

**Alt a 1 DETERMINATIONS  
COMPARING FIVE ASSAY FORMATS  
PRODUCE DIFFERENT RESULTS**

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**ABSTRACT**

The Alt a 1 content of extracts prepared from four distinct *Alternaria alternata* strains was assessed by 5 different assays employing IgG antibodies derived from a rabbit anti-Alt a 1 hypoallergen antiserum. Relative reactivities were determined by parallel line bioassay from multiple dose-response curves for each extract, using in-house freeze-dried *A. alternata* extracts as references. The resulting Alt a 1 reactivities (summarized below) varied considerably from assay to assay, with only two formats (radial immunodiffusion and Western blot) displaying consistent patterns of reactivity for all five extracts.

Assay	Mean Relative Reactivities for <i>Alternaria</i> Extracts				
	M1 Reference	GM1	GM96	GM101	GM102
ELISA inhibition, <i>Unmodified Ab</i>	1.00	0.94	0.73	0.22	0.52
ELISA inhibition, <i>Biotinylated Ab</i>	1.00	3.10	2.93	0.78	3.54
Double-Bind ELISA Capture w/ <i>Unmodified Ab</i> Probe w/ <i>Biotinylated Ab</i>	1.00	0.96	0.17	0.043	0.004
Radial Immunodiffusion	1.00	1.00	0.26	0.00	1.60
SDS-PAGE Immunoblot 30-35 kd band intensity	++	++	+/-	+/-	++++

These results suggest that all critical reagents (specific antibodies, reference extracts and purified antigens) should be incorporated into a wide variety of test configurations to validate assay methods for well-defined allergens such as *Alternaria* Alt a 1.

**INTRODUCTION**

Accurate quantitation of major allergenic proteins or IgE determinants by immunoassay methods is an important step in the optimization and standardization of allergenic extracts.

Because most extracts are complex mixtures of water-soluble macromolecules, assay reagents designed to capture and detect individual components present in these mixtures must display high levels of avidity, specificity and consistency to produce an effective and robust test. However, the analytical accuracy of such a test is not guaranteed by the mere inclusion of suitable reagents but must be confirmed using the best available reference materials in numerous assay formats and configurations.

Assays for specific allergenic components are often developed and reported using a single test format based on performance criteria, optimization of rare reagents or investigator preferences and experience.

In this study, three ELISA configurations, including double-bind (sandwich) and competitive binding (inhibition) modes, are compared with radial immunodiffusion and SDS-PAGE immunoblot procedures to establish a valid laboratory assay for Alt a 1, a major allergenic protein from *Alternaria alternata* (*tenuis*).

**MATERIALS AND METHODS**

**A. Alternaria Extracts**

Glycerinated and freeze-dried *Alternaria* extracts were prepared with acetone-treated cellular raw materials (mycelia and spores) from each of four *A. alternata* strains possessing distinct biochemical and morphological properties (see table below). Extractions were conducted for 24 hours at 23°C, followed by clarification and sterile filtration. The extracellular fluids from the *Alternaria* cultures were also examined for Alt a 1 reactivities.

Extract	Formulation	Strain	Strain Source	Extraction Conditions
M1 Reference	Freeze-dried	ATCC # 11680	Greer Labs	1:10 w/v in Am Bicarb Dialyzed vs. dH <sub>2</sub> O
GM1	Glycerinated 1:20 w/v	ATCC # 11680	Greer Labs	1:10 w/v in Coca's Soln Diluted 1:2 w/ Glycerin
GM96	"	188701	H.M. Vijay	"
GM101	"	ATCC # 96344	J. Portnoy	"
GM102	"	ATCC # 46582	R.K. Bush	"
XPM1	Freeze-dried	ATCC # 11680	Greer Labs	1:10 w/v in Am Bicarb Dialyzed vs. dH <sub>2</sub> O
XPM96	"	188701	H.M. Vijay	"
XPM101	"	ATCC # 96344	J. Portnoy	"
XPM102	"	ATCC # 46582	R.K. Bush	"

**B. Antibody Preparations**

Rabbit anti-Alt a 1 hypoallergen serum was kindly provided by Dr. Hari Vijay. A 50% ammonium sulfate-precipitable fraction of this serum was employed directly in numerous immunoassays and was biotinylated using NHS-LC-Biotin (Pierce) in borate buffer, pH 8.4 under conventional reaction conditions. Symbols for these antibody samples used elsewhere in this poster are described below.

