ETI-DELTAK-2
(P002097)

EN: Pay attention to changes!
IT: Attenzione alle modifiche!
FR: Faire attention aux modifications!
DE: Auf die Änderungen aufpassen!
ES: ¡Atención a las modificaciones!
PT: Cuidado com as modificações!
RO: Atenție la modificări!
NO: Legg merke til endringene!
SV: Uppmärksamma vidtagna ändringar!
DA: Bemærk ændringerne!
CS: Dávejte si pozor na změny!
SK: Venujte pozornosť zmien!
PL: Należy zwrócić uwagę na zmiany!
LT: Atkreipkite dėmesį į pakeitimus!
LV: Pievērsiet uzmanību izmaiņām!
HU: Kérjük, figyeljen a változásokra!
BG: Обърнете внимание на промените!
TR: Değişikliklere dikkat edin!
EL: Προσοχή στις τροποποιήσεις!
1. INTRODUCTION

The hepatitis D virus (HDV) is a 36-nm diameter defective virus, which requires the helper functions of hepatitis B virus (HBV) for successful replication. The HDV nucleocapsid is formed of a single-stranded genome (HDV-RNA), which is strictly associated to two specific proteins (p27 and p29) giving rise to the Delta antigen (HDAg) (1), and is surrounded by an HBsAg envelope (7). Consequently, detection of HDAg requires treatment of test samples with detergents to remove HBsAg and expose HDAg antigenic determinants (8).

Hepatitis D presents three main clinical pictures, different in severity of symptoms; as a rule, hepatitis D symptoms are consistently severer than those observed in HBV infection or in other viral hepatitides. The finding of the three serological markers of hepatitis D (HDAg, anti-HD IgM and anti-HD total antibodies) after detection of HBsAg allows diagnosis and characterization of various forms of hepatitis D to be established (4, 5, 9):

– **Coinfection**, when the patient is infected by both HBV and HDV at the same time. The clinical course of the disease is acute, symptomatology is generally similar to that of HBV acute hepatitis. In most cases the disease progresses to full recovery; chronicization is rare. In acute HBV/HDV coinfection, IgM anti-HD is raised a few days to a few weeks after the onset of symptoms and seroconversion to IgG anti-HD occurs later.

– **Superinfection**, when the patient (HBsAg chronic carrier) had a previous HBV infection and is later infected by HDV. The disease is severer in this case and may evolve to a fulminant form of hepatitis (more frequent than in case of coinfection) or may chronicize. As a rule, the antibody response to superinfection is quicker and brisker than in coinfection. IgM anti-HD is present at or raised soon after the onset of symptoms, followed shortly by the IgG antibody.

– **Chronic infection**, having a severer clinical course than HBV chronic hepatitis. The persistence of IgM anti-HD indicates that hepatitis D is becoming chronic, because it correlates directly with HDV replication and with the inflammatory activity of the underlying liver disease.

The finding of HDAg in the bloodstream allows the diagnosis of ongoing HDV acute infection to be established, though HDAg is found in some but not all acute D hepatitides. Its presence is short in duration and limited to the early stage of infection; the test can give negative results because viraemia is already over at the onset of clinical symptoms. Entity and duration of HD antigenaemia are usually proportional to the severity of hepatitis (4). The finding of serum HDAg is in fact more frequent and conspicuous in fulminant and severe rather than in benign or mild forms of hepatitis to such an extent that serum HDAg represents a reliable prognostic marker of clinical evolution of the illness.

As soon as the specific antibody response appears, HDAg becomes no longer detectable in serum, because its antigenic reactivity is masked by the endogenous antibodies. Consequently, HD antigenaemia is not detectable in patients with chronic D hepatitis as persistently high anti-HD titres are consistently present (4).

Chronic HD antigenaemia can however be demonstrated in immunocompromized subjects, like those with HIV infection, as the antibody response is totally or partially suppressed (2, 6).
2. PRINCIPLE OF THE ASSAY

The method for qualitative HDAg determination is a direct, non-competitive sandwich assay, illustrated in Fig. 1, based on the ELISA technique (Enzyme-linked Immunosorbent Assay).

Fig. 1

1. **Well** coated with human IgG anti-HD.
2. HDAg added with a non-ionic detergent (Igepal® 300 CA-630) from sample or control.
3. **Enzyme tracer**: human anti-HD Fab conjugated to horseradish peroxidase (HRP).

