IMMUNODOT™
BORRELIA (LYME) WITH RECOMBINANT PROTEIN

IVD For In Vitro Diagnostic Use

INTENDED USE

The ImmunoDOT Borrelia (Lyme) test is an enzyme immunoassay (EIA) dot-blot test for the qualitative detection of *Borrelia* antibodies. Both species specific (anti-P39) and related *Borrelia burgdorferi* antibodies (IgG, IgM, and IgA) are detected in serum as an aid in the diagnosis of Lyme disease.

SUMMARY AND EXPLANATION

Lyme borreliosis is reported to occur in North America, Europe, and Asia (1) (2) (3) (4) (5). Lyme disease has been present in Europe for years, but was first recognized in the United States in 1975 in Lyme, Connecticut. The disease, transmitted through the bite of a tick infected with the spirochete, *B. burgdorferi*, exhibits a variety of symptoms which may be confused with immune and inflammatory disorders. Inflammation around the tick bite eventually causing skin lesions, erythema chronicum migrans (ECM), is the first stage of disease. *B. burgdorferi* disease is also associated with neurologic or cardiac symptoms (stage 2) or arthritic symptoms (stage 3). A definitive distinction between stages is not always seen. In some cases, these secondary symptoms may occur even though the patient does not remember a tick bite or rash.

The criteria for the diagnosis of Lyme borreliosis are not clearly defined. Unless the typical ECM lesions are present, serological diagnosis is necessary to identify patients exposed to the agent. However, cross-reactions within the *Borrelia* genus and other cross-reactions (e.g., flagellin reactions with spirochetes and membrane reactions with bacterial membrane proteins) have limited the reliability of *B. burgdorferi* serology.

For confirmation of *B. burgdorferi* specific antibodies, ImmunoDOT Borrelia (Lyme) Test also measures the specific antibody response against the 39 kilodalton (P39) protein (6) (7). Antibodies against P39, unlike the antibodies to flagellin which cross-react with other spirochetal flagellins, are specific to *B. burgdorferi* and conserved among North American and European isolates (7). Additional unpublished studies have determined that other species of *Borrelia* (*hermsii, parkeri, turicatae, and coriaceae*), *Leptospira* (*icterohaemorrhagiae and canicola*), and *Treponema* (*pallidum and phagedenis*) do not contain the *B.* burgdorferi P39 antigen. Because P39 protein is highly antigenic, but constitutes only a small fraction of the protein in the organism, it may not be detected in other assay systems (e.g., western blots, EIA, IFA, etc.) which are not enriched with P39 protein.

ASSAY PRINCIPLE

ImmunoDOT utilizes an enzyme-linked immunoassay (EIA) dot technique for the detection of antibodies. Various levels of antigens are dispensed as discrete dots onto a solid membrane. Because the *B. burgdorferi*-specific P39 protein represents a small fraction of *Borrelia* protein, assay sensitivity is improved by addition of recombinant P39 antigen to the whole *Borrelia* cell extract in the first *Borrelia*-reactive dot. To improve assay specificity serum is absorbed in a Diluent containing *E. coli* proteins. After specimen is absorbed with *E. coli* extract, an assay strip is inserted, allowing patient antibodies reactive with the test antigen to bind to the strip's solid support membrane. In the second stage, the reaction is enhanced by removal of non-specifically bound materials. During the third stage, alkaline phosphatase-conjugated anti-human antibodies are allowed to react with bound patient antibodies. Finally, the strip is transferred to enzyme substrate reagent, which reacts with bound alkaline phosphatase to produce an easily seen, distinct dot.

REAGENTS

Assay Strip: positive human antibody control, negative control, *B. burgdorferi* (strain B31) partially purified sonicate of whole organism blended with purified P39 recombinant protein, partially purified sonicate of whole organism, P39 recombinant protein, and flagellin extracted and purified from *B. burgdorferi* (strain B31)
Diluent (#1): buffered diluent (pH 6.2-7.6) containing solubilized E. coli, protein stabilizers with <0.1% NaN_3
Enhancer (#2): sodium chloride with <0.1% NaN_3
Conjugate (#3): alkaline phosphatase conjugated goat anti-human antibodies in buffered diluent (pH 6.2-8.5) with <0.1% NaN_3
Developer (#4): 5-bromo-4-chloro-3-indolyl phosphate and p-nitro blue tetrazolium chloride in buffered diluent (pH 9-11) with <0.1% NaN_3

WARNINGS AND PRECAUTIONS

For In-Vitro Diagnostic Use. ImmunoDOT reagents have been optimized for use as a system. Do not substitute other manufacturers' reagents or other ImmunoDOT Assay System reagents. Dilution or adulteration of these reagents may also affect the performance of the test. Do not use any kits beyond the stated expiration date. Analytic quality water must be used. Close adherence to the test procedure will assure optimal performance. Do not shorten or lengthen stated incubation times since these may result in poor assay performance.

