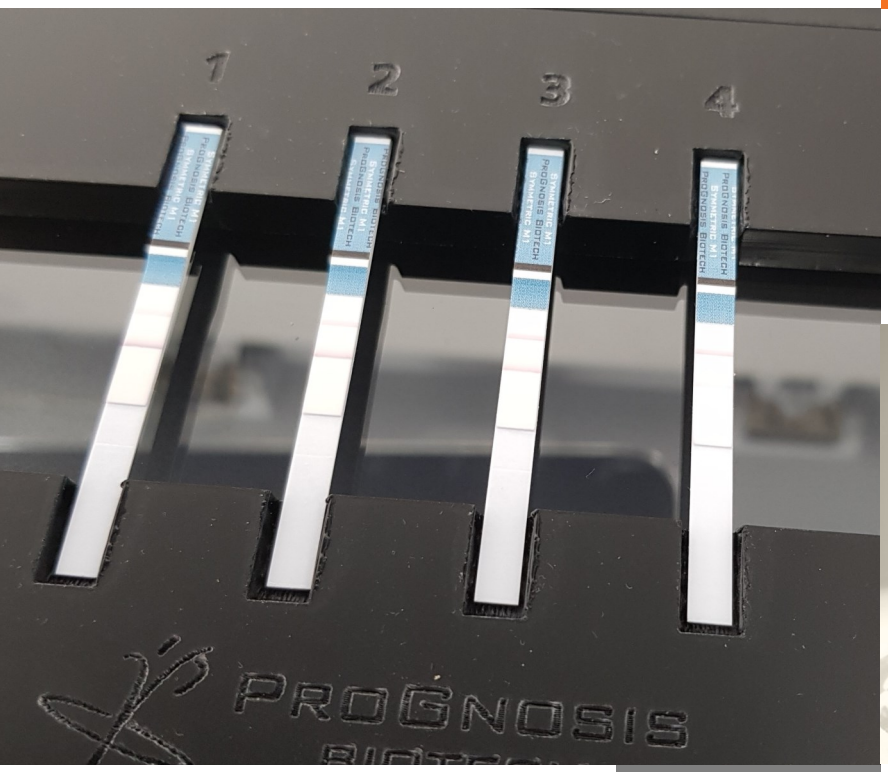
VALIDATION REPORT
SYMMETRIC M1 1000



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PROGNOSIS BIOTECH

Symmetric M1 1000 Test kit

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Symmetric M1 1000 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 bio-transformed 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 US, India and China limit in milk and milk based foods intended for direct human consumption is 0.5 µg/kg (500 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. Milk samples are added into the wells. 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

See manual S2448/S2496 V13

1.4 Method Protocol

i. Sample Preparation

- No sample preparation is necessary

ii. Immunoassay Procedure (Total time: 3min)

1.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 **200µl 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.Immediately place the appropriate number of sticks into the wells.
9.Push the START(RUN) button. The 3-minute countdown starts.
10.When the 3 minutes of 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.
11.Remove the white cotton sample-pad of the stick. Touch 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.
12.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.
13.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).

- Select from the product menu the Symmetric M1 1000 test.
- Select the type of matrix analyzed. For pasteurized and fresh cow milk choose Cow. For Ultra High Temperature milk choose UHT.
- Add the settings of the Lot that is to be used. Refer to the S-Flow manual for a detailed description of the procedure.
- 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 standard curve should have

- The LOD of the method is 47 ppt
- The LOQ of the method is 70 ppt
- IC50 = 200-450 ppt
- Each standards quadruplicates mean CV ≤ 8% .

2.2 Specificity & Cross-reactivity

The anti-Aflatoxin M1 antibody has no cross-reactions with analogous compounds (Aflatoxin M2), 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), two Aflatoxin M1-free cow raw milk samples were used (Table 1).

