

# Instructions for use

For research use only

## TOXOPLASMA GONDII IgG AVIDITY EIA

A solid-phase enzyme immunoassay for the determination of *Toxoplasma gondii* IgG antibody avidity.

Product no. 61 00 202

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### INTENDED USE

Ani Lab systems' *Toxoplasma gondii* IgG avidity EIA provides a method to detect and confirm acute phase *Toxoplasma gondii* primary infection.

### INTRODUCTION

The intracellular protozoan parasite, *Toxoplasma gondii*, causes infection in man as well as in animals. The primary hosts that harbour the intestinal, sexual stage are cats, from which the parasite is spread widely into nature. Transmission to humans occurs mainly by eating raw or rare meat, or by close contact with contaminated material (1).

Toxoplasmosis is a common infection in humans. In adolescence and adulthood, most infections are subclinical or run a mild clinical course. Primary (acquired) toxoplasmosis is typically associated with lymphadenopathy,


but other symptoms such as fever, a typical (reactive) lymphocytosis, myalgia or respiratory symptoms may occur. Chorioretinitis can be a manifestation of the acquired infection, but it more often occurs as a late sequela of congenital toxoplasmosis (2, 3). *Toxoplasma* encephalitis is a common cause of death among immunosuppressed individuals and occurs characteristically in association with AIDS (4). Toxoplasmosis may also result from transplantation of latently infected organs such as kidney or heart (2).

A clinically most important, and dreaded condition is congenital toxoplasmosis, which is a frequent consequence of primary maternal infection during pregnancy. Intrauterine infection may lead to stillbirths, intracerebral calcifications, hydrocephaly or microcephaly, and to chorioretinitis and/or psychomotor disturbances. The latter two conditions often worsen or manifest themselves not until at school age or during early adulthood (5-7). Because of the insidious or nonspecific clinical picture, the diagnosis of a *Toxoplasma* infection must be based on laboratory tests. Among the most widely used antibody tests are the Sabin-Feldman dye test, the indirect immunofluorescence test, the agglutination tests and the enzyme immunoassays for IgG, IgM or IgA. For the verification of acute primary *Toxoplasma* infections, however, the conventional assays are less than optimal due to lack of clinical diagnostic sensitivity (early antibody rises easily missed by tests employing paired sera) or of specificity (variable IgG levels; prolonged or reactivated IgM responses) (1, 3, 5).

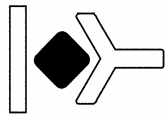
A new, uprising principle in clinical microbiology is the measurement of the antigen-binding avidity (functional affinity) of IgG (8, 9). The Ani Lab systems' *Toxoplasma Gondii* IgG Avidity EIA distinguishes the low-affinity antibodies produced at an early stage of infection from those with a higher binding affinity that reflect preexisting immunity. This type of protein-denaturing immunoassay (avidity-EIA) provides a specific and sensitive diagnosis of primary infection, as has been shown for *Toxoplasma gondii* (10-12) and for other pathogens (13-17). With a superior overall specificity (predictive value of a positive result), the *Toxoplasma* IgG Avidity EIA surpasses IgM detection in diagnostic value, irrespective of the type of IgM test:  $\mu$ -capture or indirect EIA, or immunoblotting (12).

### PRINCIPLE OF THE TEST

The principle of Ani Lab systems' *Toxoplasma Gondii* IgG Avidity EIA is based on an indirect solid-phase enzyme immunoassay with alkaline phosphatase as the marker enzyme. The assay proceeds according to the following reactions:

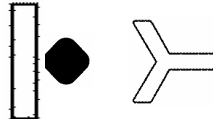
1. When present in patient serum *Toxoplasma gondii* IgG-class antibodies () will combine with the toxoplasma

antigens (●) attached to the polystyrene surface (|) in the wells of the Microstrips®.

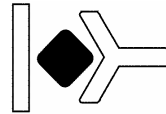


2. The wells are washed with avidity washing solution and the reference wells without the protein denaturant. IgG antibodies with low avidity are removed with the residual sample.

