	NATIONAL CENTRE FOR ANIMAL HEALTH **LABORATORY SERVICES UNIT**	**Second edition**
	STANDARD OPERATING PROCEDURE	**Version 2018.1**
	TOXICOLOGY	

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TITLE: Sample collection for Toxicological Tests

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APPROVED BY: Head, LSU

DATE: 11.06.2018

1. Purpose
The purpose of this SOP is to describe the general method for collection of different samples for toxin analysis.

2. General information/responsibility
It is not possible to comprehensively screen samples for '**poisons**' or '**toxins**'. It is up to the submitter to consider the history, clinical signs and lesions (if any) and identify specific toxins for analysis. Contact your Veterinary Laboratory if you are unsure whether a test is available for a particular toxin.
In case of suspected poisoning, it is important that an effect be demonstrated in the animal. For example, nitrate poisoning is confirmed by demonstrating the presence of nitrate in the serum or the blood of the animal, not by demonstrating the presence of nitrate in pasture plants in the paddock.
It is important that the history provided includes details of treatment with any suspected toxic compound, particularly in relation to the strength of the preparation and the time since treatment or access to the material.

3. Equipment/materials
- 3.1 Post mortem set,
- 3.2 Scalpel blade,
- 3.3 Wide mouthed glass stoppered bottle,
- 3.4 Needle and syringe,
- 3.5 Vacutainers with or without anticoagulant,
- 3.6 Vacutainer needle and adapter,
- 3.7 Clean test tubes,
- 3.8 Polythene bags etc.

4. Reagents, solution and buffer
- 4.1 Ice

5. Procedure
- 5.1 General information
  - 5.1.1 No preservatives should be added in the toxicology samples.

5.1.2 The toxicological samples should be accompanied with detail history of the case and other required information.

5.1.3 If it is vetero-legal case, the representative from the Royal Bhutan police should be involved right from conducting of the post mortem or attending of the case in live animals.

5.1.4 In case of suspected poisoning case, the following specimens should be forwarded for lab examination in a separate wide mouthed glass stopper bottle under ice without adding any preservative:

- Stomach with its content after tying both its end. In case of ruminants, about 500-1000g of well-mixed rumen contents should be sent.
- 30 cm each of ileum and colon with its contents with their ends tight.
- Liver-500-1000g in large animals and whole liver in small animals.
- Kidney-one
- Spleen or portion of it if large.
- Adipose tissue.
- Contents of urinary bladder.
- Portion of long bones especially in case of extreme putrefaction.
- Blood and other specific materials.
- Fresh/dried plants, feed etc. suspected for poisoning.

5.2 Specific specimen collection

5.2.1 Animal Feeds

5.2.1.1 *Blended Feed:* If the lot has been blended thoroughly from the various material handling operations, then the contaminated particles are assumed to be distributed uniformly throughout the lot. However, the mycotoxins may be localized due to localization of moisture in the feed leading to growth of fungi, Sampling can be done from single location. However, it is preferable to make bulk sampling of 200 g per 200 kg incremental portion is taken every 200 kg. If the bulk sample is large, mix properly and collect the required quantity of sample.

5.2.1.2 *Static lots:* Examples of static lots are commodities contained in storage bins, rail cars, or many small containers such as sacks. When drawing a sample from a bulk container, a probing pattern should be developed so that product can be collected from different locations in the lot. The sampling probe should be long enough to reach the bottom of the container when possible. Attempts should be made to use a sampling rate of 200 g per 200 kg incremental portion is taken every 200 kg.

5.2.2 Tissue samples

At least 100g of tissue should be collected, taking care to avoid contamination with soil, faeces or intestinal contents. Separate organs should be placed in separate containers.
Body fat is the preferred tissue for insecticide residue testing. For biopsy material, a minimum of 2g is required.

2.2.2.1 Blood samples

At least 8ml of blood in a blood vacuum tube, free from contamination with faeces etc.

2.2.2.2 Serum samples

At least 2ml of serum should be submitted.

2.2.2.3 Blood smears

Thick air-dried smears should be prepared, taking care to leave one end of the slide clean. They should be dry before being wrapped in paper.

2.2.2.4 Suspected toxic material

Suspected material, feedstuffs or plants should not be sent unless appropriate specimens from the affected animals have also been sent. At least 50g of material should be forwarded.

2.2.2.5 Plant material

Examination for nitrate-nitrite and cyanide are best performed in the field. Plants for identification should be pressed and dried.

2.2.2.6 Stomach contents

Nitrate and nitrite disappears rapidly from ingesta, and thus ingesta are of no value in diagnosing nitrate/nitrite poisoning. For other chemical poisons, e.g. Arsenic, lead at least 250g of ingesta should be submitted in an airtight, leak proof container.

5.3 Storage of specimen prior to dispatch

Tissues should be frozen. Herbage and ingesta samples for toxin examination should also be frozen. Other animal specimens should be chilled.

6. SAFETY

Particular care should be taken in collecting and packaging specimens for toxicology, because there is often a possibility that infectious agents are involved and these can create hazards to staff handling the materials in the laboratory. Therefore avoid contaminating the outside of any submitted containers with tissues and ensure they are leak proof.

7. TROUBLE SHOOT

NA

8. REFERENCES

Thomas B. Whitaker, Andrew B. Slate and Anders Sture Johansson. Sampling feed for mycotoxin analysis. *US Department of Agriculture, Agricultural Research Service, North Carolina State University, Raleigh, North Carolina, USA*

TITLE: Total Aflatoxin test by rapid test strip (Catalogue No. LSY-20007)

PREPARED BY: Biochemistry & Toxicology section

REVISED BY: Biochemistry & Toxicology section

APPROVED BY: Head, LSU

DATE: 11.06.2018

1. INTRODUCTION
Aflatoxins are a family of toxins produced by certain fungi that are found on agricultural crops such as maize (corn), peanuts, cottonseed, tree nuts including animal feeds. The main fungi that produce aflatoxins are *Aspergillus flavus* and *Aspergillus parasiticus*, which are abundant in warm and humid regions of the world. Aflatoxin-producing fungi can contaminate crops in the field, at harvest, and during storage.
Aflatoxins can cause liver disease in animals, they are also carcinogenic with aflatoxin B1 being the most potent carcinogen. Susceptibility varies with breed, species, age, dose, length of exposure and nutritional status. Aflatoxins may cause decreased production (milk, eggs, weight gains, etc.), are immunosuppressive, carcinogenic and mutagenic. Aflatoxins can be present in milk, meat, or eggs if consumed levels are sufficient. Hence, regular screening of animal feeds for aflatoxins are very important.

2. PRINCIPLES
The Total Aflatoxin rapid test strip is based on competitive inhibition immuno-chromatographic principle in the flow process. Aflatoxins in the sample combined with Aflatoxins specific colloidal gold labelled monoclonal antibody inhibit the combination between antibody and TAF-BSA conjugate on test line of NC membrane lead to the colour change of Test line.

When the sample has no Aflatoxin residue or concentration lower than the detection limit. T line is darker than or has same colour with C line. When concentration is equal to or higher than detection limit, T line is lighter than C line obviously or T line is invisible. No matter whether there is Aflatoxin residue in sample, C line will appear, it means the test is valid.

