint method was used to detect estrus in this group, which led to overall lower succes AI in Jerseys. We conclude that the milk P4 FPA, is a rapid and reliable diagnostic assay for detecting milk P4 levels, and consequently heat, pregnancy status, and some pathological conditions. Also the assay does not require a dedicated lab space; it can be run on the farm or in a simple farm or clinical laboratory.

Introduction

The milk progesterone (P4) assay is a useful tool for rapidly detecting oncoming heat in cows that did not conceive after artificial insemination (AI) and for identifying cows with silent estrus (McLeod et al., 1991; McDougall, 2010). Ideally, a milk P4 assay should be quantitative and simple enough to be performed on-farm (Waldmann and Rod, 2016). Ellie LLC has developed a sensitive and specific fluorescence polarization assay (FPA) that quantifies the progesterone concentration in milk.

FPA is simple to perform, and it does not require multiple separate steps, which helps end-users to avoid many potential analytical errors. With the use of portable equipment, it can be performed on-farm. It is also a homogenous assay without a solid phase, which makes it easier to consistently extract and detect hydrophobic molecules, like progesterone, when compared to solid-phase assays, like ELISA or lateral flow. Milk fat has less effect on FPA because it uses alcohol as a solvent, which also facilitates the recovery of P4. Sample preparation and results can be achieved in less than 10 minutes.

In this report, we present preliminary analytical and diagnostic performance data for the milk P4 FPA. To evaluate the assay's performance, we followed guidelines from the American Society for Veterinary Clinical Pathology – ASVCP (Flatland et al., 2010) and the U.S. Food and Drug Administration – FDA (www.fda.gov). To determine the analytical performance of the milk P4 FPA, we tested intra-assay, inter-assay, and inter-operator precision using low, medium, and high P4 concentrations. We also tested sample storage stability and the effect of adding potassium dichromate as a preservative. Furthermore, we compared the performances of the FPA and radioimmunoassay (RIA) when measuring milk P4 concentrations. To evaluate the diagnostic performance of the

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FPA, we correlated the P4 concentrations in serum and milk and monitored P4 fluctuations during at least one estrus cycle in Holstein-Friesian, Jersey, and Simmental cows.

Materials and Methods

Milk P4 FPA

The P4 concentrations in milk samples were measured with the P4 FPA Milk Assay kit (Lot No. 101) from Ellie LLC using a BioTek® Synergy™ H1 fluorescence polarization reader and 24-well black microtiter plates. The assay was performed according to the kit's manual. Testing was conducted at the Totally Vets Ltd., a veterinary clinic in Feilding, New Zealand and at our subsidiary lab at DOO Biotehnika IVD in Kraljevo, Serbia.

The P4 FPA assay is quantitative, but the results do not represent the absolute P4 concentration. Instead, the unit of measurement is Delta mP (ΔmP). ΔmP is directly proportional to the P4 concentration in milk. However, the interpretation of the results is qualitative: if a ΔmP value is below the cut-off, it indicates low P4 (i.e. an opened cow). If the ΔmP value is above the cut-off, it indicates high P4 and identifies a potentially pregnant cow, or a non-pregnant cow with an active corpus luteum, or a luteal cyst. In this manuscript, some analytical validation results are presented as mP (i.e. not converted to ΔmP). In these cases, the relationship with the milk P4 concentration is inverse.

Analytical performance of the milk P4 FPA

To assess the analytical performance of the milk P4 FPA, we used milk from Simmental cows taken 30- and 70-days post-partum to obtain samples with low and high P4 concentrations, respectively. Samples with a medium P4 concentration were created by mixing low and high P4 milk samples at a 1:1 ratio.

Intra-assay precision was determined by testing fresh milk samples, while inter-assay and inter-operator precisions were determined using aliquoted and frozen milk samples. To determine the intra-assay precision, 1 low, 1 medium and 1 high concentration sample, was tested 10 times. To determine the inter-assay precision, 10 low, 10 medium, and 10 high P4 concentration samples were tested in triplicate over 3 days. To determine the inter-operator precision, 1 low, 1 medium and 1 high concentration sample was tested 15 times each by 2 operators.

Storage stability was assessed using 16 samples of fresh milk that were aliquoted and stored at 2-8°C or -20°C for 24 hours. Before the analysis, the samples were equilibrated to room temperature (RT) for 30 min and vigorously vortexed. Bias was calculated as: $(P4^{stored} - P4^{fresh}) / P4^{fresh} \times 100$. The t-test for paired samples was used to determine whether the difference between the P4 levels in fresh and stored milk samples was significant. Spearman's ρ rank correlation analysis tested the relationship between bias and the P4 level in fresh milk samples.

To test the effects of a sample preservative, a potassium dichromate solution was added to 50 mL of fresh milk to reach a final concentration of 0.01-0.04%. Then, a paired t-test compared the P4 levels in 6 milk samples before and after the addition of the preservative.

As a preliminary calibration, a milk sample from a Simmental cow in estrus (deemed "progesterone-free" by RIA) was spiked with progesterone to reach final concentrations of 5 ng/mL, 10 ng/mL, and 15 ng/mL, and the mP value was measured for each concentration. The obtained values were used to calculate the regression equation. Next, 18 milk samples were used in a method comparison study: 9 originated from Simmental cows in estrus, and 9 originated from 2.5- to 4.5-month pregnant Simmental cows. Bland-Altman and Passing-Bablok analyses evaluated the similarity of the FPA and RIA results.