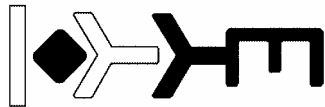
LOW  
AVIDITY  
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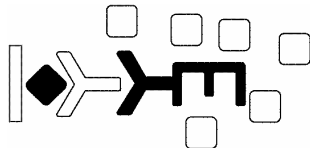
HIGH  
AVIDITY  
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3. Alkaline phosphatase conjugated anti-human IgG (Y<sub>3</sub>) is added.



4. The wells are washed and a colourless substrate, p-nitrophenylphosphate (pNPP), is added. The substrate is hydrolyzed by the enzyme to a coloured end product, p-nitrophenol.



5. The enzyme-substrate reaction is terminated with NaOH. The colour intensity is directly related to the concentration of the Toxoplasma IgG-class antibodies in the wells. Avidity results are obtained as a ratio of IgG titers.

## KIT CONTENTS

- Reagents are sufficient for 96 wells.
- Reagents are stored between +2°C and +8°C.
- The expiration date is printed on each component and on the package.
- Prewarm all reagents and Microstrips® to room temperature (RT) (+20°C – +25°C) before use.
- Avoid unnecessary exposure to light. The only light sensitive reagents are the substrate (pNPP tablets) and the avidity washing solution.

- Once the Microstrip® foil-package has been opened it has to be resealed tightly and stored at +2°C to +8°C with desiccant.
- Before use mix well the reagents by inverting the vials.
- Once opened the kit is stable for 3 months.

- 1 TOXOPLASMA GONDII COATED MICROSTRIPS®, 12 x 8 wells  
Microstrips® coated with inactivated parasites from mouse peritoneal fluid.
- 2 SAMPLE DILUENT, 2 x 125 ml  
Phosphate buffered saline, pH 7.4 ± 0.2, with proprietary additives, blue colouring agent and 15 mM sodium azide as preservative.
- 3a, b LOW and HIGH AVIDITY CONTROL, 2 x 1.0 ml  
Human serum with 15 mM sodium azide as preservative and blue colouring agent.
- 4 CONJUGATE DILUENT, 50 ml  
Phosphate buffered saline, pH 7.4 ± 0.2, with proprietary additives, red colouring agent and 15 mM sodium azide as preservative.
- 5 ANTI-HUMAN IgG-AP CONJUGATE (SHEEP), 5.0 ml concentrate  
Concentrate of alkaline phosphatase conjugated anti-human IgG, 15 mM sodium azide as preservative.
- 6 SUBSTRATE DILUENT, 90 ml  
0.9 M diethanolamine (DEA) and 0.505 mM MgCl<sub>2</sub>, pH 9.9 ± 0.2.

Refer to page 4

- 7 pNPP-SUBSTRATE, 3 tablets  
60 mg para-Nitrophenyl phosphate (pNPP) in one tablet.
- 8 TWEEN 20, WASHING SOLUTION, 5 ml concentrate  
Concentrate of Tween 20, 15 mM sodium azide as preservative.
- 9 AVIDITY WASHING SOLUTION, 50 ml  
Urea solution with proprietary additives and 0.1 % benzoic acid as preservative.

## INCUBATION COVERS, 2 pcs

Plastic sheets to cover the Microstrips® during incubation.

## REAGENT PREPARATION

Reagent	Preparation	Stability of opened/ diluted reagents (+2°C to +8°C)
1 Toxoplasma gondii coated microstrips®	Ready for use	3 months *)
2 Sample diluent	Ready for use	3 months *)
3a, b Low and high avidity control sera	Ready for use	3 months *)
4 Conjugate diluent	Ready for use	3 months *)
5 Conjugate	Dilute 1+9 (1:10) with prewarmed (+37°C) conjugate diluent (just prior to use).	Discard unused diluted conjugate solution.
6 Substrate diluent	Ready for use	3 months *)
7 pNPP-substrate	Reconstitute 1 tablet in 30 ml of prewarmed (+37°C) substrate diluent (10 minutes before the use in the assay).	Discard unused reconstituted substrate solution.
8 Tween 20 washing solution	Dilute 1+499 (1:500) with 0.01 M potassium phosphate buffered saline (PBS) pH 7.4 ± 0.2	One week
9 Avidity washing solution	Ready for use	<b>Note:</b> Avoid exposure to light

\*) The stability of the opened reagents is maximum 3 months only if they are stored properly at +2°C to +8°C. However, high environmental temperature and contamination may decrease the stability.

