

ELITEST – Lp(a) # ACK103A

Kit for the determination of lipoprotein (a)
in human plasma or serum

For in vitro use only

For research use only



Manufactured By: HYPHEN BioMed

INTENDED USE

ELITEST Lp(a) is an Enzyme Immunoassay (ELISA) for the quantitative determination of lipoprotein (a) Lp(a) levels in human plasma or serum.

CLINICAL BACKGROUND

Recently a number of new putative cardiovascular risk factors have been proposed. Among these the lipoprotein (a) (LP(a)) is emerging as an important and informative new parameter. Thus, a number of immunological assays have been developed recently for the determination of this unusual lipoprotein (Ref. 6, 9, 10, 20).

The Lp(a) lipid composition is similar to that of LDL. Whereas the LDL particle contains as sole protein the apoB100, the LP(a) particle contains an additional apoprotein called apo(a) or little (a), which is linked to the apoB by means of a disulfide bridge. The molecular weight of Lp(a) is variable and the observed phenotypes have molecular weights that can vary between 200 and 700 kDa. It has been demonstrated that size polymorphism and plasma concentration are linked (Ref. 19). Forty to fifty percent of the plasma concentration is determined by heredity. Within the population plasma levels can vary from < 0.5 mg/dl up to 200 mg/dl but remain constant through life within an individual. Diet, lifestyle or the common lipid lowering drugs (with the exception of niacin and neomycin) do not lower plasma Lp(a) concentrations to a great extent (Ref. 1, 7) although there is a general observation that women tend to have higher Lp(a) levels than men, especially around the time of the menopause (Ref. 3, 18).

Recently an impressive body of data has been accumulated to establish that Lp(a) is an independent risk factor for coronary heart disease (CHD): concentrations of Lp(a) > 30 mg/dl have been correlated directly with premature CHD (Ref. 3, 18; 16, 8), especially when LDL levels are elevated (Ref. 2). The risk for stroke (Ref. 15) as well as for restenosis after coronary artery bypass surgery reportedly correlates highly with the increases in Lp(a). Family history of premature coronary disease was linked to the presence of elevated Lp(a) levels (Ref. 4). The striking homology between Lp(a) and plasminogen has given rise to the hypothesis that the increased risk of premature atherosclerosis and thrombotic diseases associated with elevated Lp(a) levels arises from the molecular mimicry of plasminogen by apo(a) (Ref. 11, 13). By interfering with the fibrinolytic functions of plasminogen and/or plasmin, Lp(a) can promote thrombotic events. It has been shown recently that Lp(a) competes with plasminogen for the plasminogen-receptor present on a variety of cells including the endothelial cells of the aortic wall (Ref. 5, 14).

Because plasminogen cannot bind to his receptor, the plasminogen cannot be activated properly and fibrinolysis is impaired. The chronic formation and stabilization of mural thrombi is believed to play a key role in the progression and growth of atherosclerotic lesions, and acute thrombotic events often occur on the fissures of atherosclerotic plaques. Thus Lp(a) could be the "long-sought link" between thrombosis and atherosclerosis.

PRINCIPLES OF THE TEST

The wells of polystyrene microplate strips have been coated with a mouse monoclonal anti-Lp(a) (antibody to Lp(a), which constitutes the solid-phase antibody). The test sample is incubated in such well; Lp(a), if present in the sample or standard solution, will bind to the solid-phase antibody. Unbound substances are removed by washing the plate. Subsequently a sheep anti-apo B polyclonal antibody, which has been labeled with the enzyme horse-radish peroxidase (HRP), is added. This labeled antibody binds to any solid-phase antibody/Lp(a) complex previously formed, because it can bind the apo B moiety of the Lp(a) complex. Incubation with enzyme substrate produces a blue colour in the test well, which turns into yellow when the reaction is stopped with sulphuric acid. The amount of colour produced in the wells is proportional to the amount of Lp(a) originally present in the sample or standard solution.

REAGENTS SUPPLIED

Each pack contains:

- 1 sachet containing a strip-holder with **12x8 anti-Lp(a)** (mouse monoclonal) **coated test wells** and a silicagel bag as drying agent.
- 6 vials containing 0.250 ml of a prediluted **Lp(a) standard solution** at concentrations of 100, 75, 50, 25, 10 and 5 mg/dl (phosphate buffer with stabilizing proteins, containing 0.05% Kathon CG as preservative).

