EPITOPE DIAGNOSTICS, INC.

EDI™ Quantitative Fecal Calprotectin ELISA Kit

Enzyme Linked ImmunoSorbent Assay (ELISA) for the Quantitative Measurement of Human Calprotectin Level in Feces

REF KT-849 CE IVD 96

INTENDED USE

This test kit is intended for use in the quantitative determination of human calprotectin (neutrophil cytoplasmic protein S100A8/A9) levels in stool samples. It is for in-vitro diagnostic use. The test is useful for detecting inflammatory bowel disease (IBD) such as ulcerative colitis and Crohn's disease.This kit is for in vitro diagnostic use only.

SUMMARY OF PHYSIOLOGY

Quantitative determination of fecal calprotectin is an indication of the severity of bowel inflammation. Also, higher levels of calprotectin in the stool are associated with an increased risk of relapse in patients with inflammatory bowel disease (IBD). Low stool calprotectin levels correlate well with a low risk for intestinal allograft rejection. This assay uses specific monoclonal antibodies to ensure only calprotectin is detected.

ASSAY PRINCIPLE

This ELISA is designed, developed and produced for the quantitative measurement of human calprotectin in stool samples. The assay utilizes the two-site "sandwich" technique with two selected antibodies that bind to different epitopes of human calprotectin.

Assay standards, controls and patient samples are added directly to wells of a microtiter plate that is coated with antibody to calprotectin. After a short incubation period, the plate is washed and horseradish peroxidase (HRP)-conjugated human calprotectin specific monoclonal antibody is added to each well. After the second incubation period, a "sandwich" of solid-phase antibody – human calprotectin – HRP-conjugated monoclonal antibody" is formed. The unbound monoclonal antibodies and buffer matrix are removed in the subsequent washing step. For the detection of this immunocomplex, the well is then incubated with a substrate solution in a timed reaction and then measured in a spectrophotometric microplate reader. The enzymatic activity of the immunocomplex bound to the wall of each microtiter well is directly proportional to the amount of human calprotectin in the test sample. A standard curve is generated by plotting the absorbance versus the respective human calprotectin concentration for each standard on a point-to-point or 4-parameter curve fitting. The concentration of fecal human calprotectin in test samples is determined directly from this standard curve.

REAGENTS: PREPARATION AND STORAGE

This test kit must be stored at 2 – 8°C upon receipt. For the expiration date of the kit refer to the label on the kit box. All components are stable until this expiration date.

Prior to use allow all reagents to come to room temperature. Reagents from different kit lot numbers should not be combined or interchanged.

1. Calprotectin Antibody Coated Microplate (30439)

Microplate coated with anti-calprotectin antibody.

Qty: 1 x 96 well microplate
Storage: 2 – 8°C
Preparation: Ready to Use

2. Calprotectin Tracer Antibody (30440)

HRP-labeled calprotectin antibody in a stabilized protein matrix.

Qty: 1 x 0.6 mL
Storage: 2 – 8°C
Preparation: 21X Concentrate. The contents must be diluted with tracer antibody diluent (30458) and mixed well before use.

3. ELISA Wash Concentrate (10010)

Surfactant in a phosphate buffered saline with non-azide preservative.

Qty: 1 x 30 mL
Storage: 2 – 8°C
Preparation: 30X Concentrate. The contents must be diluted with 870 mL distilled water and mixed well before use.

4. ELISA HRP Substrate (10020)

Tetramethylbenzidine (TMB) with stabilized hydrogen peroxide.

Qty: 1 x 12 mL
Storage: 2 – 8°C
Preparation: Ready to Use

5. ELISA Stop Solution (30559)

2N Hydrochloric Acid (HCl)

Qty: 1 x 12 mL
Storage: 2 – 8°C
Preparation: Ready to Use

6. Calprotectin Standards Levels 1 to 7 (30571 - 30577)

Human calprotectin in a lyophilized bovine serum-based matrix with a non-azide, non-mercury preservative. Refer to vials for exact concentration for each standard.

Qty: 7 x vials
Storage: 2 – 8°C , <-20°C for long term storage
Do not exceed 3 freeze-thaw cycles.
Preparation: Must be reconstituted with 0.5 mL of demineralized water, allowed to sit for 10 minutes, and then mix microwell by inversions or gentle vortexing. Make sure that all solids are dissolved completely prior to use.

7. Calprotectin Controls (30578 - 30580)

Human calprotectin in a lyophilized bovine serum-based matrix with a non-azide, non-mercury preservative. Refer to vials for exact concentration for each standard.

Qty: 3 x vials
Storage: 2 – 8°C , <-20°C for long term storage
Do not exceed 3 freeze-thaw cycles.
Preparation: Must be reconstituted with 0.5 mL of demineralized water, allowed to sit for 10 minutes, and then mix microwell by inversions or gentle vortexing. Make sure that all solids are dissolved completely prior to use.

