1,25(OH)$_2$ Vitamin D ELISA

KAR1921

History

Resume of change :

<table>
<tr><th>Previous Version :

170929/1</th><th>Current Version :

190619/1</th></tr>
<tr><td>

VII. C. Working HRP conjugate solution:

! The working HRP conjugate solution is to be prepared absolutely —one hour before adding the solution in the plate !

Prepare an adequate volume of working HRP conjugate solution by mixing concentrated conjugate, concentrated HRP and conjugate buffer according to the number of used strips, as indicated in the below table: for example for 6 strips (48 wells): 250µL of concentrated conjugate and 50µL of concentrated HRP to 10mL of conjugate buffer.

Use a vortex to homogenize.

Keep the working HRP conjugate at room temperature (18°C to 25°C) and avoid direct sunlight or use a brown glass vial for its preparation.

<table>
<tr><th>Nb of strips</th><th>Volume of Concentrated Conjugate (µL)</th><th>Volume of Concentrated HRP (µL)</th><th>Volume of Conjugate Buffer (mL)</th></tr>
<tr><td>1</td><td>75</td><td>15</td><td>3</td></tr>
<tr><td>2</td><td>125</td><td>25</td><td>5</td></tr>
<tr><td>3</td><td>150</td><td>30</td><td>6</td></tr>
<tr><td>4</td><td>200</td><td>40</td><td>8</td></tr>
<tr><td>5</td><td>225</td><td>45</td><td>9</td></tr>
<tr><td>6</td><td>250</td><td>50</td><td>10</td></tr>
<tr><td>7</td><td>300</td><td>60</td><td>12</td></tr>
<tr><td>8</td><td>350</td><td>70</td><td>14</td></tr>
<tr><td>9</td><td>400</td><td>80</td><td>16</td></tr>
<tr><td>10</td><td>450</td><td>90</td><td>18</td></tr>
<tr><td>11</td><td>500</td><td>100</td><td>20</td></tr>
<tr><td>12</td><td>550</td><td>110</td><td>22</td></tr>
</table>

</td><td>

VII. C. Working HRP conjugate solution:

! <u>The working HRP conjugate solution is to be prepared during the incubation and minimum 1h before its use</u> (cf X.III).

Prepare an adequate volume of working HRP conjugate solution by mixing the 3 reagents in the following sequence : (1) Conjugate buffer, (2) Concentrated Conjugate, (3) Vortex, (4) Concentrated HRP, (5) Vortex.

<u>The order of addition of those 3 reagents is critical and should be rigorously respected to get reproducible Optical Densities.</u>

Prepare the solution according to the number of used strips, as indicated in the below table: for example for 6 strips (48 wells): 250 µl of concentrated conjugate and 50 µl of concentrated HRP to 10 ml of conjugate buffer.

Use a vortex to homogenize.

Until its use, keep the working HRP conjugate at room temperature and avoid direct sunlight or use a brown glass vial for its preparation.

The preparation of working HRP conjugate is not stable and must be discarded if not used.

<table>
<tr><th>Nb of strips</th><th>Volume of Conjugate Buffer (mL)</th><th>Volume of Concentrated Conjugate (µL)</th><th>Volume of Concentrated HRP (µL)</th></tr>
<tr><td>1</td><td>3</td><td>75</td><td>15</td></tr>
<tr><td>2</td><td>5</td><td>125</td><td>25</td></tr>
<tr><td>3</td><td>6</td><td>150</td><td>30</td></tr>
<tr><td>4</td><td>8</td><td>200</td><td>40</td></tr>
<tr><td>5</td><td>9</td><td>225</td><td>45</td></tr>
<tr><td>6</td><td>10</td><td>250</td><td>50</td></tr>
<tr><td>7</td><td>12</td><td>300</td><td>60</td></tr>
<tr><td>8</td><td>14</td><td>350</td><td>70</td></tr>
<tr><td>9</td><td>16</td><td>400</td><td>80</td></tr>
<tr><td>10</td><td>18</td><td>450</td><td>90</td></tr>
<tr><td>11</td><td>20</td><td>500</td><td>100</td></tr>
<tr><td>12</td><td>22</td><td>550</td><td>110</td></tr>
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Read entire protocol before use.

