**Introduction**

Fungi-Plex™ is a microbead based, flow cytometric analytical assay developed for the qualitative and quantitative detection of the mycotoxins Aflatoxin B1, Zearalenone, Ochratoxin A, Fumonisin B1, Deoxynivalenol and T2-toxin mycotoxin contaminations in food and feed. Mycotoxins are toxic agents produced by fungi, and they may pose as a health risk accumulated in both the animal feed and the food consumed by humans.

Fungi-Plex™ is a multiplexed competitive assay. The Fungi-Plex™ system uses the sensitivity of the amplified fluorescence detection by flow cytometry to measure the mycotoxins in a particle (bead) based immunoassay. Each bead provides a capture surface for a specific mycotoxin (e.g. T2) and is analogous to an individually coated well in a microtitre ELISA plate. The assay is based on the competition of the mycotoxin molecules (as antigen) and the mycotoxin-coupled phycoerythrin (PE) macromolecules.

The multi-analyte assay uses multiplexed bead particles that are fluorescently labeled with gradually increasing concentrations of a fluorescent dye. The measurements can be performed with a flow cytometry instrument which is capable of detecting the PE fluorescence (580 nm) and the clustering fluorescence (>640 nm). Properly designed flow cytometric analysis protocol and Microsoft® Excel spread sheet can be used to generate calibration curves and calculate the toxin concentrations. For your convenience, new algorythms were developed to be used to generate calibration curves and calculate the toxin concentrations.

**Principle of the Test**

Six bead populations (capture beads) with different fluorescence characteristics are coated with specific antibodies prepared against the Aflatoxin B1, Zearalenone, Ochratoxin A, Fumonisin B1, Deoxynivalenol or T2-toxin. The bead populations are mixed together to form the Fungi-Plex™ Kit that is clustered in a red channel of a flow cytometer. The capture beads are mixed with the PE-conjugated fluorescent dye. The measurements can be performed with a flow cytometry instrument which is capable of detecting the PE fluorescence (580 nm) and the clustering fluorescence (>640 nm). Properly designed flow cytometric analysis protocol and Microsoft® Excel spread sheet can be used to generate calibration curves and calculate the toxin concentrations.

**Kit Content**

- **Bead Cluster**: A9 (Brightest), A8, A7, A5, A4, A3 (Dimmest)
- **Specificity**: T2-toxin
- **Mycotoxin Standards**: Aflatoxin B1, Zearalenone, Ochratoxin A, Fumonisin B1, Deoxynivalenol

**Materials Required but not Provided**

- A flow cytometry instrument equipped with a 488 nm or 532 nm laser capable of detecting and distinguishing 576 nm, >640 nm fluorescence
- BD FACSDiva™ Software (Cat. No. 338621) for analysis
- Microcentrifuge, Microcentrifuge tube (polypropylene)
- Filter (e.g. Whatman® 359.5 % folded filter, Sigma, Cat. No. Z612936)
- Orbital shaker, vortex, pipettes

**Required for Plate-loader-equipped Flow Cytometers**

- Standard microtiter plate(s) for the BD FACSCalibur Bioanalyzer Setup (e.g. BD Falcon™ Cat. No. 353910)
- Millipore MultiScreen HTS-BV 1.2 µm clear non-sterile filter plates, [Cat. No. MSB21210 (10 pack) or MSBV1250 (50 pack)]
- Millipore MultiScreen HTS Vacuum Manifold, (Cat. No. MSVMTS00)
- a digital/microtiter shaker (e.g. MTS 2/4 digital Stirrer, IKA Works, VWR, Cat. No. 82006-096)
- Vacuum pump
- Vacuum gauge(s) and regulator

**Preparation of Fungi-Plex™ Standards**

The Mycotoxin Standard Mix (Fungi-Plex™) is lyophilized, therefore it must be reconstituted and (serially) diluted before the mixing of the Bead Mix/C and the Detection Reagent/B.

1. Reconstitute one vial of Mycotoxin Standard Mix with 0.2 ml of 50 % (v/v) acetonitrile/dH2O (add 0.1 ml acetonitrile to 0.1 ml dH2O, into the Mycotoxin Standard Mix vial) to prepare the 50x concentrated Mycotoxin Standard Mix. Leave the reconstituted mix to equilibrate for at least 30 minutes RT before the preparations of the dilutions. Shake the vial vigorously to mix the components.

2. Label seven microcentrifuge tubes and arrange them in the following order: "C50x (1:1)" , "1:2" , "1:4" , "1:8" , "1:16" , "1:32" and "1:64".

3. Dilute the Mycotoxin Standard Mix (50x conc.) in the tube of "C50x" by adding of 980 µl of the Standard Diluent Buffer to 20 µl Mycotoxin Standard Mix vial to prepare the 50x concentrated Mycotoxin Standard Mix. Leave the reconstituted mix to equilibrate for at least 30 minutes RT before the dilutions of the mix. Shake the vial vigorously to mix the components.