### EXAMPLE OF REAGENT DILUTIONS FOR DIFFERENT NUMBER OF SAMPLES

Total number of serum samples	Number of Microstrips®	Sample Diluent (ml)	Conjugate dilution (1:10)		Tween 20 (1:500)		Substrate solution		Stop solution 1M NaOH (ml)
			Conjugate + Conjugate diluent (CD)		Tween (ml)	Buffer (ml)	Substrate diluent (ml)	pNPP- tablets	
			Conj (ml)	CD (ml)					
3	6	20	0.6	5.4	0.12	59.88	30	1	6
6	9	40	0.9	8.1	0.18	89.82	30	1	9
9	12	60	1.2	10.8	0.24	119.76	30	1	12

## MATERIALS REQUIRED BUT NOT PROVIDED

- Distilled or deionized water, preferably sterile.
- 1 M NaOH (4 g NaOH in 100 ml H<sub>2</sub>O).
- 0.01 M PBS pH 7.4 ± 0.2 (0.24 g KH<sub>2</sub>PO<sub>4</sub>, 1.88 g K<sub>2</sub>HPO<sub>4</sub> × 3 H<sub>2</sub>O, 9.0 g NaCl in 1000 ml H<sub>2</sub>O).
- Graduated cylinder up to 50 ml for conjugate dilution.
- Test tubes, 5 ml, for specimen dilutions.
- Tubes or bottles to store the diluted and reconstituted reagents.
- Precision pipettes (one channel eg. 0.5-10 µl, 5-50 µl, 20-200µl, 100-1000 µl ranges and multi-channel 50-300 µl)
- Paper towels or absorbent paper.
- Timer, 60 min range.
- Microplate incubator, +37°C.
- Microplate photometer, 405 nm

## PRECAUTIONS

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Reagents are stored between +2°C and +8°C. Avoid unnecessary exposure to light. The only light sensitive reagents are the substrate (pNPP tablets) and the avidity washing solution. Storage of reagents and samples in self-defrosting freezers is not recommended. Store all working solutions in clean containers to prevent contamination.

Do not use reagents after the expiration date printed on the label.

When removing aliquots from the reagent vials, use aseptic technique to avoid contamination or incorrect results may occur. Take care not to mix the caps of reagent bottles. Use a new pipette tip for each sample.

Optimal results will be obtained by strict adherence to the test protocol. Accurate and precise pipetting, as well as following the exact time and temperature requirements, are essential.

### WARNING - POTENTIAL BIOHAZARDOUS MATERIAL:

Each donor unit used in the preparation of the control sera in the kit has been tested for the presence of antibodies to HIV (Human Immunodeficiency Virus) and HCV (Hepatitis C Virus) as well as for Hepatitis B markers and found to be non-reactive. Because no test or inactivation method can offer complete assurance that HIV, Hepatitis B Virus, Hepatitis C Virus, or other infectious agents are absent or inactive, these controls as well as specimens should be handled at the Biosafety level 2 as recommended for any potentially infectious human serum or blood specimen in the Centers for Disease Control/National Institutes of Health manual "Biosafety in Microbiological and Biomedical Laboratories", 1999. In addition, handle and dispose the Microstrips® as well as all material coming into contact with them or with specimens and control sera as if capable of transmitting infection.

Do not mix or interchange enzyme conjugate, Microstrips® and control sera from different lots. Sample and conjugate diluents are not interchangeable. Crosscontamination of reagents or samples could cause erroneous results.

Do not reuse a Microstrip® even though some wells were not used.

Do not touch the wells or splash liquid while pipetting.

Do not let wells dry once assay has started.

Never pipette by mouth.

Take care in discarding the reagents containing sodium azide. Azides are reported to react with lead and copper in plumbing to form compounds that may detonate on percussion. When disposing of solutions containing sodium azide, flush with large volumes of water. Please refer to pre-cautions and decontamination procedures as outlined by the National Institute for Occupational Safety and Health (18).

Do not let sodium hydroxide or substrate diluent come into direct contact with skin or eyes. Substrate diluent contains diethanolamine (DEA, bottle 6). Avoid contact with skin and eyes. In case of contact with eyes, rinse immediately with plenty of water and seek medical advice.

