



clarus *Cryptococcal Antigen EIA*

For the Detection of Cryptococcal Antigen – REF CRY101



Qualitative Test Procedure (condensed)	Titration Test Procedure (condensed)	Interpretation of the Results						
<ol style="list-style-type: none"> Add 50 µL of specimen, CrAg standards, and controls to anti-CrAg microwells. Incubate 30 minutes @ 22-25 °C Wash 3X – 200 µL 1X Wash Buffer. Add 100 µL Enzyme Conjugate. Incubate 30 minutes @ 22-25 °C. Wash 3X – 200 µL 1X Wash Buffer. Add 100 µL TMB Substrate. Incubate 10 minutes @ 22-25 °C. Add 100 µL Stop Solution. Read plate & record results. 	<ol style="list-style-type: none"> Label 6 test tubes 1-6. Add 50 µL of specimen to tube 1. Add 200 µL of 1X Specimen Diluent to tube 1. Add 100 µL Specimen Diluent to tubes 2-6. Transfer 100 µL of specimen from tube 1 to tube 2 and mix well. Continue serial dilution through tube 6. Test dilutions according to the <i>Qualitative Procedure</i>. Calculate titer as described in the <i>Results</i> section of this package insert. 	<p>QUALITATIVE TESTING</p> <table border="1"> <thead> <tr> <th>Blanked OD</th> <th>Result</th> </tr> </thead> <tbody> <tr> <td>$x \leq 0.265$</td> <td>Negative</td> </tr> <tr> <td>$x > 0.265$</td> <td>Positive</td> </tr> </tbody> </table> <p>TITRATION</p> <p>See Interpretation of Results – Titration Procedure</p>	Blanked OD	Result	$x \leq 0.265$	Negative	$x > 0.265$	Positive
Blanked OD	Result							
$x \leq 0.265$	Negative							
$x > 0.265$	Positive							

INTENDED USE

The clarus Cryptococcal Antigen enzyme immunoassay (CrAg EIA) is a qualitative or semi-quantitative (titration) test system for the detection of capsular polysaccharide antigens of *Cryptococcus* species complex (*Cryptococcus neoformans* and *Cryptococcus gattii*) in serum and cerebrospinal fluid (CSF). The clarus Cryptococcal Antigen Enzyme Immunoassay is an assay which can be used as an aid in the diagnosis of cryptococcosis. Test results are to be used in conjunction with information available from the patient clinical evaluation and other diagnostic procedures.

EXPLANATION

Cryptococcosis is caused by both species of the *Cryptococcus* species complex (*Cryptococcus neoformans* and *Cryptococcus gattii*) (1). Individuals with impaired cell-mediated immunity are at greatest risk of infection (2). Cryptococcosis is one of the most common opportunistic infections in AIDS patients (3). Methods of detecting cryptococcal antigen (CrAg) in serum and CSF have been utilized extensively with very high sensitivities and specificities (4-6).

BIOLOGICAL PRINCIPLES

The clarus Cryptococcal Antigen Enzyme Immunoassay (EIA) is a direct immunoenzymatic sandwich microplate assay which detects *Cryptococcus* antigens in serum and CSF. Anti-*Cryptococcus* antibodies bound to microwell plates are used as capture antibodies, and horseradish peroxidase (HRP)-conjugated anti-*Cryptococcus* antibodies are used as detect antibodies. The positive control and standard curve material are composed of cryptococcal capsular polysaccharide antigen in a buffered protein solution with a preservative.

In the qualitative procedure, specimens are analyzed undiluted. In the titration procedure, specimens are analyzed after serial dilution in specimen diluent. Either serum or CSF is added to the microwells coated with the capture antibodies and incubated. If the patient specimen contains cryptococcal antigens that are recognized by the capture antibodies, those antigens will become bound to the microwells. The microwells are washed to remove unbound patient material, and HRP-conjugated detect antibody is added to the wells. If *Cryptococcus* antigens are bound to the microwells by the capture antibodies, the detect antibody will also become bound to the microwells. The wells are then washed to remove any unbound detect antibody. Next, tetramethylbenzidine (TMB) substrate is added to the microwells, and in the presence of HRP, a blue color will develop. The reaction is stopped by the addition of a stop solution. The optical density (OD) is determined with a microplate reader at 450 nm with reference at 630 nm (reference is optional).

