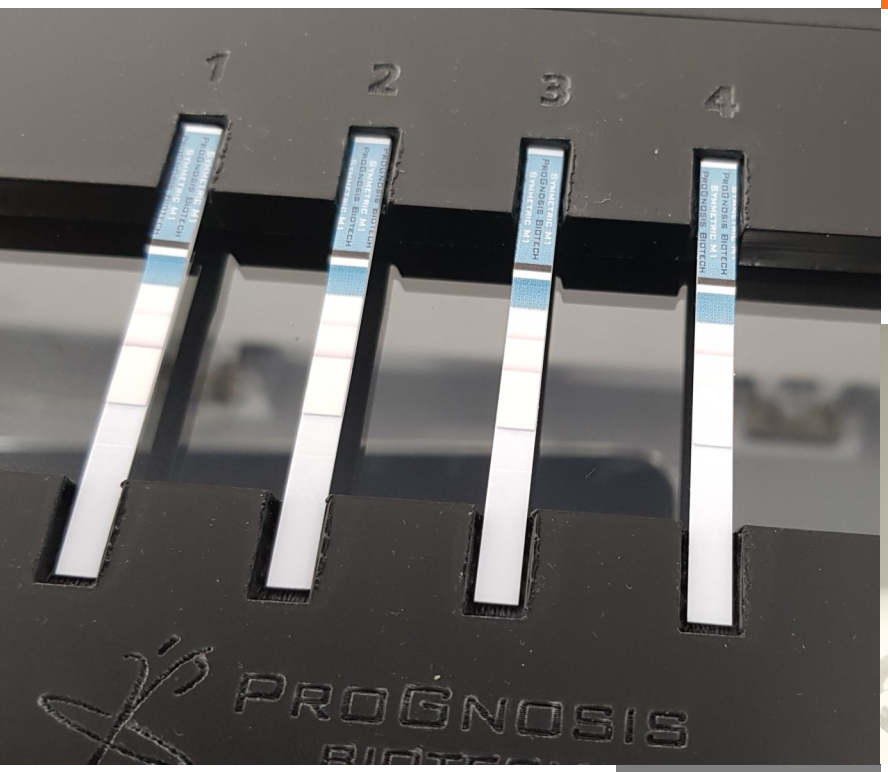
VALIDATION REPORT
SYMMETRIC M1



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PROGNOSIS BIOTECH

Symmetric M1 Test kit

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Symmetric M1 Test kit

1. Introduction

Aflatoxins are toxic metabolites of major concern to the dairy industry, generally produced by *Aspergillus flavus, A. parasiticus* and *A. nomius*. They can have immunosuppressive, mutagenic, teratogenic and carcinogenic effects. Aflatoxins that are ingested by animals in contaminated pellets and forage are biotransformed at the hepatic level into Aflatoxin M1 (AFM1). Aflatoxin is then excreted in this form into the milk used for human consumption and, it is also present in dairy products. AFM1 in milk and milk products is considered to pose certain hygienic risks for human health and as a result there are established certain limits.

1.1 Regulations

Most controlling government agencies worldwide have regulations regarding the amount of M1 allowable in human and animal foodstuffs. For example, the EU limit in milk and milk based foods intended for direct human consumption is 0.05 μg/kg (50 ppt). For more information on the aflatoxins' regulations, visit our website: **www.prognosis-biotech.com.**

1.2 Principle of the method

The quantitative lateral flow test is based on the immunochromatography assay principles. The wells of the microtiter strips contain AFM1 specific antibodies conjugated to colloidal gold. Cow, sheep or goat milk samples are added into the wells and the suspended mixture is incubated. During this step, AFM1 (if it is present) binds to the antibodies. A dipstick with two capture lines, test and control, is dipped into the mixture. The liquid starts flowing vertically on the dipstick and passes through the two lines. A valid test should always have the upper control line red. If the sample is free of AFM1, a color development occurs at the test line, indicating the absence of AFM1 in the milk sample. On the contrary, the presence of AFM1 in the sample will cause a reduced colored signal at the test line. The test line color intensity is indirectly proportionate to the concentration of AFM1 present in the samples. By utilizing S-Flow software AFM1 is accurately quantified.

