Cat#241E

HAV-IgM

IgM ANTIBODY TO HEPATITIS A VIRUS ELISA KIT

Two-step Incubation, Antibody Capture Principle

INSTRUCTIONS FOR USE

INTENDED USE

This kit is an enzyme-linked immunosorbent assay (ELISA) for qualitative determination of IgM-class antibodies to hepatitis A virus in human serum or plasma. It is intended for use in clinical laboratories for diagnosis of acute hepatitis A and managements of patients related to infection with hepatitis A virus.

SUMMARY

Hepatitis A is a self-limited disease and chronic stage or other complications are rare. Infections occur early in life in areas where sanitation is poor and living conditions are crowded. With improved sanitation and hygiene, infections are delayed and consequently the number of persons susceptible to the disease increases. Because the disease is transmitted through the fecal-oral route in dense populated regions, an outbreak can arise from single contaminated source. The cause of hepatitis A is hepatitis A virus (HAV)-non enveloped positive strand RNA virus with a linear single strand genome, encoding for only one known serotype. HAV has four major, structural polypeptides and it localizes exclusively in the cytoplasm of human hepatocytes. The infection with HAV induces strong immunological response and elevated levels first of IgM and then IgG are detectable within a few days after the onset of the symptoms. The presence of anti–HAV IgM is an important serological marker for early detection and observation of the clinical manifestation of the disease. Increasing levels of anti-HAV IgM are detectable about three weeks after exposure with highest titter after four to six weeks later. Within six months after infection IgM concentration declines to non-detectable levels.

PRINCIPLE OF THE ASSAY

This kit is a solid phase, two-step incubation, antibody capture ELISA assay in which, polystyrene microwell strips are pre-coated with antibodies directed to human immunoglobulin M proteins (anti-μ chain). The patient's serum/plasma sample is added and during the first incubation, any IgM antibodies will be captured in the wells. After washing out all the other components of the sample and in particular IgG antibodies, the specific HAV IgM captured on the solid phase is detected by the addition of HAV antigens conjugated to horseradish peroxidase (HRP-Conjugate). During the second incubation, the HRP-conjugated antigens will specifically react only with the HAV IgM antibodies and after washing to remove unbound HRP-conjugate, Chromogen solutions are added to the wells.

In presence of the (anti-**μ**)-(HAV-IgM)-(antigen-HRP) immunocomplex, the colorless Chromogens are hydrolyzed by the bound HRP conjugate to a blue-colored product. The blue color turns yellow after stopping the reaction with sulfuric acid. The amount of color intensity can be measured and is proportional to the amount of antibody captured in the wells, and to the sample respectively. Wells containing samples negative for HAV-IgM remain colorless.

Assay principle scheme: Capture ELISA

Ab(p)+IgM(s) → [Ab(p)–IgM(s)]+(Ag)ENZ→ [Ab(p)–IgM(s)–(Ag)ENZ]			→blue→yellow	(+)
Ab(p)	→ [Ab(p)	] + (Ag)ENZ→ [Ab(p)	] → no color	(-)
Incubation 1	**Incubation 2**	**Immobilized Complex**	**Coloring**	**Results**
20 min.	40 min.		15 min.	

Ab(p)–pre-coated anti-IgM antibodies (anti-μ chain);
IgM(s) – HAV IgM antibodies in sample;
(Ag)ENZ– HRP conjugated HAV antigens;

COMPONENTS

96 tests

- **MICROWELL PLATE** 1plate

Blank microwell strips fixed on white strip holder. The plate is sealed in aluminium pouch with desiccant.
8×12/12×8-well strips per plate. Each well contains anti-IgM antibodies (anti-μ chain). The microwell strips can be broken to be used separately. Place unused wells or strips in the plastic sealable storage bag together with the desiccant and return to 2~8°C.

- **NEGATIVE CONTROL** 1vial

Yellowish liquid filled in a vial with green screw cap.
0.5ml per vial.
Protein-stabilized buffer tested non-reactive for HAV IgM.
Preservatives: 0.1% ProClin 300.
Ready to use as supplied.
Once open, stable for one month at 2-8°C.

- **POSITIVE CONTROL** 1vial

Red-colored color liquid filled in a vial with red screw cap.
0.5ml per vial.
Purified anti-HAV IgM antibodies diluted in protein-stabilized buffer.
Preservatives: 0.1% ProClin 300.
Ready to use as supplied.
Once open, stable for one month at 2-8°C.