The presence of HDAg allows the enzyme tracer to bind to the solid phase. The enzyme activity is therefore proportional to the HDAg concentration present in samples or controls.

Enzyme activity is measured by adding a colourless chromogen/substrate solution. The enzyme action on chromogen/substrate produces a colour which is measured with a photometer.

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1. incubation (1 hour, 37°C)
2. washing
3. incubation (1 hour, 37°C)
4. washing
5. chromogen/substrate incubation (30 min, R.T.)
6. stop solution

**Fig. 1**
3. REAGENTS AND ACCESSORIES

3.1. Reagents provided in the kit

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coated strips</td>
<td>12 strips</td>
</tr>
<tr>
<td>Enzyme tracer</td>
<td>1 vial, 0.4 mL</td>
</tr>
<tr>
<td>Negative control</td>
<td>1 vial, 3.3 mL</td>
</tr>
<tr>
<td>Positive control</td>
<td>1 vial, 2.5 mL</td>
</tr>
<tr>
<td>Nonidet P-40</td>
<td>1 vial, 8 mL</td>
</tr>
<tr>
<td>Tracer diluent</td>
<td>1 vial, 14.7 mL</td>
</tr>
<tr>
<td>Wash buffer</td>
<td>1 vial, 40 mL</td>
</tr>
<tr>
<td>Chromogen/substrate</td>
<td>1 vial, 16 mL</td>
</tr>
<tr>
<td>Stop solution</td>
<td>1 vial, 30 mL</td>
</tr>
<tr>
<td>Number of tests</td>
<td>96</td>
</tr>
</tbody>
</table>

STORAGE: Upon receipt, store all reagents at 2-8°C, away from intense light. Do not freeze. Once opened, the reagents of this kit are stable for eight weeks when properly stored, unless otherwise stated. The kit is stable for one work week when used throughout the day for eight hours at room temperature and stored overnight at 2-8°C. Reagents should not be used past the expiry date. The expiry date of the kit is reported on the external label. The expiry date of each component is reported on the respective vial label.

Specific reagents (see §4.a) from different batches must not be mixed. Common reagents (see §4.b) are interchangeable between batches.

3.2. Materials provided with the kit

– 2 precut cardboard sealers suitable for 1 to 12 strips
– 2 cardboard sealers suitable for 12 strips (one plate)
– pouch sealer.

3.3. Equipment and materials required, but not supplied

– Vertical reading photometer (such as ETI-SYSTEM reader or equivalent) with the following instrument specifications:
  - wavelength: dual wavelength, 450 nm and 620-630 nm
  - bandwidth: ≤ 10 nm
  - absorbance range: 0 absorbance units to ≥ 2.5 absorbance units
  - repeatability: better than or equal to 0.005 absorbance units, or 1%, whichever is greater
  - linearity or trueness: better than or equal to 0.010 absorbance units, or 2%, whichever is greater
  - drift: less than 0.005 absorbance units per hour.
– Thermostatically-controlled humid chamber with the following specifications:
  - temperature: 37°C ± 1°C.
– Manual or automatic equipment for rinsing wells (such as ETI-SYSTEM washer or equivalent) with the following instrument specifications:
  - volume dispensed: 300-370 μL
  - number of wash cycles: 5
  - soak time: 30 seconds
  - aspirate the last aliquot of dispensed liquid: yes.
– Micropipettes with disposable tips (50, 100 μL) (50 μL: trueness ± 3%, precision 2%; 100 μL: trueness ± 2%, precision 1%).
– Glassware.
– Distilled water.

ETI-LAB or ETI-MAX 3000 equipment may be used for automated test processing.
4. COMPOSITION AND PREPARATION OF REAGENTS

All serum and plasma units used to produce the components provided in this kit have been tested for the presence of HBsAg, anti-HCV and anti-HIV-1/2 and found to be non-reactive, except for the material positive for anti-HD, which is reactive for HBsAg and anti-HCV. The presence of HBsAg and HCV in the final reagents is however excluded, because HBsAg is removed by purification and HCV-RNA is found negative.