Sodium azide may react with lead and copper plumbing to form highly explosive metal azides. It may be harmful if enough is ingested (more than supplied in kit). On disposal of liquids, flush with a large volume of water to prevent azide build-up (8). This dilution is not subject to GHS, US HCS and EU Regulation 2008/1272/EC labeling requirements.

The safety data sheet (SDS) is available at support.genbio.com or upon request.

Human source material. Material used in the preparation of this product has been tested and found non-reactive for hepatitis B surface antigen (HBsAg), antibodies to hepatitis C virus (HCV), and antibodies to human immunodeficiency virus (HIV-1 and HIV-2). Because no known test method can offer complete assurance that infectious agents are absent, handle reagents and patient samples as if capable of transmitting infectious disease (9). Follow recommended Universal Precautions for bloodborne pathogens as defined by OSHA (10), Biosafety Level 2 guidelines from the current CDC/NIH Biosafety in Microbiological and Biomedical Laboratories (11), WHO Laboratory Biosafety Manual (12), and/or local, regional and national regulations.

STORAGE

Store reagents and assay strips at 2-8°C. Reagents must be at room temperature (15-30°C) before use. Avoid contamination of reagents which may produce invalid results. Assuming good laboratory practices are used, opened reagents remain stable as indicated by the expiration date.

SPECIMEN COLLECTION AND HANDLING

ImmunoDOT Borrelia (Lyme) Test is performed on serum. Hemolyzed or lipemic serum has not been shown to be an acceptable specimen. Single specimens are used to assess exposure to *B. burgdorferi*. Two specimens collected at different times from the same individual are used to show seroconversion. The test requires approximately 10 µL of serum. Serum is collected according to standard practices and may be stored at 2-8°C for up to five days. Serum may be frozen below -20°C for extended periods.

MATERIALS PROVIDED

Assay Strips	Conjugate (#3)
Diluent (#1)	Developer (#4)
Enhancer (#2)	Reaction Vessels

MATERIALS REQUIRED BUT NOT PROVIDED

Workstation	Timer
Pipets	Specimen collection apparatus (e.g., finger sticking device, venipuncture equipment)
Positive Control serum	Absorbent toweling to blot dry assay strips
	Analytic quality water

SET-UP

1. Turn on Workstation and adjust to appropriate temperature if necessary. Refer to Workstation Instructions.
2. Remove 4 Reaction Vessels (per test) from the product box and insert into appropriate slots in Workstation. For the large Workstation, add water up to the fill line of the provided rinse container. For the small Workstation, use an appropriate container and sufficient water to cover all reactive windows of the assay strip.
3. Place 2 mL Diluent (#1) in Reaction Vessel #1; 2 mL Enhancer (#2) in Reaction Vessel #2; 2 mL Conjugate (#3) in Reaction Vessel #3; and 2 mL Developer (#4) in Reaction Vessel #4.
4. Appropriately label the Assay Strips.
5. If the large Workstation is used, insert the label end of the assay strip into the Strip Holder, one per groove, taking care not to touch the assay windows.

ASSAY PROCEDURE

1. Add 10 µL serum to Reaction Vessel #1 and incubate in the Workstation for 30-60 minutes.
2. Prewet Assay Strip by immersing in water for 30-60 seconds.
3. Using several (5-10) quick up and down motions with the Assay Strip, mix thoroughly in Reaction Vessel #1. Let stand for 5 minutes.
4. Remove Assay Strip from Reaction Vessel and swish in the water. Use a swift back and forth motion for 5-10 seconds allowing for optimal washing of the Assay Strip's membrane windows.