Correlation between serum and milk P4

Serum and milk samples were collected from 6 cows in estrus and 34 pregnant Simmental cows (a total of 40 serum samples and 40 milk samples). Serum P4 concentrations were measured using a commercial RIA, run by the Institute for Application of Nuclear Energy (INEP), Serbia, and milk P4 concentrations were measured by FPA.

Diagnostic performance of the milk P4 FPA

To determine the diagnostic performance of the assay, we used three groups of animals:

1. Twenty Holstein-Friesian cows were from a farm in Feilding, New Zealand. This farm maintains approximately 400 milking cows that are freely held on a pasture. Reproduction management on this farm includes a synchronization protocol and AI. Bulls identified cows that did not conceive after the first AI.
2. Fourteen Jersey cows were from a farm in Feilding, New Zealand. The farm maintains approximately 800 milking cows that are freely held on a pasture. Reproduction management on this farm does not include a synchronization protocol. Tail-paint technique identified animals in heat that were then artificially inseminated. Tail paint identified cows, again, that did not conceive after the first AI.
3. Five Simmental cows were from several mini-farms in Serbia's mountainous Dragacevo region. All cows were under the full supervision of a veterinarian and carefully examined daily throughout the experiment. It should be noted that this part of the experiment evaluated the assay's ability to monitoring P4 concentrations in milk during an estrus cycle, but not its ability as an "early non-pregnancy test". Three cows were synchronized and two were not. One cow displayed "silent estrus".

Pregnancy status

Three methods were used to determine the pregnancy status of the cows:

1. Transrectal ultrasonography at 30-33 days post-AI (Holstein-Friesian cows [n=8], Jersey cows [n=14], Simmental cow [n=1]).
2. IDEXX pregnancy-associated glycoprotein (PAG) iELISA at 30 days post-AI (Holstein-Friesian cows [n=4].
3. Heat detection at 18-25 days post-AI in 8 Holstein-Friesian cows and in 4 Simental cows during the estrus cycle.

Synchronization protocol in Holstein-Friesian cows

Briefly, synchronization was facilitated with intramuscular injections of prostaglandin 2α (PGF2α) 23 days before TAI and gonadotropin-releasing hormone (GnRH), 10 days before TAI. Non-cycling cows were also treated with a vaginal progesterone device (CIDR) for one week. Three days before TAI, all cows were injected with PGF2α and equine chorionic gonadotropin (ECG). All cows were anrtificially inseminated and received 2 mL of GnRH.

Milk sample collection

Milk samples were taken from the healthy quarter of the udder. After discarding the first 10-15 jets, 50 ml of milk was collected in a clean container. The milk samples were tested for P4 on the day of collection.

Foremilk samples from the Holstein-Friesian group were collected at afternoon milking (7-17 days after AI) with 2-4 days between collections, and the samples were collected daily between days 17 and 23. In the Jersey group, foremilk samples were collected at morning milking (1 day after AI) with 2-4 days between collections, and the samples were collected daily between days 18 and 24. The last sample was taken on day 26. In the Simmental group, the foremilk samples were collected at morning milking (one day after AI) and each day during the following month.

Statistical analyses

All statistical analyses were performed using MedCalc® software, version 16.2.1. The milk P4 cut-off value for the detection of non-pregnancy was chosen so that it yielded 100% specificity 19-23 days after AI (i.e. none of the pregnant cows were identified as non-pregnant according to the milk P4 concentration). A "P" value lower than 0.05 was considered significant.

Results and Discussion

Analytical performance of the milk P4 FPA

Intra-assay precision

Table 1 shows the FPA results for milk samples with 3 different P4 concentrations (low, medium, and high). Each sample was tested 10 times by one operator. The coefficient of variation (CV) range demonstrates the strong repeatability of the assay. The total mean CV for repeatability was 5%.

Table 1. Intra-assay precision for the milk P4 FPA.

P4 concentration	# of Tests	Mean ± SD (ΔmP)	CV (%)
Low	10	24.5 ± 1.3	5.4
Medium	10	89.1 ± 2.9	3.2
High	10	105.6 ± 6.7	6.3
Total mean CV			5.0

CV: coefficient of variation, SD: standard deviation

Inter-assay precision

Table 2 shows the FPA results for 10 milk samples with 3 different P4 concentrations each (low, medium, and high). Each sample was tested in triplicate. The coefficient of variation (CV) range demonstrates the strong reproducibility of the assay. The total mean CV for reproducibility was 8.5%.

Table 2. Inter-assay precision for the milk P4 FPA.

P4 concentration	# of Tests	Mean ± SD (ΔmP)	CV (%)
Low	30	19.3 ± 2.4	12.3
Medium	30	72.3 ± 6.1	8.5
High	30	100.6 ± 4.7	4.6
Total mean CV			8.5

Inter-operator precision

Table 3 shows the FPA results from milk samples with 3 different P4 concentrations (low, medium, and high). The coefficient of variation (CV) range demonstrates good reproducibility. The total mean CV was 8.8%.

Table 3. Inter-operator precision for the milk P4 FPA.

P4 concentration	# of Tests	Mean ± SD (ΔmP)	CV (%)
Low	15 × 2	19.3 ± 2.6	13.2
Medium	15 × 2	72.3 ± 6.0	8.3
High	15 × 2	100.6 ± 4.8	4.8
Total mean CV			8.8

CV: Coefficient of variation, SD: Standard deviation

Storage stability

The results indicate that the P4 concentration is stable under the conditions that were investigated; the median bias values are small, and the difference in the individual samples is not significant (Table 4). Nevertheless, the somewhat broad range between the minimum and maximum bias values implies that the results should be confirmed with a larger group of samples. This would allow the separate bias values that correspond to the different P4 concentrations in the fresh milk samples to be assessed.