- **HRP-CONJUGATE REAGENT** 1vials

Red-colored liquid filled in a white vial with red screw cap.
12 ml per vial.
Horseradish peroxidase-conjugated HAV antigens.
Ready to use as supplied.
Once open, stable for one month at 2-8°C.

- **STOCK WASH BUFFER** 1bottle

Colorless liquid filled in a clear bottle with white screw cap.
50ml per bottle, 20 × PBS, PH 7.4 (Contains Tween-20 as a detergent)
DILUTE BEFORE USE -The concentrate must be diluted **1 to 20** with distilled/deionized water before use.
Once diluted, stable for one week at room temperature or for two weeks at 2-8°C.

- **CHROMOGEN SOLUTION A** 1vial

Colorless liquid filled in a white vial with green screw cap.
7ml per vial.
Urea peroxide solution.
Ready to use as supplied.
Once open, stable for one month at 2-8°C.

- **CHROMOGEN SOLUTION B** 1vial

Colorless liquid filled in a black vial with black screw cap.
7ml per vial.
TMB solution(Tetramethyl benzidine dissolved in citric acid).
Ready to use as supplied.
Once open, stable for one month at 2-8°C.

- **STOP SOLUTION** 1vial

Colorless liquid filled in a white vial with white screw cap.
7ml per vial
Diluted sulfuric acid solution (2.0M H2SO4).

- **PLASTIC SEALABLE BAG** 1unit

For enclosing the strips not in use.

- **CARDBOARD PLATE COVER** 2sheets

To cover the plates during incubation and prevent evaporation or contamination of the wells.

- **PACKAGE INSERTS** 1copy

ADDITIONAL MATERIALS AND INSTRUMENTS REQUIRED BUT NOT PROVIDED

1. Freshly distilled or deionized water.
2. Disposable gloves and timer.
3. Appropriate waste containers for potentially contaminated materials.
4. Disposable V-shaped troughs.
5. Dispensing system and/or pipette (single or multichannel), disposable pipette tips.
6. Absorbent tissue or clean towel.
7. Dry incubator or water bath, 37±0.5°C.
8. Microshaker for dissolving and mixing conjugate with samples.
9. Microwell plate reader, single wavelength 450nm or dual wavelength 450nm and 630nm.
10. Microwell aspiration/wash system.
11. Normal saline solution to dilute samples.

SPECIMEN COLLECTION , TRANSPORTATION AND STORAGE

1. **Sample Collection:** Either fresh serum or plasma samples can be used for this assay. Blood collected by venipuncture should be allowed to clot naturally and completely – the serum/plasma must be separated from the clot as early as possible as to avoid hemolysis of the RBC. Care should be taken to ensure that the serum samples are clear and not contaminated by microorganisms. Any visible particulate matters in the sample should be removed by centrifugation at 3000 RPM for at least 20 minutes at room temperature, or by filtration on 0.22u filters. Plasma samples collected into EDTA, sodium citrate or heparin may be tested, but highly lipaemic, icteric, or hemolized samples should not be used as they could give erroneous results in the assay. Do not heat inactivate samples. This can cause sample deterioration.
2. **Transportation and Storage:** Store samples at 2-8°C. Samples not required for assaying within 3 days should be stored frozen (-20°C or lower).Multiple freeze-thaw cycles should be avoided. For shipment, samples should be packaged and labeled in accordance with the existing local and international regulations for transport of clinical samples and ethological agents.
3. **Sample preparation:** Each sample must be diluted 1:1000 with normal saline.

SPECIAL INSTRUCTIONS FOR WASHING

1. A good washing procedure is essential to obtain correct and precise analytical data.
2. It is therefore recommended to use a good quality ELISA microplate washer, maintained at the best level of washing performances. In general, no less than 5 automatic washing cycles with dispensing of 350-400µl/well, are sufficient to avoid false positive reactions and high background (all wells turn yellow).
3. To avoid cross-contaminations of the plate with sample or HRP-conjugate, after incubation do not discard the content of the wells, but allow the plate washer to aspirate it automatically.
4. Anyway, we recommend calibrating the washing system on the kit itself in order to match the declared analytical performances. Assure that the microplate washer's liquid dispensing channels are not blocked or contaminated, and sufficient volume of Wash buffer is dispensed each time into the wells.
5. In case of manual washing, we suggest to perform at least 5cycles, dispensing 350-400µl/well and aspirating the liquid for 5times. If poor results (high background) are observed, increase the washing cycles or soaking time per well.
6. In any case, the liquid aspirated out the strips should be treated with a sodium hypochlorite solution(final concentration of 2.5%) for 24 hours, before liquids are disposed in an appropriate way.
7. The concentrated Washing solution should be diluted **1 to 20** before use. For one plate, mix 50 ml of the concentrate with 950ml of water for a final volume of 1000ml diluted Wash Buffer. If less than a whole plate is used, prepare the proportional volume of solution.