5. Place Assay Strip into Reaction Vessel #2. Mix thoroughly with several (5-10) quick up and down motions. Let stand for 5 minutes.
6. Remove Assay Strip from Reaction Vessel #2 and swish in water as described (Step #4).
7. Place Assay Strip into Reaction Vessel #3. Mix thoroughly with several (5-10) quick up and down motions. Let stand for 15 minutes.
8. Remove Assay Strip from Reaction Vessel #3 and swish in water as described (step #4). DO NOT remove the Assay Strip from the water.
9. Allow the Assay Strip to stand in the water for 5 minutes.
10. Remove Assay Strip from water and place into Reaction Vessel #4. Mix thoroughly with several (5-10) quick up and down motions. Let stand for 5 minutes.
11. Remove Assay Strip from Reaction Vessel #4 and swish in water as described (step #4).
12. Blot and allow Assay Strip to dry. It is imperative that tests of borderline specimens be interpreted after the Assay Strip has been allowed to dry.

READING THE ASSAY STRIP

Positive	A dot with an EASILY SEEN, distinct border is visible in the center of the window. The outer perimeter of the window must be white to pale gray.
Negative	If no dot is seen or a dot is difficult to see, interpret it as negative.

Each window of the assay strip is interpreted independently. Reactions fall into three categories:

Nonreactive	Negative reaction
Weakly reactive	The dot is not easily seen and is interpreted as negative
Reactive	Positive reaction ("dot")

Weakly reactive samples are sometimes seen and are negative reactions. Weak reactions may either indicate low level autoantibodies, nonspecific cross reacting antibodies which are found in normal subjects, or low level true reactions.

QUALITY CONTROL

The top two membrane windows of the Assay Strip are reagent controls. The top window is a positive reagent control and must be positive for further interpretation. The next window is the reagent negative control and must be negative for further interpretation. Reagent controls assure that reagents are active and that the test has been performed properly. If either reagent control is invalid, the test must be repeated. The intensity of the positive control dot must not be used as a calibrator. Positive reactions in the antigen windows of the strip may be either darker or lighter than the positive control depending on the antibody titer.

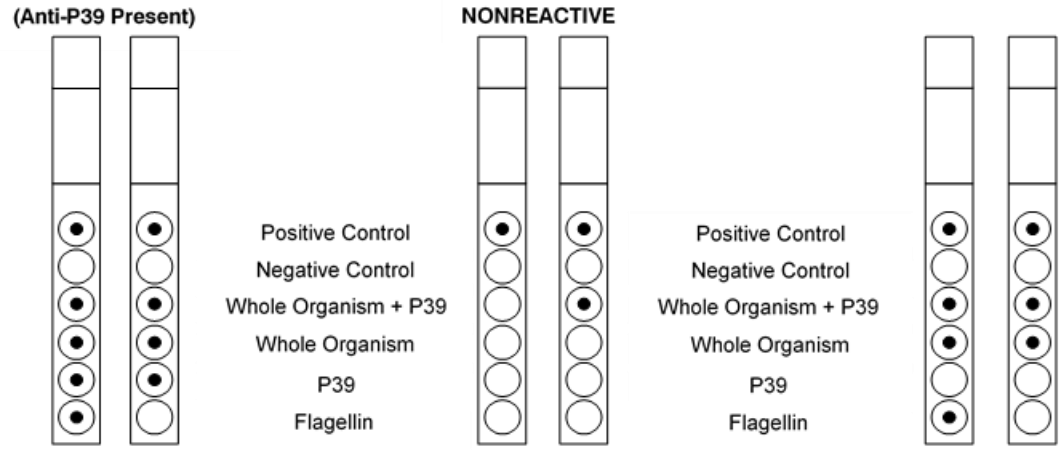
A positive control serum (Product No. 3905), reactive in the total *Borrelia* and P39 windows is available separately. Although other windows may be reactive, if the total *Borrelia* or P39 windows are nonreactive, the control test is invalid. The performance of each kit lot may be confirmed upon receipt by running a determination using the positive control serum and obtaining a positive result. The control serum should be tested in accordance with laboratory guidelines.

The assay's reagent temperature is between 42-48°C. Due to heat transfer loss, the Workstation temperature is set higher. The appropriate Workstation temperature setting is listed in the Workstation's package insert. (Contact Technical Services for additional guidance if an alternate heat source is used.)