Table 4. Storage stability of P4 in milk samples refrigerated or frozen for 24h (n=16). P4 and bias values are presented as median (min-max), while P-values are given for statistical analyses.

Storage	P4 ΔmP	Bias %	t-test	ρ
Fresh	68.3 (13.9, 114.9)	/	/	/
2-8°C	68.9 (13.3, 111.5)	-1.3 (-39.7, 29.0)	0.561	0.485
-20°C	69.8 (11.3, 121.5	4.3 (-48.8, 57.9)	0.402	0.387

(ρ) Spearman's ρ rank correlation

Effect of potassium dichromate

P4 concentrations before the addition of potassium dichromate did not significantly change after the preservative was added (P=0.415).

Table 5. Milk P4 FPA results (ΔmP) from samples before and after the addition of potassium dichromate.

Sample	Before	After
1	14	13
2	19	10
3	19	15
4	19	21
5	46	50
6	63	61

Comparison between FPA and RIA in milk samples

RIA and FPA results from milk samples were compared using Bland-Altman (Figure 1) and Passing-Bablok analyses (Figure 2). Both analyses indicate proportional bias. Nevertheless, the bias does not seem significant from a diagnostic standpoint, since both methods show an analogous pattern of discrimination between pregnant and cows in estrus (Figure 3).

Figure 1. Plot of the differences between RIA and FPA versus the mean of the two measurements

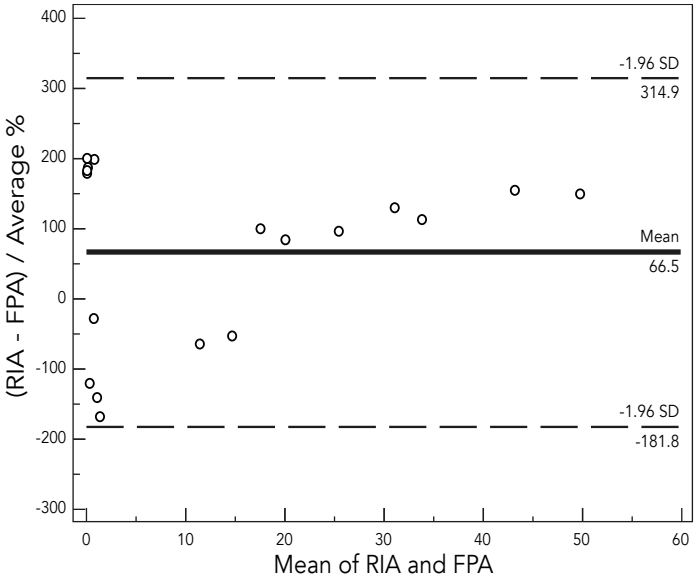


Figure 2. Passing-Bablok Regression line

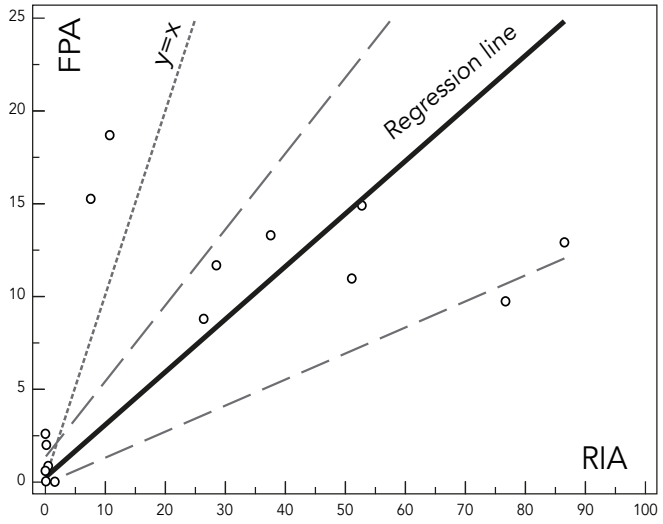
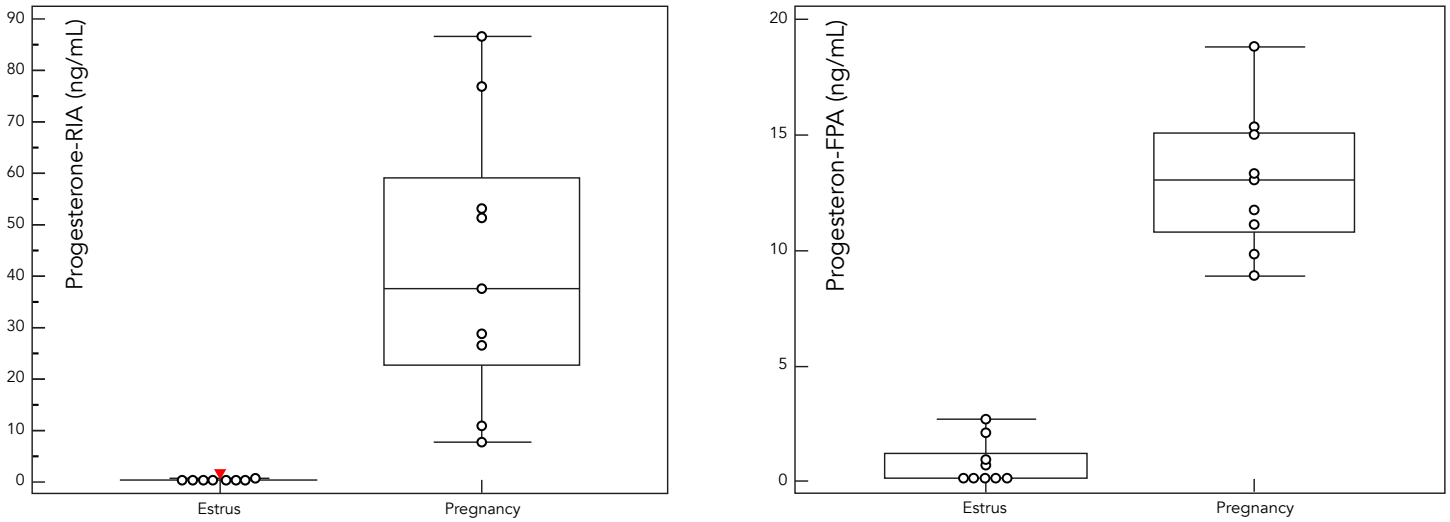