1,25(OH)$_2$ Vitamin D ELISA

I. INTENDED USE

Immunoenzymetric assay for the measurement of 1,25(OH)$_2$ Vitamin D in serum.
For Research Use Only. Not for Use in Diagnostic Procedures.

II. GENERAL INFORMATION

A. Proprietary name : DIAsource 1,25(OH)$_2$ Vitamin D ELISA

B. Catalog number : KAR1921 : 96 tests

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

For technical assistance or ordering information contact :
Tel: +32 (0) 10 84.99.11 Fax: +32 (0) 10 84.99.91

III. BACKGROUND

Biological activities

Vitamin D is mainly synthesized in the skin from 7-dehydrocholesterol and is partially from dietary and supplementation origin. In the liver, Vitamin D is hydroxylated on carbon 25 to produce the intermediate 25OH Vitamin D. 25OH Vitamin D is further metabolized before it can carry out the functions of Vitamin D on intestine, kidneys, bone and other organs and tissues. This subsequent reaction takes place in the kidneys and in other tissues. Thus 25OH Vitamin D is further hydroxylated in the 1α-position to produce 1α,25-dihydroxyvitamin D (1,25(OH)$_2$ Vitamin D). In addition to the above-mentioned tissues, placenta of pregnant women and macrophage cells in case of sarcoidis can also produce some amount of 1,25(OH)$_2$ Vitamin D.

1,25(OH)$_2$ Vitamin D is the active form of Vitamin D with regard to the known functions whereas 25OH Vitamin D and Vitamin D itself can be excluded as being physiologically functional. 1,25(OH)$_2$ Vitamin D stimulates the intestinal absorption of both calcium and phosphorus. It also stimulates bone resorption and mineralization thereby preventing the development of rickets and osteomalacia.

1,25(OH)$_2$ Vitamin D is also be active in other tissues responsible for Calcium transport (placenta, kidney, mammary gland,...) and endocrine glands such as parathyroid glands. 1,25(OH)$_2$ Vitamin D is rapidly metabolized and its halflife is approximately 12h in plasma. Its main metabolite is calcitroic acid, a C-23 carboxylic derivative, essentially without any biological activity. In addition to this pathway, 1,25(OH)$_2$ Vitamin D undergoes 24-hydroxylation to produce 1,24,25-trihydroxyvitamin D. This compound has less biological activity than its parent and this metabolic route is considered as a minor pathway.

The levels of 1,25(OH)$_2$ Vitamin D in plasma or serum is 100 to 1000 less than that of 25OH Vitamin D. Due to its low concentrations and the presence of many similar metabolites, the measurement of 1,25(OH)$_2$ Vitamin D requires extraction and separation by chromatography.

IV. PRINCIPLES OF THE METHOD

Only samples and controls, not the calibrators, are extracted with a mixture of solvents and applied on cartridges to separate $1,25(OH)_2$ Vitamin D from the other Vitamin D metabolites. After elution of the $1,25(OH)_2$ Vitamin D from the samples and controls cartridges, the calibrators, eluted samples and eluted controls are incubated directly in microtiterplate coated with anti-$1,25(OH)_2$ Vitamin D antibodies.
After an overnight incubation at 4°C, the microtiter plate is washed and the working conjugate solution is added and incubated for 1 hour at 4°C.
The microtiterplate is then washed to stop the competition reaction. The Chromogenic solution (TMB) is added and incubated for 15 minutes at room temperature (18°C to 25°C). The reaction is stopped with the addition of Stop Solution and the microtiterplate is read at the appropriate wavelength.
The amount of $1,25(OH)_2$ Vitamin D is determined colourimetrically by measuring the absorbance, which is inversely proportional to the $1,25(OH)_2$ Vitamin D concentration.
A calibration curve is plotted and the $1,25(OH)_2$ Vitamin D concentrations of the samples are determined by dose interpolation from the calibration curve.