Antibody	Description	Symbol
Rabbit Serum	High titer Rabbit anti-Alt a 1 serum Immunogen = Vijay hypoallergen major components = 16, 18, 30 Kd	Rab Serum
Ammonium Sulfate Fraction	IgG-enriched Rabbit anti-Alt a 1 50% ammonium sulfate cut Dialyzed to remove residual Am Sulf	Rab IgG
Biotinylated Am Sulf Fraction	Biotinylated IgG-enriched anti- [Protein/Bi] ratios (mg/mg) = 2.0-5.0 Dialyzed to remove residual NHS-LC-Bi	Bi-Rab IgG

**C. Enzyme-Linked Immunosorbent Assays**

Conditions common to the four ELISA methods performed in this study were as follows:

Component	Description
Plates	Corning, product # 25805-96
Coating buffer	0.05 M Carbonate buffer, pH 9.6
Wash buffer	PBS-0.05% Tween 20, pH 7.4
Sample diluent	PBS-0.05% Tween 20-0.29 M NaCl, pH 7.4
Probe diluent	PBS-0.05% Tween 20-0.05% Calf gelatin, pH 7.4
Substrate	pNPP at 1.0 mg/mL in Diethanolamine buffer, pH 9.8

Conditions specific to individual ELISA formats are tabulated below.

Assay Format	Coating Reagent	Sample	Probe Reagent(s)
Double-Bind	Rab IgG, 1.0 µg/mL protein 15-24 hr at 23°C	Alternaria extracts, 0.05-200 µg/mL protein 2 hr at 23°C	Bi-Rab IgG, 0.17 µg/mL protein 15-24 hr at 23°C Avidin-AP (Zymed) @ 1:4000 dilution 1 hr at 23°C
Inhibition w/ Rab Serum	Alt M1 Reference, 0.7 µg/mL protein 15-24 hr at 23°C	Rab Serum, 1:250,000 dilution + Alternaria extracts, 0.2-20.0 µg/mL protein 4-6 hr at 23°C	Goat anti-Rabbit IgG-AP (Sigma), 1:4000 dilution 15-24 hr at 23°C
Inhibition w/ Rab IgG	Alt M1 Reference, 0.7 µg/mL protein 15-24 hr at 23°C	Rab IgG, 1.0 µg/mL protein + Alternaria extracts, 0.2-20.0 µg/mL protein 4-6 hr at 23°C	Goat anti-Rabbit IgG-AP (Sigma), 1:2000 dilution 15-24 hr at 23°C
Inhibition w/ Bi-Rab IgG	Alt M1 Reference, 0.7 µg/mL protein 15-24 hr at 23°C	Bi-Rab IgG, 0.17 µg/mL protein + Alternaria extracts, 0.2-20.0 µg/mL protein 15-24 hr at 23°C	Avidin-AP (Zymed), 1:4000 dilution 1 hr at 23°C

#### D. Radial Immunodiffusion Assays

Agarose gels (1.0%) at 60°C were mixed with Rab IgG (final concentration = 0.16% v/v) and cast onto glass or plastic (GelBond, FMC) surfaces. Alternaria extracts (10 µL, 3-6 µg protein) were added to 3 mm wells in the gels and allowed to diffuse for 72 hours at 23°C. The gels were then equilibrated with 0.15 M NaCl, washed 3 times with distilled water, and dried completely. Precipitation circles were visualized using conventional Coomassie Blue or Silver staining procedures.

#### E. SDS-PAGE Immunoblot Analyses

Acrylamide gels (12.0%) were cast using a Bio-Rad Mini-Protean II system and loaded with 20 µL (3-6 µg protein) of Alternaria extracts under non-reducing conditions. Gels were run at constant voltage (200 V) and processed as described below.