- 1 vial containing 0.25 ml of lyophilized control serum **Level I** (exact potency is stated on flyer) (human serum).
- 1 vial containing 0.25 ml of lyophilized control serum **Level II** (exact potency is stated on flyer) (human serum).
- 2 vials containing 25 ml of concentrated **sample diluent** (phosphate buffer with stabilizing proteins, containing 0.05% Kathon CG as preservative), to be diluted 10 x before use.
- 1 vial containing 0.4 ml of concentrated **conjugate** (sheep anti-apo B polyclonal antibody labeled with HRP, containing 0.05% kathon CG as preservative), to be diluted 100 x (procedure A) or 40 x (procedure B) before use.
- 1 vial containing 20 ml of **conjugate diluent** (phosphate buffer with stabilizing proteins, containing 0,05% Kathon CG as preservative).
- 1 vial containing 0.3 ml of concentrated **TMB substrate solution** (tetramethylbenzidine dissolved in dimethyl sulfoxide), to be diluted 100 x before use.
- 1 vial containing 20 ml of **substrate buffer** (phosphate citrate buffer containing 0.006% hydrogen peroxide); ready to use.
- 2 vials containing 30 ml of concentrated **wash solution** (phosphate buffer containing detergent and 0.17% Kathon CG as preservative) to be diluted 25 x before use.
- 4 adhesive plate sealers.**
- 1 plastic minigrip bag** for storage of unused strips.

MATERIALS REQUIRED BUT NOT PROVIDED

- Distilled or deionized water.
- Sulphuric acid of analytical grade in the range of 1 to 2 mol/liter (e.g. from Merck or meeting the American Chemical Society Standards).
- Precision pipettes with disposable tips to deliver 10 or 100 µl (optionally 45 µl).
- Optionally a multichannel pipette to deliver 100 µl can be used together with disposable V-shaped troughs for addition of conjugate, substrate and sulfuric acid.
- Timer.
- Water bath set at 37°C with direct warming (i.e. the bottom of the wells must be in contact with the water) or a 37°C incubator with a relative humidity > 80%.
- Microplate washer (alternatively, washing can be performed manually, e.g. by using a repeating syringe delivering 0.3 ml volumes and an aspirating device).
- Absorbent tissues.
- Photometric reading: microplate reader, equipped with a 450 nm filter and preferably also with a 620 nm or 690 nm filter.

SAFETY

All blood components and biological materials should be considered as being potentially infectious and should be handled as such. Human serum components, which are used for the reagents of the kit, have been tested by immunoassays and were found to be non-reactive for hepatitis b surface antigen, antibodies against HIV-1, HIV-2 and HCV.

Avoid contact and inhalation of TMB substrate. If substrate comes into contact with skin, wash thoroughly with water.

Samples and all materials used in the assay must be considered potentially able to transmit infectious agents. They should be disposed of in accordance with established safety procedures.

STORAGE AND STABILITY

- If kept at 2° to 8°C, all test reagents, including the coated test wells, are stable until the expiration date given on the pack. Do not freeze reagents.
- All reagents and the sachet containing the test wells must be brought to room temperature (20-25°C) approximately 30 minutes before use and must be returned to the refrigerator immediately after use.
- Unused test wells, stored at 2-8°C, are stable for 8 weeks if stored in the plastic minigrip bag with silicagel.
- Diluted wash solution is stable for 2 weeks, if kept at 2-8°C.
- Diluted conjugate is stable for 8 hours at room temperature (20-25°C) if kept in the dark.
- Diluted substrate is stable for 1 hour at room temperature (20-25°C) if kept in the dark.
- After using some of the contents of vials containing standard solutions, sample diluent, concentrated conjugate, conjugate diluent, concentrated substrate, substrate buffer, and concentrated wash solution, the contents are stable until the expiration date if kept at 2-8°C and stored in the closed original vial.

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SPECIMEN COLLECTION

1. Serum or citrate plasma can be used.
2. Before storage serum should be separated from the blood clot, or plasma from the blood cells by centrifugation at 2000 to 5000 g for 20 minutes.
3. Insoluble material should be removed from all plasma samples by centrifugation before testing.
4. Undiluted samples can be stored at 2-8°C for 1 week, 1/200 diluted samples are stable for 24 hours at 2-8°C.
5. The samples may be stored at -20°C up to 1 month.
6. Repeatedly (more than 5 times) frozen and thawed samples may produce erroneous results.