V. REAGENTS PROVIDED

Reagents	96 Tests Kit	Colour Code	Reconstitution
Microtiterplate with 96 breakable wells coated with anti-$1,25(OH)_2$Vitamin D antibodies	96 wells	blue	**Ready** for use
CONJ | **CONC** $1,25(OH)_2$Vitamin D Concentrated Conjugate	1 vial 1 mL	blue	**Dilute** 40 x with conjugate buffer
HRP | **CONC** Concentrated HRP	1 vial 0.2 mL	yellow	**Dilute** 200 x with conjugate buffer
CAL | **N** Calibrators - N = 0 to 5 (see exact values on vial labels) in phosphate buffer with bovine casein and gentamycin	6 vials lyophilised	yellow	**Add** 1mL distilled water
WASH | **SOLN** | **CONC** Wash solution (TRIS-HCl)	1 vial 10 mL	brown	**Dilute** 200 x with distilled water (use a magnetic stirrer)
CONTROL | **N** Controls - N = 1 or 2 in human plasma with gentamycin	2 vials lyophilised	silver	**Add** 3mL distilled water
INC | **BUF** Incubation Buffer with proclin	1 vial 20 mL	green	**Ready** for use
CONJ | **BUF** Conjugate Buffer with casein and proclin	1 vial 30 mL	red	**Ready** for use
ELU | **SOLN** Elution Solution: contains methanol	1 vial 30 mL	white	**Ready** for use
CHROM | **TMB** Chromogenic solution TMB (Tetramethylbenzydine)	1 vial 25 mL	brown	**Ready** for use
STOP | **SOLN** Stop solution HCl 1.5 N	1 vial 12 mL	white	**Ready** for use
PLATE | **COVER** Adhesive Strips	4		
GEL Silica cartridges	42		**Store** at R.T.

Note : Use the Calibrator 0 for dilution of samples with values above the highest calibrator (dilute before extraction step).

VI. SUPPLIES NOT PROVIDED

The following material is required but not provided in the kit:
1 Distilled water
2 Diisopropylether ("for analysis"; GC purity ≥ 99%)
3 Cyclohexane ("for analysis"; GC purity ≥ 99.5 %)
4 Ethyl acetate("for analysis"; GC purity ≥ 99.5 %)
5 Ethanol absolute ("for analysis"; GC purity ≥ 99.9 %)
6 Dichloromethane ("for analysis"; GC purity ≥ 99.8 %)
7 Pipettes for delivery of: 50µL, 100µL, 150µL, 200 µL, 1mL and 3 mL (the use of accurate pipettes with disposable plastic tips is recommended)
8 Glass tubes (12 x 75 mm) for extraction and for elution (closed with a cap for the extraction step).
9 Glass tubes (16 x 100 mm) or (12 x 120 mm), or polypropylene tubes (falcon 2097), for the washing of the cartridges.
10 Vortex mixer
11 Magnetic stirrer
12 Centrifuge operating at 800 g
13 Microtiterplate reader capable of reading at 450 nm and 650 (bichromatic reading)

VII. REAGENT PREPARATION

A. Calibrators: Reconstitute the calibrators with 1mL distilled water.
B. Controls: Reconstitute the controls with 3mL distilled water, **carefully to avoid overflow.**

C. **Working HRP conjugate solution:**
! The working HRP conjugate solution is to be prepared during the incubation and minimum 1h before its use (cf X.III).
Prepare an adequate volume of working HRP conjugate solution by mixing the 3 reagents in the following sequence : (1) Conjugate buffer, (2) Concentrated Conjugate, (3) Vortex, (4) Concentrated HRP, (5) Vortex.
The order of addition of those 3 reagents is critical and should be rigorously respected to get reproducible Optical Densities.
Prepare the solution according to the number of used strips, as indicated in the below table: for example for 6 strips (48 wells): 250 µl of concentrated conjugate and 50 µl of concentrated HRP to 10 ml of conjugate buffer.
Use a vortex to homogenize.
Until its use, keep the working HRP conjugate at room temperature and avoid direct sunlight or use a brown glass vial for its preparation.
The preparation of working HRP conjugate is not stable and must be discarded if not used.

Nb of strips	Volume of Conjugate Buffer (mL)	Volume of Concentrated Conjugate (µL)	Volume of Concentrated HRP (µL)
1	3	75	15
2	5	125	25
3	6	150	30
4	8	200	40
5	9	225	45
6	10	250	50
7	12	300	60
8	14	350	70
9	16	400	80
10	18	450	90
11	20	500	100
12	22	550	110

D. Working Wash solution: Prepare an adequate volume of Working Wash solution by adding 199 volumes of distilled water to 1 volume of Wash Solution (200x). Use a magnetic stirrer to homogenize. Discard unused

Working Wash solution at the end of the day.