STORAGE AND STABILITY

The components of the kit will remain stable through the expiration date indicated on the label and package when stored between 2-8 °C ; **do not freeze**. To assure maximum performance of this HAV-IgM ELISA kit, during storage protect the reagents from contamination with microorganism or chemicals.

PRECAUTIONS AND SAFETY

This kit is intended **FOR IN VITRO USE ONLY** IVD

FOR PROFESSIONAL USE ONLY

The ELISA assay is a time and temperature sensitive method. To avoid incorrect result, strictly follow the test procedure steps and do not modify them.

1. Do not exchange reagents from different lots, or use reagents from other commercially available kits. The components of the kit are precisely matched as to achieve optimal performance during testing.
2. Make sure that all reagents are within the validity indicated on the kit box and are of the same lot. Never use reagents beyond the expiry date stated on reagents labels or on the kit box.
3. **CAUTION - CRITICAL STEP:** Allow the reagents and samples to stabilize at room temperature (18-30°C) before use. Shake reagent gently before, and return to 2-8°C immediately after use.
4. Use only sufficient volume of sample as indicated in the procedure steps. Failure to do so, may cause in low sensitivity of the assay.
5. Do not touch the bottom exterior of the wells; fingerprints or scratches may interfere with microwell reading.
6. When reading the results, ensure that the plate bottom is dry and there are no air-bubbles inside the wells.
7. Never allow the microplate wells to dry after the washing step. Immediately proceed to the next step. Avoid the formation of air-bubbles when adding the reagents.
8. Avoid assay steps long time interruptions. Assure same

working conditions for all wells.

9. Calibrate the pipette frequently to assure the accuracy of samples/reagents dispensing. Always use different disposal pipette tips for each specimen and reagents as to avoid cross-contaminations. Never pipette solutions by mouth.
10. The use of automatic pipettes is recommended.
11. Assure that the incubation temperature is 37°C inside the incubator.
12. When adding samples, avoid touching the well's bottom with the pipette tip.
13. When reading the results with a plate reader, it is recommended to determine the absorbance at 450nm or at 450nm with reference at 630nm.
14. All specimens from human origin should be considered as potentially infectious.
15. Materials from human origin may have been used in the kit. These materials have been tested with tests kits with accepted performance and found negative for antibodies to HIV ½, HCV, TP and HBsAg. However, there is no analytical method that can assure that infectious agents in the specimens or reagents are completely absent. Therefore, handle reagents and specimens with extreme caution as if capable of transmitting infectious diseases. Strict adherence to GLP (Good Laboratory Practice) regulations can ensure the personal safety. Never eat, drink, smoke, or apply cosmetics in the assay laboratory.
16. Bovine derived sera may have been used in this kit. Bovine serum albumin (BSA) and fetal calf sera (FCS) are derived from animals from BSE/TSE free-geographical areas.
17. The pipette tips, vials, strips and sample containers should be collected and autoclaved for 1hour at 121°C or treated with 10% sodium hypochlorite for 30minutes to decontaminate before any further steps for disposal.
18. The Stop solution (2M H_2SO_4) is a strong acid. Corrosive. Use it with appropriate care. Wipe up spills immediately or wash with water if come into contact with the skin or eyes. ProClin 300 used as a preservative can cause sensation of the skin.
19. The enzymatic activity of the HRP-conjugate might be affected from dust, reactive chemical, and substances like sodium hypochlorite, acids, alkalins etc. Do not perform the assay in the presence of such substances.
20. Materials Safety Data Sheet (MSDS) available upon request.
21. If using fully automated microplate processing system, during incubation, do not cover the plates with the plate cover. The tapping out of the remainders inside the plate after washing, can also be omitted.