PREPARATION AND MANIPULATION PROCEDURES :

Preparations:

1. Washing solution should be prepared by diluting concentrated wash solution (10) 25 x with distilled or deionized water, e.g. by diluting 24 ml to 600 ml. Prepare at least 50 ml of diluted wash solution for each test well strip.
Note: - Salt crystals may be formed in the concentrated wash solution after storage at 2-8°C. These crystals should be completely redissolved, by warming at 37°C, before dilution.
- Wash solution must be at room temperature (20-25°C) when used.
2. Dilute sample diluent 1/10 with distilled water.
Note: - Salt crystals may be formed at 2-8°C. These crystals should be completely dissolved by warming at room temperature, before dilution.
- Wash solution must be at room temperature (15-30°C) when used.
3. Reconstitute the lyophilized controls:
 - carefully open the vials,
 - add 250 µl distilled water,
 - leave for 10-15 minutes,
 - then vortex for 5 seconds,- dilute 1:200 in sample diluent (add 10 µl reconstituted control to 2 ml sample diluent),
 - vortex this dilution for 30 seconds,
 - after reconstitution these controls are stable for 4 weeks when stored at 2-8°C.
4. Conjugate should be prepared by diluting concentrated conjugate (6).
 - **PROCEDURE A (37°C incubation):** Dilute the concentrated conjugate 100 x, e.g. by diluting 10 µl to 1 ml per strip or 120 µl to 12 ml per plate.
 - **PROCEDURE B (25°C incubation):** Dilute the concentrated conjugate 40 x, e.g. by diluting 25 µl to 1 ml per strip or 300 µl to 12 ml per plate.
5. Substrate should be prepared by diluting concentrated TMB substrate (8) 100 x with substrate buffer (9), e.g. by diluting 10 µl to 1 ml per strip or 120 µl to 12 ml per plate.
Note: Concentrated TMB should be melted completely (melting point 18°C).
6. Predilute samples 1/200 by adding 10 µl sample to 2 ml diluted sample diluent.

Directions for washing:

Incomplete washing will adversely affect the test outcome. Operating instructions for washing equipment should be carefully followed. Contamination of wash solution and washer can cause extensive problems.

- Therefore:
- Store diluted wash solution at 2-8°C. Preferably use freshly prepared wash solution.
 - Pre-rinse the washer with diluted wash solution.
 - At the end of the day rinse the washer with purified water. Leave this solution in the washer until further use.

In case contaminations occur, disinfect wash bottles and washer overnight with 4% formaldehyde solution. If no suitable automatic washer is available, washing can be performed manually as follows: aspirate completely the liquid from all wells by lowering an aspiration tip gently to the bottom of each well. Take care not to scratch the inside of the well surface. After aspiration, fill the wells with 0.3 ml of diluted wash solution. Leave to soak for a minimum of 30 seconds, then aspirate the liquid. Perform these steps four times. After the last aspiration the washing procedure is completed by inverting the plate and tapping it dry on absorbent tissue.

Remarks and precautions:

1. Do not use the kit beyond the expiration date.
2. Do not combine strip plates and conjugate from packs that have different lot numbers.
3. All vessels used to prepare conjugate and substrate solutions must be cleaned thoroughly and finally rinsed with distilled water.
4. Do not touch the top of the plates with your fingers to avoid contamination.
5. Avoid microbial contamination of reagents. Take care to use clean glass ware.
6. Ensure that the samples and standards are homogeneous before use.
7. Use a new pipette-tip for each specimen aliquoted.
 - 8. To avoid contamination, do not touch the edges of the wells with the pipette tips when adding sample or conjugate.
9. Do not expose substrate to strong light during incubation or storage. Substrate solution must be almost colourless when used.
10. Solutions containing TMB, sulphuric acid or peroxide should not be in contact with metals or metal-ions, to avoid unwanted colour formation.

11. Make sure no air-bubbles in the wells are present; remove any detected by tapping gently.
12. If the wells can not be filled with conjugate or substrate immediately after washing, the strips may be placed upside down on a wet absorbent tissue for no longer than 15 minutes.