Figure 3. Milk P4 concentration in cows in estrus and in pregnant cows determined with RIA and FPA

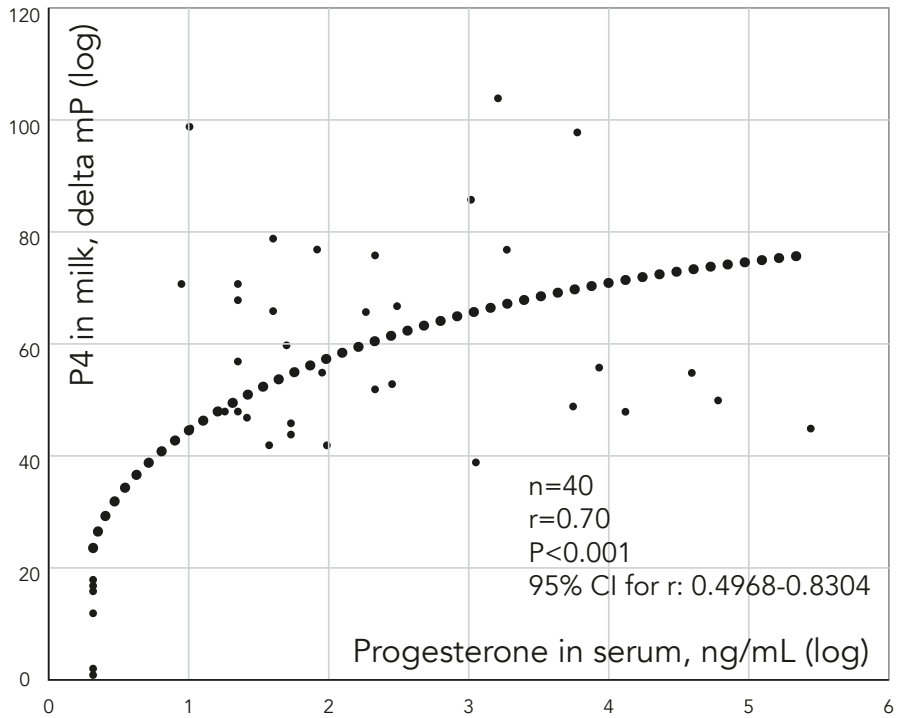


The FPA has a greater resolution when determining P4 concentrations in milk samples, which makes it easier to discern cows in estrus versus pregnant cows. This is illustrated by the wider difference between milk P4 concentrations of cows in estrus and pregnant cows obtained via FPA (Figure 3). This resolution leads to a more robust assay. Small pipetting errors when performing the assay do not change the diagnostic outcome.

Comparison of FPA in milk and RIA in serum samples

After it is biosynthesized in the ovaries, placenta and adrenal glands, P4 is released into the bloodstream (Wiltbank et al., 2014) and secreted into the milk, where it reaches higher concentrations than in the serum. Therefore, P4 serum and milk concentrations display a positive correlation (Roelofs et al., 2006); this was confirmed by our results (Figure 4).

Figure 4. Correlation between P4 concentration in the serum and milk samples.



Diagnostic Performance

Holstein-Friesian Cows

The P4 concentration in milk reliably discriminates pregnant from non-pregnant cows between 19 and 23 days after AI (Figure 5). Figure 5 shows that pregnant Holstein-Friesian cows without signs of heat (n=6) or with signs of heat at day 21 (n=3) had a high P4 level. The pregnancy status of these cows was confirmed by ultrasound or PAG on day 30 after AI.