As, however, no test method can offer absolute assurance that pathogens are absent, all specimens of human origin should be considered potentially infectious and handled with care.

a) SPECIFIC KIT REAGENTS

4.1. Coated strips
Removable wells are coated with human IgG anti-HD. The wells are ready to use and should be stored at 2-8°C.
Bring the coated wells to room temperature before opening the pouch to avoid development of condensed water. Place unused wells in the pouch, securely reseal and store at 2-8°C.

4.2. Enzyme tracer (50x conjugate)
The vial contains 0.4 mL anti-HD Fab (human), conjugated to horseradish peroxidase (HRP), TRIS buffer, BSA, stabilizers and preservatives.
Before use, dilute the solution 1:50 with tracer diluent (4.6) (e.g. 100 μL tracer + 4.9 mL tracer diluent). Prepare only the amount of working enzyme tracer needed for the run and keep the concentrated enzyme tracer at 2-8°C.
The working enzyme tracer can be stored for one month at 2-8°C after preparation.

4.3. Negative control
The vial contains 3.3 mL human serum/plasma non-reactive for anti-HD and preservatives. The reagent is ready to use and should be stored at 2-8°C.

4.4. Positive control
The vial contains 2.5 mL recombinant HDAg (3), human serum/plasma, BSA, phosphate-citrate buffer and preservatives. The reagent is ready to use and should be stored at 2-8°C.

4.5. Nonidet P-40
The vial contains 8 mL of a 2% solution of non-ionic detergent Igepal® 300 CA-630, PBS buffer at pH 7.4, preservatives and an inert blue dye. The reagent is ready to use and should be stored at 2-8°C.

4.6. Tracer diluent
The vial contains 14.7 mL human serum/plasma, newborn calf serum, PBS buffer and preservatives. The reagent is ready to use and should be stored at 2-8°C. The solution is used to dilute the enzyme tracer (4.2).

b) REAGENTS COMMON TO OTHER ETI KITS

4.7. Wash buffer (25x)
The vial contains 40 mL PBS buffer, Tween® 20 and preservatives. Before use, dilute the vial contents to one litre with distilled water. The working wash buffer is stable for one week at 2-8°C, and is used to rinse wells.
If crystallization occurs at 2-8°C, warm the wash buffer to 37°C and mix well before diluting.
4.8. Chromogen/substrate
The vial contains 16 mL of a tetramethylbenzidine/hydrogen peroxide system. The reagent is ready to use and should be stored at 2-8°C, away from the light. The solution should be colourless or have a slightly blue tinge. If the chromogen/substrate turns a darker blue, it may have become contaminated and should be discarded.

4.9. Stop solution
The vial contains 30 mL 0.4N sulphuric acid solution. The reagent is ready to use and should be stored at 2-8°C.

5. SPECIMEN COLLECTION AND PREPARATION
Either human serum or plasma may be used. The anticoagulants citrate, EDTA and heparin have been tested and may be used with this assay. Blood should be collected aseptically by venipuncture, allowed to clot, and the serum separated from the clot as soon as possible. Samples having particulate matter, turbidity, lipaemia, or erythrocyte debris may require clarification by filtration or centrifugation before testing. Grossly haemolyzed or lipaemic samples as well as samples containing particulate matter or exhibiting obvious microbial contamination should not be tested. If the assay is performed within 48 hours of sample collection, the samples should be kept at 2-8°C; otherwise they should be aliquoted and stored deep-frozen (−20°C or below). If samples are stored frozen, mix thawed samples well before testing. Performance is not affected by samples that have undergone up to four freeze-thaw cycles.

Results obtained in heparinized plasma positive specimens may score absorbance values above those of serum samples.