TEST PROCEDURE A (37°C incubation)

Before starting the assay, adjust the temperature of the water bath or the incubator to 37°C. Be sure all reagents are at room temperature. Dilute the sample diluent 1/10 with distilled water.

1. Take the strip-holder with the required number of strips, taking into account that for each test run 6 standards and one blank (sample diluent) should be included. Place unused strips in the plastic minigrp bag with the silicagel bag. During the test-run the strips stay in the strip-holder and can be marked on one edge.
2. Add 10 µl of the appropriate specimen (1:200 diluted, cfr. preparations 7), standard (100, 75, 50, 25, 10, 5 mg/dl) or controls (1:200 diluted, cfr preparations 4) to each well.
3. Add 100 µl of sample diluent to each test well reserved for specimen, standards and controls.
Add 100 µl of sample diluent to one test well reserved as blank. Make sure specimen and standards are adequately mixed with the sample diluent.
4. Cover the strips with an adhesive sealer. Incubate for 120 minutes at 37°C. (**Note:** prepare conjugate solution during incubation, see Preparations 5) (1:100 dilution).
5. Wash each well 4 times (see Directions for washing).
6. Add 100 µl prepared conjugate solution to each well, tap the strip-holder carefully to mix.
7. Cover the strips with a new adhesive sealer. Incubate for 60 minutes at 37°C. (**Note:** prepare substrate solution during incubation, see Preparations 6).
8. Wash each well 4 times (see Directions for washing).
9. Add 100 µl prepared substrate solution to each well, tap the strip-holder carefully to eliminate air bubbles that may occur.
10. Incubate for 30 minutes at 20-25°C.
11. To stop the reaction, add 100 µl sulphuric acid to each well, in the same sequence and at the same time intervals as the substrate solution. Tap the strip-holder carefully to ensure thorough mixing.
12. Blank the reader and read (within 15 minutes after step 11) the absorbance of the solution in the wells at 450 nm. For dual wavelength analysis, 690 nm or 620 nm should be used as a reference wavelength.

ASSAY PROCEDURE B (25°C incubation)

Be sure all reagents are at room temperature. Dilute the sample diluent 1/10 with distilled water.

1. Take the strip-holder with the required number of strips, taking into account that for each test run 6 standards and one blank (sample diluent) should be included. Place unused strips in the plastic minigrp bag with the silicagel bag. During the test-run the strips stay in the strip-holder and can be marked on one edge.
2. Add 10 µl of the appropriate specimen (1:200 diluted, cfr. preparations 7), standard (100, 75, 50, 25, 10, 5 mg/dl) or controls (1:200 diluted, cfr preparations 4) to each well.
3. Add 100 µl of sample diluent to each test well reserved for specimen, standards and controls.
Add 100 µl of sample diluent to one test well reserved as blank. Make sure specimen and standards are adequately mixed with the sample diluent.
4. Cover the strips with an adhesive sealer. Incubate for 60 minutes at 20-25°C. (**Note:** Prepare conjugate solution during incubation, see Preparations 5). (1:40 dilution)
5. Wash each well 4 times (see Directions for washing).
6. Add 100 µl prepared conjugate solution to each well, tap the strip-holder carefully to mix.
7. Cover the strips with a new adhesive sealer. Incubate for 60 minutes at 20-25°C. (**Note:** prepare substrate solution during incubation, see Preparations 6).
8. Wash each well 4 times (see Directions for washing).
9. Add 100 µl prepared substrate solution to each well, tap the strip-holder carefully to eliminate air bubbles that may occur.
10. Incubate for 30 minutes at 20-25°C.
11. To stop the reaction add 100 µl sulphuric acid to each well, in the same sequence and at the same time intervals as the substrate solution. Tap the strip-holder carefully to ensure thorough mixing.
12. Blank the reader and read (within 15 minutes after step 11) the absorbance of the solution in the wells at 450 nm. For dual wavelength analysis, 690 nm or 620 nm should be used as a reference wavelength.