1.3 Kit Characteristics

Refer to the instruction manual S2148/S2196 vN3.

1.4 Method Protocol

i. Sample Preparation

- No sample preparation is necessary

ii. Immunoassay Procedure (Total time: 10min)

1. Place the equipment of automatic release (test top) on the incubator and connect it to the appropriate input, located at the left side of the incubator. Plug in the **One-touch** Incubator and wait until the temperature has been stabilized at 40°C.
2. Before opening the reagents, take the kit out of the fridge (at least for half an hour) and wait until the temperature of the reagents reaches the ambient temperature.
3. Open one plastic pot and take out as many test strips and microwells as milk samples to be tested (no more than the reader positions per experiment). If needed, using scissors, carefully cut the number of reaction wells.

4. The pot with dipsticks should always be well closed after reagents have been taken out. - A pot with dipsticks should be emptied before another is opened.
5. Shake the milk samples vigorously or vortex.
6. Place the microwell(s) in the incubator.
7. Place a new tip on the micropipette and dispense **100µl** of milk into each of the microwells. Using the same pipet tip, aspirate the sample up and down about 10 times to completely mix the lyophilized gold particles in the milk, while avoiding bubbles. The sample should turn into a **uniform pink color**. After mixing the particles, remove and discard the pipet tip. In case of more than 3 samples, an 8 channel multipipette should be used. **The ideal temperature of the milk sample is between 4 and 18°C.**
8. Push the START (RUN) button. The 5-minute countdown starts.
9. Place the appropriate number of sticks into the automatic release equipment.
10. When the 5 minutes are over, the stick will be automatically dropped into the strips and the **second incubation** of 5 minutes will start. **Always check if the sticks have been dropped into the wells.**
11. When the 5 minutes of the second incubation are over, i.e. after the sound-signal, press START (STOP)* again to stop the ringing tone and take the dipsticks out of the microwells.
12. Remove the white cotton sample-pad of the stick. Hold the stick with your hand from the colorful pad and remove the white pad with your hands. Do not use a paper towel or any other material.
13. Place the stick inside the plastic holder in order to be scanned. In case of S-Flow scanner, the sticks must be facing up. In case of Epson scanner, the sticks must be facing down (inverted). The colored side must be facing the orange sticker.
14. Use S-Flow software to quantify results as soon as possible and no later than 10 minutes after the end of analysis.

1.5 Data Analysis

♦ Automatically

The S-Flow software will use a Lot specific curve to calculate the results in parts per trillion (ppt).

1. Select from the product menu the Symmetric M1 test.
2. Select the type of matrix analyzed. For pasteurized and fresh cow milk choose Cow. For fresh goat and fresh sheep milk choose Goat and Sheep, respectively. For Ultra High Temperature milk choose UHT.
3. Add the settings of the Lot that is to be used. Refer to the S-Flow manual for a detailed description of the procedure. A simple visual interpretation of the stick is NOT possible.
4. Press Scan.

Results (in **ppt**) are shown on the main window of S-Flow automatically in 5-10 seconds.

2. Immunoassay Specifications

2.1 General Specifications

- The LOD of the method is 6 ppt
- The LOQ of the method is 8 ppt
- IC50 = 22.5-50 ppt
- Quadruplicates value at 50ppt CV (%) ≤ 8.

2.2 Specificity & Cross-reactivity

The cross-reaction of the anti-Aflatoxin M1 antibody with Aflatoxin M2 is 10% and 0% with other mycotoxins (Ochratoxin A, Zearalenone, Deoxynivalenol and Fumonisin B1) and other unrelated compounds, such as antibiotics (Benzylpenicillin, Cefalonium, Oxytetracycline, Erythromycin, Neomycin, Enrofloxacin, Sulfadiazine, Trimethoprim and Dapsone).

3. Validation

3.1 Determination of the Limit of Detection (LOD) and the Limit of Quantification (LOQ)

For the determination of LOD (2xSd) and LOQ (3xSd), three Aflatoxin M1-free cow raw milk samples were used and the results are reported in Table 1. Each sample was analyzed twenty times (n=20).