6. ASSAY PROCEDURE
Bring all reagents to room temperature (20-25°C) before assaying.
Perform all assay steps in the order given and without any appreciable delays between the steps.
Number sufficient wells to run 3 replicates of the negative control and 2 replicates of the positive control with each run of samples in singlicate. Negative and positive controls must be run with each plate of patient specimens. Controls and samples should be subjected to the same process and incubation time.
Prepare one substrate blank well containing chromogen/substrate only.
A clean, disposable tip should be used for dispensing each control and sample.
Identify wells with data sheet for testing controls and samples. Dispense controls and samples as illustrated in the scheme overleaf.
Adjust the thermostatically-controlled humid chamber to 37° ± 1°C.
1. Dispense 50 μL Nonidet P-40 (4.5) into all wells. Dispense 100 μL negative controls, positive controls and samples into their respective wells.
2. Apply a cardboard sealer in order to prevent evaporation. Gently tap the reaction wells to release any air bubbles trapped in the liquid.
3. Incubate for one hour ± 5 min at 37° ± 1°C.
4. Prepare the working enzyme tracer solution (4.2 + 4.6) before the end of the first incubation.
5. When incubation has been completed, remove and discard the cardboard sealer. Aspirate the liquid and rinse each well five times with a volume of wash buffer ranging from 0.30 to 0.37 mL. Avoid overflows from the reaction wells. Regardless of whether an automatic or semiautomatic washer is used, the soak time between each wash cycle should be 30 seconds. After rinsing, turn the strips mouth down on to blotting paper and gently tap to remove any excess liquid. Leave the strips overturned on blotting paper until the following reagent is pipetted, to avoid evaporation.
6. Dispense 100 μL working enzyme tracer solution into all wells except for the blank well. Repeat step 2.
7. Incubate for one hour ± 5 min at 37° ± 1°C.
8. Repeat step 5.
9. Dispense 100 μL chromogen/substrate solution into all wells.
10. Incubate for 30 ± 2 min at room temperature, away from direct or intense light.
11. Dispense 100 μL stop solution into all wells in the same order and at the same rate as for the chromogen/substrate.
12. Measure the absorbance of specimens with a photometer at 450/630 nm within one hour of adding the stop solution. Samples at very high concentrations of analyte, however, should preferable be read shortly after addition of the stop solu-
tion, because of the possible formation of precipitates that lower the initial absorbance values. Such phenomenon, however, does not result in sample misclassification.

When dedicated equipment is used, absorbance values are provided automatically after selecting the suitable protocol. When another vertical reading photometer is used, blank the instrument with the blank, record the absorbance at 450/630 nm for each specimen and subtract the 630 nm absorbance value from the 450 nm absorbance value.

ETI-LAB or ETI-MAX 3000 equipment may be used for automated test processing.

7. CALCULATION OF RESULTS

7.1. Calculation of cut-off value
The cut-off value is determined by adding 0.100 to the mean absorbance for the negative control values (NCx).
Cut-off value = NCx + 0.100.

7.2. Run validation criteria
The following criteria should be used to validate quality control when evaluating results. Always calculate and evaluate mean control values for each plate, even if plates are combined to form a single batch.
The absorbance value for the blank well must range between 0.000 and 0.150.
0.000 ≤ BLK ≤ 0.150.
The mean absorbance for the negative control must be less than 0.100.
NCx < 0.100.
Each negative control absorbance value must be less than 0.110.
NCx < 0.110.
If any one of the negative control absorbance values does not meet these criteria, it should be discarded and the mean value recalculated using the remaining two values. If more than one negative control absorbance value does not meet these criteria, the run is invalid and must be repeated.
The difference between the mean positive control absorbance value and the mean negative control absorbance value must be greater than or equal to 0.500.
PCx – NCx ≥ 0.500.
If not, the run is invalid and must be repeated.

7.3. Interpretation of results
The presence or absence of HDAG is determined by comparing the absorbance value of unknown samples to that of the cut-off value.
The unknown samples with absorbance values greater than or equal to the cut-off value should be considered reactive for HDAG. The unknown samples with absorbance values less than the cut-off value should be considered non-reactive.
Samples with absorbance values within ±10% of the cut-off value must be retested in order to confirm the initial result. Samples which are repeatedly reactive should be considered positive. Samples which are non-reactive at the second test should be considered negative.
7.4. Calculation example
The following data must only be considered an example and should not be employed instead of the data obtained by the user.

Absorbance for the negative control

<table>
<thead>
<tr>
<th>Negative control sample No.</th>
<th>Net absorbance at 450/630 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.017</td>
</tr>
<tr>
<td>2</td>
<td>0.013</td>
</tr>
<tr>
<td>3</td>
<td>0.015</td>
</tr>
</tbody>
</table>

Mean absorbance, NC̄ = 0.015.

Absorbance for the positive control

<table>
<thead>
<tr>
<th>Positive control sample No.</th>
<th>Net absorbance at 450/630 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.852</td>
</tr>
<tr>
<td>2</td>
<td>1.712</td>
</tr>
</tbody>
</table>

Mean absorbance, PC̄ = 1.782.