REAGENTS

- Anti-CrAg Microwells** (192 wells, REF CRYMW1): Stripwell plates featuring breakaway polystyrene microwells. The wells are coated with anti-CrAg monoclonal antibodies.
- CrAg Positive Control** (1.5 mL, REF CRYP1): Cryptococcal antigen in a buffered protein solution containing less than 0.1% sodium azide as a preservative
- 20X Wash Buffer** (50 mL, REF EIAWB1): Concentrated wash buffer with a preservative.
- Enzyme Conjugate** (2 x 10 mL, REF CRYE2C): Anti-CrAg monoclonal antibodies conjugated to horseradish peroxidase (HRP) in a buffered blocking solution containing less than 0.1% sodium azide as a preservative
- 10X Specimen Diluent** (2 x 10 mL, REF EIASD1): Concentrated buffered protein solution containing less than 1.0% sodium azide as a preservative
- TMB Substrate** (2 x 10 mL, REF EIATMB): Buffered solution containing urea peroxide and tetramethylbenzidine (TMB).
- Stop Solution** (2 x 10 mL, REF EIAS1): 2N sulfuric acid. CAUTION: AVOID CONTACT WITH SKIN. FLUSH WITH WATER IF CONTACT OCCURS.

MATERIALS NOT PROVIDED

- Pipettor capable of delivering up to 200 µL and disposable tips
- Distilled or deionized water
- Spectrophotometer microplate reader capable of reading absorbance at 450 nm and 630 nm
- Squirt bottle, EIA plate washer, or multi-channel pipettor for washing
- Timer
- Graduated cylinder for dilutions of wash buffer and specimen diluent

WARNINGS and PRECAUTIONS

- For in vitro diagnostic use only.
- Specific standardization is necessary to produce our high-quality reagents and materials. IMMY cannot guarantee the performance of its products when used with materials purchased from other manufacturers. Do not interchange reagents from different kit lot numbers or other manufacturers.
- The user assumes full responsibility for any modification to the procedures published herein.
- Always wear gloves when handling reagents in this kit as some reagents are preserved with 0.095% (w/w) sodium azide. Sodium azide should not be flushed down the drain, as this chemical may react with lead or copper plumbing to form potentially explosive metal azides. Excess reagents should be discarded in an appropriate waste receptacle.
- Avoid contact with Stop Solution (2 N sulfuric acid). If exposed, immediately flush with copious amounts of water.
- Avoid splashing when dispensing reagents into the microwells as this causes erroneous results.
- Inadequate washing can cause excessive background reactivity in any EIA protocol.
- Use only protocols described in this package insert. Incubation times or temperatures other than those specified may give erroneous results.
- Maintain proper pipetting techniques and pattern throughout procedure to ensure optimal and reproducible results.

REAGENT PREPARATIONS

- The entire kit, including the microwell plates, should be at room temperature (22-25 °C) for/during use.
- Prepare a 1X solution of wash buffer by mixing 19 parts DI water with 1 part 20X wash buffer.
- Prepare a 1X specimen diluent solution by mixing 9 parts DI water with 1 part 10X specimen diluent.

REAGENT STABILITY AND STORAGE

The entire clarus *Cryptococcus* Antigen EIA test kit should be stored at 2-8 °C until the expiration dates listed on the reagent labels. All reagents should be returned to the refrigerator promptly after use.

Unused microwells should be placed in the re-sealable Mylar bags, sealed immediately after opening, and returned to the refrigerator promptly. Care should be taken to ensure the desiccant pouch remains in the bag with unused microwells.

