APPLICATION NOTE

AUTOMATION OF THE IDEXX MILK PREGNANCY TEST

Introduction

Accurate and timely detection of pregnancy in dairy cows is an essential component of today's reproductive management programs, as a high reproductive efficiency is a prerequisite for high life-time production from dairy animals. Laboratory methods for pregnancy determination using milk as starting sample have numerous advantages over traditional methods, such as rectal palpation or transrectal ultrasound, as they are safer, equally or less expensive, and do not require trained personnel or special equipment on-farm. Pregnancy-associated glycoproteins (PAGs) constitute a large family of glycoproteins expressed in the outer epithelial cell layer (chorion/trophectoderm) of the placenta of cows and other eutherian species. PAGs can be detected in milk of pregnant cows, thus providing a convenient, specific and sensitive method for pregnancy diagnosis.

The IDEXX Milk Pregnancy Test, for use in bovine milk samples, is an enzyme-linked immunoassay for the detection of PAGs in bovine milk as a marker for pregnancy from ≥ 35 days post-breeding. The test can be used ≥ 60 days post calving.

Material

- Crocodile ELISA MiniWorkstation (Titertek-Berthold)
- Milk Pregnancy Test (Part number 99-41209, IDEXX)
- Adhesive plate covers
- Precision micropipettes or multi-dispensing micropipettes, with suitable disposable tips
- Distilled or deionized water

Methods

All reagents were brought up to room temperature for 30 minutes prior to use. Wash Solution was prepared following the manufacturer's instructions.

Controls and samples were pipetted according to the manufacturer's instructions. 3 samples known to be positive, and 3 samples known to be negative, were used. All controls and samples were run in triplicate.

The plate should be tightly sealed with adhesive cover during incubations to avoid evaporation. Manual steps were included in the program of the Crocodile to allow the user to put or remove the sealing, as needed.

In order to reduce the amount of user intervention and improve automation, the assay was tested covering the plate only in the first incubation (2 h at 37°C) and leaving the plate uncovered in the other incubation steps (20-30 minutes at room temperature); no significant evaporation was observed, and the assay performed as expected.

The Crocodile ELISA miniWorkstation was programmed with the steps summarized in Table 1.

Results

For the assay to be valid, the Positive Control mean minus the Negative Control mean must be greater than or equal to 0.500, and the Negative Control mean must be less than or equal to 0.200. Both criteria were fulfilled running the assay in the Crocodile ELISA miniWorkstation.

Pregnant or open (not pregnant) status is determined by the corrected OD values (S-N: OD of the Sample minus OD of the Negative Control) for each sample: if the S-N value is less than 0.100, the animal is considered not pregnant (open); if it is equal to or greater than 0.250, the animal is considered pregnant; and if is less than 0.250 but greater than or equal to 0.100, the animal should be re-checked to confirm pregnancy status. All negative samples were determined as negative, and all positive samples were determined as positive. There were no false positives or false negatives. Results are summarized in Figure 1:

ID	OD	S-N	Result
Negative Control	0,136	0,000	
Positive Control	1,992	1,856	
Negative Sample #1	0,170	0,034	NEGATIVE
Positive Sample #1	2,129	1,993	POSITIVE
Positive Sample #2	1,233	1,097	POSITIVE
Positive Sample #3	3,717	3,581	POSITIVE
Negative Sample #2	0,099	-0,037	NEGATIVE
Negative Sample #3	0,106	-0,030	NEGATIVE

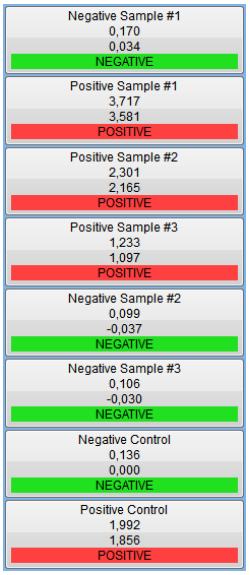

***Figure 1**. Left: Table displaying the results for controls and samples; OD values correspond to OD (450 nm) - OD (620 nm). Data shown are averages of triplicate measurements. Right panel: screenshot of data reduction software (MikroWin).*

Summary:

The assay fulfilled both validation conditions and all positive and negative samples were correctly determined. The assay procedure is simple and involves only the addition of controls and samples, while the instrument is processing all necessary dispense, wash, incubation and reading steps. The addition of manual steps in the Crocodile Control Software allows the user to remove the cover when required, thus providing an easy and convenient integration of manual and automated steps. In consequence, the Crocodile ELISA miniWorkstation, in combination with the MikroWin data reduction software, provides a convenient and easy-to-use method to automate the IDEXX Milk Pregnancy Test.