APPLICATION
It is used for testing Total Aflatoxin residue in grain, feed and edible oil sample etc.
Sensitivity: 1ppb (ng/ml)/ Detection limit: Grain, feed, edible oil: 5ppb

3. OBJECTIVE
To provide the procedures for the screening of aflatoxin in various ingredients like grain, feed, edible oil.

4. APPARATUS
4.1 Homogenizer/ Blender or a tightly sealing jar with lid
4.2 Oscillator

4.3 Centrifuge
4.4 Scale pipette
4.5 Balance (0.01g)
4.6 Single channel pipettes (20ul-200ul, 100ul-1000ul)
4.7 Centrifuge tube (10ml)
4.8 TAF Test card 50 pieces

5. REAGENTS, SOLUTION AND BUFFER

5.1 Methanol, n-hexane

6. PROCEDURE

6.1 Sample Preparation

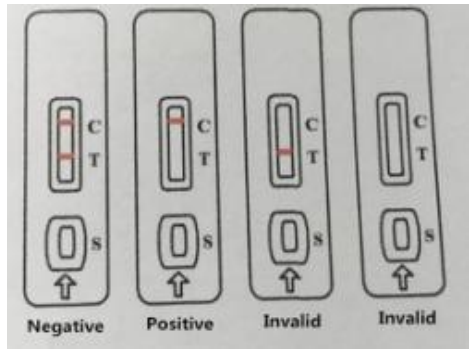
Weigh 1±0.05g crushed feed sample into 15ml centrifuge tube, add 3ml of Sample extracting buffer, shake vigorously by hand or vortex for 3 min, centrifuge at 4000r/min at room temperature for 10 min. Take upper layer clear liquid to test.

6.2 Operation procedures

6.2.1 Take 200µl of the test samples into micro wells, then repeatedly absorb for 5 times, mix the sample with reagent in the micro wells completely until no solid judge by eyes (very important step).
6.2.2 Incubate for 3 min at room temperature (20-25^{0}C), then insert the test strips into the micro wells with the MAX end fully dipped in to the mixture solution.
6.2.3 Insert the test strips into micro wells for 5-8 min then read the result, it is invalid other time.

7. RESULT AND INTERPRETATION

7.1 Negative: Red T line is darker than or has same colour with C line. It means there is no Aflatoxin residue in sample or the residue is lower than detection limit.
7.2 Positive: T line is lighter than the T line or T line is invisible. It means Aflatoxin residue is higher than or equal to detection limit.
7.3 Invalidation: C line isn't seen wine red. It means the test card is out of efficacy, out of date or improper operation. Please run the test again using another package. If the invalid tests keep happening, please contact the local distributor.



8. WASTE DISPOSAL

Wastes should be disposed as per the SOP for waste and chemicals.

9. RISK ASSESSMENT

Methanol is flammable. Caution must be taken in its use and storage.

10. TROUBLESHOOTING

- NA

11. REFERENCES

Catalogue: LSY-20007 Total Aflatoxins rapid test strip, http: //www.lsybt.com/en/content/?786.html accessed on 28/02/2018 at 2.30PM

TITLE: Quantitative Aflatoxin detection by ELISA

PREPARED BY: Biochemistry & Toxicology section

REVISED BY: Biochemistry & Toxicology section

APPROVED BY: Head, LSU

DATE: 11.06.2018

1. INTRODUCTION

Aflatoxins are toxic and carcinogenic. They are metabolites of the fungi *Aspergillus flavus* and *Aspergillus parasiticus*. There are four principle types of aflatoxin: B_1, B_2, G_1 and G_2, which are named for their respective innate fluorescent properties. Aflatoxin B_1 is the most frequently encountered of the group and the most toxic. Aflatoxins can be found mainly in cereals, corn, peanuts, cotton seed, nuts and animal feeds.
Different countries follow permissible limits of Aflatoxin in feeds. The US Food and Drug Administration action levels of aflatoxin are as follows: (1) 300ppb for feeder cattle; (2) 200ppb for finishing swine; (3) 100ppb for breeding beef cattle, swine and mature poultry; and (4)20ppb for humans, and for immature animals and dairy animals.

Hence, quantitative analysis is essential for the feeds to be fit for consumption by the animals.

2. PRINCIPLES

The AgraQuant® Total Aflatoxin Assay is a direct competitive enzyme-linked immunosorbent assay (ELISA).
Aflatoxins are extracted from a ground sample with 70% methanol. The extracted sample and enzyme-conjugated aflatoxin are mixed and added to the antibody-coated microwell. Aflatoxins in samples and control standards are allowed to compete with enzyme-conjugated aflatoxin for the antibody binding sites. After a washing step, an enzyme substrate is added and blue color develops. The intensity of the color is inversely proportional to the concentration of aflatoxin in the sample or standard. A stop solution is then added which changes the color from blue to yellow. The microwells are measured optically using a microwell reader with an absorbance filter of 450nm and a differential filter of 630nm. The optical densities of the samples are compared to the OD's of the standards and an interpretative result is determined.

3. APPLICATION

The AgraQuant® Total Aflatoxin Assay is a direct competitive enzyme-linked immunosorbent assay (ELISA) that determines a quantitative level for the presence of total aflatoxin (B1, B2, G1 and G2) and is intended for use in grains, cereals, nuts, animal feeds and other commodities.

4. OBJECTIVE

The objective of the SOP is to describe the method of the kit to measure the concentration of the Aflatoxins in a sample.

5. APPARATUS

5.1 Romer Series II® Mill or equivalent
5.2 Blender or a tightly sealing jar with lid
5.3 Balance, 400g
5.4 Graduated cylinder: 100mL
5.5 Container with a minimum 125mL capacity
5.6 Whatman#1 filter paper, or equivalent
5.7 Filter funnel
5.8 8-channel and single channel pipettes capable of pipetting 100µL and 200µL with tips
5.9 Wash bottle
5.10 Absorbent paper towels
5.11 3 reagent boats for use as reagent containers for an 8-channel pipettes
5.12 Microwell reader with a 450nm filter and a 630nm filter

6. REAGENTS, SOLUTION AND BUFFER

6.1 96 antibody coated microwells (12 eight-well strips) in a microwell holder (sealed in a foil pouch)
6.2 96 non-coated dilution microwells (12 eight-well strips marked with blue at base)
6.3 5 vials of 1.5mL of each aflatoxin standard (0, 4, 10, 20 and 40 ppb)
6.4 1 bottle of 25mL of aflatoxin conjugate (green-capped bottle)
6.5 1 bottle of 15mL of substrate solution (blue-capped bottle)
6.6 1 bottle of 15mL of stop solution (red-capped bottle)
6.7 70% methanol
6.8 Distilled or de-ionized water

7. PROCEDURE

7.1 Sample Preparation / Extraction

7.1.1 Obtain a representative sample and grind it using a Romer Series II® Mill so that 75% will pass through a 20-mesh screen, then thoroughly mix the subsample portion.
7.1.2 Weigh out 20 g of ground sample into a clean jar that can be tightly sealed.
7.1.3 Add 100 mL of 70/30 (v/v) methanol/water extraction solution and seal jar. Note: Samples should be extracted in a ratio of 1:5 (w:v) of sample to extraction solution respectively.
7.1.4 Shake or blend for 3 minutes.
7.1.5 Allow sample to settle, then filter the top layer of extract through a Whatman #1 filter and collect the filtrate.