SPECIMEN COLLECTION AND PREPARATION

Collect samples aseptically using established techniques by qualified personnel. When handling patient specimens, adequate measures should be taken to prevent exposure to potentially present etiologic agents. The use of specimens other than serum and cerebrospinal fluid (CSF) has not been established. Hyperlipemic, hemolyzed, icteric, and/or contaminated samples should not be tested.

For optimal results, sterile samples should be used. Specimens should be tested as soon as possible. The maximum storage time permissible at 2-8 °C has not been established. Samples may be frozen and thawed up to two times after storage at ≤ 20 °C. Specimens may be stored for up to one week at ≤ -20 °C, provided they are not repeatedly thawed and refrozen, as this may affect test results (do not store in a frost-free freezer). A very low positive specimen could become negative after storage at < -20 °C for one week. Specimens in transit between labs should be maintained at 2-8 °C or ≤ -20 °C. (7)

Specimens should be brought to room temperature (22 to 25 °C) prior to testing.

PROCEDURE

REFER TO REAGENTS SECTION FOR A LIST OF MATERIALS PROVIDED.

QUALITATIVE PROCEDURE

- A. Bring all kit components to room temperature.
- B. Snap off enough of the anti-CrAg microwells (CRYMW1) to run all specimens and controls. Insert them into a microwell holder.
- C. Place remaining wells back into bag with desiccant pouch and store at 2-8 °C.
- D. Pipette 50 µL of each specimen into the anti-CrAg microwells, recording the location of each.
- E. Pipette 50 µL of 1x Specimen Diluent (EIASD1) into a microwell to serve as a blank.
- F. Pipette 50 µL of Positive Control (CRYPC1) into a microwell.
- G. Mix by shaking gently 10-15 seconds on the countertop.
- H. Incubate the plate at room temperature (22-25 °C) for 30 minutes.
- I. Using a pipettor, aspirate the contents from the wells, and discard into a biohazard receptacle.
- J. Fill all wells with at least 200 µL of 1X Wash Buffer (EIAWB1). This can be accomplished using a squirt bottle, EIA plate washer, or multichannel pipettor. If using a squirt bottle, direct the stream against the sides of the wells to avoid foaming. Dump the plate contents.
- K. Repeat Step J two more times for a total of three washes. After the final wash, strike the plate on a clean stack of paper towels or other absorbent material hard enough to remove as much wash buffer as possible.
- L. Add 100 µL of Enzyme Conjugate (CRYECC) to each well.
- M. Mix by gently shaking 10-15 seconds on the countertop.
- N. Incubate the plate at room temperature (22-25 °C) for 30 minutes.
- O. Repeat Steps I-K.
- P. Add 100 µL of TMB Substrate (EIATMB) to each microwell. Start a timer for 10 minutes with the addition of the substrate to the first well.
- Q. Mix by gently shaking 10-15 seconds on the countertop.
- R. Incubate the plate at room temperature (22-25 °C) for 10 minutes.
- S. Add 100 µL of Stop Solution (EIAS1) to each microwell in the same order as step P.
- T. Mix by gently shaking 10-15 seconds on the countertop.
- U. Read and record the results (see READING THE TEST).

TITRATION PROCEDURE

- 1. Prepare a dilution series of each specimen to be titrated as outlined in the following procedure:
 - A. Combine 50 µL of specimen and 200 µL of 1X Specimen Diluent to a test tube labeled #1 and mix.
 - B. Pipette 100 µL of 1X Specimen Diluent in each of 5 tubes labeled #2-#6.
 - C. Transfer 100 µL of the 1:5 diluted patient specimen from tube #1 into tube #2 and mix well.
 - D. Transfer 100 µL from tube #2 into tube #3 and mix well. Continue this dilution procedure through tube #6. Further dilutions may be made if necessary.

Tube Number	#1	#2	#3	#4	#5	#6
Dilution Factor	1:5	1:10	1:20	1:40	1:80	1:160

- 2. Perform the assay on the above dilutions following the Qualitative Procedure outlined above.
- 3. Read and record the results (see READING THE TEST).