Table 1. Aflatoxin M1-free cow raw milk samples for the determination of LOD and LOQ.

	Aflatoxin M1 result (ppt)		
Sample (n=20)	Raw milk A	Raw milk B	Raw milk C
1	1.6	1.4	6.4
2	1.5	3.3	1.9
3	5.1	5.7	1.8
4	1.7	4.8	2.1
5	1.5	1.0	3.2
6	0.7	2.7	0.6
7	2.2	6.9	3.0
8	0.5	1.2	1.3
9	2.4	5.0	1.8
10	2.8	0.6	6.7
11	0.4	4.8	2.5
12	0.6	1.2	1.1
13	5.7	2.1	3.9
14	3.1	0.5	2.5
15	0.0	2.6	0.0
16	0.0	0.9	2.4
17	5.6	3.2	1.5
18	2.3	0.1	2.4
19	2.8	1.9	1.6
20	4.4	2.6	0.6
Mean	**2.2**	**2.6**	**2.4**
SD	**1.7**	**1.9**	**1.7**
Mean Sd	**1.8**		
LOD (2xSd)	**5.9**		
LOQ (3xSd)	**7.7**		

The LOD and LOQ were defined as 2 x Standard Deviation and 3 x Standard Deviation of the aflatoxin M1-free cow raw milk samples, respectively. It was found that LOD and LOQ is 6 ppt and 8 ppt, respectively.

3.2 Determination of Recovery (%)

For the determination of Recovery (%) at different cow milk types (pasteurized milk and milk powder) and other animal species (sheep and goat milk), Aflatoxin M1-free samples were collected and spiked at two different levels (50 and 100ppt). All samples, blank and spiked, were analyzed twenty times (n=20) and the recoveries (%) are shown in Table 2. The mean recoveries (%) of each milk type are reported in Table 3.

Table 2. Recovery (%) of different milk types at different levels.

Milk sample	spike (ppt)	Mean result	SD	CV(%)	Recovery (%)
Cow	0	<8	-	-	-
	50	49.9	1.9	3.8	**99.8**
	100	101.5	2.8	2.8	**101.5**
Sheep	0	<8	-	-	-
	50	48.5	1.3	2.6	**97.0**
	100	92.2	3.4	3.6	**92.2**
Goat	0	<8	-	-	-
	50	47.7	1.1	2.3	**95.3**
	100	93.6	3.7	3.9	**93.6**
Pasteurized milk	0	<8	-	-	-
	50	51.2	1.4	2.7	**102.3**
	100	103.2	2.9	2.8	**103.2**
Milk powder	0	<8	-	-	-
	50	50.1	1.6	3.1	**100.2**
	100	96.8	2.4	2.5	**96.8**

Table 3. Mean recoveries (%) of all milk types.

Milk Types	Mean Recovery (%)
Cow	**100.7**
Sheep	**94.6**
Goat	**94.4**
Pasteurized milk	**102.8**
Milk powder	**98.5**

3.3 Repeatability

The repeatability of the test was evaluated analyzing two different samples eight times and the results are reported in Table 4.

Table 4. Coefficients of Variation of the concentration (ppt) of two different samples analyzed in eight different tests.

Sample (n=8)	Mean result (ppt)	Sd	CV (%)
Cow milk 1	**53.4**	**1.6**	**3.0**
Cow milk 2	**30.6**	**1.5**	**4.8**

3.4 Performance Evaluation

- *Proficiency Tests*

Table 5. Ring Test results

Test	Assigned value (ng/kg)	Result (ng/kg)	Z-Score
A.I.A. Ring Test Lotto RT M1 260923 Aflatoxin M1 in Lyophilized Milk September 2023	15.49	16.20	0.21
	23.92	24.40	0.09
	41.90	41.50	-0.05
	52.78	49.70	-0.44
A.I.A. Ring Test Lotto RT M1 280323 Aflatoxin M1 in Lyophilized Milk March 2023	12.21	10.52	-0.76
	21.50	20.72	-0.20
	40.93	41.17	0.04
	53.85	57.03	0.39

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