Note: Commodity extracts should have a pH of 6-8. Excessive alkaline or acidic conditions may affect the test result and should be adjusted before testing.

7.2 Assay

Note: All reagents and kit components must be at room temperature 18-30°C (64-86°F) before use. It is recommended that an 8-channel pipette be used to perform the assay. No more than 48 samples and standards total (6 test strips) should be run in one experiment when using an 8-channel pipette. If an 8-channel pipette is not used (i.e. using only single channel pipettes), it is recommended that no more than a total of 16 samples and standards (2 test strips) be run in any one experiment.

7.2.1 Place the appropriate number of blue-bordered Dilution Strips in a microwell strip holder. One Dilution Well will be required for each standard, (i.e. 0, 4, 10, 20, & 40 ppb) or sample.

7.2.2 Place an equal number of Antibody Coated Microwell strips in a microwell strip holder. Return unused microwell strips to the foil pouch with the desiccant packet and reseal pouch with tape.

7.2.3 Measure the required amount of Conjugate from the green-capped bottle (~240 µL/well or 2 mL/strip) and place in a separate container (e.g. reagent boat when using the 8-channel pipette). Using an 8-channel pipette, dispense 200 µ L of Conjugate into each blue-bordered Dilution Well.

7.2.4 Using a single channel pipette, add 100 µ L of each standard or sample into the appropriate Dilution Well containing 200µL of Conjugate. Use a fresh pipette tip for each standard or sample. Note: Make sure the pipette tip has been completely emptied. Using an 8-channel pipette with fresh tips for each 8-well strip, mix each well by carefully pipetting it up and down 3 times and immediately transfer 100µL of the contents from each Dilution Well into a corresponding Antibody Coated Microwell. Incubate at room temperature for 15 minutes. Note: Do not agitate the plate to mix as it may cause well-to-well contamination.

7.2.5 Empty the contents of the microwell strips into a waste container. Wash by filling each microwell with distilled or deionized water, and then dumping the water from the microwell strips. Repeat this step 4 times for a total of 5 washes. Note: Take care not to dislodge the strips from the holder during the wash procedure.

7.2.6 Lay several layers of absorbent paper towels on a flat surface and tap microwell strips on towels to expel as much residual water as possible after the fifth wash. Dry the bottom of the microwells with a dry cloth or towel.

7.2.7 Measure the required amount of Substrate from the blue-capped bottle (~120 µL/well or 1 mL/strip) and dispense into a separate container (e.g. reagent boat for an 8-channel pipettor). Pipette 100µL of the Substrate into each microwell strip using an 8-channel pipettor. Incubate at room temperature for 5 minutes.

7.2.8 Measure the required amount of Stop Solution from the red-capped bottle (~120µL/well or 1mL/strip) and dispense into a separate container (e.g. reagent boat for an 8-channel pipette). Pipette 100µL of Stop Solution into each microwell strip using an 8-channel pipette. The color should change from blue to yellow.

7.2.9 Read the strips with a microwell reader using a 450 nm filter. Record OD readings for each microwell.

Note: Air bubbles should be eliminated prior to reading strips as they may affect analytical results.

Additional Notes: Ratio of Conjugate to Standard/Sample should remain at 2:1, but volumes

of Conjugate and Standards/Samples can be reduced, e.g. using 100µL and 50µL, respectively. The content to be transferred from dilution well to antibody coated well remains the same, as 100µL. Do not return unused reagents to their original bottles. Carefully keep track of the position of Samples and Standards during the assay. Do not mix the assay microwells by shaking at any time during test.

8. RESULT AND INTERPRETATION

Using either the unmodified OD values or the OD values expressed as a percentage of the OD of the zero (0) standard, construct a dose-response curve using the five standards. Since the amount of aflatoxin in each standard is known, the unknowns can be measured by interpolation from this standard curve. Results can also be easily calculated using the Romer® Log/Logit spreadsheet that is provided (free of charge) upon request. If the Log/Logit regression model is used for results interpretation, the linearity coefficient (r^2) of the calibration curve should be no less than 0.985. An OD value of less than 0.5 absorbance units for 0ppb standard may indicate deterioration of reagents.
If a sample contains aflatoxin levels higher than the highest standard (>40 ppb), the filtered extract should be further diluted in 70% methanol such that the diluted sample results are in a range of 5 - 20ppb and reanalyzed to obtain accurate results. The dilution factor must be included when the final result is calculated.
Levels of aflatoxin are as reported as parts per billion (ppb) for in feed type.

9. WASTE DISPOSAL

Wastes should be disposed as per the SOP for disposal of biohazard waste and chemicals.

10. RISK ASSESSMENT

10.1 Methanol is flammable. Caution must be taken in its use and storage.
10.2 The Stop Solution contains acid. Avoid contact with skin or eyes. If exposed, flush with water.
10.3 Consider all materials, containers and devices that are exposed to the sample or standards to be contaminated with toxin. Wear protective gloves and safety glasses when using the kit.

11. TROUBLESHOOTING

- N/A

12. REFERENCES

The AgraQuant® Total Aflatoxin Assay, a direct competitive enzyme-linked immunosorbent assay (ELISA), Romer Labs Singapore Pte Ltd. All Rights Reserved. This document is the property of Romer Labs Singapore Pte Ltd
PI-000041-1 Finalized 9/27/02

TITLE: Quantitative Detection of Ochratoxin by ELISA

PREPARED BY: Biochemistry & Toxicology section

REVISED BY: Biochemistry & Toxicology section

APPROVED BY: Head, LSU

DATE: 11.06.2018

1. INTRODUCTION
Ochratoxin A is a toxic metabolite produced by several molds of the *Aspergillus flavus* and *Penicillium* genera, including *Aspergillus ochraceus*. The fungal species has the potential to produce Ochratoxin A, a known nephrotoxin and carcinogen. It has been frequently detected in human foods and animal feed, mainly in cereal products, although a range of commodities has been reported to contain the toxin. It causes various toxicities in animals including poultry.

2. PRINCIPLES
The Helica™ Ochratoxin A Assay is a solid phase direct competitive enzyme immunoassay. An Ochratoxin specific antibody optimized to cross react primarily with Ochratoxin A (see crossreactivity information) is coated to a polystyrene microwell. Toxins are extracted from a ground sample with 70% methanol. The extracted sample and HRP-conjugated Ochratoxin are mixed and added to the antibody-coated microwell. Ochratoxin from the extracted sample and HRPconjugated Ochratoxin compete to bind with the antibody coated to the microwell. Microwell contents are decanted and non-specific reactants are removed by washing. An enzyme substrate (TMB) is added and color (blue) develops. The intensity of the color is directly proportional to the amount of bound conjugate and inversely proportional to the concentration of Ochratoxin in the sample and standards. Therefore, as the concentration of Ochratoxin in the sample or standard increases, the intensity of the blue color will decrease. An acidic stop solution is added which changes the chromogen color from blue to yellow. The microwells are measured optically by a microplate reader with an absorbance filter of 450 nm (OD450). The optical densities of the samples are compared to the OD's of the kit standards and an interpretative result is determined.

