

**Instructions for use**
**IBDX® AMCA IgG ELISA Kit**

Semi-quantitative ELISA for anti-Mannobioside  
Carbohydrate antibodies in human serum or plasma  
**96 determinations**

**CE** Store at 2-8 °C  
Reagents included in the kit are  
for *in vitro* diagnostic use only

**REF** CAT No **M704100**







**IVD** *In vitro* diagnostic device

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	<b>LOT</b> Batch number		<b>REF</b> Catalogue number
	<b>Manufacturer</b>		<b>Temperature limitation</b>
	<b>Expiration date</b>		<b>Consult operating instructions</b>

**INTENDED USE**

The IBDX AMCA IgG ELISA kit is an indirect solid phase enzyme-linked immunosorbent assay (ELISA) for the detection of circulating IgG class antibodies against Mannobioside antigen in human serum. It is intended as an aid for the diagnosis and risk assessment of Crohn's disease (CD). Use of the AMCA IgG kit together with the other IBDX kits is recommended to obtain optimal results.

**INTRODUCTION**

Inflammatory bowel disease (IBD) is a general term used to describe diseases that cause inflammation of the intestines. Two prominent classes of IBD are Crohn's disease (CD) and ulcerative colitis (UC), both of which occur worldwide and are characterized by chronic intestinal inflammation of unknown aetiology.

Other gastrointestinal functional disorders (OGD) with symptoms similar to those of IBD are irritable bowel syndrome (IBS) and celiac. IBS is a functional disorder of the colon, a non-inflammatory disease diagnosed only by ruling out all other disease options. Celiac denotes sensitivity to gluten, which is controlled through maintaining a strict diet. Other non-IBD categories are caused by bacterial and viral infections.

Though CD and UC share several symptoms, the course, complications, and management of these diseases considerably differ, in particular regarding their surgical aspects. Consequently, the differential diagnosis of these diseases is pivotal to establish proper treatment.

The major commercially applied serologic markers for IBD are anti-*Saccharomyces cerevisiae* antibodies (ASCA) and atypical peri-nuclear antineutrophil cytoplasmic antibodies (pANCA). ASCA are directed against phosphomannan in the cell walls of the yeast *S. cerevisiae*. Their prevalence amounts to 48%–69% among CD patients and 15% among UC patients (Reumaux, D., et al., 2003; Sendid, B., et al., 1996). pANCA are autoantibodies directed at antigens found mostly in azurophilic granules of neutrophils with a prevalence of up to 85% among UC patients and 5%–28% among CD patients as well as healthy people (Reumaux, D., et al., 2003).

The bacterial intestinal microbiota plays major roles in human physiology and IBD. IBDX kits which are based on serological antibodies to fungi are an excellent tool to study fungal microbiota dysbiosis in IBD (Sokol et al. 2016). Laminaribioside, Chitobioside, Mannobioside, anti-Chitin and anti-Laminarin are novel sugar antigens, known as innate immunity triggers and part of the cell wall of *Candida Albicans* and *Saccharomyces cerevisiae*. Antibodies recognizing these antigens are novel serologic markers in Crohn's disease, including ASCA-negative CD patients (Dotan, I., 2006, Seow et al. 2009). *gASCA* is an improved ASCA assay based on immobilized purified Mannan polysaccharide. Anti-chitobioside carbohydrate antibodies (ACCA), anti-mannobioside carbohydrate antibodies (AMCA), and anti-laminaribioside carbohydrate antibodies (ALCA), anti-Chitin (anti-C) and anti-Laminarin (anti-L) provide high specificity while exploiting a cost-effective standard enzyme immunoassay format. Together, ALCA, AMCA, ACCA, anti-C and anti-L identify 33%-56% of the ASCA negative Crohn's disease patients (Dotan, I., et al., 2006; Ferrante, M., et al., 2006, Malickova et al.2010). A sera positive result with multiple antigens predicts a more complicated and severe disease course and high risk for abdominal surgery (Rieder, F., et al., 2010).