Reusable glassware must be washed out and rinsed free of detergents.

## SPECIMEN COLLECTION AND HANDLING

Blood is collected aseptically by venipuncture and allowed to clot in a sterile tube. The serum is separated by centrifugation. Use of sterile or aseptic techniques will preserve the integrity of the specimen. Serum samples are refrigerated (+4°C) upon collection or, if the test cannot be performed within 48 hours, frozen (-20°C). Samples should not be repeatedly frozen and thawed.

Heat-treatment of specimens (30 minutes at +56°C) may slightly change the absorbance level of serum samples.

Microbially contaminated, grossly hemolyzed or hyperlipemic serum may give erroneous results.

Long storage of the serum (over one year in frozen stage) may cause the formation of lipid aggregates (19). These aggregates may elevate the absorbance of negative sera and diminish the absorbance of positive sera.

**TEST PROCEDURE**

## OUTLINE OF PROCEDURE

**STEP I** Add 100 µl sample/reagent blank/controls

Incubate 60 min., +37°C

Wash

**STEP II** Add 100 µl conjugate solution

Incubate 60 min., +37°C

Wash

**STEP III** Add 100 µl substrate solution

Incubate 30 min., +37°C

**STEP IV** Add 100 µl 1 M NaOH

Measure at 405 nm

## PRELIMINARY PREPARATIONS

- Bring the reagents and Microstrips® to RT.
- Prewarm the incubator to +37°C.
- If washing solution is stored diluted, bring it to RT.

## SPECIMEN AND CONTROL DILUTION

Specimen:

Dilute each serum into sample diluent five times at 4-fold steps: 1:50; 1:200; 1:800; 1:3200; 1:12800. If the IgG titre is very high, the dilution series is started from 1:200 and if the IgG titre is very low, the dilution series is advised to start from 1:12.5.

Controls:

Dilute the controls 3a and 3b further into the sample diluent four times at 4-fold steps to obtain dilutions 1:4; 1:16; 1:64; 1:256.

## STEP I

1. Place an appropriate number of coated Microstrips® needed for the samples (1 strip for blank solution, 2 strips for controls and 1 strip/sample) to be tested into the Microstrip® frame.
2. Pipette 100 µl of the diluted samples into the wells: the four highest dilutions (1:200 to 1:12800) serially into wells A-D in the strip (for PBS-Tween washing) and the four lowest dilutions (1:50 to 1:3200) into wells E-H (for avidity washing).
3. After pipetting the specimens pipette sample diluent (this is the reagent blank) to all eight wells of the first strip and diluted controls (like specimens) to strips 2 and 3 as follows:

Low and high avidity control strips:

wells A-D 1:4; 1:16; 1:64, 1:256  
wells E-H undiluted control; 1:4, 1:16; 1:64

4. Cover the Microstrips® tightly with plastic sheet.
5. Incubate for 60 minutes at +37°C.

## WASHING

7. Empty the wells by shaking out the liquid and gently tap the inverted Microstrips® a few times on a clean paper towel.
8. Rows A-D: Add 150 µl of PBS-Tween washing solution  
Rows E-H: Add 150 µl of avidity washing solution  
Allow the washing solution to stand **exactly** 5 minutes in all wells at RT (+20°C - +25°C). See the NOTE below.
9. Empty the wells by shaking out the liquid.
10. Repeat the washing twice (3x5 minutes in total).
11. After the third washing, tap the inverted Microstrips® a few times and turn them upside down on a paper towel for a moment.

## STEP II

1. Pipette 100 µl of the diluted conjugate into each well. See the NOTE below.
2. Cover the Microstrips® tightly with plastic sheet.
3. Incubate for 60 minutes at +37°C.

## WASHING

4. Empty the wells by shaking out the liquid and gently tap the inverted Microstrips® a few times on a clean paper towel.
5. Add 150 µl PBS-Tween washing solution into each well. When using a pipette cut off a few millimeters from the end of the pipette tips to avoid too high pressure and foaming during pipetting. See the NOTE below.
6. Empty the wells by shaking out the liquid.
7. Perform the washing three times in total.
8. After the third washing, tap the inverted Microstrips® a few times and turn them upside down on a paper towel for a moment.