3. APPLICATION
The Helica Ochratoxin A Assay is a competitive enzyme-linked immunoassay intended for the quantitative detection of Ochratoxin A levels in grains, cereals, coffee, and other commodities including animal feeds.

4. OBJECTIVE
To describe the procedures for estimation of Ochratoxin concentration in a sample.

5. APPARATUS
5.1 Grinder sufficient to render sample to particle size of fine instant coffee

5.2 Collection Container: Minimum 125 mL capacity
5.3 Balance: 20g measuring capability
5.4 Graduated cylinder: 100 mL
5.5 Filter Paper: Whatman #1 or equivalent
5.6 Filter Funnel
5.7 Pipette with tips: 100µl and 200µl
5.8 Wash bottle
5.9 Absorbent paper towels
5.10 Microplate reader with 450 nm filter

6. REAGENTS, SOLUTION AND BUFFER

6.1 1 pouch Antibody coated microwells: 96 wells (12 eight well strips) in a microwell holder coated with a mouse anti-Ochratoxin antibody
6.2 1 plate Dilution wells (green): 96 non-coated wells (12 eight well strips) wells in a microwell holder.
6.3 6 vials Ochratoxin A Standards: 1.5 mLl/vial of Ochratoxin A at the following concentrations: 0.0, 0.4, 1.0, 2.0, 4.0, and 8.0 ng/mL in organic solution.
6.4 2 bottle Ochratoxin A HRPconjugate: 2 x 12 mL of Ochratoxin A conjugated to peroxidase in buffer with preservative
6.5 1 bottle Substrate Reagent: 15 mL stabilized tetramethylbenzidine (TMB)
6.6 1 bottle Stop Solution: 15 mL Acidic Solution
6.7 Methanol: 70 mL reagent grade per sample
6.8 Distilled or deionized water: 30 mL per sample

7. PROCEDURE

Bring all reagents to room temperature (19º - 27ºC) before use. Store reagents at 2 to 8°C, and do not use beyond expiration date(s). Never freeze kit components.

7.1 Extraction procedure

Note: The sample must be collected according to established sampling techniques

7.1.1 Prepare the Extraction Solution (70% Methanol) by adding 30 mL of distilled or deionized water to 70 mL of methanol (reagent grade) for each sample to be tested.
7.1.2 Grind a representative sample to the particle size of fine instant coffee (50% passes through a 20 mesh screen).
7.1.3 Weigh out a 20 g ground portion of the sample and add 100 mL of the Extraction Solvent (70% methanol). Note: The ratio of sample to extraction solvent is 1:5 (w/v).
7.1.4 Mix by shaking in a sealed container or in a blender for a minimum of 2 minutes.
7.1.5 Allow the particulate matter to settle, then filter 5 – 10mL of the extract through a Whatman #1 filter paper (or equivalent) and collect the filtrate to be tested. The sample is now ready for testing.

7.2 Assay Procedure

Note: It is recommended that a multi-channel pipette be utilized to perform the assay. If a single channel pipette is used, it is recommended that no more than a total of 16 samples

and standards (2 test strips) are run.

7.2.1 Bring all the reagents to room temperature before use.

7.2.2 Place one Dilution Well in a micro well holder for each Standard and Sample to be tested. Place an equal number of Antibody Coated Microtiter Wells in another microwell holder.

7.2.3 Dispense 200µl of the Conjugate into each Dilution Well.

7.2.4 Using a new pipette tip for each, add 100µl of each Standard and Sample to appropriate dilution well containing Conjugate. Mix by priming pipette at least 3 times. Note: Operator must record the location of each Standard and Sample throughout test.

7.2.5 Using a new pipette tip for each, transfer 100µl of contents from each Dilution Well to a corresponding Antibody Coated Microtiter Well. Incubate at room temperature for 15 minutes.

7.2.6 Decant the contents from microwells into a discard basin. Wash the microwells by filling each with distilled or deionized water, then decanting the water into a discard basin. Repeat wash for a total of 5 washes.

7.2.7 Tap the microwells (face down) on a layer of absorbent towels to remove residual water.

7.2.8 Measure the required volume of Substrate Reagent (1 mL/strip or 120µ l/well) and place in a separate container. Add 100µl to each microwell. Incubate at room temperature for 5minutes.

7.2.9 Measure the required volume of Stop Solution (1 mL/strip or 120 µ l/well) and place in a separate container. Add 100µl in the same sequence and at the same pace as the Substrate was added.

7.2.10 Read the optical density (OD) of each microwell with a microtiter plate reader using a 450 nm filter. Record the optical density (OD) of each microwell.

8. RESULT AND INTERPRETATION

Construct a dose-response curve using either the unmodified OD values or the OD values expressed as a percentage of the OD of the zero (0.0) standard against the Ochratoxin A content of the standard. Unknowns are measured by interpolation from the standard curve. The sample dilution results in a standard curve from 2 ppb to 40 ppb. If a sample contains Ochratoxin A at greater than the highest standard, it should be diluted appropriately in 70% methanol and retested. The extra dilution step should be taken into consideration when expressing the final result. The information contained on the label of a standard vial refers to the contents of that vial.

However, the sample has been diluted at a 5:1 ratio with 70% methanol, and so the level of Ochratoxin shown by the standard must be multiplied by 5 in order to indicate the ng of Ochratoxin per gram of commodity (ppb) as follows:

Standard ng/mL	Commodity (ppb)
0.0	0.0
0.4	2.0
1.0	5.0
2.0	10.0
4.0	20.0

8.0	40.0

9. WASTE DISPOSAL

Waste should be disposed as per the SOP for disposal of biohazard waste and chemicals.

10. RISK ASSESSMENT

10.1 Standards are flammable. Caution should be taken in the use and storage of these reagents.

10.2 The Stop Solution contains acid. Do not allow to contact skin or eyes. If exposed, flush with water.

10.3 Consider all materials, containers and devices that are exposed to sample or standards to be contaminated with toxin. Wear protective gloves and safety glasses when using this kit.

11. TROUBLESHOOTING

Samples tested should have a pH of 7.0 (±1.0). Excessive alkaline or acidic conditions may affect the test results.

12. REFERENCES

OCHRATOXIN A ASSAY FOR GRAINS CAT. NO. 941OCH01M – 96 Helica Biosystems, Inc. v.06 08-06-07

TITLE: Quantitative Detection of Fumonisin by ELISA

PREPARED BY: Biochemistry & Toxicology section

REVISED BY: Biochemistry & Toxicology section

APPROVED BY: Head, LSU

DATE: 11.06.2018

1. INTRODUCTION
The Fumonsins are a recently discovered group of structurally related toxic metabolites of several fungal species. Of primary importance is their production by *Fusarium moniliforme*, which is a common contaminant of corn in many parts of the world. Ingestion of fumonisins in contaminated animal feed and food for human consumption has been linked to both toxic and carcinogenic effects. The three most abundant fumonisins are FB_1, FB_2, and FB_3.