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

Sample (N=20)	Concentration (ppt)		
	COW RAW A	COW RAW B	
1	11,7	6,4	
2	41,6	19,5	
3	14,3	45,2	
4	0,9	20,9	
5	22,8	0,2	
6	68,4	6,5	
7	27,5	35,4	
8	9,9	16,7	
9	14,6	0,2	
10	1,1	7,2	
11	6,8	43,1	
12	62,1	1,2	
13	30,5	29,7	
14	29,1	69,1	
15	7,7	13,7	
16	82,1	62,9	
17	55,7	32,3	
18	18,1	5,7	
19	5,9	79,9	
20	2,6	13,9	
	25,6		MEAN
	23,4		SD

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 47 ppt and 70 ppt, respectively.

3.2 Determination of Recovery (%)

For the determination of Recovery (%) at three different levels (70, 500, 750 ppt), different Aflatoxin M1-free milk samples were spiked (Table 2-6).

i. Cow raw milk sample

Table 2. Cow raw milk sample.

Cow raw milk (N=20)	Blank sample	
1	<70	
2	<70	
3	<70	
4	<70	
5	<70	
6	<70	
7	<70	
8	<70	
9	<70	
10	<70	
11	<70	
12	<70	
13	<70	
14	<70	
15	<70	
16	<70	
17	<70	
18	<70	
19	<70	
20	<70	
	<70	**MEAN**

Table 3. Recovery (%) of Cow raw milk sample at three different levels.

Cow raw milk (N=20)	Spike at the LOQ level (%) 70 ppt		Spike 500 ppt		Spike 750 ppt	
1	70,4		518,4		749,1	
2	72,4		501,3		795,4	
3	81,2		479,0		730,8	
4	87,1		512,6		758,2	
5	77,2		491,2		782,3	
6	65,8		486,1		801,4	
7	93,2		482,7		755,3	
8	82,2		513,7		749,8	
9	52,5		521,8		741,6	
10	76,4		479,3		768,3	
11	82,9		498,2		774,5	
12	94,8		492,7		766,4	
13	77,6		510,2		769,9	
14	59,3		484,7		745,1	
15	84,3		469,9		761,6	
16	75,4		482,8		720,5	
17	68,0		481,6		751,1	
18	76,3		503,3		723,2	
19	84,7		485,3		774,5	
20	68,8		506,6		731,7	
	76,5	**Average**	**495,1**	**Average**	**757,5**	**Average**
	10,3	**SD**	**14,7**	**SD**	**21,7**	**SD**
	13,5	**CV(%)**	**3,0**	**CV(%)**	**2,9**	**CV(%)**
	109,3	**Recovery (%)**	**99,0**	**Recovery (%)**	**101,0**	**Recovery (%)**
Mean Recovery (%)				**103,1**		

ii. UHT Cow milk sample

Table 4. UHT cow milk sample.

UHT milk (N=20)	Blank sample	
1	<70	
2	<70	
3	<70	
4	<70	
5	<70	
6	<70	
7	<70	
8	<70	
9	<70	
10	<70	
11	<70	
12	<70	
13	<70	
14	<70	
15	<70	
16	<70	
17	<70	
18	<70	
19	<70	
20	<70	
	<70	**MEAN**

Table 5. Recovery (%) of UHT cow milk sample at three different levels.

Cow raw milk (N=20)	Spike at the LOQ level (%) 70 ppt		Spike 500 ppt		Spike 750 ppt	
1	58,4		499,7		744,2	
2	45,3		488,2		741,2	
3	66,4		518,4		750,7	
4	70,4		500,7		761,1	
5	69,5		486,7		759,0	
6	55,4		503,4		745,6	
7	41,2		496,2		761,5	
8	82,4		489,7		821,8	
9	88,7		514,4		779,8	
10	74,8		518,7		823,8	
11	61,9		488,0		751,7	
12	76,4		491,8		754,9	
13	101,2		479,7		746,8	
14	68,3		526,3		751,8	
15	52,8		493,8		747,7	
16	56,6		475,8		766,0	
17	79,6		512,7		752,1	
18	84,7		490,3		810,7	
19	91,4		488,8		787,6	
20	100,5		512,2		803,8	
	71,3	**Average**	**498,8**	**Average**	**768,1**	**Average**
	16,7	**SD**	**13,8**	**SD**	**26,2**	**SD**
	23,4	**CV(%)**	**2,8**	**CV(%)**	**3,4**	**CV(%)**
	101,9	**Recovery (%)**	**99,8**	**Recovery (%)**	**102,4**	**Recovery (%)**
Mean Recovery (%)				**101,4**		