## STEP III

1. Pipette 100 µl of the substrate solution into each well. See NOTE below.
2. Incubate for exactly 30 minutes at +37°C.

## STEP IV

1. Pipette 100 µl of 1 M NaOH into each well. See NOTE below.
2. Mix carefully. This is to stop the enzyme-substrate reaction.

**NOTE:** The use of an 8-channel multipipetting device will minimize the time factor between Microstrips® and improve results.

**MEASURING**

Readings should be taken within one hour of terminating the enzyme-substrate reaction. Readings are recorded as absorbance units.

Blank the photometer at 405 nm against air and measure the absorbances.

Most microplate readers can be blanked against the reagent blank. This means that the absorbance of the reagent blank is automatically subtracted from the absorbance of the samples.

**RESULTS**

**Quality Control Values**

Before proceeding to calculate the results, make sure that the absorbance values obtained for the reagent blank are  $\leq 0.500$ .

The avidity percentages of the controls should fall within the guidelines: the low avidity control < 15 %, the high avidity control > 30 %. If these values are not obtained, the test is recommended to be repeated.

**Calculation of the Results**

Two titration curves are obtained for the controls and for each serum.

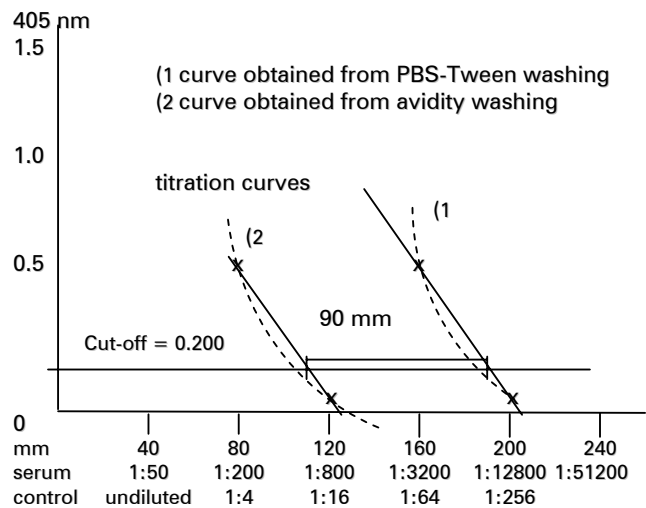
1. Calculate the mean of the reagent blank absorbances and subtract the mean from all control and sample absorbances.
2. Plot the curves to millimetre paper so that each doubling in serum dilution corresponds to a fixed distance, 20 mm, on the x-axis and the absorbances are in the Y-axis.
3. Cut-off for avidity assay is 0.200 abs units. Draw this line to the millimetre paper and measure the distance (in mm) between the straight lines connecting the points in titration curves. (See example 1.)  
The corresponding avidity percentages are listed in table I.

**Table I.** Distances between titration curves and the corresponding avidities.

End-point distance (mm)	Avidity (%)	End-point distance (mm)	Avidity (%)	End-point distance (mm)	Avidity (%)
1	96.6	51	17.1	101	3.0
2	93.3	52	16.5	102	2.9
3	90.1	53	15.9	103	2.8
4	87.1	54	15.4	104	2.7
5	84.1	55	14.9	105	2.6
6	81.2	56	14.4	106	2.5
7	78.5	57	13.9	107	2.5
8	75.8	58	13.4	108	2.4