INTERPRETATION

The first dot, nearest the label is the positive reagent control. The second dot is the negative reagent control. The third dot measures both anti-P39 and anti-whole organism (*Borrelia*) responses. The fourth dot measures anti-whole organism. The fifth dot measures anti-P39. The bottom, or sixth dot, measures anti-flagellin. Because of the complex antibody cross-reactivities reported between *B. burgdorferi* antigens and other agents, the following reporting guideline is recommended:

Whenever both P39 and whole organism (dots 3,4,and 5) are reactive, the result is reported as:	Whenever all *Borrelia* dots are nonreactive, the result is reported as nonreactive for *Borrelia* antibodies. Dot 3 (whole organism and P39) may exhibit a weak reaction while the other *Borrelia* dots are nonreactive. This result is also reported as:	All other outcomes are initially specified as reactive against the specific antigen (e.g., reactive against whole organism and nonreactive against P39, reactive against flagellin, etc.) or may simply be reported as:
"Specific B. burgdorferi detected"	**"Borrelia antibody not detected"**	**"Borrelia antibody detected"**



- Negative results in suspected early Lyme disease should be repeated in 4-6 weeks.
- Whole organism or flagellin positive results should be interpreted with caution. Sera from patients with other spirochetal diseases may also give positive results.
- If the reaction pattern obtained is not illustrated by one of the six examples above, contact GenBio Technical Service at (800) 288-4368

In all cases an additional test to clarify the result is recommended before a definitive interpretation is reported. In cases in which clarification is not possible, another specimen collected two to six weeks later to measure an antibody response titer rise may provide additional information for diagnosis.

1. **Flagellin:** Because significant cross-reactivity between spirochetal and bacterial flagellin is possible, if the bottom dot (Dot 6), flagellin, is reactive and P39 (Dot 5) is nonreactive, an alternative test method such as western blot (13) is recommended. If syphilis is suspected, a non-treponemal (e.g. RPR) test is recommended.
2. **Whole Organism:** Because of the known lack of specificity of the antibody response against whole *Borrelia* organism, if P39 (Dot 5) is nonreactive, a second serology method using a different type of antigen preparation to clarify the reactivity is recommended. Although the western blot method (13) has been shown to be no more reliable than any other method for Lyme borreliosis serological diagnosis, the method does provide additional information about antigen reactivity and may help clarify such indeterminate samples.

LIMITATIONS

- The diagnosis of Lyme disease should be based on interpretation of test results in combination with the patient's clinical signs and symptoms, other clinical and laboratory test results, and epidemiological data.
- Anti-p39 positive results should be interpreted in conjunction with other positive serological test results for Lyme disease.
- Negative results do not rule out the diagnosis of Lyme disease. Some patients may not produce significant humoral response to *B. burgdorferi,* early antibiotic therapy may suppress antibody response, P39 may not be expressed by all

strains of *B. burgdorferi*, and/or detectable antibodies to P39 may not be produced in some Lyme disease patients, especially in early disease stages. Negative results in suspected early Lyme disease should be repeated in 4-6 weeks.

- The continued presence or absence of antibodies cannot be used to determine the success or failure of therapy.
- Testing should not be performed as a screening procedure for the general population. The predictive accuracy of a positive or negative serologic result depends on the pretest likelihood of Lyme disease being present. Testing should only be done when other laboratory tests and clinical evidence suggest the diagnosis of Lyme disease.
- A single positive result only indicates previous immunologic exposure; level of antibody response is unreliable in determining active infection or disease stage.
- Whole organism or flagellin positive results should be interpreted with caution. Sera from patients with other spirochetal diseases (syphilis, yaws, pinta, leptospirosis, relapsing fever, periodontal disease, etc.) may also give positive results. If syphilis is suspected, a non-treponemal (e.g., RPR) test may be used to rule out this cause.
- Patients with connective tissue autoimmune diseases (rheumatoid arthritis, ANA, SLE, etc.) may have antibodies which cross-react with whole organism antigen(s).
- Patients with other bacterial and viral infections such as Rocky Mountain Spotted Fever, EBV, CMV, and HIV may also have antibodies which cross-react with *B. burgdorferi* antigens.

EXPECTED RESULTS

In general, three types (stages) of Lyme disease are recognized: erythema chronicum migrans (ECM), neurologic, and arthritic. Antibody levels are generally low or absent during early (ECM) infection. Most symptomatic patients will have either no antibody or highly cross-reactive antibody during the first 1-2 weeks after tick bite and the antibody titer will rise and become more specific with time. Highest antibody levels are seen in chronic arthritis subjects.

