# ELISA for Detection of *Rhizopus oryzae* Fucomannan

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## Introduction

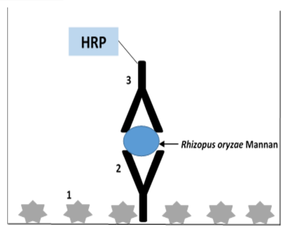
Mucormycosis is an invasive fungal infection that can cause high morbidity and mortality in immunocompromised patients. It is caused by fungi of the taxonomic order, Mucorales (1). Patients at risk for mucormycosis include those with hematopoietic stem cell or organ transplants, diabetes mellitus, trauma, and infants with low birth weight (2).

Lack of a rapid diagnostic test is the most frequently cited need for improved care of the mucormycosis patient. Mortality rates from mucormycosis increases from 47% when diagnosed after four weeks from the onset of symptoms, to 66% after twelve weeks (3). Rapid initiation of antifungal therapy is critical for treating mucormycosis. An effective diagnostic can help prevent i) direct tissue injury from Mucorales infections ii) angioinvasion, and iii) progression to dissemination.

**The goal of this project was to optimize the sensitivity in our Enzyme-Linked Immunosorbent Assay (ELISA) for diagnosis of mucormycosis.** Preliminary studies in the Kozel lab show that cell wall fucomannan (FM) is shed into blood, serum and BALF during mucormycosis infection and can be used as a biomarker for diagnosis.

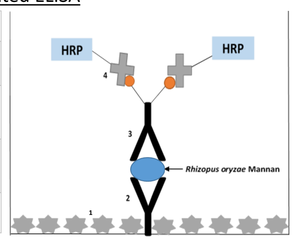
Standard ELISA

| Variables Optimized |
|---|
| 1. **Blocking Buffer:** various blocking buffer reagents were tested in the assay to increase sensitivity and reduce background. |
| 2. **Primary Antibody concentration:** The primary antibody used in this assay was mAb 2DA6. The primary antibody "captures" fucomannan from solution. |
| 3. **Conjugated Secondary Antibody concentration:** The secondary antibody used in the ELISA was 2DA6 conjugated to horseradish peroxidase (HRP).HRP is an enzyme that yields a colored product when substrate is added. The secondary antibody "detects" captured fucomannan. |




Biotinylated ELISA

| Variables Optimized |
|---|
| 1. **Blocking Buffer:** Various blocking buffers were tested in the assay to increase sensitivity and reduce background. |
| 2. **Primary Antibody Concentration:** The primary antibody used in this assay was mAb 2DA6. The primary antibody "captures" fucomannan from solution. |
| 3. **Conjugated Secondary Antibody Concentration:** The secondary antibody, 2DA6, is conjugated to biotin. Biotin is a binding site for streptavidin-HRP. The secondary antibody "detects" captured fucomannan |
| 4. **Indicator:** Streptavidin-HRP binds to 2DA6-biotin. The outcome is enhanced because there are more HRP molecules per fucomannan molecule in the well. |




## Literature Cited

1. George Petrikkos, Anna Skiada, Olivier Lortholary, Emmanuel Roilides, Thomas J. Walsh, Dimitrios P. Kontoyiannis; Epidemiology and Clinical Manifestations of Mucormycosis, Clinical Infectious Diseases, Volume 54, Issue suppl_1, 1 February 2012, Pages S23–S34.
2. Thomas J. Walsh, Maria N. Gamaletsou, Michael R. McGinnis, Randall T. Hayden, Dimitrios P. Kontoyiannis; Early Clinical and Laboratory Diagnosis of Invasive Pulmonary, Extrapulmonary, and Disseminated Mucormycosis (Zygomycosis), Clinical Infectious Diseases, Volume 54, Issue suppl_1, 1 February 2012, Pages S55–S60.
3. Georgios Chamilos, Russell E. Lewis, Dimitrios P. Kontoyiannis; Delaying Amphotericin B–Based Frontline Therapy Significantly Increases Mortality among Patients with Hematologic Malignancy Who Have Zygomycosis, Clinical Infectious Diseases, Volume 47, Issue 4, 15 August 2008, Pages 503–509.

## Results

**Table 1. Primary antibody optimization.** An ELISA was done to determine the optimal concentration of mAb 2DA6 antibody for capture of fucomannan for both the standard and biotinylated ELISA's.

| *mAb 2DA6 (µg/ml)* | *LOD[1]* | *Background* |
|---|---|---|
| *10* | 0.5 | 0.6 |
| *5* | 0.6 | 0.4 |
| *2.5* | 0.4 | 0.4 |
| *1.3* | 0.1 | 0.4 |
| *0.6* | 1.0 | 0.1 |

[1]Limit of detection (LOD; ng Fm/ml using $OD_{450}$ = 0.5) was determined through log-log analysis.

**Table 2 and Table 3. Optimization of blocking buffer.** Various blocking buffer reagents were tested in the standard and biotinylated ELISA to determine the reagent that caused greatest sensitivity and reduced background.

**Standard ELISA**

| *Blocking Buffer* | *LOD[1]* | *Background* |
|---|---|---|
| *PBST (0.05% Tween 20)* | 1.6 | 0.2 |
| *PBST (0.5% Tween 20)* | 1.7 | 0.1 |
| *1% Fish gelatin +0.1% Tween 20* | 1.0 | 0.2 |

[1]Limit of detection (LOD; ng Fm/ml using $OD_{450}$ = 0.5) was determined through log-log analysis.