9	73.2	59	12.9	109	2.3
10	70.7	60	12.5	110	2.2
11	68.3	61	12.1	111	2.1
12	66.0	62	11.7	112	2.1
13	63.7	63	11.3	113	2.0
14	61.6	64	10.9	114	1.9
15	59.5	65	10.5	115	1.9
16	57.4	66	10.2	116	1.8
17	55.5	67	9.8	117	1.7
18	53.6	68	9.5	118	1.7
19	51.8	69	9.2	119	1.6
20	50.0	70	8.8	120	1.6
21	48.3	71	8.5	121	1.5
22	46.7	72	8.2	122	1.5
23	45.1	73	8.0	123	1.4
24	43.5	74	7.7	124	1.4
25	42.0	75	7.4	125	1.3
26	40.6	76	7.2	126	1.3
27	39.2	77	6.9	127	1.2
28	37.9	78	6.7	128	1.2
29	36.6	79	6.5	129	1.1
30	35.4	80	6.3	130	1.1
31	34.2	81	6.0	131	1.1
32	33.0	82	5.8	132	1.0
33	31.9	83	5.6	133	1.0
34	30.8	84	5.4	134	1.0
35	29.7	85	5.3	135	0.9
36	28.7	86	5.1	136	0.9
37	27.7	87	4.9	137	0.9
38	26.8	88	4.7	138	0.8
39	25.9	89	4.6	139	0.8
40	25.0	90	4.4	140	0.8
41	24.1	91	4.3	141	0.8
42	23.3	92	4.1	142	0.7
43	22.5	93	4.0	143	0.7
44	21.8	94	3.8	144	0.7
45	21.0	95	3.7	145	0.7
46	20.3	96	3.6	146	0.6
47	19.6	97	3.5	147	0.6
48	18.9	98	3.3	148	0.6
49	18.3	99	3.2	149	0.6
50	17.7	100	3.1	150	0.6

Example 1.



Low avidity control → 90 mm = 4.4 % AVIDITY

**Interpretation of the Results**

- < 15 % Low avidity.  
Acute, primary infection by *Toxoplasma gondii*.
- 15 - 30 % Borderline avidity.  
Primary infection during the last six months is possible.
- > 30 % High avidity.  
Excludes primary *Toxoplasma gondii* infection within the last three months.

When low or borderline avidity is obtained the test should be repeated and the average result of two separate avidity measurements should be used.

The result of a single assay does not constitute sufficient proof for the diagnosis of recent infection. A recent infection can only be diagnosed based on a combination of clinical and serological data.

Note that low avidities are not obtained after reactivation or reinfection.

**LIMITATIONS OF THE PROCEDURE**

A serum sample obtained during the acute phase of infection, when only IgM antibodies are present, may be negative by this procedure.

The test result should be used in conjunction with information available from the clinical status and other test results.

**PERFORMANCE CHARACTERISTICS**

**Precision**

The total variation was determined by running 3 independent test runs done by 3 technicians. Three samples having different avidity % levels were tested as 3 parallels in each test run (n=9, for low avidity control (LAC) and high avidity control (HAC) n=3).

**Table 2.** Total variation of samples as avidity percentages.

Sample	Mean	SD	CV %
LAC	8.3	2.0	24.4
HAC	48.3	2.9	5.9
A511	7.2	1.8	24.7
1561	13.0	3.2	24.9
PO	48.8	3.8	7.8

**Clinical evaluations**

*Toxoplasma* IgG avidity was evaluated (12) in a large study consisting 16733 pregnant women. The sera were obtained in 1st, 2nd and 3rd trimesters of pregnancy (total

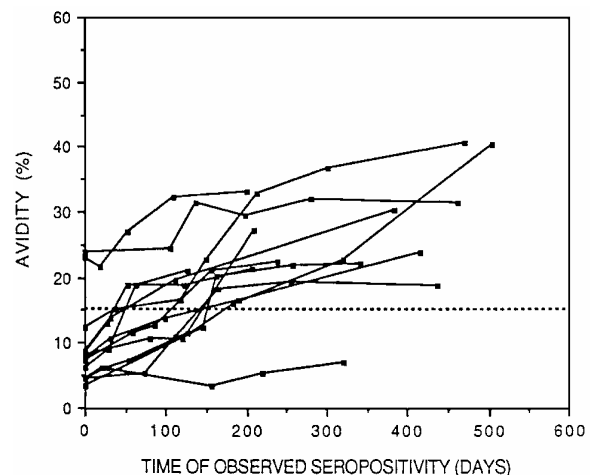
of 44181 sera). Complete sets of 3 sera were obtained from 70,5% of the subjects.

The sera were first tested for IgG using two commercially available ELISA tests. All sera containing IgG were further tested with  $\mu$ -capture IgM and the resulting positive sera with indirect IgM assay and with *Toxoplasma gondii* avidity. All these indirect IgM positive sera were also tested with immunoblotting and found positive. IgG avidity was tested from 240  $\mu$ -capture negative sera as control.