Step	Description
Gel soak	10 mM Tris, pH 7.5 for 0.5 hr at 23°C
Blot	Passive transfer to nitrocellulose (Nitro-Bind, MSI) for 15-24 hr using 10 mM Tris transfer buffer
Block	TBS-0.5% Tween 20, pH 8.0 for 2 hr at 23°C
Rinse	Distilled water
Serum diluent	TBS-0.05% Tween 20-0.29 M NaCl, pH 8.0
Serum	Rab Serum at 1:5,000 for 3 hr at 23°C
Wash	TBS-0.05% Tween 20, pH 8.0, 3 times for 5-10 minutes
Probe diluent	TBS-0.05% Tween 20-0.05% Calf gelatin, pH 8.0
Probe reagent	GAR IgG-AP at 1:1:2000 for 2 hr at 23°C
Wash	TBS-0.05% Tween 20, pH 8.0, 3 times for 5-10 minutes
Rinse	Distilled water
Substrate	BCIP (0.25 mg/mL)/NBT (0.35 mg/mL) in Tris buffer, pH 9.5 for 5-20 minutes at 23°C
Stop	Distilled water

## RESULTS

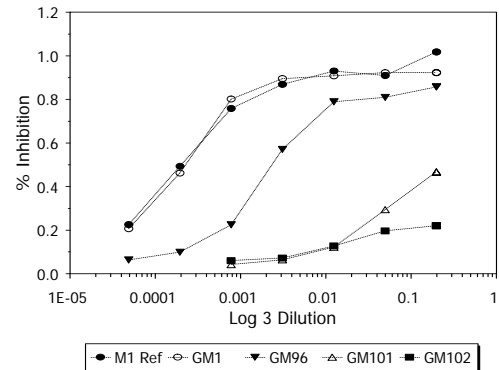
#### A. Protein Yields

The protein concentrations of Alternaria extracts and culture filtrates determined by Bradford assay were consistent across the four *A. alternata* strains for all three product groups.

Glycerinated Extract	[Protein] mg/mL	Freeze-dried Extract	[Protein] mg/mL	Culture Filtrate	[Protein] mg/mL
GM1	0.821	XPM1	0.522	CF1	0.386
GM96	0.797	XPM96	0.584	CF96	0.478
GM101	0.952	XPM101	0.592	CF101	0.371
GM102	1.198	XPM102	0.637	CF102	0.306
		M1 Reference	0.709		

#### B. Double-Bind ELISA

Dose-response curves for Alternaria samples in the double-bind ELISA format are illustrated below.



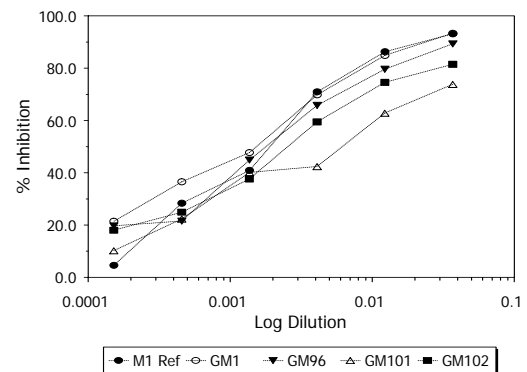
Mean relative potency values determined by parallel line bioassay analyses (tabulated below) indicate that glycerinated and freeze-dried M1 extracts exhibit high levels of Alt a 1 reactivity compared to analogous M96, M101 and M102 products based on double-bind ELISA.

Number of Assays Run	Mean Relative Reactivities for Glycerinated Alternaria Extracts				
	M1 Reference	GM1	GM96	GM101	GM102
6	1.000	0.963	0.167	0.043	0.004

Number of Assays Run	Mean Relative Reactivities for Freeze-Dried Alternaria Extracts				
	M1 Reference	XPM1	XPM96	XPM101	XPM102
2	1.000	1.072	0.310	0.072	0.049

#### C. ELISA Inhibition w/ Rab Serum or Rab IgG

Similar dose-response curves were observed for Alternaria extracts in the ELISA inhibition assays using Rab Serum or Rab IgG as primary antibody. Typical inhibition curves and mean relative potency values (represented below) showed that the Alt a 1 reactivities of M101 extracts remained low relative to M1, but that both M96 and M102 products exhibited higher relative reactivities by ELISA inhibition compared to double-bind ELISA.