The number of antibody positive subjects in a population depends on several factors: 1) prevalence of the causative agent, 2) assay used to detect antibody, and 3) clinical screening criteria to select tested subjects. Because early assays lacked accuracy (14), the number of antibody positive subjects in a population at present (1991) is highly dependent on the assay used. Whenever a suitably accurate test is used, few positives should be detected in a randomly screened population in a non-endemic area. On the other hand, if patients with typical ECM signs in an endemic region are tested, many positive results are expected.

Disagreement between assays which do not use an absorbent and those assays like ImmunoDOT which do use an absorbent are expected. Fawcett (15) (16) has shown that assays using an absorbent were equally sensitive to those without an absorbent and that the absorbed assays were significantly more specific. In the course of primary disease, the highly specific anti-P39 antibody may appear after earlier, non-specific antibody. Anti-P39 was positive in all Stage 3 (arthritic) cases tested.

PERFORMANCE CHARACTERISTICS

REPRODUCIBILITY

Because ImmunoDOT products are discrete, qualitative tests, within assay reproducibility is not applicable. Between assays reproducibility is demonstrated by testing the same series of positive samples. In all cases, the samples interpreted as positive in the ImmunoDOT product are repeatedly positive (six replicates) in the ImmunoDOT product.

CORRELATION

The specificity of ImmunoDOT Borrelia (Lyme) Test using 196 sera from asymptomatic blood donors collected from areas other than the upper Midwestern and Northeastern United States (hyperendemic regions) is 100% specific.

The specificity was also determined using 168 sera from non-Lyme, symptomatic patients (Table 1), with autoimmune disease, pneumonia, mononucleosis, lymphadnopathy, rheumatoid arthritis, syphilis, and AIDS. No samples were positive for anti-P39 (100% specificity); however, all syphilis samples reacted with the dots containing *Borrelia* antigens other than P39.

Table 1: Specificity - Lyme Disease Negative Patients

Sample	Nonreactive	Borrelia +	B. burgdorferi +
SLE	5	0	0
JRE	11	0	0
ANA+	36	2	0
CMV IgM+	25	0	0
Heterophile+	24	1	0
RF +	24	1	0
HIV +	15	0	0
RMSF+	12	0	0
Syphilis+	0	12	0

Sera from eighty-four patients at three sites diagnosed with Lyme borreliosis were used to measure assay sensitivity. Diagnoses were based on epidemiological, clinical, and serological criteria. These studies are presented in Table 2.

Table 2: Sensitivity - Diagnosed Lyme Disease Patients

Sample	Nonreactive	Borrelia +	B. burgdorferi +
Site 1	0	45	41
Site 2	1	11	11
Site 3-Early (ECM)*	18	4	0
Site 3-Late	0	5	5
Totals	19	65	57

* All 22 samples were EIA and western blot negative.

Assay sensitivity for the samples which were not classified as to stage of disease was 98% (56/57). The one nonreactive sample was also nonreactive in other EIA tests, IFA, and western blot. Fifty-two (91%) were anti-P39 reactive. Consistent with expectations that antibody is either absent or at low titer in early cases, four of 22 sera (18%) from early Lyme cases with ECM, but nonreactive in EIA and western blot analysis, were ImmunoDOT reactive for *Borrelia* antibody. None were anti-P39 reactive. All sera from the five late stage cases were positive.

Assay specificity is 100% in asymptomatic normals. Specificity in Lyme disease negative patients other than those with syphilis is 97%. None were anti-P39 reactive. All 12 syphilis subjects contained anti-*Borrelia*, but none were anti-P39 reactive.

Eighty-seven samples were evaluated at Site 1 using the ImmunoDOT and anticomplement immunofluorescence (ACIF) methods. The comparative results are shown in Table 3.

Table 3: Correlation to ACIF

ACIF	Nonreactive	Borrelia +	B. burgdorferi +
Reactive	0	45	41
Nonreactive	38	4	0

Twenty-four proficiency samples from a state public health laboratory and a national proficiency program were also used to evaluate ImmunoDOT performance. The expected results (reactive or nonreactive) were based on consensus. That is, the samples were classified as reactive if almost all reported results were reactive, and vice-versa for nonreactive specimens. ImmunoDOT detected 14 out of 14 nonreactive samples correctly and identified 10 of 10 reactive samples as *Borrelia* antibody reactive, but only identified five of the ten as anti-P39 reactive. These results indicate that the five samples without anti-P39 are either cross-reactive or lack specific anti-P39, presumably due to the early stage of disease.