Glycominds offers the IBDX panel including *gASCA* IgG, ALCA IgG, ACCA IgA and AMCA IgG, anti-C IgA, anti-L IgA ELISA kits to aid in the diagnosis and risk assessment of CD. When used concurrently with atypical pANCA (IFA), these kits provide a cost effective, accurate and sensitive tool to aid in differential diagnosis and prognosis of CD.

**PRINCIPLES OF THE PROCEDURE**

Diluted serum samples (1:101) and pre-diluted controls/calibrator react for 30 minutes with Mannobioside immobilised in microtiter wells. After washing away unbound serum components, antibodies specifically bound to antigen, if present in sample, are detected using enzyme labelled anti-human IgG. After 30 minutes incubation, unbound conjugate is removed by washing, and chromogenic substrate is added for 30 minutes. A colour develops if specific antibodies are present. Subsequently, a stop solution is added to terminate the enzymatic reaction and, thereafter, absorbance of the calibrator, controls and samples can be evaluated spectrophotometrically. Optical density (OD) is directly proportional to the amount of bound antibody. Arbitrary units are calculated based on sample OD and calibrator OD.

Samples are considered positive for the presence of AMCA IgG antibodies if the sample units' value is greater than the cut-off value.

## KIT CONTENTS

C304100	AMCA Microwell Plate 96 well	<b>1 Plate</b> in sealed pouch with desiccant
G300023	IBDX™ Sample Diluent	<b>25 mL</b> clear solution in 60 mL white bottle
G300022	Wash Concentrate 20x Sufficient for 1000mL	<b>50 mL</b> clear solution in 60 mL white bottle
C304025	AMCA HRP IgG Conjugate horseradish peroxidase	<b>14 mL</b> red solution in 15 mL white bottle
G300024	TMB Chromogen 3,3',5,5'-Tetramethylbenzidine	<b>14 mL</b> clear solution in 15 mL amber bottle
G300021	HRP Stop Solution	<b>14 mL</b> clear solution in 15 mL white bottle
C304140	AMCA IgG Calibrator	<b>1 mL</b> clear solution in 1 mL blue capped tube
C304130	AMCA IgG Negative Control	<b>1 mL</b> clear solution in 1 mL blue capped tube
C304120	AMCA IgG Positive Control	<b>1 mL</b> clear solution in 1 mL blue capped tube
M704300	Instructions for use	<b>1 IFU</b>

## OTHER REQUIRED MATERIALS

10 mm X 60 mm tubes for dilution; 10 µL, 100 µL, and 1000 µL pipettes, with appropriate disposable pipette tips; 1 L container for diluted Wash Concentrate; microplate reader with 450 nm filter; General laboratory apparatus; Distilled or deionized water.

## STORAGE AND STABILITY

On arrival, store the kit at 2 - 8°C. Reagents remain stable until the expiration date when stored and handled as indicated. Follow good laboratory practices to minimize microbial and cross contamination of reagents once opened. It is important to protect the unused wells from excess moisture. Do not use kits beyond their expiration date.

Diluted Wash Concentrate is stable for 21 Days at 2 - 8°C. Exposure to 50°C or more for more than few hours will destroy the kit. Do not freeze.

## PATIENT SAMPLES

### Specimen collection and storage

Patient samples, serum should be collected by venipuncture as prescribed by local regulatory requirements. Following collection, the serum should be separated immediately from red blood cells. NCCLS Document H18-A recommends the following storage conditions for samples: 1) Store samples at room temperature for no longer than 8 hours. 2) Refrigerate the sample at 2 - 8°C if the assay will not be completed within

8 hours. 3) If the assay will not be completed within 48 hrs, or for shipment of the sample, freeze at -20°C or lower. Frozen specimens must be mixed well after thawing and prior to testing. Repeated freezing and thawing may affect results. Addition of azide or other preservatives to the test samples may adversely affect the results. Microbially contaminated, heat-treated, or specimens containing visible particulate should not be used. Grossly hemolyzed or lipemic serum or specimens should be avoided.