ASSAY PROCEDURE

Step1 **Reagents Preparation:** Allow all reagents and samples to reach room temperature. (18-30°C) for at least 15-30minutes. Check the Wash buffer concentrate for the presence of salt crystals. If crystals have formed in the solution, resolubilize by warming at 37°C until crystals dissolve. Dilute the stock wash buffer **1 to 20** with distilled or deionized water. Use only clean vessels to dilute the Wash buffer. Mark three wells as Negative control **(e.g. B1, C1, D1),** two wells as Positive control **(e.g. E1, F1)** and one Blank well. (**e.g. A1**, neither samples or HRP-Conjugate should be added into the Blank well). If the results will be determined by using dual wavelength plate reader, the requirement for use of Blank well could be omitted. Use only number of strips required for the test.

Step2 **Diluting Sample:** Dilute the specimen 1:1000 with normal saline. Do not dilute the Controls, as they are ready for use as supplied

Step3 **Adding Sample:** Add **100µl** of samples into each well and **100µl** Positive and Negative controls into their respective wells. **Note: Use a separate disposal pipette tip for each specimen, Negative and Positive Controls as to avoid cross-contamination**

Step4 **Incubating (1) Sample:** Cover the plate with the plate cover and incubate for **20 minutes at 37**°C. It is recommended to use thermostat-controlled water tank to assure the temperature stability and humidity during the incubation. If dry incubator is used, do not open the door frequently.

Step5 **Washing (1):** At the end of the incubation remove and discard the plate cover. Wash each well **5**times with diluted Washing buffer. Each time allow the microwells to soak for **30-60 seconds**. After the final washing cycle, turn down the plate onto blotting paper or clean towel, and tap it as to remove any remaining liquids.

Step6 **Adding Conjugate:** Add **100µl** of HRP-Conjugate Reagent into each well except the blank.

Step7 **HRP-Conjugate Incubation (2):** Cover the plate with the plate cover and incubate for **40 min at 37°C**.

Step8 **Washing (2):** Remove and discard the plate cover. Aspirate the liquid and rinse each well **5times** with Wash buffer (as step 5). After the final washing cycle, turn the plate and tap out any remainders.

Step8 **Coloring:** Add **50µl** of Chromogen A and **50µl** Chromogen B solution into each well including the **Blank** and mix by tapping the plate gently. Incubate the plate at **37°C for 15minutes avoiding light**. The enzymatic reaction between the Chromogen solutions and the HRP-Conjugate produces blue color in Positive control and HAV-IgM positive sample wells.

Step9 **Stopping Reaction:** Using a multichannel pipette or manually, add **50µl** Stop solution into each well and mix gently. Intensive yellow color develops in Positive control and HAV IgM positive sample wells.

Step10 **Measuring the Absorbance:** Calibrate the plate reader with the Blank well and read the absorbance at **450nm**.If a dual filter instrument is used, set the reference wavelength at **630nm**. Calculate the Cut-off value and evaluate the results.

(**Note:** read the absorbance within **5**minutes after stopping the reaction)

INTERPRETATION OF RESULTS AND QUALITY CONTROL

Each microplate must be considered separately when calculating and interpreting results of the assay, regardless of the number of plates concurrently processed. The results are calculated by relating each sample's optical density (OD) value to the Cut-off value (C.O.) of the plate. If the Cut-off reading is based on Single filter plate reader, the results must be calculated by subtracting the Blank well OD value from the print report values of samples and controls. In case the reading is based on dual filter plate reader, do not subtract the Blank well OD from the print report values of samples and controls

1. Calculation of Cut-off value (C.O.) = *Nc × 2.1

***Nc** = the mean absorbance value of the three negative controls

Important: If the mean OD value of the negative controls is lower than 0.05, take it as 0.05.

Example:
Calculation of Nc:

Well No	B1	C1	D1
Negative controls OD value	0.02	0.012	0.016

Nc=0.016 (Nc is lower than 0.05, so take it as 0.05)
Calculation of Cut-off value: (C.O.)= 0.05 x 2.1= 0.105

If one of the Negative control values does not meet the Quality control range specifications, it should be discarded, and the mean value is calculated again using the remaining two values. If more than one negative control OD value does not meet the Quality control range specifications, the test is invalid and must be repeated.

2. Quality control range:

1. The absorbance of the Blank well, which contains only Chromogens and Stop solution, is less than 0.080 at 450 nm.
2. The OD value of the Positive control must be equal to, or greater than 0.800 at 450/630nm, or at 450nm after blanking.
3. The OD value of the Negative control must be less than 0.100 at 450/630nm or at 450nm after blanking.

