# Confirmatory test of HBsAg

## *KAPG4SAO*

DIAsource ImmunoAssays S.A. - Rue du Bosquet, 2 - B-1348 Louvain-la-Neuve - Belgium

# Confirmatory test of HBsAg

Test kit for the confirmation of the existence of HBsAg in the specimens

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*FOR RESEARCH USE ONLY*

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## 1) Intended Use

Confirmatory test of HBsAg is a test kit for the confirmation of the existence of HBsAg in the specimens. When Confirmatory test of HBsAg is used in combination with HBsAg Elisa, it can confirm the presence or absence of HBsAg in specimens which are positive for HBsAg in screening test.

## 2) Summary and Test Explanation

Hepatitis B surface antigen (HBsAg) is a serological marker typical of hepatitis B infection. Viral hepatitis of type B is usually accompanied by the appearance of hepatitis B surface antigen in the blood which can be generally detected days weeks as early as 2 to 3 weeks before clinical symptoms begin appear.

Its titer reaches a peak at the time when jaundice and changes in the levels of liver-specific enzymes like ALT and AST are observed and gradually decreases following its elimination. When serum or plasma samples react positive in a HBsAg screening assay, an in vitro confirmation of the presence of hepatitis B surface antigen (HBsAg) have to be always carried out.

Assays for HBsAg are routinely used to screen of blood donation in order to reduce hepatitis B transmission by blood transfusion. Furthermore, these assays are used to diagnose a suspected HBV infection and to monitor the status of infected individuals i.e. a chronic hepatitis B virus infection.

## 3) Test description

Confirmatory test of HBsAg is specially formulated from high potency and strong neutralizing antiserum which contains antibody to HBsAg. Confirmatory test of HBsAg adopts to the basic principles of the neutralization of antibody with antigen. Even the specimen with very high level of HBsAg still can be accurately confirmed without diluting the specimen in advance. After the first incubation of the procedure, the plate only absorbed part of the HBsAg content in the specimen. Then the unbound HBsAg is removed with the serum by washing the plate. Therefore, the anti-HBs reagent needs to neutralize only that part of HBsAg which has been coupled to the plate.Then only limited quantity of HBsAg left to react with enzyme labeled antibody that added to the test later on.

## 4) Description of Materials Provided & Product Code system

- Item 1 - 2 in the following reagent table should be refrigerated at 2-8°C. For long period storage, freezing at -20°C is recommended.

| ITEMS | Components | Description | Qt. per 96 tests |
|---|---|---|---|
| (1) | CONTROL + <br> Anti-HBs Positive Control | One bottle (3ml), containing guinea pig antiserum against human HBsAg ad and ay subtype and protein stabilizers. Preservatives: 0.003% Gentamycin and 0.01% Thimerosal. | 1 bottle, 3 ml |
| (2) | CONTROL - <br> Anti-HBs Negative Control | One bottle (3ml), containing normal human serum and protein stabilizers. Preservatives: 0.003% Gentamycin and 0.01% Thimerosal. | 1 bottle, 3 ml |

● **OTHER MATERIALS AND DEVICES NEEDED**

| ITEMS | Components |
|---|---|
| (1) | HBsAg Elisa or equivalent HBsAg screening kits. |
| (2) | Accessories for performing HBsAg test. |

## 4.1) Storage condition and Stability of the kit and components

| Kit/components | Storage temp. | State | Stability |
|---|---|---|---|
| Anti-HBs Positive Control | +2～+8°C | Original | 12 months |
| | | Once open | 1 month |
| Anti-HBs Negative Control | +2～+8°C | Original | 12 months |
| | | Once open | 1 month |

# 5) Instructions for Use

## 5.1) Warnings:

5.1.1) This reagent kit is for professional use only.

5.1.2) This reagent kit is for *research use only*.

5.1.3) 

Bring all kit reagents and samples to room temperature (+20 to +30°C) and mix carefully before use.

5.1.4)  Do not use reagent beyond its expiration date.

5.1.5) Do not interchange reagents between different lots.

5.1.6) Do not pipette with the mouth.

5.1.7) Do not smoke or eat in areas where specimens or reagents are handled.

5.1.8) All kit components and specimens should be regarded as potential health hazards. It should be used and discarded according to your laboratory's safety procedures. Such safety procedures probably include the wearing of protective gloves and avoiding the use of aerosols.