READING THE TEST

- A. Reading the plate should take place within 15 minutes of test completion. Carefully wipe the undersides of the wells with a clean, lint-free tissue and measure the absorbance of each microwell as outlined below.
 - 1. A dual wavelength reader is preferred, with absorbances read at 450 nm and 630 nm. Blank on the 1X Specimen Diluent well.
 - 2. If a single wavelength reader is used, read the absorbance at 450 nm. Blank on the 1X Specimen Diluent well.
- B. Disinfect and retain microwell holder. Discard used assay materials as biohazard waste.

QUALITY CONTROL

At the time of each use, kit components should be visually inspected for obvious signs of microbial contamination, freezing or leakage. Discard if these conditions are found.

The Positive Control and Negative Control should be assayed with each batch of patient specimens to provide quality assurance of the reagents. The positive and negative controls are intended to monitor for substantial reagent failure.

Reagent	Acceptable Blanked OD Parameters
Positive Control	> 0.265
Negative Control	< 0.100

If the blanked OD of the Positive Control is not within these parameters, patient test results should be considered invalid, and the assay should be repeated.

Additional controls may be tested according to guidelines or requirements of local, state, and/or federal regulations or accrediting organizations.

RESULTS

INTERPRETATION OF RESULTS – QUALITATIVE PROCEDURE

Blanked OD	Result
x ≤ 0.265	Negative
x > 0.265	Positive

A positive result implies the presence of antigen to *Cryptococcus* species complex, however, all test results should be considered in conjunction with other clinical and laboratory data by the physician.

Negative results do not rule out the diagnosis of cryptococcal disease.

INTERPRETATION OF RESULTS – TITRATION PROCEDURE

Note: Use only absorbance values which have been obtained from the semi-quantitative assay to calculate EIA titers.

- 1. Select the **highest** absorbance value within the acceptable range of 0.265-2.500 to calculate the EIA titer.
- 2. If the 1:5 dilution of the specimen yields an absorbance of < 0.265, repeat the assay on the undiluted specimen.
- 3. Using the selected absorbance, calculate the EIA titer as outlined below

CALCULATION OF EIA TITER

$$(Blanked OD) \times Multiplication Factor = EIA Titer$$

Multiplication Factors*						
Tube Number	#1	#2	#3	#4	#5	#6
Dilution Factor	1:5	1:10	1:20	1:40	1:80	1:160
Multiplication Factor	50	100	200	400	800	1600

* Use a multiplication factor of 10 to calculate an EIA titer for undiluted specimens.

Example Calculations

For example, if a patient had a blanked OD absorbance of 1.65 from tube #3 at a 1:20 dilution, the multiplication factor would be 200.

$$(Blanked OD) \times Multiplication Factor = EIA Titer$$

$$1.65 \times 200 = 330, \text{ which is reported as } 1:330$$

LIMITATIONS OF THE PROCEDURE

- The assay performance characteristics have not been established for matrices other than serum and CSF.
- A negative result does not preclude diagnosis of cryptococcosis.
- The clarus Cryptococcal Antigen EIA is not intended for monitoring therapy.
- The clarus Cryptococcal Antigen EIA cannot be used as the sole means of determining cryptococcal disease. Test results must be used in conjunction with information available from the patient clinical evaluation and other diagnostic procedures.
- Testing should not be performed as a screening procedure for the general population. The predictive value of a positive or negative result depends on the pretest likelihood of cryptococcal disease being present. Testing should only be done when clinical evidence suggests the diagnosis of cryptococcal disease.
- Specimens may be stored for up to one week at ≤ -20 °C. Reductions in OD values after one week of specimen storage at ≤ -20 °C and after multiple freeze thaws may occur. Since a reduction in OD values was observed, it is possible that a fresh, very low-positive specimen (near 0.300 Blanked OD) could become negative if it is stored for one week.
- In clinical studies, specimens were stored for a maximum of five days at 2- 8 °C. However, the maximum storage time permissible at 2-8 °C has not been established.
- The performance of the clarus Cryptococcal Antigen EIA has not been established for manual reading and/or visual result determination.
- Reproducibility of the titer values produced with the titration procedure has not been established.
- Titer results are not numerically equivalent between assays. Antigen titer results should not be compared between different assays.
- Samples from patients with trichosporonosis, a factor known to cause false positives with cryptococcal EIA antigen testing, have not been tested.