2. PRINCIPLES
The HELICA BIOSYSTEMS fumonisin ELISA is a solid phase direct competitive enzyme immunoassay. A fumonisin-specific antibody optimized to cross react with the three-fumonisin subtypes is coated to a polystyrene microwell. Toxins are extracted from a ground sample with 90% methanol. The extracted sample and HRP-conjugated fumonisin are mixed and added to the antibody-coated microwell. Fumonisin from the extracted sample and HRP-conjugated fumonisin compete to bind with the antibody coated to the microwell. Microwell contents are decanted and non-specific reactants are removed by washing. An enzyme substrate (TMB) is added and color (blue) develops. The intensity of the color is directly proportional to the amount of bound conjugate and inversely proportional to the concentration of fumonisin in the sample or standard.
Therefore, as the concentration of fumonisin in the sample or standard increases, the intensity of the blue color will decrease. An acidic stop solution is added which changes the chromogen color from blue to yellow. The microwells are measured optically by a microplate reader with an absorbance filter of 450nm (OD450). The optical densities of the samples are compared to the OD's of the kit standards and an interpretative result is determined.

3. APPLICATION
The HELICA BIOSYSTEMS fumonisin ELISA is a competitive enzyme-linked immunoassay intended for the quantitative detection of fumonisins in maize and feeds.

4. OBJECTIVE
To describe the procedure for measuring fumonisin concentration in a sample.

5. APPARATUS
5.1 Grinder sufficient to render sample to particle size of fine instant coffee
5.2 Collection Container: Minimum 125ml capacity
5.3 Balance: 20g measuring capability

5.4 Graduated cylinder: 100ml
5.5 Filter Paper: Whatman #1 or equivalent
5.6 Filter Funnel
5.7 Pipette with tips: 100µl and 200µl
5.8 Wash bottle
5.9 Absorbent paper towels
5.10 Microplate reader with 450nm filter

6. REAGENTS, SOLUTION AND BUFFER

6.1 1 pouch Antibody coated microwells: 96 wells (12 eight well strips) in a microwell holder coated with a mouse anti-fumonisin monoclonal antibody.
6.2 1-plate Dilution wells (green): 96 non -coated wells (12 eight well strips) in a microwell holder.
6.3 6vials Fumonisin Standards: 1.5ml/v ial of fumonisin at the following concentrations: 2.5, 7.5, 20.0, 50.0, 150.0 ng/mL in aqueous solution
6.4 2 bottles Fumonisin HRP-conjugate: 2x12mL of binary fumonisin HR-conjugate in buffer with preservative.
6.5 1 bottle Substrate Reagent: 15ml stabilized tetramethylbenzidine (TMB)
6.6 1 bottle Stop Solution: 15ml Acidic Solution
6.7 1 pouch washing buffer: PBS with 0.05% Tween 20, bring 1 liter with distilled water and store refrigerated.
6.8 Methanol: 36ml reagent grade per sample
6.9 Distilled or deionized water: 4ml per sample

7. PROCEDURE

Bring all reagents to room temperature (19º - 27ºC) before use. Store reagents at 2 to 8°C, and do not use beyond expiration date(s). Never freeze kit components.

7.1 Extraction process

Note: The sample must be collected according to established sampling techniques

7.1.1 Prepare the Extraction Solution (90% Methanol) by adding 4ml of distilled or deionized water to 36ml of methanol (reagent grade) for each sample to be tested.
7.1.2 Grind a representative sample to the particle size of fine instant coffee (50% passes through a 20 mesh screen).
7.1.3 Weigh out a 20g ground portion of the sample and add 40ml of the Extraction Solvent (90% methanol).Note: The ratio of sample to extraction solvent is 1:2 (w/v).
7.1.4 Mix by shaking in a sealed container or in a blender for one minute.
7.1.5 Allow the particulate matter to settle, then filter 5 - 10ml of the extract through a Whatman #1filter paper (or equivalent) and collect the filtrate to be tested.
7.1.6 Dilute the sample extract 1:20 in distilled water (e.g. 0.1 mL + 1.9 mL)
7.1.7 Diluted sample is now ready for testing.

7.2 Assay procedure

Note: It is recommended that a multi-channel pipette be utilized to perform the assay. If a

single channel pipette is used, it is recommended that no more than a total of 16 samples and standards (2 test strips) are run.

7.2.1 Bring all the reagents to room temperature before use. Reconstitute the PBS-Tween packet by washing out the contents with a gentle stream of distilled water into a 1-Liter container.

7.2.2 Place one Dilution Well in a microwell holder for each Standard and Sample to be tested. Place an equal number of Antibody Coated Microtiter Wells in another microwell holder.

7.2.3 Dispense 100µl of the Conjugate solution A (green) into the appropriate dilution wells followed by 100uL of Conjugate solution B (clear).

7.2.4 Using a new pipette tip for each, add 100µl of each Standard and Sample to appropriate Dilution Well containing Conjugate. Mix by priming pipette 3 times. Note: Operator must record the location of each Standard and Sample throughout test.

7.2.5 Using a new pipette tip for each, transfer 100µl of contents from each Dilution Well to a corresponding Antibody Coated Microtiter Well. Incubate at room temperature for 10 minutes. The mixing wells contain enough solution to run each standard and/or sample in duplicate if so desired.

7.2.6 Decant the contents from microwells into a discard basin. Wash the microwells by filling each with PBS Tween wash buffer then decanting the water into a discard basin. Repeat wash for a total of 3 washes.

7.2.7 Tap the microwells (face down) on a layer of absorbent towels to remove residual water.

7.2.8 Measure the required volume of Substrate Reagent (1 ml/strip or 120 µl/well) and place in a separate container. Add 100µl to each microwell. Incubate at room temperature for 10 minutes.

7.2.9 Measure the required volume of Stop Solution (1 ml/strip or 120 µl/well) and place in a separate container. Add 100µl in the same sequence and at the same pace as the Substrate was added.

7.2.10 Read the optical density (OD) of each microwell with a microtiter plate reader using a 450nm filter. Record the optical density (OD) of each microwell.

8. RESULT AND INTERPRETATION

Construct a dose-response curve using either the unmodified OD values or the OD values expressed as a percentage of the OD of the zero (0.0) standard against the fumonisin content of the standard. Unknowns are measured by interpolation from the standard curve. The information contained on the label of a standard vial refers to the contents of that vial. However, the sample has been diluted at a 2:1 ratio with 90% methanol followed by a dilution of 20:1 in distilled water, and so the level of fumonisin shown by the standard must be multiplied by 40 in order to indicate the ng of fumonisin per gram of commodity (ppm) as follows:

Standard ng/mL	Commodity ug/gm (ppm)
0.0	0.0
2.5	0.1
7.5	0.3

20.0	0.8
50.0	2.0
150.0	6.0

The sample dilution results in a standard curve from 0.0 ppm to 6.0 ppm. If a sample contains fumonisin at greater than the highest standard, it should be diluted appropriately in distilled water and retested. The extra dilution step should be taken into consideration when expressing the final result.

SAMPLE	HPLC	HELICA ELISA
1	<0.1 ppm	<0.1 ppm
2	0.6 +/- 0.2 ppm	0.72 +/- 0.06 ppm
3	2.0 +/- 0.3 ppm	1.98 +/- 0.12 ppm
4	3.5 +/- 0.4 ppm	3.30 +/- 0.35 ppm

9. WASTE DISPOSAL

Waste should be disposed as per the SOP for disposal of biohazard waste and chemicals.