#### ELISA Inhibition w/ Rab Serum:

Number of Assays Run	Mean Relative Reactivities for Glycerinated Alternaria Extracts				
	M1 Reference	GM1	GM96	GM101	GM102
8	1.000	0.905	0.681	0.191	0.502

Number of Assays Run	Mean Relative Reactivities for Freeze-Dried Alternaria Extracts				
	M1 Reference	XPM1	XPM96	XPM101	XPM102
2	1.000	1.316	1.086	0.377	0.578

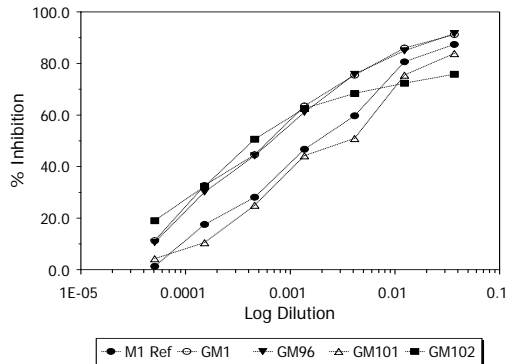
#### ELISA Inhibition w/ Rab IgG:

Number of Assays Run	Mean Relative Reactivities for Glycerinated Alternaria Extracts				
	M1 Reference	GM1	GM96	GM101	GM102
6	1.000	0.979	0.733	0.246	0.528

Number of Assays Run	Mean Relative Reactivities for Freeze-Dried Alternaria Extracts				
	M1 Reference	XPM1	XPM96	XPM101	XPM102
2	1.000	1.265	1.061	0.301	0.540

## D. ELISA Inhibition w/ Bi-Rab IgG

ELISA inhibitions employing Bi-Rab IgG as primary antibody produced Alt a 1 reactivity patterns which varied considerably from those derived from the other four assay formats included in this study, as summarized below. Immunoblot studies (not shown) confirmed that biotinylation does not alter the antigenic specificity of Rab IgG, and that biotinylated proteins do not bind non-specifically to immobilized *Alternaria* components.



Number of Assays Run	Mean Relative Reactivities for Glycerinated <i>Alternaria</i> Extracts				
	M1 Reference	GM1	GM96	GM101	GM102
4	1.000	3.102	2.930	0.782	3.538

Number of Assays Run	Mean Relative Reactivities for Freeze-Dried <i>Alternaria</i> Extracts				
	M1 Reference	XPM1	XPM96	XPM101	XPM102
2	1.000	1.698	1.384	0.262	1.386

## E. Radial Immunodiffusion (RID)

Interestingly, M102 extracts, which displayed the lowest Alt a 1 reactivities by double-bind ELISA, possessed the highest Alt a 1 levels in the RID assays. Culture filtrates of the four *Alternaria* strains demonstrated a similar pattern of reactivity.

Number of Assays Run	Mean Diameters <sup>1</sup> and Relative Reactivities <sup>2</sup> for Glycerinated <i>Alternaria</i> Extracts				
	M1 Reference	GM1	GM96	GM101	GM102
4	10.5 mm (1.00)	10.0 mm (0.95)	5.0 mm	0.0 mm	14.25 mm (2.43)

Number of Assays Run	Mean Diameters <sup>1</sup> and Relative Reactivities <sup>2</sup> for Freeze-Dried <i>Alternaria</i> Extracts				
	M1 Reference	XPM1	XPM96	XPM101	XPM102
2	11.25 mm (1.00)	12.0 mm (1.13)	6.25 mm (0.18)	5.5 mm	17.0 mm (3.18)

Number of Assays Run	Mean Diameters <sup>1</sup> and Relative Reactivities <sup>2</sup> for <i>Alternaria</i> Culture Filtrates				
	M1 Reference	CF1	CF96	CF101	CF102
2	12.5 mm (1.00)	16.0 mm (2.11)	10.0 mm (0.56)	7.0 mm (0.26)	26.0 mm (7.27)

1 Values for undiluted samples at protein concentrations listed above (see Protein Yields)

2 Determined by parallel line bioassay analysis (log 2 relative potency)

## F. SDS-PAGE Immunoblots

Rabbit anti-(Alt a 1 hypoallergen) antibodies bound to epitopes present on multiple proteins produced by each *A. alternata* strain, based on immunoblot analyses. A prominent immunoreactive band was observed for M1 and M102 products at 30-35 kD, consistent with the molecular weight of 31 kD reported for intact Alt a 1. The intensities of the 30-35 kD bands for the *Alternaria* extracts and culture filtrates correlated closely with their RID reactivities, as illustrated below.

Number of Assays Run	30-35 kD Band Intensities for <i>Alternaria</i> Extracts and Culture Filtrates				
	M1 Reference	GM1 XPM1	GM96 XPM96 CF96	GM101 XPM101 CF101	GM102 XPM102 CF102
4	Moderate	Moderate	Low	Very Low	High

## INTERPRETATIONS

### A. Assay Differences

Immunoassays rely on specific interactions between primary, secondary or tertiary structural regions on antigens and antibodies.