E. **Extraction solvent**: 2 ml for each control or sample to be tested are needed. **Prepare a fresh solution** of diisopropylether, cyclohexane and ethyl acetate: 50/40/10 volume/volume).
Be careful : exact proportion of volume have to be strictly respected.

F **Washing solvent** : 1 ml for each control or sample to be tested is needed. **Prepare a fresh solution** of diisopropylether, cyclohexane, ethyl acetate and ethanol absolute (50/40/10/1 volume/volume).
Be careful : exact proportion of volume have to be strictly respected .

VIII. STORAGE AND EXPIRATION DATING OF REAGENTS

- Before opening or reconstitution, all kits components are stable until the expiry date, indicated on the label, if kept at 2 to 8°C; **except the cartridges which must be stored at room temperature (18°C to 25°C)**.
- After their reconstitution, **calibrators** are stable for 4 weeks at 2-8°C. For longer storage periods, aliquots should be made and kept at –20°C for 4 months maximum. Avoid subsequent freeze-thaw cycles.
- After their reconstitution, **controls** are stable for 3 days at 2-8°C. For longer storage periods, aliquots should be made and kept at –20°C for 1 month maximum. Avoid subsequent freeze-thaw cycles.
- Freshly prepared Working Wash solution should be used on the same day.
- After its use, discard working HRP conjugate.
- **Use freshly prepared extraction solvent and washing solvent, do not store them**.
- Alterations in physical appearance of kit reagents may indicate instability or deterioration.

IX. SPECIMEN COLLECTION AND PREPARATION

- The kit is suitable for serum samples.
- Serum samples must be kept at 2-8°C.
- If the test is not run within 24 hrs, storage in aliquots, at -20°C is recommended.
- Avoid subsequent freeze-thaw cycles.
- After thawing, the samples should be vortexed and centrifuged.

X. PROCEDURE

I. Extraction step : ! Only for controls and samples !

1. Label glass tubes (12x75 mm) for extraction: 2 controls and up to 40 samples.
2. Add 0.5mL control or sample in the respective tubes.
3. Dispense 2mL extraction solvent in each tube.
4. Tubes are closed with a cap and placed on a shaker for 1 hour at 1200 rpm.
5. Centrifuge each tube for 5 minutes at room temperature (18°C – 25°C at 800 g).
6. Supernatants are needed for the next step of separation.

II. Separation step : ! Only for controls and samples !

1. Label glass tubes (16 x 100 mm) or (12 x 120 mm), or polypropylene tubes (falcon 2097), for washing cartridges: 2 controls and up to 40 samples.
2. Put one silica cartridge in each tube.
3. Apply 1.6mL of supernatant (2 x 0.8mL), obtained after extraction step, on cartridge. Let draw by gravity.
4. Wash cartridges with 1mL washing solvent (cf: reagent preparation).
   ! Be careful: never apply vacuum on cartridges, just let solvent draw by gravity.
5. Add 500µL dichloromethane on each cartridge, let draw by gravity.
6. Add 500µL of distilled water on each cartridge and centrifuge each tube for 5 minutes at room temperature (18°C – 25°C at 800 g).
7. Label glass tubes (12 x 75 mm) for elution of $1,25(OH)_2$ Vitamin D. After centrifugation, transfer cartridges in the corresponding glass tubes.
8. Apply 300µL elution solution on each cartridge to elute $1,25(OH)_2$ Vitamin D and centrifuge for 5 minutes at room temperature (18°C – 25°C at 800 g).
9. **Vortex** the eluted fraction.

Note : After this step, samples must be incubated in coated microtiterplate immediately to avoid degradation.

III. Incubation step :

1. Select the required number of strips for the run. The unused strips should be resealed in the bag with a desiccant and stored at 2-8°C.
2. Secure the strips into the holding frame.
3. Vortex briefly reconstituted calibrators, extracted controls and extracted samples.
4. Pipette 150µL of Incubation Buffer into all wells.
5. Pipette 50µL of each Calibrator (not extracted), eluted controls and eluted samples into the appropriate wells.
6. Incubate for 18±2 hours, at 2-8°C.Cover the plate with a lid or a sealing film.

Prepare the Working HRP conjugate solution 60 min +/- 15 min before washing the wells after the overnight incubation.