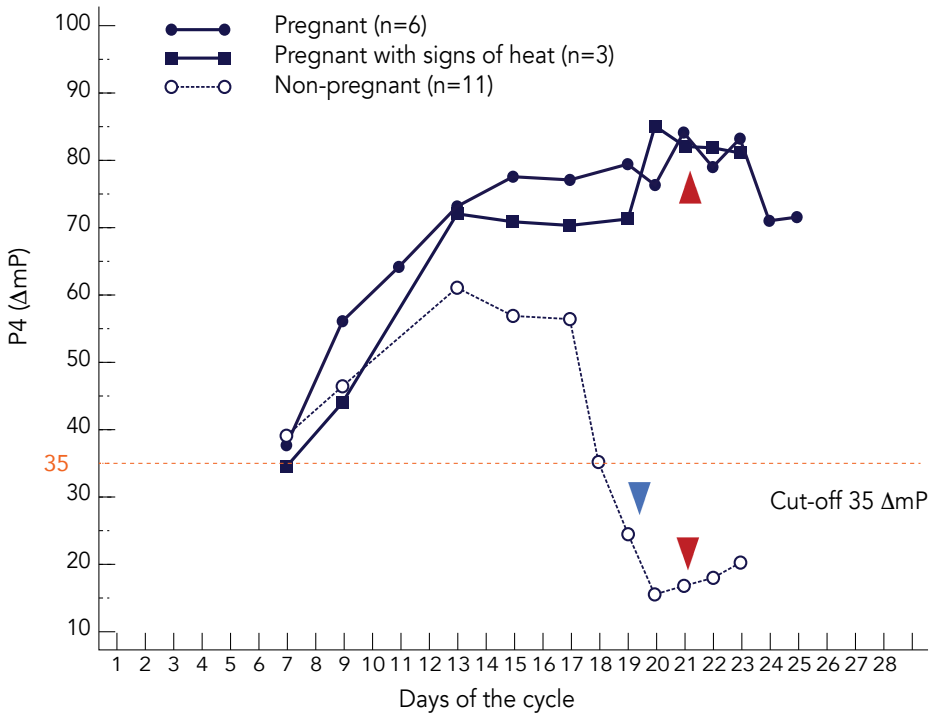
It is important to note that a low P4 FPA measurement accurately classifies cows as non-pregnant; therefore, AI repetition is specifically targeted. Furthermore, non-pregnant cows (n=11) had a significant decrease in P4 on day 19 (median value), ranging between days 15 and 22, and signs of heat appearing 2 to 3 days after the decline.

To analyze the diagnostic performance of the milk P4 FPA as a "non-pregnancy test," we calculated its sensitivity and specificity between days 19 and 23 of the AI cycle when the majority of "open" Holstein-Friesian cows were expected to have low P4 levels (Figure 6). The sensitivity range was between 60 to 91% and specificity was 100% when the milk P4 FPA was performed only once between days 19 and 23 after the first AI (Figure 6). Maximum sensitivity (91%) at 100% specificity was achieved on day 22 (Figure 6). Based on these results, it is possible that a combination of assay results gathered from cows that exhibit high P4 levels on two consecutive days, or twice on days 20 and 22 only, would yield an even higher diagnostic sensitivity and accuracy. Ideally, the sensitivity and accuracy could reach 100% at 100% specificity.

It should be noted, again, that the investigated Holstein-Friesian cows were synchronized so that a period of pre-estrus and estrus P4 decline could be precisely determined in open cows.

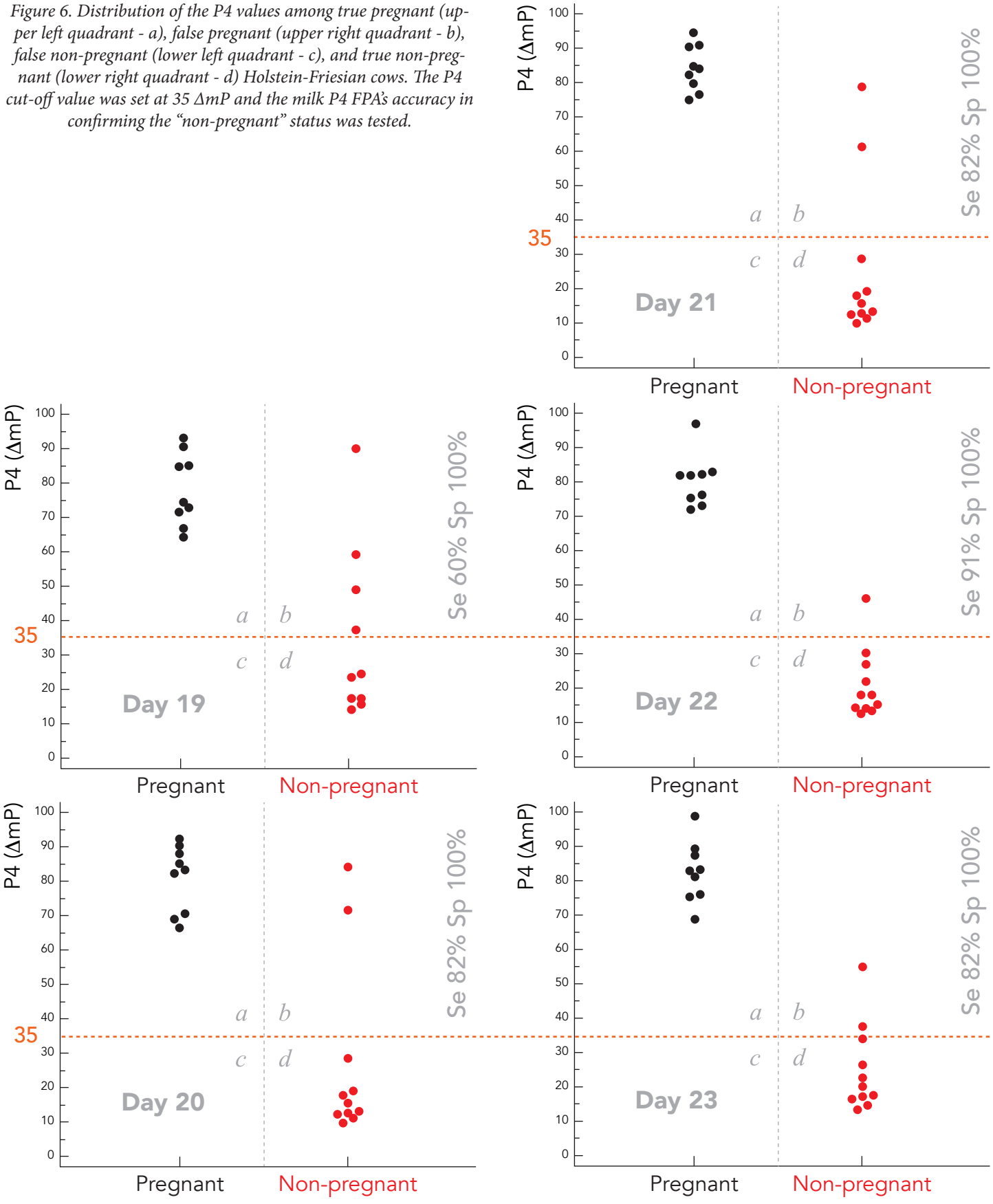
Most importantly, the 35 ΔmP cut-off value yielded 100% specificity throughout the study, which would prevent repeated AI of already pregnant cows.