5.1.9) Potential infectious specimens and non-acid containing spills or leakages should be wiped up thoroughly with 5% sodium hypochlorite or treated in accordance with your practice for potential bio-hazard control.

5.1.10) Prior to disposing used specimens and kit reagents as general waste; it should be treated in accordance with the local practice of potential bio-hazardous waste or treated as follows:

Both liquid and solid waste should be autoclaved at +121°C for at least 30 minutes.

Solid waste can also be incinerated.

Non-acidic liquid waste can be treated with sodium hypochlorite diluted to a final concentration of 1%.

Acidic liquid wastes must be neutralized before treatment with sodium hypochlorite as mentioned above and should stand for 30 minutes to obtain effective disinfection.

5.1.11) 

2N Sulfuric Acid is an irritant to skin, eyes, respiratory tract and mucous membranes. Avoid contact of the 2N sulfuric acid with skin and mucous membranes. In case of contact, flush immediately with abundant amounts of water. In case of inhalation, find fresh air and seek medical attention in case of pain.

5.1.12) 

Chromogenic TMB concentrate contains organic solvent, which is flammable. Chromogenic TMB concentrate contains dimethyl sulfoxide, an irritant to skin and mucous membranes.

5.1.13) 

Although all human sourced material are tested non reactive for Anti-HCV and Anti-HIV, and inactivated at +56°C for one hour, the reagent shall be handled as potential infectious material.[*2]

## 5.2) Specimen Collection and Preparation for Analysis

5.2.1) No special preparation of the patient is required prior to blood collection. Blood should be collected by approved medical techniques.

5.2.2) Either serum or plasma specimens can be used with this test kit. Whole blood specimen should be separated as soon as possible in order to avoid hemolysis. Any particulates (e.g. fibrin clots, erythrocytes) contained in the specimen should be removed prior to use.

5.2.3) Specimens must be stored at +2 to +8°C and avoid heat-inactivation to minimize deterioration. For long-term storage, they should be frozen below -20°C. Storage in self-defrosting freezer is not recommended.

5.2.4) Frozen specimens must be thoroughly thawed and mixed homogenously before test.

5.2.5) Avoid multiple freeze-thaw procedures.

**WARNING**

1.The specimen must not contain any compounds of AZIDE, which inhibits the peroxidase activity.

2. Incompletely coagulated sera and microbial-contaminated specimens should not be used.

## 5.3) Storage conditions and Stability of Reagents

5.3.1) 

The kit must be stored at +2 to +8°C. Do not freeze.

5.3.2) 

Return reagents to +2 to +8°C immediately after use.

## 5.4) Test Procedure

5.4.1) 

Bring all reagents and specimens to room temperature (+20 to +30°C) before test

5.4.2) Write down the relative numbers of specimens and the wells on the data sheet. For each specimen and control, four wells are needed and divide the four wells into 2 groups, A group and B group (2 Wells for A and 2 wells for B) . Reserve one well for blank.

5.4.3) Add 50 µl of specimen or controls into each marked well individually.

5.4.4) Tear off the protective backing of the adhesive slip and press the slip on the plate to seal it.

5.4.5) 

Incubate the plate at room temperature ( 20 to 30°C ) for 20±4 hours.

5.4.6) At the end of incubation, wash the plate by following the plate washing procedure of HBsAg Elisa.

5.4.7) Add 50µl Anti HBs positive control to A group and equal volume of Anti HBs negative control to B group.

5.4.8) Cover the plate with the adhesive slip and incubate at 40±1°C for 1 hour.

5.4.9) Remove the slip and add 50µl of Anti-HBs · HRPO conjugate solution into each well (except the blank) Seal the plate with a new adhesive slip.

5.4.10) Incubate the plate at 40±1°C for 1 hour.

5.4.11) Repeat the step 5.4.6) to wash the plate.

5.4.12) Add 100 µl pre-mixed chromogenic TMB concentrate and substrate buffer solution (1:1) into each well. Avoided-light incubation at room temperature for half an hour.

5.4.13) Stop the reaction by adding 100µl of 2N $H_2SO$ and detect the OD 450/620-690nm (450nm reading wavelength with 620 – 690 nm reference wavelength)*[1].

## 5.5) Calculations of Results

Following the calculation methods of HBsAg Elisa to obtain the result:

5.5.1) Calculation the Negative Control mean (NCx). All values of Negative Control should meet the following criteria, otherwise those tests will be invalided.