## PROCEDURAL NOTES

Allow samples and components to reach room temperature prior to the assay. Take care to agitate samples gently in order to ensure homogeneity and mix the components well prior to use in the assay

Dilution should be performed immediately before use.

Do not use kit components beyond their expiration date.

Do not interchange or mix kit components from different lots.

Have all reagents and samples ready before start of the assay. Once started, the assay should be performed without interruption and incubations should be accurately timed to achieve reliable and consistent results.

Perform the assay steps in the order indicated.

The assay calibrator and controls are manufactured from diluted human serum. Although these materials have been tested for presence of biohazardous agents, proper precautions should be taken while handling. Treat all human source material as potentially infectious. Normal clinical laboratory safety procedures should be maintained at all times. Operators should wear gloves and protective clothing when handling any patient sera or serum-based products.

### Automated washing:

Consider excess volumes required for setting up the instrument and dead volume of automated equipment.

### Manual washing:

Discard liquid from wells by inverting the plate. Vigorously tap the microwell frame with wells downside on clean absorbent paper. Pipette 300 µL of diluted Wash Concentrate into each well, wait for 20 seconds. Repeat the whole procedure three more times.

### TMB Chromogen:

Avoid exposure of the TMB chromogen solution to light!

## PREPARATION

### Dilute wash concentrate:

Dilute the Wash Concentrate 1:20 with distilled water (e.g., 50 mL plus 950 mL). Prepare 50 mL of diluted Wash Concentrate (1x) per 8 wells or 600 mL for 96 wells.

### Samples:

Dilute serum samples 1:101 with IBDX™ Sample Diluent (5 µL serum+ 500 µL sample diluent). Mix well! Dilution should be performed immediately before use. **It is recommended that samples be run in duplicate.**

### Microwell plate:

The microwell plate is sealed in a foil pouch with desiccant. The plate consists of a frame and 12 (8 well) strips. Allow the sealed microwell plate to reach room temperature before opening. Calculate the number of wells required for the assay. Remove unused strips from the frame, replace and store in the foil pouch, together with desiccant, seal tightly and store at 2 - 8°C.

## ASSAY PROCEDURE

**ALL REAGENTS MUST BE BROUGHT TO ROOM TEMPERATURE (20-26°C) PRIOR TO BEGINNING THE ASSAY.**

**Print the Assay Scheme – last page**

1. **Prepare** reagents and samples. Dilute patients' samples (1:101) in IBDX Sample Diluent.
2. **Add 50 µL** each of the *AMCA* IgG Calibrator, the *AMCA* IgG Positive Control, the *AMCA* IgG Negative Control and the diluted (1:101) patient samples to different wells.
3. Cover the wells and **incubate** for **30 minutes** at room temperature on a level surface.
4. **Wash** step: Thoroughly aspirate the contents of each well. Add to all wells 300 µL of the diluted Wash Concentrate; then aspirate. Repeat this cycle three more times for a total of four washes. Invert the plate and tap it on absorbent material to remove any residual fluid after the last wash. It is important to completely empty each well. Maintain the same procedural sequence for the aspiration as was used for the sample addition.
5. **Add 100 µL** of the *AMCA* HRP IgG Conjugate to each well. Conjugate should be pipetted using standard aseptic conditions and good laboratory techniques. Remove only the amount necessary for the assay. **TO AVOID POTENTIAL MICROBIAL AND/OR CHEMICAL CONTAMINATION, NEVER RETURN UNUSED CONJUGATE TO THE BOTTLE.**
6. **Incubate** wells for **30 minutes** as described in step 3.
7. **Wash** step. Repeat step 4.
8. **Add 100 µL** of TMB Chromogen to each well.
9. **Incubate** for **30 minutes** at room temperature (keep away from direct light).
10. **Add 100 µL** of HRP Stop Solution to each well. Proceed following the same procedural sequence and timing as was used for addition of the TMB Chromogen.
11. Gently tap plate to mix wells. Thereafter **incubate** for **2 – 5 minutes** at room temperature on a level surface.
12. **Read** the absorbance (OD) of each well at 450 nm within one half hour of stopping the reaction.