Cut-off value (NC̄ + 0.100) = 0.015 + 0.100 = 0.115.

Positive – Negative control difference (P − N) = 1.782 − 0.015 = 1.767.

In the given example, the P − N difference meets the run validation criteria because it is greater than 0.500; thus the technique is acceptable and data should be considered valid.

Screening of unknown samples
Sample no. 1 = absorbance 0.910
Sample no. 2 = absorbance 0.053.

Sample no. 1 should be considered reactive for HDAg and sample no. 2 non-reactive, as the cut-off value is 0.115.

8. SPECIFIC PERFORMANCE CHARACTERISTICS

8.1. Analytical specificity
Analytical specificity may be defined as the ability of the assay to accurately detect specific analyte in the presence of potentially interfering factors in the sample matrix (e.g., anticoagulants, haemolysis, effects of sample treatment), or cross-reactive antibodies. Controlled studies of potentially interfering substances or conditions showed that the assay performance was not affected by anticoagulants (EDTA, heparin, citrate), haemolysis (up to 100 mg/dL haemoglobin), lipaemia (up to 3000 mg/dL triglycerides), bilirubinaemia (up to 20 mg/dL bilirubin), freezing of samples, or by antibodies to other infectious agents.
8.2. Precision
Different sample pools (from A to D), containing different titres of specific analyte, were assayed to determine repeatability and reproducibility of the assay (i.e., within- and between-assay variability). The variability shown in the tables below did not result in sample misclassification.

<table>
<thead>
<tr>
<th>Repeatability</th>
<th>Pool A</th>
<th>Pool B</th>
<th>Pool C</th>
<th>Pool D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of determinations</td>
<td>26</td>
<td>26</td>
<td>26</td>
<td>26</td>
</tr>
<tr>
<td>Mean absorbance</td>
<td>0.278</td>
<td>0.620</td>
<td>0.956</td>
<td>1.079</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>0.022</td>
<td>0.050</td>
<td>0.075</td>
<td>0.066</td>
</tr>
<tr>
<td>Coefficient of variation, %</td>
<td>8.0</td>
<td>8.1</td>
<td>7.8</td>
<td>6.2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Reproducibility</th>
<th>Pool A</th>
<th>Pool B</th>
<th>Pool C</th>
<th>Pool D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of determinations</td>
<td>26</td>
<td>26</td>
<td>26</td>
<td>26</td>
</tr>
<tr>
<td>Mean absorbance</td>
<td>0.301</td>
<td>0.628</td>
<td>1.049</td>
<td>1.036</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>0.035</td>
<td>0.077</td>
<td>0.147</td>
<td>0.184</td>
</tr>
<tr>
<td>Coefficient of variation, %</td>
<td>11.5</td>
<td>12.3</td>
<td>14.0</td>
<td>17.7</td>
</tr>
</tbody>
</table>

8.3. Diagnostic specificity
Diagnostic specificity is defined as the probability of the assay of scoring negative in the absence of specific analyte. Diagnostic specificity was assessed by testing 715 specimens selected from an expected HDAg-negative population (blood donors; dialysis patients; intravenous drug users; patients affected by infectious diseases with similar symptomatology, such as acute or chronic HBV infection, and past HDV infection; patients affected by HIV and HCV infections; patients affected by other hepatic diseases; subjects positive for rheumatoid factor or heterophile antibodies). Seven positive results were observed at first test in the population studied - diagnostic specificity: 99.02% (95% confidence interval: 98.00-99.61%). At retest, those samples scored correctly negative.

8.4. Diagnostic sensitivity
Diagnostic sensitivity is defined as the probability of the assay of scoring positive in the presence of specific analyte. Diagnostic sensitivity was assessed by testing 27 specimens selected from an expected HDAg-positive population (patients affected by acute HDV infection). No negative results were observed in the population studied - diagnostic sensitivity: 100% (95% confidence interval: 87.23-100%).