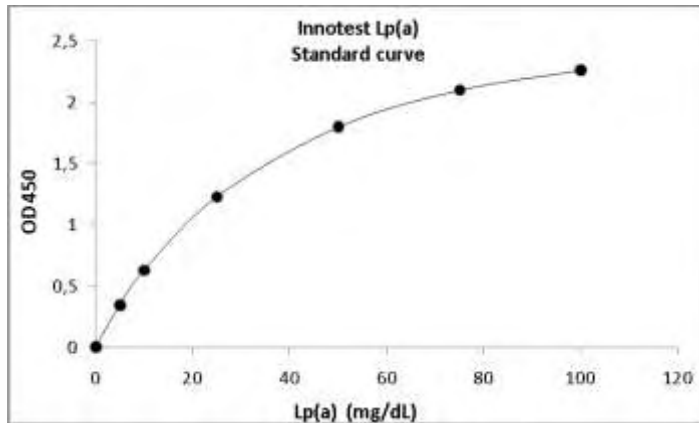
CALCULATION OF THE RESULTS

Construct the standard curve by plotting the mean absorbance values obtained for each of the Lp(a) standard solutions on the vertical (Y) axis versus the corresponding Lp(a) concentrations (100, 75, 50, 25, 10, 5 mg/dl) on the horizontal (X) axis, using rectilinear graph paper.

Draw the best fitting curve through these points.

Using the mean absorbance value for each sample to be tested, determine the corresponding concentration of Lp(a) in mg/dl from the standard curve.

If, in an initial assay, a sample is found to contain a Lp(a) concentration above 60 mg/dl, the sample can be further diluted (1/2) with sample diluent. The value read from the



standard curve must then be multiplied by 2.

A typical standard curve is shown below:

If more accurate readings of low values are desired, a computer assisted data reduction program should be used. A cubic spline program is the recommended program

SPECIFIC PERFORMANCE CHARACTERISTICS

Specificity

In this assay configuration no interference has been found from plasminogen (up to 500 mg/dl), LDL (up to 500 mg/dl), apo AI, apo AII, apo CII, apo CIII, apo E or human albumin (respectively at 500 mg/dl, 200 mg/dl, 50 mg/dl, and 10 g/l). No reaction with these antigens is observed when they are spotted on nitrocellulose (at equal concentrations) and blotted with the coating monoclonal antibody directed against the apo(a).

Expected values and clinical background

The concentrations of Lp(a) have been determined in 232 healthy normolipemic patients using the ELITEST Lp(a). The sera were collected from the patients at a clinic located in the northern part of Belgium.

The population consisted of 130 females and 113 men and the mean age of this group is 56.0 ± 11.0 years, with a range of 23 to 83 years. The mean Lp(a) concentration in this population is 13.6 ± 16.9 mg/dl, with a median of 7.1 mg/dl and a range from 0.4 to 90 mg/dl.

We did not observe any statistically significant values between males and females, and we did not observe any effect of aging on the Lp(a) levels. Studies performed in a larger group of patients (Ref. 17) described an increase of the Lp(a) values in women coinciding with the time of the menopause.

Results

It has been demonstrated that elevated Lp(a) values can be associated with an increased risk for the development of premature coronary heart disease (Ref. 16, 8, 2). A threshold value of 30 mg/dl is generally accepted as being the level above which the risk for developing premature coronary heart disease is increased.

We have investigated the Lp(a) concentrations in a large group of patients (916) in whom the presence of coronary heart disease (CHD+) was confirmed by coronary angiography. The patients were recruited at the same clinic where the control samples were collected.

The results of the patients suffering from CHD (CHD+) are compared with the results from the control group that is described in the "expected values" section (CHD-).

The results are summarized in the following table:

	CHD-	CHD+
Age, years	56.0 ± 11.0	61.4 ± 9.8*
Lp(a) mean, mg/dl	13.6 ± 16.9	21.5 ± 24.9**
Lp(a) median, mg/dl	7.1	10.7
Lp(a) range, mg/dl	0.4 - 90.0	0.4 - 156.0
Number of patients	232	916
% of population with Lp(a) >30 mg/dl	15	25

*statistically different Student t-test, with $p < 0.001$

**statistically different Mann Whitney Wilcoxon test with $p < 0.001$

Quality controls

The reproducibility of standard curves parameters and control sera should be within established limits of laboratory acceptability.

The customer can set up in-house controls following these instructions: three fresh sera or plasma are selected. One in the low (5 to 10 mg/dl), one in the medium (20 to 30 mg/dl) and one in the high concentration range (40 to 50 mg/dl). These sera or plasma are aliquoted into small portions (e.g. Eppendorf vials containing 100 µl of sample) and stored at -20°C. Each time the assay is performed one of these fractions is thawed and used only one day. These aliquoted fractions can be stored for a maximum of 1 year at -20°C and can serve as an internal control for the assay. Variability on the samples must be followed and the inter assay CV must preferably be lower than 10%.