10. RISK ASSESSMENT

10.1 The Stop Solution contains acid. Do not allow to contact skin or eyes. If exposed, flush with water.

10.2 Consider all materials, containers and devices that are exposed to sample or standards to be contaminated with fumonisin. Wear protective gloves and safety glasses when using this kit.

11. TROUBLESHOOTING

- N/A

12. REFERENCES

FUMONISIN ELISA ASSAY (Cat. No. 951FUM01C) Helica Biosystems, Inc.
V.01 03-01-10

TITLE: Quantitative Detection of T-2 Toxin by ELISA

PREPARED BY: Bio-chemistry & Toxicology section

REVISED BY: Bio-chemistry & Toxicology section

APPROVED BY: Head, LSU

DATE: 11.06.2018

1. INTRODUCTION
T-2 toxin is a type A trichothecene. T-2 toxin is produced by fungi of the *Fusarium* genus, and the most important producer is *Fusarium sporotrichioides*. This mycotoxin occurs in grains such as wheat, maize, oats, barley, rice, beans and soyabeans as well as in some cereal-based products. T-2 toxin inhibits protein synthesis and affects the actively dividing cells such as those lining the gastrointestinal tract, skin, lymphoid and erythroid cells. The effects of T-2 toxin to animals include weight loss or poor weight gain, bloody diarrhoea, dermal necrosis or beak lesions, haemorrhage and decreased production (weight gain, eggs, milk, etc.).

2. PRINCIPLES
The T-2 Toxin Assay is a direct competitive enzyme-linked immunosorbent assay (ELISA). T-2 toxin is extracted from a ground sample with 70 % methanol. The extract is further diluted at 1:10 using de-ionized or distilled water. The diluted extract and enzyme-conjugated T-2 toxin are mixed in dilution microwells and transferred to the antibody-coated microwells. T-2 toxin in samples and control standards are allowed to compete with enzyme-conjugated T-2 toxin for the antibody binding sites. After a washing step, an enzyme substrate is added and blue color develops. The intensity of the color is inversely proportional to the concentration of T-2 toxin in the sample or standard. A stop solution is then added which changes the color from blue to yellow. The microwells are measured optically using a microwell reader with an absorbance filter of 450 nm and a differential filter of 630 nm. The optical densities of the samples are compared to the OD's of the standards and an interpretative result is determined.

3. APPLICATION
The T-2 Toxin Assay is a direct competitive enzyme-linked immunosorbent assay (ELISA) that determines a quantitative level for the presence of T-2 toxin and is intended for use in grains, cereals, nuts, animal feeds and other commodities.

4. OBJECTIVE
To describe the procedure to measure T-2 Toxin concentration in a sample.

5. APPARATUS
5.1 Mill/Grinder or equivalent

5.2 Blender or a tightly sealing jar with lid

5.3 Balance, 400 g
5.4 Graduated cylinder: 100mL
5.5 Container with a minimum 125mL capacity
5.6 Whatman#1 filter paper, or equivalent
5.7 Filter funnel
5.8 MycoSep 112 column
5.9 8-channel and single channel pipette capable of pipetting 100µL and 200µL with tips
5.10 Timer
5.11 Wash bottle
5.12 Absorbent paper towels
5.13 3 reagent boats for use as reagent containers for an 8-channel pipettor
5.14 Microwell reader with a 450nm filter.

6. REAGENTS, SOLUTION AND BUFFER

6.1 96 antibody coated microwells (12 eight-well strips) in a microwell holder (sealed in a foil pouch)
6.2 96 non-coated dilution microwells (12 eight-well strips marked with green at base)
6.3 5 vials of 1.5mL of each T-2 toxin standard. Standard concentrations are 0, 25, 100, 250 and 500 ppb, respectively. Standards need further dilution of 1:10 with deionized or distilled water before assay.
6.4 1 bottle of 25mL T-2 toxin conjugate (green-capped bottle)
6.5 1 bottle of 15mL of substrate solution (blue-capped bottle)
6.6 1 bottle of 15mL of stop solution (red-capped bottle)
6.7 100% methanol: ACS grade methanol
6.8 70% methanol or ACS grade methanol for making 70 % methanol
6.9 Distilled or de-ionized water for making 70 % methanol
6.10 84% acetonitrile

7. PROCEDURE

7.1 Sample Preparation / Extraction

7.1.1 Obtain a representative sample and grind it using a mill/ginder so that 95 % will pass through a 20-mesh screen, then thoroughly mix the subsample portion.
7.1.2 Weigh out 20 g of ground sample into a clean jar that can be tightly sealed.
7.1.3 Add 100 mL of 70 % methanol and seal jar. **Note:** Samples should be extracted in a ratio of 1:5 (w:v) of sample to extraction solution respectively.
7.1.4 Shake or blend for 3 minutes.
7.1.5 Allow sample to settle, then filter the top layer of extract through a Whatman #1 filter and collect the filtrate. **Note:** Commodity extracts should have a pH of 6-8. Excessive alkaline or acidic conditions may affect the test results and should be adjusted before testing.
7.1.6 Dilute the sample extract 1:10 with deionized or distilled water. For example, add 1 mL of extract to 9 mL of distilled or deionized water.
7.1.7 The sample is ready for testing without further preparation.

7.2 Assay

Note: All reagents and kit components must be at room temperature 18-30 C (64-86 F) before use. It is recommended that an 8-channel pipette be used to perform the assay. No more than 48 samples and standards total (6 test strips) should be run in one experiment when using an 8-channel pipette. If an 8-channel pipette is not used (i.e. using only single channel pipettes), it is recommended that no more than a total of 16 samples and standards (2 test strips) be run in any one experiment.

7.2.1 Dilute kit standards (i.e. 0, 25, 100, 250, & 500 ppb) 1:10 with deionized or distilled water in test tubes. For example, add 0.1 mL of standard to 0.9 mL of deionized or distilled water and mix.

7.2.2 Place the appropriate number of green bordered Dilution Wells in a microwell strip holder. One Dilution

7.2.3 Well will be required for each standard or sample.

7.2.4 Place an equal number of Antibody Coated Microwell strips in a microwell strip holder. Return unused microwell strips to the foil pouch with the desiccant packet and reseal pouch with tape.

7.2.5 Measure the required amount of Conjugate from the green-capped bottle (~240 L/well or 2 mL/strip) and place in a separate container (e.g. reagent boat when using the 8-channel pipette). Using an 8-channel pipette, dispense **200 µL of Conjugate** into each green-bordered Dilution Well.

7.2.6 Using a single channel pipette, add **100 µL of each diluted standard or sample** into the appropriate Dilution Well containing 200 µL of Conjugate. Use a fresh pipette tip for each standard or sample. **Note:** Make sure the pipette tip has been completely emptied.

7.2.7 Using an 8-channel pipette with fresh tips for each 8-well strip, mix each well by carefully pipetting it up and down 3 times and immediately transfer **100 µL of the contents from each Dilution Well** into a corresponding Antibody Coated Microwell. Incubate at room temperature for **10 minutes. Note:** Do not agitate the plate to mix as it may cause well-to-well contamination.