HBsAg Elisa

$NC \leqq 0.1$ (Absorbance)

5.5.2) Calculation the average test value of group A (Ax) and B (Bx) of each specimen and control individually. The mean of B group values should meet the following criteria, otherwise it is impossible to confirm this specimen.

HBsAg Elisa : $Bx \geqq NCx + 0.05$

5.5.3) Percentage of neutralization can be calculated by the following formula:

$$\frac{Bx - Ax}{Bx - NCx} \times 100\ \% = \text{Neutralization Percentage}$$

**Note:** 1. Not to calculate the Neutralization Percentage with negative samples.

2. A weak-positive sample in the HBsAg Elisa may be negative tested in the confirmation test.

## 5.6) Interpretation of Results

5.6.1) A specimen is confirmed positive for HBsAg if the mean of B group values is equal to or greater than cutoff value (NCx + 0.05) and Neutralizing Percentage is equal to or greater than 50 %.

**Note: The Positive control must be confirmed HBsAg POSITIVE at first, or the whole experiment will be considered invalid and should be repeated again.**

5.6.2) In cases of samples with high HBsAg concentrations (e.q. saturated OD without neutralization antibodies) and neutralization percentages lower than 50 %, the test should be repeated after 1:100 dilution of the sample.

## 5.7) Troubleshooting

If the result cannot be reproduced, perform a preliminary troubleshooting by checking the possibilities listed below:

5.7.1) Improper washing procedure.

5.7.2) Contamination with positive specimen.

5.7.3) Wrong volume of sample, conjugate or substrates.

5.7.4) Contamination of the well rim with conjugate.

5.7.5) Improper specimen, such as hemolyzed serum or plasma, specimen containing sediments and specimen not thoroughly mixed before use.

5.7.6) Wrong incubation time or temperature.

5.7.7) Obstructed or partial obstructed washer aspirate/dispense head and needles.

5.7.8) Insufficient aspiration.

## 5.8) Limitations and Interferences

5.8.1) This reagent kit is to be used for un-pooled human serum or plasma only.

5.8.2) The reagent kit has not been validated for use with cadaveric samples.

5.8.3) A negative HBsAg result without other evidence should not be used to exclude an HBV infection.

5.8.4) Interfering Substances:

The following results were obtained in respective experiments:

1. No interferences with different anticoagulants such as lithium heparin, EDTA, citrate have been observed.
2. Samples containing potential interfering substances [e.g. triglycerides (lipemia), hemoglobin (hemolysis), bilirubin (icterus), monoclonal immunoglobulin components, elevated levels of autoimmune antibodies (rheumatoid factor-RF, antinuclear antibodies-ANA, or antimitochondrial antibodies-ANA)] and samples from pregnant women did not interfere with the Confirmatory test of HBsAg assay.

## 5.9) Performance Characteristics

### 5.9.1) Analytical Specificity

Spiking experiment with HBsAg material were performed with paired non-reactive serum and plasma with the three anticoagulants in order to show equivalence in the test results between serum and different types of plasma in the Confirmatory test of HBsAg.

The lipemic, hemolytic, icteric samples and samples with high monoclonal and elevated levels of autoimmune antibodies do not interfere with the test result. Pregnancy is not influencing the test result HBsAg. No false positive and false negative results are observed with samples with these characteristics.

### 5.9.2) Analytical Sensitivity

The neutralization capacity was determined using the HBsAg *ad* and *ay* reference preparations of the Paul Ehrlich Institute, Langen/Germany with 95% Confidence Interval.

The confirmatory activity of Ad subtype is 0.019 PEI U/ml.

The confirmatory activity of Ay subtype is 0.075 PEI U/ml.

The Confidence Interval has to be 95%.

### 5.9.3) Diagnostic Specificity

20 false positive samples which were reactive with the HBsAg Elisa assay were tested with the Confirmatory test of HBsAg assay to evaluate the diagnostic specificity.

