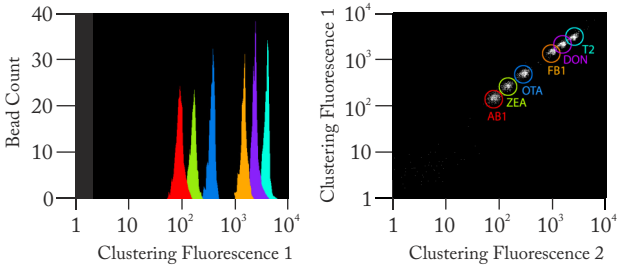
Application Protocol for Fungi-Plex™ Kit

Introduction

Fungi-Plex™ is a microbead based, flow cytometric analytical assay developed for the qualitative and quantitative detection of the mycotoxin 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 microtiter 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 of 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 and integrated into our FCAP Array™ software for clustering the acquired bead populations and processing the clusterized reporter fluorescence data.



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 mycotoxins and then incubated with the mycotoxin standards (included in the Kit) or the samples. Following the acquisition with a flow cytometer, the acquired FCS list mode sample data can be analyzed using the FCAP Array™ Analysis Software. The kit provides the quantitative analysis of maximum 88 samples and the generation of six standard curves.

Kit Content

for maximum 88 tests
(Store the following items (A-F, as described below) at 2-8 °C.
Do not freeze them!)

A Fungi-Plex™ Bead Mix: One vial, 0,15 ml (50× concentrated, it must be diluted with the Wash Buffer /F/ prior to use).

The vial contains the capture beads with discrete fluorescence intensity. The six bead populations can be detected at >640 nm (e.g. the FL3, FL4 in BD FACSCalibur™; Red, Far Red, Near Infra Red in BD FACS Array™ flow cytometers). The individual bead clusters can be distributed from the brightest to dimmest.

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

B Detection Reagent: One vial, 0,1 ml (100× concentrated, it must be diluted with the Assay Diluent Buffer /D/ prior to use).

The vial contains the PE-conjugated mycotoxin mixture. After the dilution with the Assay Diluent Buffer, the reagent should be formulated for the use at 50 ul/test. The PE fluorescence can be detected in the FL2 channel of a BD FACSCalibur™, and Yellow in BD FACS ArrayTM flow cytometers.

C Mycotoxin Standard Mix (Fungi-Plex™): One vial, lyophilized powder.
This vial contains the lyophilized mycotoxins. The mycotoxins must be reconstituted using 0,2 ml of 50 % (v/v) acetonitrile/distilled water (dH_2O) to prepare a 50x concentrated bulk standard mix. The standards should be prepared by serial dilution of that bulk with the Standard Diluent Buffer /E/.

D Assay Diluent Buffer: One vial, 15 ml
(10× concentrated, it must be diluted in dH_2O prior to use)

The Assay Diluent Buffer is used to dilute the Detection Reagent /B/.

E Standard Diluent Buffer: 1 vial, 20 ml (ready to use)

The Standard Diluent Buffer is used to dilute the Mycotoxin Standard Mix /C/.

F Wash Buffer: One vial, 15 ml
(10× concentrated, it must be diluted in dH_2O prior to use)

The Wash Buffer is phosphate buffered saline containing detergent. The Wash Buffer is used for the wash steps (described below) and to resuspend the washed beads for the analysis. The buffer is also used to dilute the Fungi-Plex™ Bead Mix /A/.