## DATA PROCESSING

The result for each sample is calculated by dividing the average OD of the sample by the average OD of the *AMCA* IgG Calibrator, multiplied by the number of units denoted on the *AMCA* IgG Calibrator tube label.

$$\text{Sample Value (units)} = \frac{\text{Average Sample OD}}{\text{AMCA IgG OD}} \times \text{AMCA IgG Calibrator (units)}$$

The result correlates with the quantity of antibody present in the patient sample in a non-linear fashion.

Please note that the calibrator units ARE NOT the cut-off values. The cut-off values appear in the Interpretation of results section.

## QUALITY CONTROL

The *AMCA* IgG Calibrator, *AMCA* IgG Positive Control and Negative Control should be included in every run to verify assay integrity and accuracy. The *AMCA* IgG Calibrator, *AMCA* IgG Positive Control and *AMCA* IgG Negative Control are prediluted, and therefore ready for use. They do not function as a control for procedural methods associated with specimen dilution.

Assay results are considered valid when the following criteria are met: A) The absorbance of the *AMCA* IgG Positive Control must be higher than the absorbance of the *AMCA* IgG Calibrator, which must be higher than the absorbance of the *AMCA* IgG Negative Control. B) The *AMCA* IgG Positive Control must have an absorbance **higher than 1.2**, whereas the *AMCA* IgG Negative Control absorbance should be **below 0.4**.

If any of the above criteria are not met, the assay should be considered invalid and therefore repeated.

## KIT PERFORMANCE

**Calibration** – Due to lack of international reference material the *AMCA* IgG calibrator is denoted in arbitrary units.

**Analytical sensitivity** - The analytical sensitivity of the *AMCA* IgG is less than 10 units.

**Precision**

Within Assay Imprecision < 11%

Between Assay Imprecision < 15%

## INTERPRETATION OF RESULTS

### Differentiation between Crohn's Disease, Ulcerative Colitis, and non-IBD

For each sample, units for *gASCA*, *ACCA*, *ALCA*, *AMCA*, anti-C and anti-L are calculated as explained in the package insert for the individual assay. Use the following cut-off values table to determine whether the patient is positive or negative for the individual IBDX markers. These cut-off values were established on the basis of several studies using the IBDX panel on different cohorts. However, since the ELISA assay is a very sensitive technique, capable of detecting even small differences in a patients' population, the values shown below are suggested values only. Each laboratory should establish its own cut off values.

IBDX Cut-off values table:

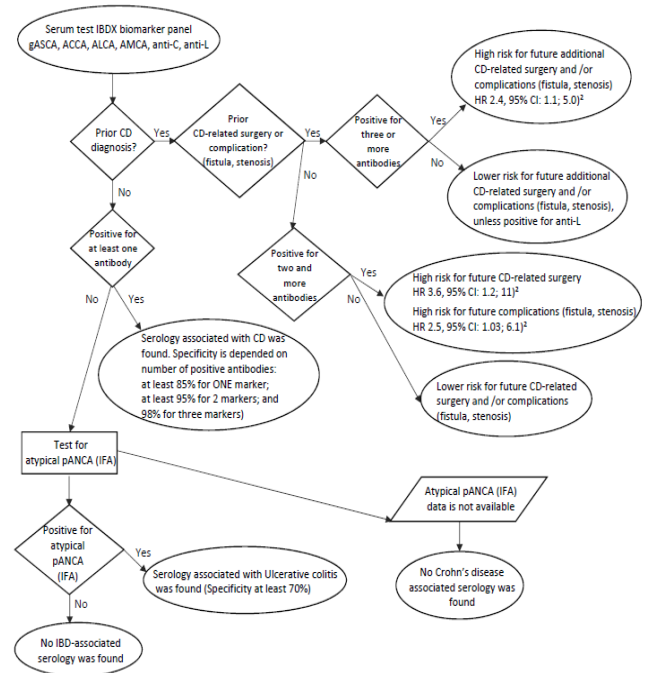
Result	Cut-off values for					
	<i>gASCA</i>	<i>ACCA</i>	<i>ALCA</i>	<i>AMCA</i>	anti-C	anti-L
<b>Negative</b>	<45	<80	<55	<90	<45	<45
<b>Equivocal*</b>	45-50	80-90	55-60	90-100	45-50	45-50
<b>Positive</b>	>50	>90	>60	>100	>50	>50