BIBLIOGRAPHY

1. **Ali, C, et al.** Clinical manifestations and epidemiological characteristics of Lyme disease in Hailin County, Heilongjiang Province. China. *Ann NY Acad Sci.* 1988, Vol. 539, p. 302.
2. *Lyme Disease-United States: 1987 and 1988.* **US Centers for Disease Control.** 1989, Morbidity Mortality Weekly Report, Vol. 38, p. 668.
3. *Lyme Disease: Canada.* **US Centers for Disease Control.** 1989, Morbidity Mortality Weekly Report, Vol. 38, p. 677.
4. *Lyme borreliosis in the Soviet Union: a cooperative US-USSR report.* **Dekonenko, E J, et al.** 1988, J Infect Dis, Vol. 158, p. 748.
5. *The global distribution of Lyme disease.* **Schmid, G P.** 1985, Rev Infect Dis, Vol. 7, p. 41.
6. *Antibody to a 39-kilodalton Borrelia burgdorferi antigen (P39) as a marker for infection in experimentally and naturally inoculated animals.* **Simpson, W J, et al.** 1991, J Clin Microbiol, Vol. 29, p. 236.
7. *Reactivity of human Lyme borreliosis sera with a 39-kilodalton antigen specific to Borrelia burgdorferi.* **Simpson, W J, Schrumpf, M E and Schwan, T G.** 1990, J Clin Microbiol, Vol. 28, p. 1329.
8. **US Centers for Disease Control.** *Manual Guide – Safety Management No. CDC–22 Decontamination of Laboratory Sink Drains to Remove Azide Salts.* Atlanta : Centers for Disease Control, 1976.
9. —. *HHS Publication No. (CDC) 93-8395, 3rd ed: Biosafety in Microbiological and Biomedical Laboratories.* Washington DC : US Government Printing Office, 1993.
10. **US Department of Labor, Occupational Safety and Health Administration.** *29 CFR Part 1910.1030, Occupational safety and health standards, bloodborne pathogens.*
11. **US Department of Health and Human Services.** *HHS Publication No. (CDC) 21-11: Biosafety in Microbiological and Biomedical Laboratories. 5th ed.* Washington DC : US Government Printing Office, 2009.
12. **World Health Organization.** *Laboratory Biosafety Manual 3rd ed.* Geneva : World Health Organization, 1991.
13. *Use of Western Blot and Enzyme-Linked Immunosorbent Assays to Assist in the Diagnosis of Lyme Disease.* **Rose, C D, et al.** 3, 1991, Pediatrics, Vol. 88, p. 465.
14. *First National Conference on Lyme Disease Testing.* **US Centers for Disease Control and US Food and Drug Administration.** 1990.
15. *Adsorption with a Soluble E. coli Antigen Fraction (SEF) Improved Specificity of ELISA Tests for Lyme Disease.* **Fawcett, P T, et al.** 5, 1991, J Rheum, Vol. 18, p. 705.

16. *An Adsorption Procedure to Increase the Specificity of Enzyme-Linked Immunosorbent Assays for Lyme Disease without Decreasing Sensitivity.* **Fawcett, P T, O'Brien, A E and Doughty, R A.** 0, 1989, Arth Rheum, Vol. 30, p. 1.

QUICK REFERENCE PROCEDURE

IMMUNODOT BORRELIA (LYME)

Set-Up

- Make sure Workstation is at temperature.
- Place reaction Vessels into slots in Workstation and add water to the rinse container.
- Place 2 mL Diluent (1) in Vessel #1; 2 mL Enhancer (2) in Vessel #2; 2 mL Conjugate (3) in Vessel #3; and 2 mL Developer (4) in Vessel #4.

Procedure

- Add 10 µL serum to Vessel #1 and incubate 30-60 min.
- Prewet assay strip in water for 30 - 60 seconds.
- Place strip in Vessel #1, mix, let stand 5 min.
- Remove strip, place in water, swish 5-10 sec.
- Place strip in Vessel #2, mix, let stand 5 min.
- Remove strip, place in water, swish 5-10 sec.
- Place strip in Vessel #3, mix, let stand 15 min.
- Remove strip, place in water, let stand 5 min.
- Place strip in Vessel #4, mix, let stand 5 min.
- Remove strip, place in water, swish, blot, dry, and read

To place an order for ImmunoDOT products, contact your local distributor, or call GenBio directly for the distributor nearest you and for additional product information.
For assistance, please call toll-free 800-288-4368.

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