**Biotinylated ELISA**

| *Blocking Buffer* | *LOD[1]* | *Background* |
|---|---|---|
| *1% Normal Goat Serum* | 0.3 | 0.2 |
| *1x Casein in PBS (pH 7.4)* | 0.3 | 0.2 |

[1]Limit of detection (LOD; ng Fm/ml using $OD_{450}$ = 0.5) was determined through log-log analysis.

**Table 4 and Table 5. Optimization of concentration of secondary antibody.** Secondary antibody was serially diluted in the assays to determine the optimal concentration of secondary antibody for detection of "captured" fucomannan.

**Standard ELISA**

| *2DA6-HRP (µg/ml)* | *LOD[1]* | *Background* |
|---|---|---|
| *8* | 0.4 | 0.5 |
| *4* | 0.9 | 0.3 |
| *2* | 1.6 | 0.2 |
| *1* | 2.2 | 0.1 |
| *0.5* | 3.0 | 0.1 |

[1]Limit of detection (LOD; ng Fm/ml using $OD_{450}$ = 0.5) was determined through log-log analysis.

**Biotinylated ELISA**

| *Biotinylated 2DA6 (µg/ml)* | *LOD[1]* | *Background* |
|---|---|---|
| *2* | 0.02 | 0.5 |
| *1* | 0.2 | 0.6 |
| *0.5* | 0.1 | 0.3 |
| *0.25* | 2.9 | 0.2 |

[1]Limit of detection (LOD; ng Fm/ml using $OD_{450}$ = 0.5) was determined through log-log analysis.

**Table 5 and Table 6. Limit of Detection of fucomannan by optimized standard ELISA and optimized biotinylated ELISA.** Once all variables in the standard and biotinylated ELISA's were optimized, both were repeated four times each with the optimized reagents and concentrations. Results were assessed using two different endpoints: i) a limit of detection using $OD_{450}$ = 0.5 and ii) a limit of detection using $OD_{450}$=(standard deviation of the average background)*3 + (average background).

**Optimized Standard ELISA**

| *Analysis Method* | *Endpoint 1 (CV)* | *Endpoint 2 (CV)* |
|---|---|---|
| | *LOD[1]* | *LOD[2]* |
| *Log-log* | 1.31 (13.2%) | 0.90 (48.6%) |
| *4 parameter* | 1.39 (15.3%) | 0.88 (59.8%) |

[1]Limit of detection (LOD; ng Fm/ml using $OD_{450}$ = 0.5).

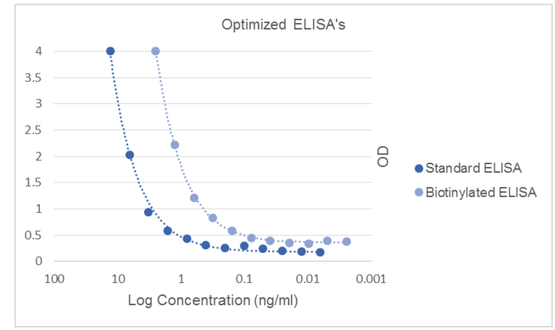
[2]Limit of detection (LOD; ng Fm/ml using OD450= (Standard deviation of average background)*3 + (average background)).

**Optimized Biotinylated ELISA**

| *Analysis Method* | *Endpoint 1 (CV)* | *Endpoint 2 (CV)* |
|---|---|---|
| | *LOD[1]* | *LOD[2]* |
| *Log-log* | 0.10 (21.6%) | 0.05 (35.9%) |
| *4 parameter* | 0.13 (23.4%) | 0.04 (75.6%) |

[1]Limit of detection (LOD; ng Fm/ml using $OD_{450}$ = 0.5).

[2]Limit of detection (LOD; ng Fm/ml using OD450= (Standard deviation of average background)*3 + (average background)).




**Graph 1:** After optimizing all components in the Biotinylated and Standard ELISA, the biotinylated assay showed a 10-fold increase in sensitivity.

## Conclusion

| Variables Optimized For Standard ELISA | Variables Optimized For Biotinylated ELISA |
|---|---|
| 1. **Blocking Buffer:** The optimal blocking buffer with high signal and reduced background was PBST (0.05% Tween 20) | 1. **Blocking Buffer:** The optimal blocking buffer with high signal and reduced background was 1x Casein in PBS (pH 7.4). |
| 2. **Primary Antibody concentration:** The optimal concentration of mAb 2DA6 for capture of fucomannan was 3µg/ml. | 2. **Primary Antibody Concentration:** The optimal concentration of mAb 2DA6 for capture of fucomannan was 3µg/ml. |
| 3. **Conjugated Secondary Antibody concentration:** The optimal concentration of 2DA6-HRP for detection of captured fucomannan was 4µg/ml. | 3. **Conjugated Secondary Antibody Concentration:** The optimal concentration of 2DA6-biotin for detection of captured fucomannan was 1µg/ml. |
| | 4. **Indicator:** The optimal concentration of streptavidin-HRP was 0.25µg/ml. |

Through optimization of the standard and biotinylated ELISA a 10-fold increase in sensitivity was obtained with the biotinylated ELISA. Due to the high background in the biotinylated ELISA, there was a compromise between the protocol that yields the greatest sensitivity vs. the protocol that produces the lowest amount of background. The protocol that was chosen for this experiment was the protocol with the greatest sensitivity.

## Acknowledgements

*This poster was made possible by a grant from the National Institute of General Medical Sciences (GM103440) from the National Institutes of Health.*

*NIH grant R43AI140817, Kathleen Zayac, Michael Mash.*

## Competing Interests

T.R.K. and A.R.B-M. have equity interests in DxDiscovery, which is a University of Nevada, Reno spin-off company focused on early stage, antibody-based diagnostic development for global health.

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