7.2.8 Empty the contents of the microwell strips into a waste container. Wash by filling each microwell with distilled or deionized water, and then dumping the water from the microwell strips. Repeat this step 4 times for a total of 5 washes. **Note:** Take care not to dislodge the strips from the holder during the wash procedure.

7.2.9 Lay several layers of absorbent paper towels on a flat surface and tap microwell strips on towels to expel as much residual water as possible after the fifth wash. Dry the bottom of the microwells with a dry cloth or towel.

7.2.10 Measure the required amount of Substrate from the blue-capped bottle (~120 L/well or 1 mL/strip) and dispense into a separate container (e.g. reagent boat for an 8-channel pipettor). Pipette **100 µL of the Substrate** into each microwell strip using an 8-channel pipettor. Incubate at room temperature for **5 minutes**.

7.2.11 Measure the required amount of Stop Solution from the red-capped bottle (~120 L/well or 1 mL/strip) and dispense into a separate container (e.g.

reagent boat for an 8-channel pipette). Pipette **100 µL of Stop Solution** into each microwell strip using an 8-channel pipette. The color should change from blue to yellow.

7.2.12 Read the strips with a microwell reader using a 450 nm filter with a 630nm differential filter. Record OD readings for each microwell. **Note:** Air bubbles should be eliminated prior to reading strips as they may affect analytical results.

7.2.13 **Additional Notes:** Ratio of **Conjugate** to **Standard/Sample** should remain at **2:1**, but volumes of **Conjugate** and **Standards/Samples** can be reduced, e.g. using 100 **µ**L and 50 **µ**L, respectively. The content to be transferred from dilution well to antibody coated well remains the same as 100 L. Do not return unused reagents to their original bottles. Carefully keep track of the position of Samples and Standards during the assay. Do not mix the assay microwells by shaking at any time during test.

8. RESULT AND INTERPRETATION

Using either the unmodified OD values or the OD values expressed as a percentage of the OD of the zero (0) standard, construct a dose-response curve using the five standards. Since the amount of T-2 in each standard is known, the unknowns can be measured by interpolation from this standard curve.

Results can also be easily calculated using the Romer® Log/Logit spreadsheet that is provided (free of charge) upon request. If the Log/Logit regression model is used for results interpretation, the linearity coefficient (r^2) of the calibration curve should be no less than 0.985. An OD value of less than 0.5 absorbance units for 0 ppb standard may indicate deterioration of reagents.

Samples containing less than 25 ppb should be reported as "< 25 ppb". Samples containing greater than 500 ppb should be reported as "> 500 ppb". Samples containing greater than 500 ppb should be further diluted with deionized or distilled water such that the diluted sample results are in the range of 25-500 ppb and re-analysed to obtain accurate results. The dilution factor must be included when the final result is calculated.

Levels of T-2 toxin (type A trichothecene. T-2 toxin) are reported as parts per billion (ppb) in feed type.

9. WASTE DISPOSAL

Waste should be disposed as per the SOP for disposal of biohazard waste and chemicals.

10. RISK ASSESSMENT

10.1 The Stop Solution contains acid. Avoid contact with skin or eyes. If exposed, flush with water.

10.2 Consider all materials, containers and devices that are exposed to the sample or standards to be contaminated with toxin. Wear protective gloves and safety glasses when using the kit.

11. TROUBLESHOOTING

11.1 For quantitation of samples above 500 ppb, samples should be diluted such that the diluted sample results are in a range of quantitation

11.2 Cross Reactivity

Compound	**Cross reactivity %**
T-2	100
HT-2	44
T-2 Triol	1.6
T-2 Tetraol	<0.04
Verrucarol	<0.04

12. REFERENCES

The AgraQuant®T-2 toxin, a direct competitive enzyme-linked immunosorbent assay (ELISA), Romer Labs Singapore Pte. Ltd.3791 Jalan Bukit Merah #08-08 e-Centre@redhill, Singapore, 159471

TITLE: Quantitative Detection of Zearalenone Toxin by ELISA

PREPARED BY: Biochemistry & Toxicology section

REVISED BY: Biochemistry & Toxicology section

APPROVED BY: Head, LSU

DATE: 11.06.2018

1. INTRODUCTION

Zearalenone is produced by *Fusarium graminearum*. Grain infected with this organism often will have a pink color because of pigment that may be simultaneously produced with Zearalenone. Most often Zearalenone is found in corn however, it is also found in other important crops such as wheat, barley, sorghum and rye throughout the world. The major effects of Zearalenone are estrogenic and primarily involve the urogenital system.

2. PRINCIPLES

The AgraQuant® Zearalenone Assay is a direct competitive enzyme-linked immunosorbent assay (ELISA). Zearalenone is extracted from a ground sample with 70% methanol. The diluted extracts and enzyme-conjugated Zearalenone are mixed and added to the antibody-coated microwell. Zearalenone in samples and control standards are allowed to compete with enzyme-conjugated Zearalenone for the antibody binding sites. After a washing step, an enzyme substrate is added and blue color develops. The intensity of the color is inversely proportional to the concentration of Zearalenone in the sample or standard. A stop solution is then added which changes the color from blue to yellow. The microwells are measured optically using a microwell reader with an absorbance filter of 450nm and a differential filter of 630nm. The optical densities of the samples are compared to the OD's of the standards and an interpretative result is determined.

3. APPLICATION

The AgraQuant® Zearalenone Assay is a direct competitive enzyme-linked immunosorbent assay (ELISA) that determines a quantitative level for the presence of Zearalenone and is intended for use in grains, cereals, nuts, animal feeds and other commodities.

4. OBJECTIVE

To outline the procedure for measuring Zearalenone concentration in a sample.

5. APPARATUS

5.1 Mill/Grinder or equivalent
5.2 Blender or a tightly sealing jar with lid
5.3 Balance, 400 g
5.4 Graduated cylinder: 100mL

5.5 Container with a minimum 125mL capacity
5.6 Whatman#1 filter paper, or equivalent
5.7 Filter funnel
5.8 MycoSep 112 column
5.9 8-channel and single channel pipettes capable of pipetting 100µL and 200µL with tips
5.10 Timer
5.11 Wash bottle
5.12 Absorbent paper towels
5.13 3 reagent boats for use as reagent containers for an 8-channel pipette
5.14 Microwell reader with a 450nm filter.