INTERFERENCE

This assay was not evaluated for potential interference related to specimen pretreatment with 2-mercaptoethanol or with specimens including the following substances: bloody CSF, cloudy CSF, white blood cells, xanthochromic CSF, bilirubin, protein, systemic lupus erythematosus (SLE), sarcoidosis, or *N.meningitidis*.

This assay was evaluated for the potential of interference due to serum conditions including icteric, hemolyzed, and lipemic samples. These samples exhibited no interference in the assay.

CROSS REACTIVITY ANALYSIS

The clarus CrAg EIA was evaluated for cross-reactivity against a panel of patient specimens across a variety of different pathologies. The results of this testing are shown in the table below.

Pathology	Number of Specimens	% Positive
Penicilliosis	5	0 % (0/5)
Sporotrichosis	6	0 % (0/6)
HAMA	5	0 % (0/5)
Syphilis	10	0 % (0/10)
Rubella	5	0 % (0/5)
Mycoplasmosis	10	0 % (0/10)
Toxoplasmosis	7	0 % (0/7)
CMV	10	0 % (0/10)
Blastomycosis	10	0 % (0/10)
Coccidioidomycosis	10	0 % (0/10)
Histoplasmosis	10	0 % (0/10)
Candidiasis	10	0 % (0/10)
Aspergillus GM+	10	10 % (1/10)
Rheumatoid Factor	10	0 % (0/10)

Additionally, cross-reactivity was assessed by testing crude culture filtrate antigens at a range of concentrations using the clarus CrAg EIA. At high concentrations (> 0.1 mg/mL), antigens from *Paracoccidioides brasiliensis* exhibited some cross-reactivity.

Antigens from the following organisms were tested and exhibited no cross-reactivity:

Aspergillus terreus *Aspergillus fumigatus*
Aspergillus niger *Aspergillus flavus*

HIGH DOSE HOOK EFFECT (PROZONING)

Although rare, extremely high concentrations (> 1.0 mg/mL) of cryptococcal antigen can result in weaker test results and, in extreme instances, yield negative test results. If prozoning is suspected in weakly positive or negative test results, the specimen should be diluted following the titration procedure to rule out false negative results.

EXPECTED VALUES

The frequency of cryptococcosis is dependent on several factors including: patient population, type of institution, and epidemiology. In these studies, the IMMY clarus Cryptococcal Antigen EIA exhibited 97.6% agreement positive and 98.1% agreement negative with another manufacturer's EIA (serum and CSF combined).

PERFORMANCE CHARACTERISTICS

COMPARISON TO ANOTHER MANUFACTURER'S CRYPTOCOCCAL ANTIGEN EIA

The clarus CrAg EIA was evaluated in a multi-site study using 1782 patient specimens (426 CSF, 1356 serum) that were submitted to US reference and clinical laboratories for cryptococcal antigen testing. These specimens were tested using the clarus CrAg EIA (CRY101), and another manufacturer's EIA. The results of this comparison are shown in the tables below.

The combined data were analyzed for percent agreement positive and percent agreement negative. The results of these analyses are shown in the table below.

Serum Data	Other Manufacturer's EIA	
	Pos.	Neg.
IMMY clarus CrAg EIA	131	28
	2	1195

Serum Data	Calculated	95% CI
% Positive Agreement	98.5% (131/133)	94.7-99.6%
% Negative Agreement	97.7% (1195/1223)	96.7-98.4%

CSF Data	Other Manufacturer's EIA	
	Pos.	Neg.
IMMY clarus CrAg EIA	29	3
	2	392

CSF Data	Calculated	95% CI
% Positive Agreement	93.5% (29/31)	79.3-98.2%
% Negative Agreement	99.2% (392/395)	97.8-99.7%