Figure 5. P4 in milk samples from a group of synchronized pregnant and non-pregnant Holstein-Friesian cows from New Zealand.



The median values of pregnant (n=6), pregnant with heat (n=3), and non-pregnant (n=11) cows are shown. In non-pregnant cows, P4 significantly decreased (blue arrow) on day 19 (median value) and heat (red arrow) appeared on day 21 (median value). The horizontal line represents the 35 ΔmP cut-off value.

Figure 6. Distribution of the P4 values among true pregnant (upper left quadrant - a), false pregnant (upper right quadrant - b), false non-pregnant (lower left quadrant - c), and true non-pregnant (lower right quadrant - d) Holstein-Friesian cows. The P4 cut-off value was set at 35 ΔmP and the milk P4 FPA's accuracy in confirming the "non-pregnant" status was tested.



Jersey cows

The P4 concentration in milk reliably distinguished pregnant and non-pregnant Jersey cows between 19 and 23 days after AI (Figure 7). However, three cows that had high P4 values (above the 35 ΔmP cut-off) between days 19 and 23 were non-pregnant on day 30. This was most likely caused by a miscalculated time of estrus during AI (Jerseys were not synchronized), or loss of fetus after day 23. In line with this, one of the three cows tested "non-pregnant" on day 26 according to the milk P4 FPA. In four other non-pregnant Jersey cows, a decrease in the P4 concentration between days 18 and 23 was reliably followed by heat (Figure 7).

Interestingly, in comparison with Holsteins, Jersey cows had a smaller spread in P4 concentration between pregnant and non-pregnant animals (Figure 7). This is likely due to the influence of matrix on the FPA performance, or it could be a physiological characteristic of the breed. Jersey cows are known to have a different milk composition; thus, measuring the concentration of hydrophobic molecules like P4 in milk is more challenging. Further optimization of the assay could lead to even higher accuracy. It is also important to note that the milk P4 FPA was 100% specific without a single misdiagnosis of pregnant animals.

Figure 7. Milk P4 measurements from non-synchronized pregnant and non-pregnant Jersey cows from New Zealand. The graph shows the median values of pregnant (n=7) and non-pregnant (n=4+3) cows. In non-pregnant cows, P4 decreased below the cutoff value on day 19 (indicated with a blue arrow), and heat appeared on day 21 (indicated with a red arrow). The horizontal line represents the 35 ΔmP cutoff value.