3. Interpretations of the results:

(S = the individual absorbance (OD) of each specimen)

Negative Results (S/C.O. <1): samples giving absorbance less than the Cut-off value are negative for this assay, which indicates that no IgM class antibodies to HAV have been detected with this HAV IgM ELISA kit. Therefore, there are no serological indications for recent infection, and the patient is probably not infected with hepatitis A virus .

Positive Results (S/C.O.≥1) :samples giving an absorbance greater than, or equal to the Cut-off value are initially positive, which indicates that IgM class antibodies to HAV have probably been detected with this HAV IgM ELISA kit. Retesting in duplicates of any reactive sample is recommended. Repeatedly reactive samples can be considered positive for IgM antibodies to HAV and therefore there are serological indications for current infection with hepatitis A virus.

Borderline: (S/CO =0.9-1.1) Samples with absorbance to Cut-off ratio between 0.9 and 1.1 are considered borderline. Retesting of these samples in duplicates is recommended. Repeatedly reactive samples can be considered positive for IgM antibodies to HAV.
Follow-up and supplementary testing of any positive samples with other HAV tests is required to confirm the infection state.

TEST PERFORMANCE AND EXPECTED RESULTS

The **clinical sensitivity** of this kit was evaluated by testing samples obtained from 739 (288 children and 451 adults) individuals suspected for infection with HAV during outcome. Another group of samples from 1950 healthy blood donors was tested in order to determine the clinical specificity of the test. These evaluation studies were carried out in direct comparison with another commercially available HAV IgM ELISA kit used as a confirmation assay. The evaluation results are given below.

Clinical Specificity:

	CHILDREN			ADULTS		
	Tested	specificity	False positive	Tested	specificity	False positive
Donors	1220	>99%	5	730	>99%	4

Clinical Sensitivity:

	CHILDREN				SENSITIVITY
	Tested	-	+	confirmed	
Inapparent infection	148	3	145	145	100%
Anicteric /icteric	140	15	35	35	100%
TOTAL	288	18	180	180	100%

	ADULTS				
	Tested	-	+	confirmed	SENSITIVITY
Inapparent infection	238	192	46	46	100%
Anicteric /icteric	213	120	190	190	100%
TOTAL	451	312	236	236	100%

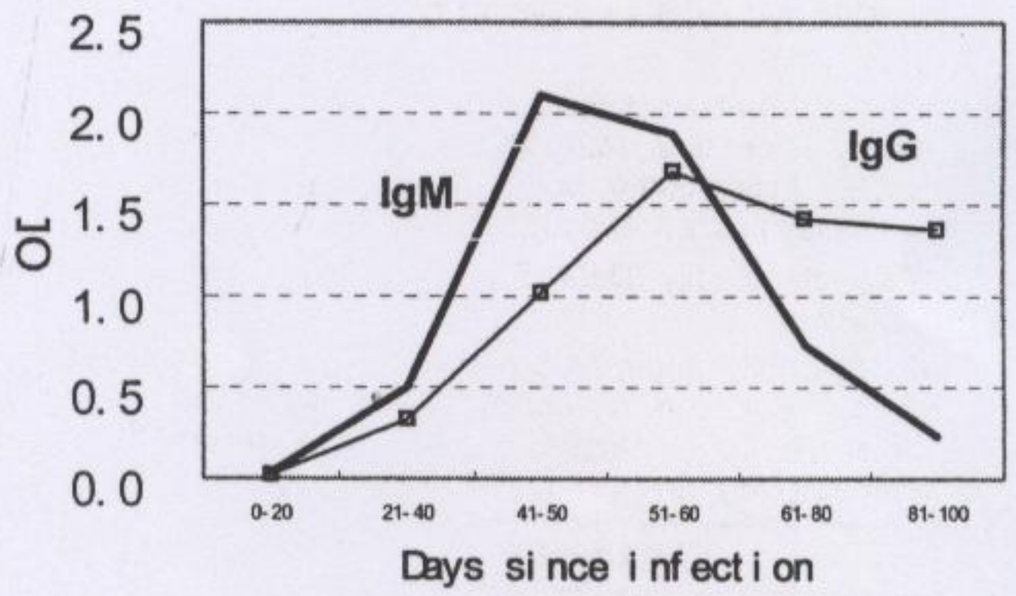
Linearity of sample dilution- undiluted sample:

Sample dilution index	OD
1:1	2.543
1:500	2.234
1:5000	1:042
1:50 000	0.673
1:500 000	0.036

Follow-up of individuals infected with HAV:

Days since infection	OD
0-20	0.031
21-40	0.521
41-50	2.143
51-60	1.890
61-80	0.736
81-100	0.231

Follow-up of individuals infected with HAV:



Comparisons of this HAV IgM ELISA kit performance characteristic in follow-up of individuals infected with HAV, and reference anti-HAV IgG ELISA kit (squares).

Analytical Specificity:

1. No cross reactivity observed with samples from patients confirmed to be infected with HBV, HCV, HIV, CMV, and TP.
2. No interferences from elevated levels of rheumatoid factors up to 2000U/ml were observed during clinical testing.
3. The assay performance characteristics are unaffected from elevated concentrations of bilirubin, hemoglobin, and triolein.

Reproducibility	Within run			Between run		
Specimen	Test	MeanOD	CV%	Test	MeanOD	CV%
Weak positive	10	0.428	8.1	10	0.395	8.5
Moderate positive	10	0.916	7.3	10	0.856	7.6
Strong positive	10	2.172	4.6	10	2.982	5.1

LIMITATIONS

1. Non- repeatable reactive results may be obtained with any ELISA test due to the general characteristics of this diagnostic method. A negative result with an antibody detection test does not preclude the possibility of infection. Antibodies may be undetectable during the early stages of the disease and in some immunosuppressed individuals.
2. If, after retesting of the initially reactive samples, the assay results are negative , these samples should be considered as non-repeatable (false positive) and interpreted as negative. As with many very sensitive ELISA assays, false positive results can occur due to the several reasons, most of which are related but not limited to inadequate washing step.
3. Any positive results must be interpreted in conjunction with the patient clinical information and other laboratory results
4. Common sources for mistakes: kits beyond the expiry date, bad washing procedures and wrong washing buffer concentration, contaminated reagents, incorrect assay procedure steps, insufficient aspiration during washing, failure to add samples or reagents, equipment, timing, volumes, sample nature and quality.
5. The prevalence of the marker will affect the assay's predictive values.
6. False negative results can occur from inhibition of specific IgM in the presence of high titers of specific IgG. The removal of IgG can be helpful to prevent false negative results and methods for this are given elsewhere.
7. This kit is intended ONLY for testing of individual serum or plasma samples. Do not use it for testing of cadaver samples, saliva, urine or other body fluids, or pooled (mixed) blood.

INDICATIONS OF INSTABILITY OR DETERIORATION OF THE REAGENTS

1. Values of the Positive or Negative controls ,which are out of the indicated Quality control range, are indicator of possible deterioration of the reagents and/or operator or equipment errors. In such case, the results should be considered as invalid and the samples must be retested. In case of constant erroneous results classified as due to deterioration or instability of the reagents, immediately substitute the reagents with new ones.
2. If after mixing of the Chromogen A and B solutions into the wells, the, the color of the mixture turns blue within few minutes, do not continue carrying out the testing and replace the reagents with fresh ones.

VALIDITY

Please do not use this kit beyond the expiration indicated on the kit box and reagent labels.

REFERENCES:

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SUMMARY OF THE ASSAY PROCEDURE:	
Dilute the sample with normal saline	1:1000
Add sample	100µl
Incubate	20minutes
Wash	5times
Add HRP-Conjugate	100µl
Incubate	40minutes
Wash	5times
Coloring	50µl A + 50µl B
Incubate	15minutes
Stop the reaction	50µl stop solution
Read the absorbance	450nm or450/630 nm

SUMMARY OF THE MAJOR COMPONENTS OF THE KIT:	
Microwell plate	One/ 96 wells
Negative/ Positive control	One each/ 0.5ml
HRP-Conjugate	Two/ 6ml
Wash Buffer	One/ 50ml
Chromogen A/B Stop solution	One each/7ml
Note: the components of individual kits are not interchangeable	

Example of controls/samples dispensing scheme

	1	2	3	4	5	6	7	...	...	12
A	Blank	S3								
B	Neg.	...								
C	Neg	...								
D	Neg.									
E	Pos.									
F	Pos.									
G	S1									
H	S2									

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