**None of these samples could be confirmed with the Confirmatory test of HBsAg assay. The diagnostic specificity was 100%.**

### 5.9.4) Diagnostic Sensitivity

#### 5.9.4.1) HBV infected individuals

All more than 300 positive specimens could be confirmed with both the Confirmatory test of HBsAg and the Dade Behring Enzygnost HBsAg confirmatory assay

#### 5.9.4.2) Commercial seroconversion panels

1. The diagnostic sensitivity determined in the 15 commercial seroconversion panels to following results:

| Sample | No. of sample | Reactive | Sensitivity |
|---|---|---|---|
| HBsAg positive serum and plasma | 15 | 15 | 100% |

Diagnostic sensitivity = 15/15 = 100%

2. The results of the testing of 15 seroconversion panels with Confirmatory test of HBsAg assay as following tables:

| | HBsAg Confirmed Results from Initial Draw Date | | Reference Assay vs. Confirmatory test of HBsAg Assay |
|---|---|---|---|
| Panel ID | Reference Conf. Assay (# Days) | Confirmatory test of HBsAg Assay (# Days) | Difference in Bleed #s* |
| PHM 903 | 6 | 10 | -1 |
| PHM 904 | 7 | 18 | -1 |
| PHM 906 | 137 | 150 | -1 |
| PHM 909 | 9 | 9 | 0 |
| PHM 910 | 35 | 42 | -1 |
| PHM 918 | 7 | 12 | -1 |
| PHM 923 | 15 | 21 | -1 |
| PHM 924 | 29 | 29 | 0 |
| PHM 925 | 8 | 14 | -1 |
| PHM 926 | 13 | 15 | -1 |
| PHM 927 | 4 | 7 | -1 |
| PHM 928 | 9 | 14 | -1 |
| PHM 929 | 14 | 18 | -1 |
| PHM 931 | 19 | 19 | 0 |
| PHM 932 | 61 | 61 | 0 |
| Over all | ---- | ---- | Sum= - 11 bleed #s |
| | ---- | Average | -11/15 = - 0.73 bleed #s |

**Summary of the evaluation of all tested serocoversion panel:**

The results have shown that the Confirmatory test of HBsAg is nearly equivalent to the reference assay. On average the Confirmatory test of HBsAg test is only 0.73 bleed #s later in the 15 tested seroconversion panels in comparison with the reference assay.

### 5.9.5) Precision

#### 5.9.5.1) Intra-assay reproducibility

Intra-assay precision was determined using the HBsAg Elisa positive control sample provided and two patient serum samples of different HBsAg titer were confirmed with the Confirmatory test of HBsAg in replicates of 10 in a single run over 3 days.

| **Sample-ID** | **Day 1 Neutralization Percentage [%]** | **Day 2 Neutralization Percentage [%]** | **Day 3 Neutralization Percentage [%]** |
|---|---|---|---|
| 1 | 82,44 | 69,40 | 78,15 |
| 1 | 80,31 | 67,53 | 74,61 |
| 1 | 82,22 | 73,42 | 74,84 |
| 1 | 76,84 | 71,54 | 73,06 |
| 1 | 82,51 | 74,33 | 80,27 |
| 1 | 86,37 | 59,82 | 78,39 |
| 1 | 84,19 | 72,48 | 79,55 |
| 1 | 80,86 | 61,65 | 81,28 |
| 1 | 81,42 | 66,55 | 77,83 |
| 1 | 79,83 | 65,61 | 84,36 |
| **M** | **81,71** | **68,23** | **77,90** |
| **SD** | **2,77** | **4,93** | **3,41** |
| **CV** | **3,39** | **7,23** | **4,37** |

**Table 1:** within run precision of patient sample 1.

| Sample-ID | Day 1 Neutralization Percentage [%] | Day 2 Neutralization Percentage [%] | Day 3 Neutralization Percentage [%] |
|---|---|---|---|
| 2 | 88,63 | 78,79 | 67,89 |
| 2 | 82,15 | 69,58 | 66,05 |
| 2 | 81,94 | 77,06 | 66,80 |
| 2 | 76,80 | 68,50 | 72,90 |
| 2 | 81,41 | 73,03, | 61,63 |
| 2 | 76,69 | 67,29 | 64,75 |
| 2 | 87,26 | 75,71 | 70,50 |
| 2 | 79,44 | 67,01 | 70,64 |
| 2 | 81,49 | 75,97 | 70,29 |
| 2 | 80,68 | 72,21 | 64,90 |
| **M** | **82,01** | **72,51** | **67,63** |
| **SD** | **3,67** | **4,28** | **3,45** |
| **CV** | **4,48** | **5,90** | **5,10** |