7. Aspirate the liquid from each well.
   Wash the plate 3 times by:
   - dispensing 0.35mL of Wash Solution into each well and
   - aspirating the content of each well
8. Pipette 200µL of Working HRP conjugate solution into each well.
9. Incubate for 1 hour at 4°C.Cover the plate with a lid or a sealing film.
10. Aspirate the liquid from each well.
    Wash the plate 3 times by:
    - dispensing 0.35 ml of Wash Solution into each well and
    - aspirating the content of each well
11. Pipette 200µL of the Chromogenic solution into each well within 15 minutes following the washing step.
12. Incubate the microtiterplate for 15 minutes at room temperature (18°C to 25°C), avoid direct sunlight.
13. Pipette 100µL Stop Solution into each well.
14. Read absorbances at 450 nm (reference filter 630nm or 650 nm) within 1 hour and calculate the results as described in section XI.

XI. CALCULATION OF RESULTS

1. Read the plate at 450 nm against a reference filter set at 650 nm (or 630 nm).
2. Calculate the mean of duplicate determinations.
3. We recommend the use of computer assisted methods to construct the calibration curve. 4-parameter logistic function curve fitting is the preferred method. Reject obvious outliers.
4. By interpolation of the sample OD values, determine the $1,25(OH)_2$ Vitamin D concentrations of the samples from the calibration curve.

XII. TYPICAL DATA

The following data are for illustration only and should never be used instead of the real time calibration curve.

$1,25(OH)_2$ Vitamin D ELISA	**OD units**
Calibrator :	
0 pg/mL	2.93
3 pg/mL	2.52
12 pg/mL	1.85
50 pg/mL	1.11
120 pg/mL	0.57
180 pg/mL	0.36

Note : 1 pg/mL = 2.4 pmol/L

XIII. PERFORMANCE AND LIMITATIONS

A. Detection Limit

Twenty zero calibrators were assayed along with a set of other calibrators. The detection limit, defined as the apparent concentration two standard deviations below the average OD at zero binding, was 0.8 pg/mL.

B. Specificity

Cross reactivity of the $1,25(OH)_2$ Vitamin D ELISA assay was determined by testing sera with spiked and unspiked cross reactants. The results are summarized in the following table:

Compound and concentration	**Cross-Reactivity (%)**
$1,25(OH)_2$-Vitamin.D3at 200 pg/mL	114
$1,25(OH)_2$-Vitamin.D2 at 200 pg/mL	108
25OH-Vitamin-D3at 1µg /mL	0.004
25OH-Vitamin-D2 at 1µg /mL	

24,25$(OH)_2$-Vitamin.D3 at 200 ng/mL	0.0003
25,26$(OH)_2$-Vitamin.D3 at 400ng/mL	0.03
	0.02

The effect of potential interfering substances on samples using the DIAsource 1,25$(OH)_2$ Vitamin D ELISA test was evaluated. Different levels of Hemoglobin, Bilirubin (conjugated and unconjugated), Triglyceride and Vitamin C in serum samples were tested on samples with different 1,25$(OH)_2$ Vitamin D Concentration. Our acceptance criteria was to have interference of less than 15%. The tested substances did not affect the performance of the DIAsoure 1,25$(OH)_2$ Vitamin D ELISA.

Substance	1,25$(OH)_2$ VitaminD (pg/mL)	Concentration of Interferent (mg/dL)	Mean % Variation
Hemoglobin	31.8	250	5.0%
		500	
	186.5	250	
		500	
Bilirubin Conjugated	31.8	50	-12.3%
	186.5	50	
Bilirubin Unconjugated	31.8	50	-0.4%
		100	
	186.5	50	
		100	
Triglyceride	31.8	50	-1.0%
		100	
		250	
	186.5	50	
		100	
		250	
Vitamin C	31.8	100	4.9%
		1000	
	186.5	100	
		1000	

C. Precision

INTRA-ASSAY PRECISION

Sample	N	<X> ± SD (pg/mL)	CV (%)
A	13	18.3 ± 2.5	13.9
B	13	168.9 ± 8.4	5.0

INTER-ASSAY PRECISION

Sample	N	<X> ± SD (pg/mL)	CV (%)
A	13	26.7 ± 3.5	13.2
B	13	83.4 ± 14.6	17.5

SD: Standard Deviation; CV: Coefficient of variation

D. Accuracy

The sample was diluted with Calibrator 0, before extraction step.