8.5. High-dose saturation effect
Whenever samples containing extremely high antigen concentrations are tested, the saturation effect can mimic concentrations lower than real. However, a well-optimized two-step sandwich method excludes grossly underestimated results, because the analytical signals remain consistently high (saturation curve). Analysis of possible prozone effect was evaluated by testing four high-titred HDAg-positive samples. All samples resulted in very high absorbance values that would be expected with high-titred sera, indicating no prozone effect and, therefore, no sample misclassification.
9. LIMITATIONS OF THE PROCEDURE
Bacterial contamination or heat inactivation of the specimens may affect the absorbance values of the samples with consequent alteration of HDAg levels. Diagnosis of an infectious disease should not be established on the basis of a single test result. A precise diagnosis, in fact, should take into consideration the patient's clinical history, symptomatology, as well as serological data. Serological data, however, should be interpreted with care in immunocompromized patients.

10. WARNINGS AND PRECAUTIONS
A skillful technique and strict adherence to the instructions are necessary to obtain reliable results. In particular, precise pipetting, aspiration and washing are essential. Non-repeatedly reactive samples might derive from various methodological factors, such as:
- cross-exchange of vial caps
- use of the same tip when withdrawing from different vials or dispensing different samples
- exposure of reagents or samples to intense heat or heavy sources of bacterial contamination
- inadequate rinsing of wells
- contamination of well rims by tracer or samples
- use of reagents from different master lots.
In addition the following precautions are required:
- to avoid contamination, use a clean, dedicated dispenser for the enzyme tracer solution
- wash buffer should be stored in clean containers to prevent contamination with enzyme-inactivating substances.

11. SAFETY PRECAUTIONS
- Handle with care chromogen/substrate and stop solution. Avoid chromogen/substrate and stop solution coming into contact with oxidizing agents or metallic surfaces.
- Do not eat, drink, smoke or apply cosmetics in the assay laboratory.
- Do not pipette solutions by mouth.
- Avoid direct contact with all potentially infectious materials by using protective clothing such as lab coats, protective glasses and disposable gloves. Wash hands thoroughly at the end of each assay.
- Avoid splashing or forming an aerosol. Any reagent spills should be washed with a 5% sodium hypochlorite solution and disposed of as though potentially infectious.
- All samples, biological reagents and materials used in the assay must be considered potentially able to transmit infectious agents. They should therefore be disposed of in accordance with the prevailing regulations and guidelines of the agencies holding jurisdiction over the laboratory, and the regulations of each Country. Disposable materials must be incinerated; liquid waste must be decontaminated with sodium hypochlorite at a final concentration of 5% for at least half an hour. Liquid waste containing acid must be neutralized before treatment. Any materials to be reused must be autoclaved using an overkill approach (USP 24, 2000, p. 2143). A minimum of one hour at 121°C is usually considered adequate, though the users must check the effectiveness of their decontamination cycle by initially validating it and routinely using biological indicators.
SCHEME OF THE ASSAY

1 - DISPENSE REAGENTS INTO THE STRIP WELLS ACCORDING TO THE FOLLOWING SCHEME, LEAVING AN EMPTY WELL FOR THE BLANK:

<table>
<thead>
<tr>
<th>REAGENTS</th>
<th>WELLS</th>
<th>NEGATIVE CONTROL (3x)</th>
<th>POSITIVE CONTROL (2x)</th>
<th>SAMPLES (1x)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NONIDET P-40</td>
<td>50 μL</td>
<td>50 μL</td>
<td>50 μL</td>
<td>50 μL</td>
</tr>
<tr>
<td>NEGATIVE CONTROL</td>
<td>100 μL</td>
<td>–</td>
<td>100 μL</td>
<td>–</td>
</tr>
<tr>
<td>POSITIVE CONTROL</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>100 μL</td>
</tr>
<tr>
<td>SAMPLES</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

2 - INCUBATE FOR ONE HOUR AT 37°C.

3 - ASPIRATE THE LIQUID. REPEATEDLY WASH WITH WASH BUFFER.

4 - DISPENSE 100 μL WORKING ENZYME TRACER INTO ALL WELLS, EXCEPT FOR THE BLANK WELL.

5 - INCUBATE FOR ONE HOUR AT 37°C.

6 - ASPIRATE THE LIQUID. REPEATEDLY WASH WITH WASH BUFFER.

7 - DISPENSE 100 μL CHROMOGEN/SUBSTRATE INTO ALL WELLS.

8 - INCUBATE FOR 30 MIN AT ROOM TEMPERATURE IN THE DARK.

9 - DISPENSE 100 μL STOP SOLUTION INTO ALL WELLS.

10 - READ ABSORBANCE VALUES WITH A PHOTOMETER AT 450/630 nm.