\* Repeating the sample assay is recommended

Results should then be reported as follows:

- i) If the patient is positive for one of the markers, report that serology associated with CD was found (specificity at least 85%).
- ii) If the patient is already diagnosed with CD and do not have prior CD complications (fistula and/or stenosis) and prior CD-related surgery and is positive for two or more of the markers, report as follows:
  - a) Serology associated with CD was found (specificity at least 95%).
  - b) High risk for future CD-related surgery with HR of 3.6 (95% CI: 1.2 to 11) (Rieder, F., et al., 2010).
  - c) High risk for future CD-related complication (fistula, stenosis) with HR of 2.5 (95% CI: 1.03 to 6.1) (Rieder, F., et al., 2010).
- iii) If the patient is already diagnosed with CD and had either prior CD complications (fistula and/or stenosis) or prior CD-related surgery and is positive for three or more of the markers, or is positive to anti-L report as follows:
  - a) Serology associated with CD was found (specificity at least 98%).
  - b) High risk for future additional CD-related complication or surgery with HR of 2.4 (95% CI: 1.1 to 5) (Rieder, F., et al., 2010).
- iv) If the patient is diagnosed with CD and negative for all markers report on a Lower risk for future CD-related complications (fistula, stenosis) and/or abdominal surgery.
- v) If the patient is not yet diagnosed with CD and negative for all markers, report that no serology associated with CD was found.
- vi) In order to exclude the possibility of ulcerative colitis (UC), it is recommended to further test IBDX panel negative samples for atypical pANCA (IFA, not provided by Glycominds).
  - a) If patient is found *positive* for IFA and *negative* for IBDX panel, report that serology associated with UC was found.
  - b) If patient is found *negative both* for IFA and for the IBDX panel, report that no IBD-associated serology was found.

The following flowchart summarizes IBDX panel interpretation:



## PERFORMANCE

### Differentiation between Crohn's Disease, Ulcerative Colitis and non-IBD

#### Study I (Seow et al. 2009)

Sera of 915 Canadian patients (517 CD, 301 UC and 97 healthy controls (HC)) were tested retrospectively in a blinded fashion for the presence of IBDX markers and for atypical pANCA. IBD (IFA) patients' diagnosis was based on clinical, endoscopic, histological and radiological criteria.

The following tables outline the diagnostic performance for each assay independently:

CD vs. UC						
	<i>gASCA</i>	<i>ACCA</i>	<i>ALCA</i>	<i>AMCA</i>	<i>Anti-C</i>	<i>Anti-L</i>
Sensitivity (%)	60.7	8.7	19.7	12.2	10.25	17.99
Specificity (%)	85.38	97.01	92.36	96.7	96.68	97.67
Positive predictive value (%)	87.71	83.33	81.6	86.3	88.33	90.29
Negative predictive value (%)	55.87	38.22	40.12	39.06	38.79	40.7
Efficiency (%)	73.06	52.86	56.05	54.4	53.4	57.33

IBD vs. HC		gASCA	ACCA	ALCA	AMCA	Anti-C	Anti-L
Sensitivity (%)		42.77	6.46	15.04	8.58	7.05	12.22
Specificity (%)		89.69	96.91	93.81	96.91	97.94	97.94
Positive predictive value (%)		97.33	94.83	95.52	96.05	96.77	99.05
Negative predictive value (%)		15.16	10.56	11.18	10.78	10.72	11.39
Efficiency (%)		66.23	51.69	54.42	52.75	52.5	55.6

### Study II (Rieder et al. 2010)

The sera of 824 German patients (363 CD, 130 UC, 74 other GI disease, and 257 non-IBD)

were tested retrospectively in a blinded fashion for the presence of IBDX markers and for atypical pANCA. IBD (IFA) patients' diagnosis was based on clinical, endoscopic, histological and radiological criteria.