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Table 1.1. Summary of steps programmed in the Crocodile Control Software

#	Step name	Description and parameters
1	**Incubator ON**	**Incubation** Incubator On, Temperature: 37°C
2	**Incubator heat up**	**Manual** "Insert plate when the incubator reaches 37°C and press Continue", Duration: 00:10:00, Mode: User Continue, Position: Insert Position
3	**Sample Incubation**	**Shaking** For 02:00:00, at Incubator, with 1 mm Amplitude at 5 Hz
4	**Incubator OFF**	**Incubation** Incubator Off
5	**Remove adhesive cover**	**Manual** "Please remove cover", Duration: 00:02:00, Mode: user continue, Position: Insert position, Alarm Notification: sound of choice, 0 %
6	**Wash Solution priming**	**Washing** Method: Prime Washer, Wash Solution Inlet: 1, Cycles: 3, Volume: 800 µL
7	**Wash**	**Washing** Method: Standard, Wash Solution Inlet: 1, Cycles: 4, Volume: 300 µL, Delay: 1 s, Wait: 200 ms, Dispenser Depth: 1300 (Plate Offset: -50), Aspiration Depth: 2910* (Plate Offset: 20), Sweep: 4 mm @ 2 mm/s
8	**Aspiration**	**Washing** Method: Aspirate Only, Cycles: 1, Delay: 1s, Wait: 500 ms, Dispenser Depth: 1300 (Plate Offset: -50), Aspiration Depth: 2920* (Plate Offset: 20), Sweep: 4 mm @ 2 mm/s
9	**Detector priming**	**Dispensing** Volume: 850 µL, Inlet: 1, Method: Priming
10	**Detector distribution**	**Dispensing** Volume: 100 µL, Inlet: 1, Method: Standard
11	**Mix**	**Shaking** For 00:00:10 at Shaker Position with 1 mm Amplitude at 5 Hz
12	**Detector incubation**	**Manual** Message: "Incubating at Room Temperature", Duration: 00:30:00, Mode: Auto Continue, Position: Insert Position
13	**Wash**	**Washing** Method: Standard, Wash Solution Inlet: 1, Cycles: 4, Volume: 300 µL, Delay: 1 s, Wait: 200 ms, Dispenser Depth: 1300 (Plate Offset: -50), Aspiration Depth: 2910* (Plate Offset: 20), Sweep: 4 mm @ 2 mm/s
14	**Aspiration**	**Washing** Method: Aspirate Only, Cycles: 1, Delay: 1s, Wait: 500 ms, Dispenser Depth: 1300 (Plate Offset: -50), Aspiration Depth: 2920* (Plate Offset: 20), Sweep: 4 mm @ 2 mm/s

* Aspiration depth may have to be optimized for individual Crocodile instruments

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Table 1.2. Summary of steps programmed in the Crocodile Control Software.

#	Step name	Description and parameters
15	**Conjugate priming**	**Dispensing** Volume: 850 µL, Inlet: 2, Method: Priming
16	**Conjugate distribution**	**Dispensing** Volume: 100 µL, Inlet: 2, Method: Standard
17	**Mix**	**Shaking** For 00:00:10 at Shaker Position with 1 mm Amplitude at 5 Hz
18	**Conjugate incubation**	**Manual** Message: "Incubating at Room Temperature", Duration: 00:30:00, Mode: Auto Continue, Position: Insert Position
19	**Wash**	**Washing** Method: Standard, Wash Solution Inlet: 1, Cycles: 4, Volume: 300 µL, Delay: 1 s, Wait: 200 ms, Dispenser Depth: 1300 (Plate Offset: -50), Aspiration Depth: 2910* (Plate Offset: 20), Sweep: 4 mm @ 2 mm/s
20	**Aspiration**	**Washing** Method: Aspirate Only, Cycles: 1, Delay: 1s, Wait: 500 ms, Dispenser Depth: 1300, Aspiration Depth: 2920*, Sweep: 4 mm @ 2 mm/s
21	**Substrate priming**	**Dispensing** Volume: 850 µL, Inlet: 3, Method: Priming
22	**Substrate distribution**	**Dispensing** Volume: 100 µL, Inlet: 3, Method: Standard
23	**Mix**	**Shaking** For 00:00:10 at Shaker Position with 1 mm Amplitude at 5 Hz
24	**Substrate incubation**	**Manual** Message: "Incubating at Room Temperature", Duration: 00:20:00, Mode: Auto Continue, Position: Insert Position
25	**Stop solution priming**	**Dispensing** Volume: 850 µL, Inlet: 4, Method: Priming
26	**Stop solution distribution**	**Dispensing** Volume: 100 µL, Inlet: 4, Method: Standard
27	**Mix well**	**Shaking** For 00:00:10 at Shaker Position with 1 mm Amplitude at 5 Hz
28	**Measure**	**Reading** Reference Measurement, Filter 1: 450 nm, Filter 2: 620 nm

* Aspiration depth may have to be optimized for individual Crocodile instruments