DILUTION TEST

Sample Dilution	Theoretical Concentration (pg/mL)	Measured Concentration (pg/mL)	Slope	Y-Intercept	R	Recovery (%)
1/1	118.9	118.9	0.99	1.12	0.99	100
1/2	59.5	60.7				102
1/4	29.7	29.3				99
1/8	14.9	16.6				112

Conversion factor :

From pg/mL to pmol/L : x 2.4

From pmol/L to pg/mL : x 0.42

To the best of our knowledge, no international reference material exists for this parameter.

RECOVERY TEST

Added 1,25(OH)2-Vitamin D (pg/mL)	Recovered 1,25(OH)2-Vitamin D (pg/mL)	Recovered (%)
52.4	54.1	103
104.7	111.1	106
157.1	155.8	99

XIV. INTERNAL QUALITY CONTROL

- If the results obtained for Control 1 and/or Control 2 are not within the range specified on the vial label, the results cannot be used unless a satisfactory explanation for the discrepancy has been given.
- If desirable, each laboratory can make its own pools of control samples, which should be kept frozen in aliquots. Controls which contain azide will interfere with the enzymatic reaction and cannot be used.
- Acceptance criteria for the difference between the duplicate results of the samples should rely on Good Laboratory Practises
- It is recommended that Controls be routinely assayed as unknown samples to measure assay variability. The performance of the assay should be monitored with quality control charts of the controls.

It is good practise to check visually the curve fit selected by the computer

XV. PRECAUTIONS AND WARNINGS

Safety

For *research* use only.

The human blood components included in this kit have been tested by European approved and/or FDA approved methods and found negative for HBsAg, anti-HCV, anti-HIV-1 and 2. No known method can offer complete assurance that human blood derivatives will not transmit hepatitis, AIDS or other infections. Therefore, handling of reagents, serum or plasma specimens should be in accordance with local safety procedures.

All animal products and derivatives have been collected from healthy animals. Bovine components originate from countries where BSE has not been reported. Nevertheless, components containing animal substances should be treated as potentially infectious.

Avoid any skin contact with all reagents, Stop Solution contains HCl. In case of contact, wash thoroughly with water.

Do not smoke, drink, eat or apply cosmetics in the working area. Do not pipette by mouth. Use protective clothing and disposable gloves.

XVI. BIBLIOGRAPHY

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Stop Solution	100µL	100µL
Read on a microtiterplate reader. Record the absorbance of each well at 450 nm (versus 630 or 650 nm).		

DIAsource Catalogue Nr : KAR1921	P.I. Number : 1700551/RUO	Revision nr : 190619/1

Revision date : 2019-06-19

XVII. SUMMARY OF THE PROTOCOL

	CALIBRATORS µL	**SAMPLE(S) CONTROLS µL**
EXTRACTION		
Calibrators	-	-
Samples / Controls	-	500
Extraction solvent	-	2000
Shaking	1 hour at 1200 rpm	
Centrifugation	5 minutes at 800 g	
SEPARATION		
Supernatant from extraction step	-	1600
CARTRIDGE		
Supernatant	1600µL	
Washing Solvent	1000µL	
Dichloromethane	500µL	
Distilled water	500µL	
Centrifugation	5 minutes at 800 g	
Elution solution	300µL	
Centrifugation	5 minutes at 800 g	
	Vortex	
INCUBATION STEP		
In microtiterplate		
Incubation Buffer	150µL	150µL
Calibrators	50µL	-
Extracted samples	-	50µL
	Cover the plate with a lid or sealing film	
	Incubate 18 ± 2 h (overnight) at 4°C (2-8°C)	
	Prepare working HRP solution 1 hour before next step	
	Aspirate the contents of each well	
	Wash 3 times with 350µL of Wash Solution and aspirate	
Working HRP Conjugate	200µL	200µL
Cover the plate with a lid or sealing film and Incubate for 1 hour at 4°C (2-8°C). Aspirate the contents of each well. Wash 3 times with 350 µl of Wash Solution and aspirate.		
TMB	200µL	200µL
Incubate for 15 min at room temperature (18°C to 25°C).		

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