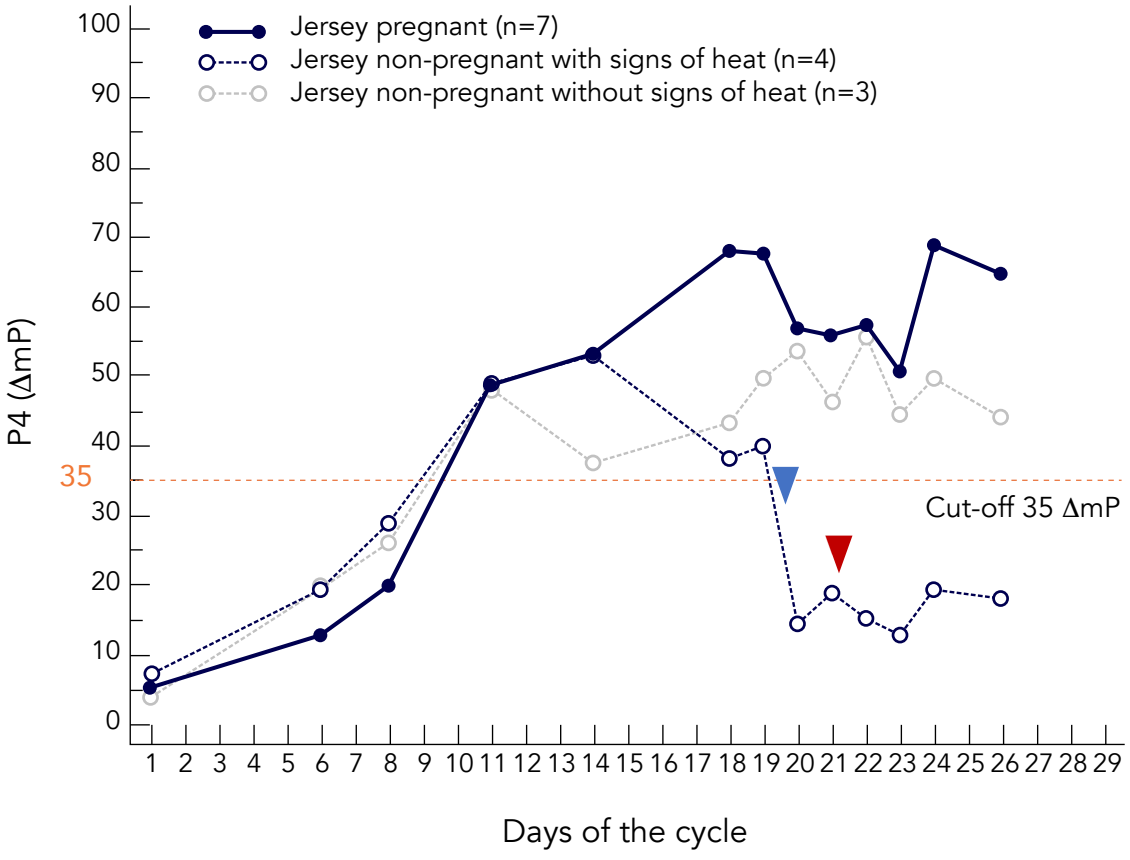


Figure 8. Distribution of the P4 values among true pregnant (upper left quadrant - a), false pregnant (upper right quadrant - b), false non-pregnant (lower left quadrant - c), and true non-pregnant (lower right quadrant - d) Jersey cows. The P4 cut-off value was set at 35 ΔmP, and the accuracy of confirming the "non-pregnant" status was tested. Six consecutive days are shown with cross-sectional P4 values for all cows.

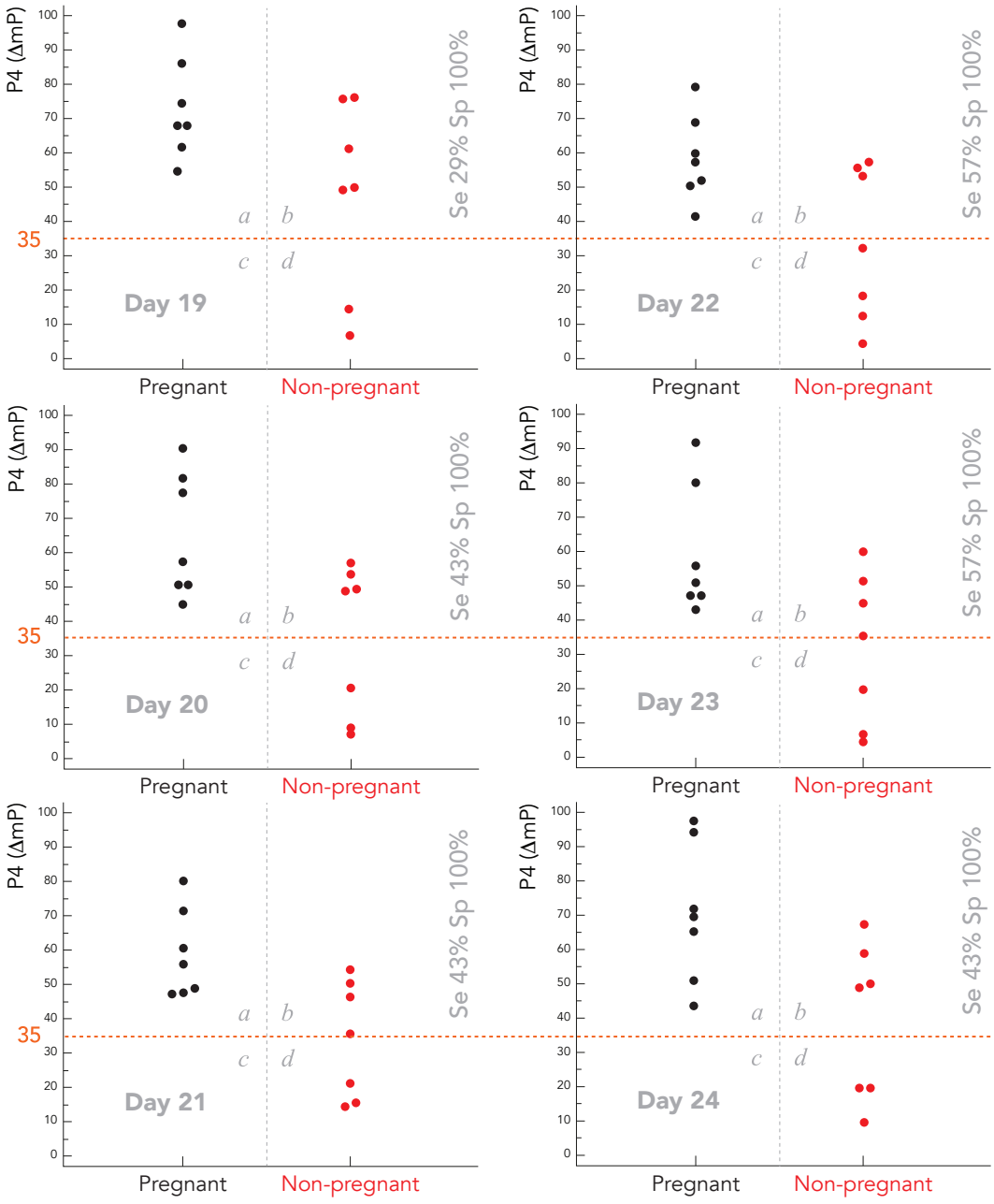
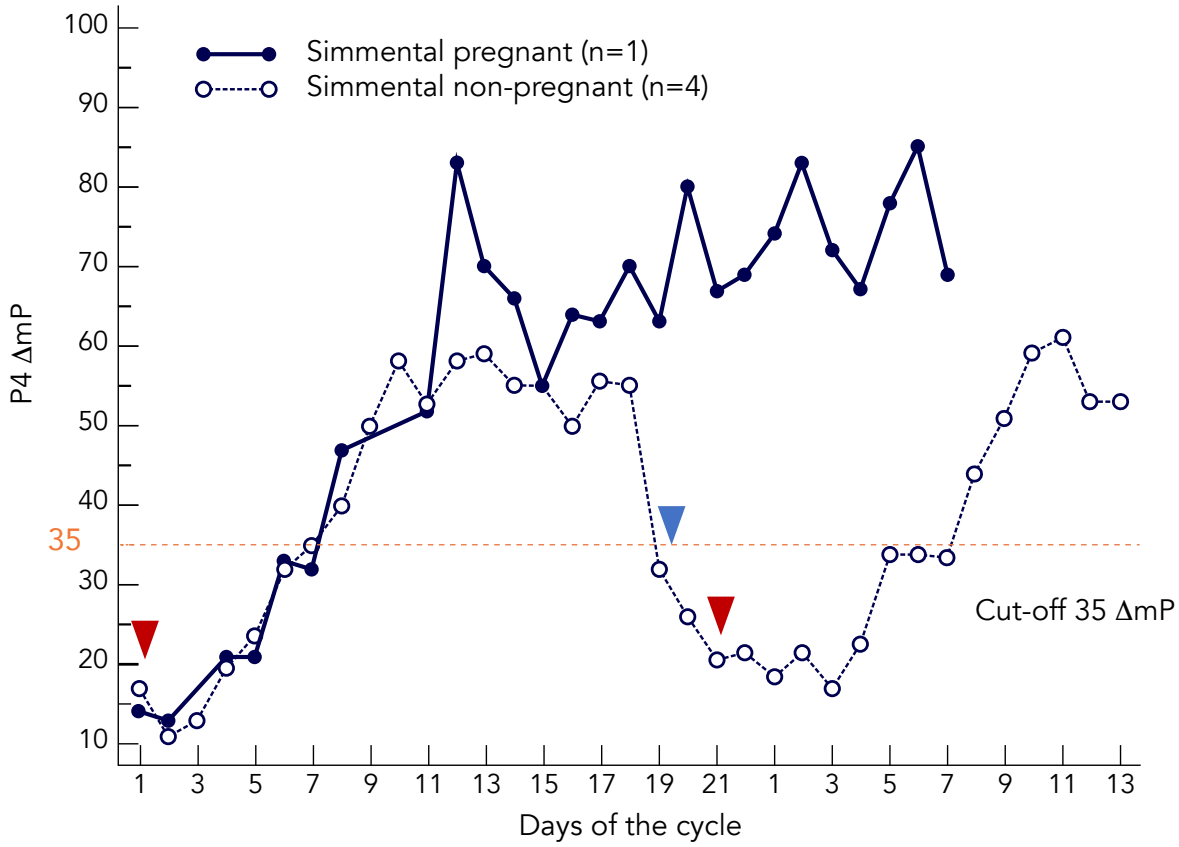