6. REAGENTS, SOLUTION AND BUFFER

6.1 96 antibody coated microwells (12 eight-well strips) in a microwell holder (sealed in a foil pouch)
6.2 96 non-coated dilution microwells (12 eight-well strips marked with blue at base)
6.3 5 vials of 1.5mL of each Zearalenone standard (0, 25, 100, 300 and 1000 ppb)
6.4 1 bottle of 25mL of Zearalenone conjugate (green-capped bottle)
6.5 1 bottle of 15mL of substrate solution (blue-capped bottle)
6.6 1 bottle of 15mL of stop solution (red-capped bottle)
6.7 100% methanol: ACS grade methanol
6.8 70% methanol or ACS grade methanol for making 70 % methanol
6.9 Distilled or de-ionized water for making 70 % methanol
6.10 84% acetonitrile

7. PROCEDURE

7.1 Sample Preparation / Extraction

7.1.1 Obtain a representative sample and grind it using a Grinder or Mill so that 75% will pass through a 20-mesh screen, then thoroughly mix the subsample portion.
7.1.2 Weigh out 20 g of ground sample into a clean jar that can be tightly sealed.
7.1.3 Add 100 mL of 70% methanol and seal jar. **Note:** Samples should be extracted in a ratio of 1:5 (w:v) of sample to extraction solution respectively.
7.1.4 Vigorously shake or blend for 3 minutes.
7.1.5 Allow sample to settle, then filter the top layer of extract through a Whatman #1 filter and collect the filtrate. **Note:** Commodity extracts should have a pH of 6-8. Excessive alkaline or acidic conditions may affect the test results and should be adjusted before testing.
7.1.6 Dilute the sample extract 1:5 with 70% methanol. For example, add 1 ml of extract to 4 ml of 70% methanol.
7.1.7 The sample is ready for testing without further preparation.
7.1.8 Optional method for sample preparation: weigh 20g of ground sample into a suitable container, and add 500ml of 70% methanol; vigorously shake for 3 minutes; filter the top layer of extract through a Whatman#1 filter paper. The sample is ready for testing (with no further dilutions needed). **Note:**

Commodity extracts should have a pH of 6-8. Excessive alkaline or acidic conditions may affect the test results and should be adjusted before testing.

7.2 Assay

Note: All reagents and kit components must be at room temperature 30°C (64-86°F) before use. It is recommended that an 8-channel pipette be used to perform the assay. No more than 48 samples and standards total (6 test strips) should be run in one experiment when using an 8-channel pipette. If an 8-channel pipette is not used (i.e. using only single channel pipettes), it is recommended that no more than a total of 16 samples and standards (2 test strips) run in any one experiment.

7.2.1 Place the appropriate number of blue-bordered Dilution Strips in a microwell strip holder. One Dilution Well will be required for each standard, (i.e. 0, 25, 100, 300, & 1000 ppb) or sample.

7.2.2 Place an equal number of Antibody Coated Microwell strips in a microwell strip holder. Return unused microwell strips to the foil pouch with the desiccant packet and reseal pouch with tape.

7.2.3 Measure the required amount of Conjugate from the green-capped bottle (~240 L/well or 2 mL/strip) and place in a separate container (e.g. reagent boat when using the 8-channel pipette). Using an 8-channel pipette, dispense **200µL of Conjugate** into each blue-bordered Dilution Well.

7.2.4 Using a single channel pipette, add **100µL of each standard or sample** into the appropriate Dilution Well containing 200**µ**L of Conjugate. Use a fresh pipette tip for each standard or sample. **Note:** Make sure the pipette tip has been completely emptied.

7.2.5 Using an 8-channel pipettor with fresh tips for each 8-well strip, mix each well by carefully pipetting it up and down 3 times and immediately transfer **100 µL of the contents from each Dilution Well** into a corresponding Antibody Coated Microwell. Incubate at room temperature for **10 minutes. Note:** Do not agitate the plate to mix as it may cause well-to-well contamination.

7.2.6 Empty the contents of the microwell strips into a waste container. Wash by filling each microwell with de- ionized water, and then dumping the water from the microwell strips. Repeat this step 4 times for a total of 5 washes. **Note:** Take care not to dislodge the strips from the holder during the wash procedure.

7.2.7 Lay several layers of absorbent paper towels on a flat surface and tap microwell strips on towels to expel as much residual water as possible after the fifth wash. Dry the bottom of the microwells with a dry cloth or towel.

7.2.8 Measure the required amount of Substrate from the blue-capped bottle (~120 **µ**L/well or 1 mL/strip) and dispense into a separate container (e.g. reagent boat for an 8-channel pipettor). Pipette **100µL of the Substrate** into each microwell strip using an 8-channel pipettor. Incubate at room temperature for **5 minutes**.

7.2.9 Measure the required amount of Stop Solution from the red-capped bottle (~120**µ**L/well or 1 mL/strip) and dispense into a separate container (e.g. reagent boat for an 8-channel pipette). Pipette **100µL of Stop Solution** into each microwell strip using an 8-channel pipettor. The color should change from blue to yellow.

7.2.10 Read the strips with a microwell reader using a 450 nm filter with a differential

filter of 630nm. Record OD readings for each microwell.

Note: Air bubbles should be eliminated prior to reading strips as they may affect analytical results. **Additional Notes:** Ratio of **Conjugate** to **Standard/Sample** should remain at **2:1**, but volumes of **Conjugate** and **Standards/Samples** can be reduced, e.g. using 100 **µ**L and 50 **µ**L, respectively. The content to be transferred from dilution well to antibody coated well remains the same as 100 **µ**L. Do not return unused reagents to their original bottles. Carefully keep track of the position of Samples and Standards during the assay. Do not mix the assay microwells by shaking at any time during test.

8. RESULT AND INTERPRETATION

Using either the unmodified OD values or the OD values expressed as a percentage of the OD of the zero (0) standard, construct a dose-response curve using the five standards. Since the amount of Zearalenone in each standard is known, the unknowns can be measured by interpolation from this standard curve. Results can also be easily calculated using the Romer® Log/Logit spreadsheet that is provided (free of charge) upon request. If the Log/Logit regression model is used for results interpretation, the linearity coefficient (r^2) of the calibration curve should be no less than 0.985. An OD value of less than 0.5 absorbance units for 0ppb standard may indicate deterioration of reagents.

If a sample contains Zearalenone levels higher than the highest standard (> 1000ppb), the filtered extract should be further diluted in 70% methanol such that the diluted sample results are in a range of 25 – 1000ppb and re-analyzed to obtain accurate results. The dilution factor must be included when the final result is calculated. Levels of Zearalenone are reported as parts per billion (ppb) in feed type.

9. WASTE DISPOSAL

Waste should be disposed as per the SOP for disposal of biohazard waste and chemical.

10. RISK ASSESSMENT

10.1 The Stop Solution contains acid. Avoid contact with skin or eyes. If exposed, flush with water.

10.2 Consider all materials, containers and devices that are exposed to the sample or standards to be contaminated with toxin. Wear protective gloves and safety glasses when using the kit.

11. TROUBLESHOOTING

For quantitation of samples above 1000ppb samples should be diluted such that the diluted sample results are in a range of quantitation). Note: several commodity matrices have been validated in the quantitation range of 40-1000ppb, e.g. corn/soy blend, malted barley, sorghum, soybean, etc.

12. REFERENCES

The AgraQuant® Zearalenone ELISA, a direct competitive enzyme-linked immunosorbent assay (ELISA), Romer Labs Singapore Pte. Ltd.3791 Jalan Bukit Merah #08-08 e-Centre@redhill, Singapore, 159471

TEST CATEGORIZATION TOXICOLOGY

Sl. No.	Procedure / SOP	DVL	SVL/ TVH	RLDC/ NVH	NCAH
1	Qualitative Mycotoxin detection -Aflatoxin		X	X	X
2	Quantitative Mycotoxin detection - Aflatoxin - Ochratoxin - Fumonisin - Trichothecenes - Zearalenone				X