Materials Required but not Provided

The following items are also required:

- A flow cytometry instrument equipped with a 488 nm or 532 nm laser capable of detecting and distinguishing 576 nm, >640 nm fluorescence
  Note: We recommend a dual-laser flow cytometer equipped with 488 nm or 532 nm and 633 nm or 635 nm lasers.
- 12 × 75 mm sample acquisition tubes for a flow cytometer (e.g. BD Falcon™ Cat . No. 352008)
- FCAP Array™ Software (Cat. No. 338621) for analysis (recommended)
- Microcentrifuge, Microcentrifuge tube (polypropylene)
- filter (*e.g.* Whatman® 595 ½ folded filter, Sigma, Cat. No. Z612936)
- orbital shaker, vortex, pipettes

Required for Plate-loader-equipped Flow Cytometers

- Standard microtiter plate(s) for the BD FACSArray Bioanalyzer Setup (e.g. BD Falcon™ Cat . No . 353910)
- Millipore MultiScreen HTS-BV 1,2 µm clear non-sterile filter plates, [Cat. No. MSBVN1210 (10 pack) or MSBVN1250 (50 pack)]
- Millipore MultiScreen HTS Vacuum Manifold, (Cat. No. MSVMHTS00)
- 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 (if not using the recommended manifold)

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/dH_2O (add 0,1 ml acetonitrile to 0,1 ml dH_2O, 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 of RT before the preparations of the dilutions. Shake the vial rigorously 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.) solution in the tube of "C50x" by adding of 980 µl of the Standard Diluent Buffer to 20 µl Mycotoxin Standard Mix (50x conc.).
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.

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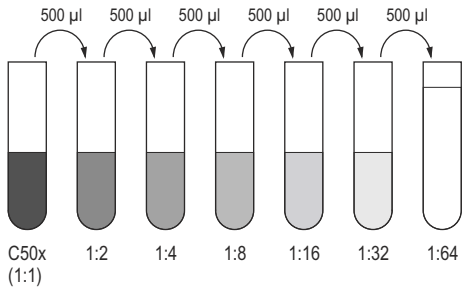
6. We recommend that the first eight pieces of wells (of a plate) or the tubes in an experiment should be used for the standards. The standards should be run in order from the lowest concentration (Standard Diluent Buffer, 0 ng/ml) to the highest concentration (/1:1/ = C50x).

Preparation of the Fungi-Plex™ Standards



Mycotoxin standards

Std	Aflatoxin B1		Zearalenone		Ocratoxin A		Fumonisin B1		Deoxynivalenol		T2 toxin	
	µg/kg	ng/ml	µg/kg	ng/ml	µg/kg	ng/ml	µg/kg	ng/ml	µg/kg	ng/ml	µg/kg	ng/ml
1:1	48	0.64	1200	16	48	0.64	6000	80	7500	100	2400	32
1:2	24	0.32	600	8	24	0.32	3000	40	3750	50	1200	16
1:4	12	0.16	300	4	12	0.16	1500	20	1875	25	600	8
1:8	6	0.08	150	2	6	0.08	750	10	937,5	12,5	300	4
1:16	3	0.04	75	1	3	0.04	375	5	468,75	6,25	150	2
1:32	1.5	0.02	37.5	0.5	1.5	0.02	187.5	2.5	234,38	3,125	75	1
1:64	0.75	0.01	18.75	0.25	0.75	0.01	93.75	1.25	117,19	1,5625	37.5	0.5
0	0	0	0	0	0	0	0	0	0	0	0	0

Preparation of Fungi-Plex™ Bead Mix

Because the Fungi-Plex™ Bead Mix is 50× concentrated, therefore it must be diluted to the optimal concentration before the starting of an experiment.

1. Vortex the Fungi-Plex™ Bead Mix stock vial for at least 15 seconds.
2. Determine the total volume of diluted beads needed for the experiment.

   Each tube or well requires 50 µl of the diluted beads. The total volume of diluted beads can be calculated by multiplying the number of the tests by 50 (µl).
3. Pipette the Fungi-Plex™ Bead Mix and Wash Buffer into a tube labeled "Bead Mix 1x".

   The volume of the Fungi-Plex™ Bead Mix can be calculated by the multiplying of the number of tests by 1 µl. The volume of the Wash Buffer can be calculated by the multiplying of the number of the tests by 49 µl.

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 equlibrated 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/dH$_2$O.
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.

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