Table 6. Mean Recoveries (%) of all matrices.

Matrix	Mean Recovery (%)
COW RAW MILK	103,1
UHT COW MILK	101,4

3.3 Reproducibility

The coefficients of variation of reproducibility of the concentrations (ppt) (Table 7) of two different samples ran eight times in 8 different tests are reported:

Table 7. Coefficients of Variation of the concentration (ppt) of two different samples ran in eight different tests.

Cow raw milk 1	Results (ppt)	
N=8	407,2	
	406,6	
	444,9	
	431,7	
	426,0	
	422,1	
	416,2	
	426,5	
	422,6	**MEAN**
	2,8	**CV(%)**
Cow raw milk 2	**Results (ppt)**	
N=8	220,7	
	232,2	
	245,8	
	215,6	
	245,9	
	222,8	
	264,4	
	257,3	
	238,1	**MEAN**
	7,1	**CV(%)**

3.4 Stability

The coefficients of variation (%) of the concentrations (ppt) of a cow raw milk sample spiked at three different levels (70, 250, 750 ppt) during an accelerated stability experiment are reported (Table 8 & 9) :

Table 8. Coefficients of Variation of the concentration (ppt) of cow raw milk sample spiked at three different levels - 1 day.

Cow raw milk (N=8)	Spike at the LOQ level 70 ppt		Spike 250 ppt		Spike 750 ppt	
1	79,4		269,6		758,3	
2	54,6		255,4		770,6	
3	76,6		228,6		741,7	
4	55,6		258,7		718,6	
5	75,8		239,0		751,5	
6	79,4		238,2		761,6	
7	81,5		242,9		770,2	
8	92,8		256,4		799,3	
	74,5	**Average**	**248,6**	**Average**	**759,0**	**Average**
	12,2	**SD**	**12,7**	**SD**	**22,0**	**SD**
	16,4	**CV(%)**	**5,1**	**CV(%)**	**2,9**	**CV(%)**

Table 9. Coefficients of Variation of the concentration (ppt) of cow raw milk sample spiked at three different levels after storage 30 days in 50°C.

Cow raw milk (N=8)	Spike at the LOQ level 70 ppt		Spike 250 ppt		Spike 750 ppt	
1	66,8		218,9		725,6	
2	73,9		231,7		714,3	
3	54,3		243,9		699,7	
4	33,8		254,9		725,0	
5	69,2		229,3		688,4	
6	58,1		248,7		732,6	
7	45,0		221,7		734,1	
8	75,4		245,4		745,8	
	59,6	**Average**	**236,8**	**Average**	**720,7**	**Average**
	13,7	**SD**	**12,4**	**SD**	**17,8**	**SD**
	23,0	**CV(%)**	**5,2**	**CV(%)**	**2,5**	**CV(%)**

Antonios Ntantasios
Managing Director

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ΠΑΡΑΓΩΓΗ ΧΗΜΙΚΩΝ ΠΡΟΪΟΝΤΩΝ
ΑΡ. ΤΕΝΗ: 116245240000

PROGNOSIS BIOTECH

PROGNOSIS
BIOTECH

www.prognosis-biotech.com
E: info@prognosis-biotech.com

T: +30 2410 623922
Farsalon 153 | 41335 Larissa, Greece