4. Add 500 µl Standard Diluent Buffer to the remaining empty tubes.

5. Perform a serial dilution by the transferring of 500 µl of the volume from the "C50x (1:1)" (Top Standard) tube to the "1:2" dilution tube and mix thoroughly. Continue the preparation of the serial dilutions by the transferring of 500 µl of the volume from the "1:2" tube to the "1:4" tube and so on until the "1:64" tube.

**Materials and Reagents**

**Standard Diluent Buffer**

- 10× concentrated, it must be diluted in dH2O prior to use

**Detection Reagent**

- 100% concentrated, it must be diluted with the Assay Diluent Buffer /D/ prior to use

**Bead Mix**

- 0.15 ml of 50x concentrated, it must be diluted with the Wash Buffer /F/ prior to use

**Flow Cytometers**

- BD FACSCI1r™
- Yellow in BD FACSDiva™; Red, Far Red, Near Infra Red in BD FACSArray™ flow cytometers.
Application Protocol for Fungi-Plex™ Kit

Preparation of Fungi-Plex™ Detection Reagent

Because the Detection Reagent (PE-conjugated Mycotoxin Mixture) is 100× concentrated, therefore it must be diluted with the Assay Diluent Buffer to its optimal concentration before the starting of an experiment.

Note: Protect the conjugate mixture from the exposure to the direct light because of photobleaching and the loss of the fluorescent intensity.

1. Determine the number of the tests in the experiment.
2. Determine the total volume of the diluted Detection Reagent mixture needed for your experiment. Each tube or well requires 50 µl of the diluted Detection Reagent mixture. The total volume of the diluted Detection Reagent mixture can be calculated by the multiplying of the number of the tests (calculated above) by 50 µl. The Detection Reagent mixture should be equilibrated and mixed rigorously prior to use. However, it is somewhat viscous so take care the proper pipetting!

We recommend to prepare a few more tests than needed in the experiment.

3. Pipette the Detection Reagent mixture and the Assay Diluent Buffer into a tube labeled "DR Mix 1x". Store at 4°C, and protect from the light as possible.

Preparation of Samples

The samples should be stored in a cool (2-8 °C), dry place, protected from light.

1. Weight 5 g of the ground sample into a container and add 15 ml of 84% (v/v) acetonitrile/dH2O.
2. Shake it using an orbital shaker for 10 minutes.
3. Filter the extract through the filter.
4. Dilute the filtered sample by the adding of 240 µl of Assay Diluent Buffer to 10 µl of the sample.
5. Use 100 µl of the diluted filtrate per well or tube.

Assay Procedure

Following the preparation and dilution of the individual assay components, transfer the standards or the samples, the mixed Fungi-Plex™ Bead Mix and the diluted Detection Reagent mixture volumes to the appropriate assay wells or tubes for the incubation and analysis.

Note: Protect Fungi-Plex™ Bead Mix and conjugate mixture from the direct exposure to the light.

For Filter Plates:

1. Prepare all reagents as described above before the starting of the experiment.
2. Add 100 µl of the standard or sample to the assay wells.
3. Add 50 µl of the diluted Detection Reagent mixture to each assay well (protect from the direct exposure to the light as possible).
4. Vortex the diluted Capture Bead Mix for at least 5 seconds.
5. Add 50 µl of the diluted Capture Bead Mix to each assay well.
6. Incubate the microwell plate for 45 minutes using a /digital/ shaker at 600 RPM (do not exceed the 650 RPM!)

Note: Work at RT and protect the plate from the direct exposure to the light!
7. Insert the plate to the vacuum manifold and aspirate (do not exceed the 40 kPa!) until the wells are totally drained (2-10 seconds).
8. Add 200 µl of the Wash Buffer to each well, and aspirate again (do not exceed the 40 kPa!) until the wells are totally drained (2-10 seconds). Repeat the washing step two times.
9. Add 200 µl of the Wash Buffer to each well. Shake the microwell plate on a /digital/ shaker at 600 RPM, RT for 5 minutes to resuspend the beads.
10. Begin the analysis using a flow cytometer equipped with a high throughput plate loader device.

For Tubes:

1. Prepare all reagents as described above before the starting of the experiment.
2. Add 100 µl of the standard or sample to the assay tubes.
3. Add 50 µl of the diluted Detection Reagent mixture to each assay tube (protect from the direct exposure to the light as possible).
4. Vortex the diluted Capture Bead Mix for at least 5 seconds.
5. Add 50 µl of the diluted Capture Bead Mix to each assay tube.
6. Vortex gently the tubes and incubate the for 45 minutes (RT). Protect the tubes from the direct exposure to the light.
7. Add 1,0 ml of the Wash Buffer to each assay tube and centrifuge at 4000 x g for 5 minutes.
8. Aspirate and discard the supernatant from each assay tube.
9. Add 200 µl of the Wash Buffer to each assay tube. Vortex briefly the tubes to resuspend the beads.
10. Begin the analysis using a flow cytometer.

Note: Mix gently the tubes before the measurement.