**Table 2:** Within run precision of patient sample 2.

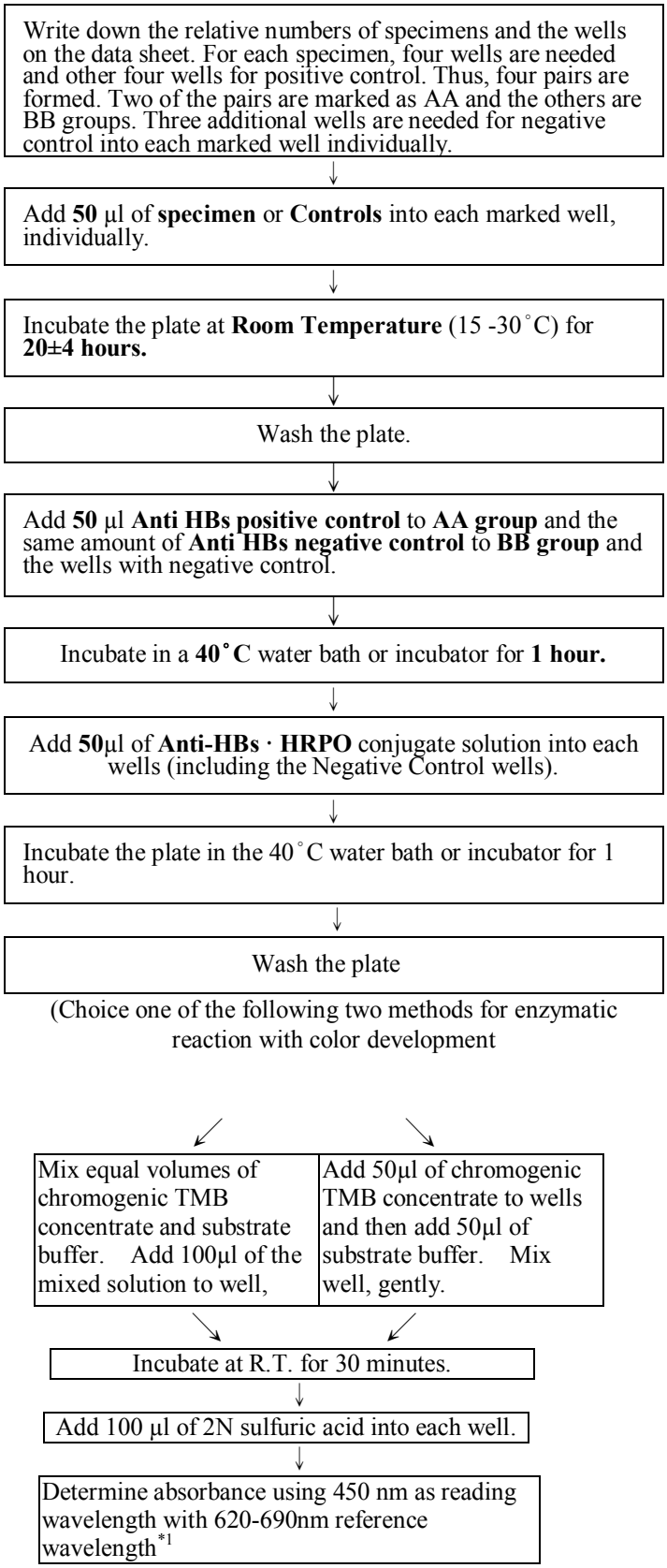
| Sample-ID | Day 1 Neutralization Percentage [%] | Day 2 Neutralization Percentage [%] | Day 3 Neutralization Percentage [%] |
|---|---|---|---|
| PC | 97,00 | 96,39 | 96,01 |
| PC | 96,40 | 95,05 | 96,02 |
| PC | 97,71 | 94,10 | 95,60 |
| PC | 96,42 | 96,16 | 94,28 |
| PC | 96,22 | 95,77 | 95,30 |
| PC | 96,55 | 94,73 | 94,16 |
| PC | 97,25 | 96,16 | 95,92 |
| PC | 96,67 | 95,35 | 96,03 |
| PC | 97,06 | 95,25 | 96,05 |
| PC | 96,64 | 96,60 | 95,01 |
| **M** | **96,79** | **95,56** | **95,44** |
| **SD** | **0.46** | **0.80** | **0.73** |
| **CV** | **0.47** | **0.83** | **0.77** |

**Table 3:** Within run precision of positive control.

**The calculated CV's ranged between 0.47% and 7.23%. (=acceptable value for an immunoassay in microtiter plate format).**