The following tables outline the diagnostic performance for each assay independently: The following tables outline the diagnostic performance for each assay independently:

CD vs. UC		gASCA	ACCA	ALCA	AMCA	Anti-C	Anti-L
Sensitivity (%)		63.1	16.8	25.6	27.3	25.0	25.6
Specificity (%)		91.5	93.1	92.3	93.1	89.5	92.7
Positive predictive value (%)		95.4	87.1	90.3	91.7	87.4	91.1
Negative predictive value (%)		47.0	28.6	30.8	31.4	29.1	30.0
Efficiency (%)		77.3	54.9	58.9	60.2	59.1	57.25

CD vs. Non-IBD		gASCA	ACCA	ALCA	AMCA	Anti-C	Anti-L
Sensitivity (%)		63.1	16.8	25.6	27.3	25.0	25.6
Specificity (%)		84.4	88.7	88.3	91.1	87.6	90.4
Positive predictive value (%)		85.1	67.8	75.6	81.1	74.4	79.3
Negative predictive value (%)		61.8	43.0	45.7	47.0	44.7	45.6
Efficiency (%)		73.75	52.75	56.9	59.2	56.3	58

## Prediction of CD complications/abdominal surgery

Both Seow et al. 2009 and Rieder et al. reported on strong association between being positive for multiple IBDX markers and having complications (fistula, stricture) and/or an abdominal surgery. Rieder et al. 2010 reported on IBDX biomarker panel prediction value in prospective study. 149 CD patients were recruited and tested prospectively in a blinded fashion for the presence of IBDX markers. All clinical assessments were collected before or within 20 days of sample procurement 142 of those completed a follow-up time of at least 3 years. 83% of the patients were within 1<sup>st</sup> year of diagnosis. 76 patients were complications and surgery free at baseline, 49 patients had complications but not surgery and 17 patients had surgery but not complications. Time to next event (complications or surgery for naïve group or surgery for prior complication group or complication for prior surgery group) was measured and HZ was calculated both in Kaplan-Meier and Cox-proportional Hazard regression.

The following table summarizes the results.

### Risk\* for CD related complications/ abdominal surgery

Number of positive markers	Complication/abdominal surgery free at baseline	Prior Complication	Prior Surgery
1	HR of 2.2 (95% CI: 0.74 to 6.5) p=0.16	HR of 1.8 (95% CI: 0.61 to 5.4) p=0.29	HR of 2.6 (95% CI: 0.58 to 12) p=0.21
2 or more	HR of 2.8 (95% CI: 1.2 to 6.4) p=0.016	HR of 2.5 (95% CI: 1.03 to 6.1) p=0.043	HR of 3.6 (95% CI: 0.12 to 12.0) p=0.023
3 or more	HR of 3.1 (95% CI: 1.2 to 8.1) p=0.019	HR of 2.6 (95% CI: 0.92 to 7.2) p=0.072	HR of 2.8 (95% CI: 0.80 to 9.6) p=0.11

\* Adjusted Time-to-event Analysis, Cox Proportional Hazard Regression Model  
Markers included in analysis are gASCA, ACCA, ALCA, AMCA., anti-C and anti-L  
\*p<0.05 chi square versus group of negative for all markers.  
HR – Hazard ratio to next event.