Figure 8 shows the sensitivity and specificity of the milk P4 FPA in more detail when cows were tested from days 19 to 24 and using a cut-off value of 35 ΔmP. It should be noted that the sensitivity of the assay is lower when applied in Jersey cows than in Holstein-Friesian cows. This may be explained by the fact that the Jersey cows were not synchronized. Consequently, the timing of insemination (the timing for the first AI was not tested with the milk P4 FPA) and all subsequent calculations could have been misinterpreted. Thus, these results should be considered with caution. However, it is interesting that the assay displayed its highest sensitivity on days 21 and 22 in Jersey cows (57%) and day 22 in Holstein-Friesian cows (91%).

Simmental cows

Milk P4 concentrations from five Simmental cows from Serbia were monitored daily for one month following AI. One pregnant cow had a continuous increase in P4 concentration from the day of insemination to the end of the testing period. Four non-pregnant cows had a decline in P4 concentration on day 19 (median value) that ranged between days 17 and 20. Among them, the cow with a silent estrus had the same typical P4 pattern as all other non-pregnant cows (Figure 9). In all cows, the assay results strongly correlated with biological status.

Figure 9. Milk P4 concentrations in samples from a group of Simmental cows from Serbia. The graph shows the values for one pregnant cow and the median values for non-pregnant cows (n=4). In non-pregnant cows, P4 decreased below the cut-off value on day 19 (indicated with a blue arrow), and heat appeared on day 21 (indicated with a red arrow). The horizontal line represents the 35 ΔmP cut-off value.



Conclusion

The data in this report unambiguously demonstrate the strong analytical and diagnostic performance of the milk P4 FPA developed by Ellie LLC. A specificity of 100% is critical to prevent a potentially dangerous repeated insemination of already-pregnant cows. Pre-estrous cows and cows with silent estrus were also consistently identified. In tightly controlled situations (e.g. the inclusion of a synchronization protocol), the assay demonstrated high sensitivity and specificity. We expect the assay's performance to facilitate a significant financial gain via the early detection of opened cows. More validation is necessary to verify the performance of the assay during real-world, high-throughput operation and under various laboratory and field conditions.

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