COMPARISON TO IMMY CrAg Lateral Flow Assay

A multi-site split-specimen comparison study was performed on both serum and CSF specimens that had been submitted for cryptococcal antigen EIA testing. A total of 1782 specimens (CSF n=426; serum n=1356) were tested in both the clarus Cryptococcal Antigen EIA and the IMMY Cryptococcal Antigen Lateral Flow Assay (LFA) according to their respective package inserts. Analysis of this data yielded percent positive agreements of 96.9% and 93.8% in serum and CSF, respectively. Analysis indicated percent negative agreements of 99.8% and 99.5% in serum and CSF, respectively.

LIMIT OF DETECTION

In order to establish the limit of detection, purified cryptococcal antigen was diluted in negative serum and CSF and 24 replicates per concentration were tested using the clarus CrAg EIA. The results of this testing are shown in the following tables:

Serum Data

Serum Data		CSF Data	
Concentration	% Positive	Concentration	% Positive
4.9 ng/mL	0% (0/24)	1.3 ng/mL	0% (0/24)
5.0 ng/mL	8% (2/24)	1.4 ng/mL	0% (0/24)
5.1 ng/mL	54% (13/24)	1.5 ng/mL	4% (1/24)
5.2 ng/mL	71% (17/24)	1.6 ng/mL	83% (20/24)
5.3 ng/mL	100% (24/24)	1.7 ng/mL	100% (24/24)

LoD (Serum)	5.3 ng/mL
LoD (CSF)	1.7 ng/mL

REPRODUCIBILITY AND PRECISION

The clarus CrAg EIA was evaluated for reproducibility and precision by spiking serum and mock CSF with cryptococcal antigen to produce a panel consisting of a negative sample, a high negative (C_s) sample, a low positive sample and a moderate positive sample. This panel was tested twice per day at three sites with a total of five operators over a five-day period in order to determine both the inter-lab and the intra-lab reproducibilities and precision of the assay. The results of this study are shown in the table below.

Description	Type	IMMY			National Reference Lab			Clinical Lab			Combined Data (3 Sites)		
		Ave. O.D.	Std Dev.	% CV	Ave. O.D.	Std Dev.	% CV	Ave. O.D.	Std Dev.	% CV	Ave. O.D.	Std. Dev.	% CV
Blank	Control	0.066	0.012	17.8	0.000	0.0027	3686.2	0.075	0.005	7.0	0.046	0.034	74.67
CRYP1	Control	1.814	0.078	4.3	1.973	0.1083	5.5	2.473	0.158	6.4	2.059	0.295	14.34
Negative	Serum	0.022	0.011	48.7	0.012	0.0058	48.8	0.019	0.005	25.4	0.018	0.009	50.25
High Negative	Serum	0.040	0.008	19.2	0.028	0.0077	27.7	0.035	0.007	20.0	0.034	0.009	26.51
Low Positive	Serum	0.420	0.041	9.7	0.338	0.0436	12.9	0.372	0.038	10.3	0.377	0.053	14.16
Moderate Positive	Serum	1.683	0.229	13.6	1.666	0.1281	7.7	1.959	0.205	10.5	1.756	0.229	13.05
Negative	CSF	0.065	0.018	27.8	0.058	0.0087	15.1	0.099	0.010	10.0	0.072	0.022	29.85
High Negative	CSF	0.179	0.020	11.1	0.174	0.0190	10.9	0.298	0.027	9.2	0.211	0.059	28.09
Low Positive	CSF	0.346	0.028	8.0	0.339	0.0319	9.4	0.533	0.039	7.3	0.397	0.092	23.27
Moderate Positive	CSF	0.629	0.039	6.2	0.606	0.0443	7.3	0.929	0.056	6.0	0.707	0.149	21.13

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INTERNATIONAL SYMBOL USAGE

	Storage 2-8 °C		Lot Number
	Manufactured by		Reference Number
	Expiration Date		In Vitro Diagnostics
	Conforms to European Union Requirements		Sufficient for "# Tests
	Protect from Humidity		

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