Individual assay formats often possess different requirements or preferences for productive antigen-antibody binding due to differences in the presentation and accessibility of critical protein structures.

As a result, use of a single antibody preparation in multiple test formats (as in this study) does not guarantee similar results or consistent patterns of reactivity.

### B. Antibody Specificities and Reactivities

The Alt a 1 hypoallergen used to prepare the rabbit antibody employed in these assays contains a minor component at 31 kD (intact Alt a 1) and two major components (16-18 kD) shown to be immunogenic but non-allergenic fragments.

Immunoblot analyses with *Alternaria* extracts and culture filtrates show that proteins at numerous molecular weights are recognized by the rabbit anti-hypoallergen IgG antibodies, and may include:

- A single immunogenic site on one protein shared by multiple molecular forms (mono-specific)
- Distinct immunogenic regions on multiple protein components (poly-specific)
- A combination of shared, cross-reactive and unique binding site specificities

Comparisons of the Alt a 1 reactivities of products derived from each *A. alternata* strain reveal consistent patterns of antigen-antibody binding between several of the assay formats employed in this study. Possible explanations for these patterns are described below.

#### 1. RID data correlates with immunoblot intensities for 30-35 kD *Alternaria* component(s)

- In RID, antibody molecules embedded in agarose gels are separated by defined distances
- An antigen molecule must bind to at least 2 different antibodies to produce a positive RID reaction
- 30-35 kD antigens are multivalent and possess suitable determinant structures and distributions to produce RID precipitin reactions
- 15-25 kD antigens detected on blots may be too small to cross-link antibodies in the RID gels
- Blot-positive components of > 40 kD may be too large or of inappropriate shape and determinant valency to produce effective RID reactions

#### 2. Double-bind ELISA data is consistent with immunoblot intensities in 15-25 kD range

- Antibody adsorption to polystyrene microwells may produce an antigen-binding solid phase which favors some IgG molecules (and Alt a 1 specificities) over others
- Antigen binding sites may be close to the ELISA plate surface and more accessible to antigens of lower molecular weight (15-25 kD) relative to larger (> 30 kD) or sterically hindered protein components

3. ELISA inhibition data is more consistent from product to product compared to the other assay formats
  - Binding of antigens to polystyrene may expose antibody binding sites normally buried or inaccessible in their native (and liquid phase) conformations
  - Determinant valency and distribution is much less critical for inhibition of antibody binding compared to the multi-site interactions required for RID and double-bind ELISA

### *CONCLUSIONS*

The development of accurate and reproducible methods to quantitate the major allergen content or biological potency of *Alternaria* extracts is complicated by several factors, including:

#### Heterogeneity of *A. alternata* antigens

- Distribution                      Cellular mats, culture fluids
- Strain variability                Composition, morphology, culture conditions
- Biochemical variability        Harvesting conditions  
Endogenous proteases
- Biochemical similarity        Homologous sequences  
Cross-reactive determinants

#### Immunoassay format preferences

- Solid phase                      Adsorption/presentation differences
- Antigen                          Conformation, size, epitope valency
- Antibody                        Protein binding specificities  
Polyclonal anti-(Alt a 1 hypoallergen) IgG  
Number of distinct antigens per strain  
Number of distinct determinants per antigen

#### Definition and availability of reference reagents

- Antigen                        Extracts or purified protein preparations
- Antibody                        Production, purification, characterization
- Reagent and assay validation criteria

The results presented in this study confirm the complex nature of Alt a 1 and the importance of assay formats and reagent configurations in the definition, optimization and validation of suitable materials and methods for Alt a 1 determinations.

Studies are in progress to complete the validation process, and include:

- Comparisons of two monoclonal anti-Alt a 1 preparations to the rabbit polyclonal antibody in ELISA and Western blot assays
- Isolation and characterization of the dominant protein allergens from each *A. alternata* strain
- Immunization of rabbits or mice with the isolated or purified allergens, and implementation of the resulting antibodies in the various assay formats
- Establishment of the source materials, methods and analytical criteria for Alt a 1 reference preparations
- Comparisons of the *in vitro* data described above with *in vivo* sensitivities of allergic patients

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