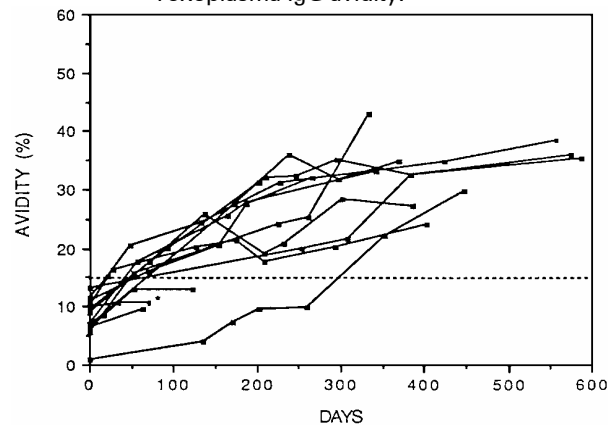
Among the 16733 women 3178 (19%) were IgG positive. In serial samples, 13 women showed diagnostic IgG seroconversions and 3 showed diagnostic IgG titer rises and were also positive by all 3 IgM tests. Altogether 226 women were IgM positive in the  $\mu$ -capture assay but only 36 also in the other two IgM assays. These women with IgM in all 3 tests were followed up. According to indirect IgM ELISA 55% remained positive for 1 year and in  $\mu$ -capture IgM ELISA 91% of the subjects were still positive after 5 months suggesting that positive IgM result alone cannot be used as a diagnostic marker for acute toxoplasmosis.

High avidity was recorded in 428, borderline in 13 and low avidity in 25 women. All the subjects with low avidity were IgM positive in the  $\mu$ -capture ELISA; 11 belonged to the IgG seroconverters and 3 had diagnostic IgG rises. After seroconversion, 10/11 (91%) reached high or borderline values by 6 months of (observed) seropositivity (Figure 1). All the initially seropositive subjects with low avidity, who were followed up, reached high or borderline values by 10 months (Figure 2).

**Figure 1.** Maturation of *Toxoplasma* IgG avidity after IgG seroconversion.

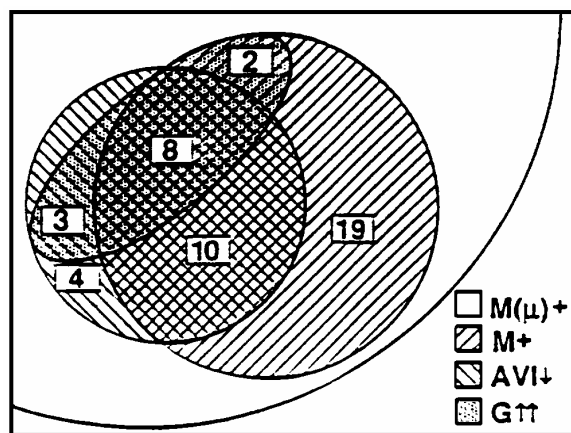


**Figure 2.** Maturation of initially low ( $\leq 15\%$ ) Toxoplasma IgG avidity.



The degree of concordance of the results in the various assays is summarized in Figure 3. Toxoplasma gondii avidity has a high sensitivity - intermediate between indirect and  $\mu$ -capture IgM ELISAs - and its positive predictive of an acute infection value is superior to that of any of the IgM tests used.

**Figure 3.** Relationship between IgG seroconversions (N = 13), positive IgM results (N = 39), and results of low IgG avidity (N = 25). The disc areas reflect the numbers of women with the indicated findings and overlapping indicates concordance between assays. G $\uparrow\uparrow$  = IgG seroconversion, AVI $\downarrow$  = low IgG avidity, M+ = positive IgM by indirect ELISA, M( $\mu$ ) + = positive IgM by  $\mu$ -capture ELISA.



26 low and borderline avidity subjects from the above material were tested using Ani Lab systems' commercial Toxoplasma gondii IgG Avidity EIA assay. Our test was found substantially equivalent to the avidity test used in the above study. In addition 21 patients with known past immunity were tested and all these subjects gave high avidity percentage as expected.

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Product number	Product description
61 00 202	Toxoplasma gondii IgG Avidity EIA Kit
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