## 5.10) Flow chart of the test procedure

**Test Procedure When test with HBsAg Elisa**



## 6) Bibliography


1. Blumberg BS, Alter HJ, Visnich S. A „new“ antigen in leukemia sera. JAMA, 1965;191:101- 106.
2. Dane DS, Cameron CH, Briggs M. Virus-like particles in serum of patients with Australia-antigen-associated hepatitis. Lancet 1970; 1: 695 - 698.
3. Aach RD, Grisham JW, Paker CW. Detection of Australia antigen by radioimmunoassay. Proc Natl Acad Sci. USA 1971;68:1056-1060.
4. Kim CY, Tikes JG. Purification and biophysical characterization of hepatitis antigen. J Clin Invest. 1973; 52:1176-1186.
5. Wolters G. Cuijpers LP, Kacaki J, Schuurs AH, Enzyme linked immunosorbent assay for hepatitis B surface antigen. J Infect. Dis 1977;136:Suppl 311-377.
6. Shih JW, Cote PJ Jr, Dapolito GM, Gerin JL. Production of monoclonal antibodies against hepatitis B surface antigen (HBsAg)by somatic cell hybrids. J Virol Methods. 1980;1:257-273.
7. Hoofnagle JH, Di Bisceglie AM. Serologic diagnosis of acute and chronic viral hepatitis. Semin Liver Dis. 1991;11:73-83


## 7) Notes

*1 The reference wavelength of the photometer to be used can be 620 nm to 690 nm. However, the user should validate the photometer in combination with HBsAg Elisa before use.

*2 Incomplete inactivation of hepatitis B virus after heat treatment at +60°C for 10 hours, J. Infect. Dis. 138:242-244.

**Revision date : 2013-11-14**

| | **Used symbols** |
|---|---|
| | Consult instructions for use |
| | Storage temperature |
| | Use by |
| LOT | Batch code |
| REF | Catalogue number |
| CONTROL | Control |
| I V D | In vitro diagnostic medical device |
| | Manufacturer |
| Σ | Contains sufficient for <n> tests |
| WASH SOLN CONC | Wash solution concentrated |
| CAL 0 | Zero calibrator |
| CAL N | Calibrator # |
| CONTROL N | Control # |
| Ag 125I | Tracer |
| Ab 125I | Tracer |
| Ag 125I CONC | Tracer concentrated |
| Ab 125I CONC | Tracer concentrated |
| | Tubes |
| INC BUF | Incubation buffer |
| ACETONITRILE | Acetonitrile |
| SERUM | Serum |
| DIL SPE | Specimen diluent |
| DIL BUF | Dilution buffer |
| ANTISERUM | Antiserum |
| IMMUNOADSORBENT | Immunoadsorbent |
| DIL CAL | Calibrator diluent |
| REC SOLN | Reconstitution solution |
| PEG | Polyethylene glycol |
| EXTR SOLN | Extraction solution |
| ELU SOLN | Elution solution |
| GEL | Bond Elut Silica cartridges |
| PRE SOLN | Pre-treatment solution |
| NEUTR SOLN | Neutralization solution |
| TRACEUR BUF | Tracer buffer |
| | Microtiterplate |
| Ab HRP | HRP Conjugate |
| Ag HRP | HRP Conjugate |
| Ab HRP CONC | HRP Conjugate concentrate |
| Ag HRP CONC | HRP Conjugate concentrate |
| CONJ BUF | Conjugate buffer |
| CHROM TMB CONC | Chromogenic TMB concentrate |
| CHROM TMB | Chromogenic TMB solution |
| SUB BUF | Substrate buffer |
| STOP SOLN | Stop solution |
| INC SER | Incubation serum |
| BUF | Buffer |
| Ab AP | AP Conjugate |
| SUB PNPP | Substrate PNPP |
| BIOT CONJ CONC | Biotin conjugate concentrate |
| AVID HRP CONC | Avidine HRP concentrate |
| ASS BUF | Assay buffer |
| Ab BIOT | Biotin conjugate |
| Ab | Specific Antibody |
| SAV HRP CONC | Streptavidin HRP concentrate |
| NSB | Non-specific binding |
| 2nd Ab | 2nd Antibody |
| ACID BUF | Acidification Buffer |
| DIST | Distributor |
| TRAY | Incubation trays |
| PMSF | PMSF solution |
| | Protect from light |
| STRIP | Dot Strip |
| SUB | Substrate |
| EXTR SOLN CONC | Extraction Buffer Concentrate |
| CART | Cartridge |
| SAV HRP | Streptavidin HRP |
| PIPETTE | Pipette |
| WASH SOLN | Wash buffer |