## REFERENCES

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## ASSAY SCHEME

	Action	Material	Description
1	<b>Preparation</b>	All reagents Patient samples	Bring to room temperature (20 - 26°C) Dilute (1:101) in IBDX Sample Diluent
2	<b>Add</b>	Pos. / Neg. Controls	50 µL each
		Calibrator	50 µL
		Diluted samples	50 µL each
3	<b>Incubate</b>		30 minutes at room temperature (20 - 26°C)
4	<b>Wash</b>		Wash with 4x 300 µL diluted Wash Concentrate (x1)
5	<b>Add</b>	HRP Conjugate	100 µL all wells
6	<b>Incubate</b>		30 minutes at room temperature (20 - 26°C)
7	<b>Wash</b>		Wash with 4x 300µL diluted Wash Concentrate (x1)
8	<b>Add</b>	TMB Chromogen	100 µL all wells
9	<b>Incubate</b>		30 minutes at room temperature (20 - 26°C). Protect from light!
10	<b>Add</b>	HRP Stop Solution	100 µL all wells
11	<b>Incubate</b>		Mix gently by tapping then let stand 2-5 minutes at room temperature (20 - 26°C)
12	<b>Read</b>		Read OD at 450 nm

## SAFETY PRECAUTIONS

- **This kit is for *in vitro* use only.** Glycominds Diagnostics (2014) Ltd. and its authorized distributors shall not be liable for damages indirectly or consequentially brought about by changing or modifying the procedure indicated. The assay should be performed by trained technical staff only.
- Some of the reagents contain small amounts of sodium azide (< 0.09 % w/v), and proclin (0.05 % v/v) as preservatives. They must not be swallowed or allowed to come into contact with eyes or skin.
- One kit component – Stop Solution – contain a hazardous reagent in an amount that requires labelling. In case of contact with eyes, rinse immediately with plenty of water and seek medical advice; after contact with skin, wash immediately with plenty of soap-suds. Wear suitable protective clothing and gloves. In case of accident or if you feel unwell, seek medical advice immediately (show the label where possible).
- Source materials derived from human body fluids or organs used in the preparation of this kit were tested and found negative for HBsAg and HIV, as well as for HCV antibodies. However, no known test guarantees the complete absence of such viral agents. Therefore, handle all components and all patient samples as if potentially hazardous.
- Since the kit contains potentially hazardous materials, the following precautions should be observed: Do not smoke, eat or drink while handling kit material. Always use protective gloves and coats. Never pipette material by mouth. Wipe up spills promptly, washing the affected surface thoroughly with a decontaminant.
- Avoid release into the environment. Observe local environmental regulations when disposing of wastes.

## TROUBLESHOOTING

Problem:	Possible causes:	Solution:
<i>Control values out of range</i>	1. Incorrect temperature, timing, improper pipetting, reagents not mixed.	1. Check that the time and temperature were correct. Pipette carefully. Repeat assay
	2. Optical pathway not clean.	2. Check for dirt or air bubbles. Wipe bottom of plate and reread.
<i>All results negative</i>	One or more reagents not added or added in wrong sequence.	Recheck procedure. Check for unused reagent. Repeat assay.
<i>All assay results yellow</i>	1. Contaminated buffers or reagents.	1. Check all solutions for turbidity.
	2. Washing solution contaminated.	2. Use clean container. Check quality of water.
	3. Improper dilution of sample.	3. Dilute sample appropriately. Repeat assay.
<i>Poor precision</i>	1. Pipette delivery CV greater than 5%.	1. Check calibration of pipette.
	2. Samples or reagents not mixed sufficiently or not equilibrated to room temperature.	2. Mix all reagents gently but thoroughly and equilibrate to room temperature.
	3. Reagent addition taking too long- and inconsistent-time intervals.	3. Develop consistent uniform technique and/or use automated equipment.
	4. Washing not consistent; trapped bubbles; washing solution left in the wells.	4. Check that all wells are filled and aspirated uniformly. Dispense liquid above level of reagent in well. After the last wash, empty the wells by tapping on an absorbent tissue.