Limitations

No interfering substances have been reported for the measurement of this lipoprotein. Neither hemoglobin (severely hemolysed sera), nor bilirubin (up to 15 mg/dl) nor triglycerides (up to 2500 mg/dl) interfere in the assay.

Although Lp(a) levels are hardly influenced by diet or lifestyle there are some situations in which Lp(a) values must be interpreted with some caution. During pregnancy it has been noted that Lp(a) levels rise during the first and second trimester, but stabilize afterwards. It has been reported also that Lp(a) can act as an acute phase reactant e.g. after surgical operations or after a myocardial infarction (Ref. 12). In situations of hepatobiliary disease Lp(a) levels are decreased (Ref. 21), while in patients with renal failure or patients suffering from familial hypercholesterolemia increased Lp(a) levels have been observed.

Detection limit

When performed as recommended in this package insert the detection limit of the assay is 2 mg/dl (sample dilution 1/200). Concentrations as low as 0.001 mg/dl can be measured in undiluted samples. Readings on the standard curve, as constructed according to the instructions given in the package insert, should then be divided by 200. It is advisable to make a minimum 1/2 dilution of the samples (divide the result by 100).

Precision

a. Standard curve (6 runs)

Standard (mg/dl)	CV (%) intra-assay	CV (%) Inter-assay
100.0	2.3	0.8
50.0	3.0	2.9
25.0	6.0	6.7
12.5	9.0	6.5
6.3	10.3	5.2
3.1	8.3	4.6

b. Samples (6 runs)

Sample	Concentration (mg/dl)	CV (%) intra-assay	CV (%) Inter-assay
1	55.0	5.4	10.2
2	31.9	9.7	7.4
3	26.5	7.2	3.4
4	23.1	5.1	5.9
5	18.7	9.1	3.6
6	6.0	4.5	3.6

LIST OF RELEVANT LITERATURE

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SUMMARY OF ASSAY PROCEDURE A (Flow Chart)

Reagent	Volume	Procedure
Reconstitute controls and dilute 1/200 Dilute sample diluent 1/10 Dilute samples 1/200		
Prediluted samples, standards, and/or controls	10 µl	Add to each appropriate test well.
Prediluted sample diluent	100 µl	Add to each test well, reserved for specimen, standards and blank. Mix and cover plate with adhesive sealer.
Incubate for 120 minutes at 37°C		
Warning : prepare conjugate before the end of incubation (1:100 dilution)		
Wash solution	4 x 0.3 ml	Wash each well 4 times.
Conjugate	100 µl	Add to each well, mix gently. Cover plate with new adhesive sealer.
Incubate for 60 minutes at 37°C		
Warning : prepare substrate before the end of incubation.		
Wash solution	4 x 0.3 ml	Wash each well 4 times.
Substrate	100 µl	Add to each well.
Incubate for 30 minutes at 20-25°C.		
Sulphuric acid	100 µl	Add to each well. Mix by tapping side of plate
Read absorbance at 450 nm within 15 minutes.		

SUMMARY OF ASSAY PROCEDURE B (Flow Chart)

Reagent	Volume	Procedure
Reconstitute controls and dilute 1/200 Dilute sample diluent 1/10 Dilute samples 1/200		
Prediluted samples, standards, and/or controls	10 µl	Add to each appropriate test well.
Prediluted sample diluent	100 µl	Add to each test well, reserved for specimen, standards and blank. Mix and cover plate with adhesive sealer.
Incubate for 60 minutes at 20-25°C.		
Warning : prepare conjugate before the end of incubation (1:40 dilution)		
Wash solution	4 x 0.3 ml	Wash each well 4 times.
Conjugate	100 µl	Add to each well, mix gently. Cover plate with new adhesive sealer.
Incubate for 60 minutes at 20-25°C		
Warning : prepare substrate before the end of incubation.		
Wash solution	4 x 0.3 ml	Wash each well 4 times.
Substrate	100 µl	Add to each well.
Incubate for 30 minutes at 20-25°C.		
Sulphuric acid	100 µl	Add to each well. Mix by tapping side of plate
Read absorbance at 450 nm within 15 minutes.		