8. Tracer Antibody Diluent (30458)
For tracer antibody dilution according to the assay procedures.
Qty: 1 x 12 mL
Storage: 2 – 8°C
Preparation: Ready to Use

9. Assay Buffer(30485)
Buffer to be used according to the assay procedures.
Qty: 1 x 12 mL
Storage: 2 – 8°C
Preparation: Ready to Use

10. Extraction Buffer Concentrate (30473)
Buffer to be used according to the assay procedures.
Qty: 1 x 120 mL
Storage: 2 – 8°C
Preparation: 5X Concentrate. The contents must be diluted with 480 mL of demineralized water and mixed well before use.

SAFETY PRECAUTIONS

The reagents are for in vitro diagnostic use only. Source material which contains reagents of bovine serum albumin was derived in the contiguous 48 United States. It was obtained only from healthy donor animals maintained under veterinary supervision and found free of contagious diseases. Wear gloves while performing this assay and handle these reagents as if they were potentially infectious. Avoid contact with reagents containing hydrogen peroxide, or sulfuric acid. Do not get in eyes, on skin, or on clothing. Do not ingest or inhale fumes. On contact, flush with copious amounts of water for at least 15 minutes. Use Good Laboratory Practices.

MATERIALS REQUIRED BUT NOT PROVIDED

1. Calprotectin Fecal Sample Collection Kit (Epitope Catalog No: KT-843)
2. Precision single channel pipettes capable of delivering 50 µL, 100 µL, 500 µL, etc.
3. Disposable pipette tips suitable for above volume dispensing.
4. Disposable plastic 100 mL and 1000 mL bottle with caps.
5. Aluminum foil.
6. Deionized or distilled water.
7. Plastic microtiter well cover or polyethylene film.
8. ELISA multichannel wash bottle or automatic (semi-automatic) washing system.
9. Spectrophotometric microplate reader capable of reading absorbance at 450 nm and 650 or 630

SPECIMEN COLLECTION & STORAGE

1. Only one fecal sample is required. Fresh fecal sample must be collected by using Epitope Diagnostics Calprotectin Fecal Sample Collection Kit (Cat. No. KT-843). This device is specially designed for easy collection of a substantially consistent amount of fecal sample into the tube pre-filled with sample extraction buffer. The collected fecal sample may be transported at ambient temperature, stored at 2-8 °C and tested within 3 days. Fecal sample may be stored below -20 °C for a longer storage period. Avoid more than three freeze - thaw cycles for each specimen. Before measuring for fecal Calprotectin, vortex to dissolve stool sample.

NOTE: The validation data of this test was generated by using Calprotectin Fecal Sample Collection Kit (Cat. No. KT-843)! To order this device, please order Calprotectin Fecal Sample Collection kit (Cat. No. KT-843). Each kit contains 50 tubes filled with extraction buffer. A different Calprotectin test result may be obtained by using a different type of fecal sample collection tube.

2. It is an alternative to collect fecal sample with a commercial stool sample collection device. The collected sample can be stored at 2-8°C for up to 6 days.

ASSAY PROCEDURE

1. Reagent Preparation

1. Prior to use allow all reagents to come to room temperature. Reagents from different kit lot numbers should not be combined or interchanged.
2. ELISA Wash Concentrate (10010) must be diluted to working solution prior to use. Please see REAGENTS section for details.
3. Reconstitute all assay calibrators level 1 to level 7 (30571-30577) and controls (30578-30580) by adding 0.5 mL of demineralized water to each vial. Allow the standards and controls to sit undisturbed for 5 minutes, and then mix well by inversions or gentle vortexing. One must make sure that all solid is dissolved completely prior to use. These reconstituted standards and controls may be stored at 2 – 8°C for up to 3 days or at –10°C or below for long-term storage. Do not exceed 3 freeze-thaw cycles.

2. Sample Preparation

1. Manual Weighing:

1. The collected sample should be diluted in two steps with 1:40 and 1:9 before measurement. Following is a detailed sample extraction process.
   1. Label and tare an empty polypropylene tube together with an inoculation loop.
   2. Weigh 50 – 100 mg of stool using the inoculation loop by placing it into the pre-tarred tube.
   3. Record the net amount of sample and break the inoculation loop; leave the lower part of the loop in the tube.
   4. For every 1 volume of the stool, add 39 volume of Extraction buffer into the tube (stool volume calculation: 100 mg stool = 100 µl stool). The following is an easy to use reference extraction procedure.

Fecal Sample Weight (mg)	Extraction Buffer Volume (mL)
50 - 54	2.0
55 - 59	2.2
60 - 64	2.4
65 - 69	2.6
70 - 74	2.8
75 - 79	3.0
80 - 84	3.2
85 - 89	3.4
90 - 94	3.6
95 - 99	3.8
100 - 104	4.0

   5. Vortex to dissolve stool sample. Let the sample set at room temperature vertically for 30 min for sedimentation or centrifuge the sample at 3000 x g for 5 minutes.
   6. Transfer 0.15 mL clear supernatant (no particles) to a clean tube with 1.2 mL Extraction Buffer. Mix the sample by gently vortexing. This extracted sample is ready to be measured for fecal Calprotectin.

2. Using EDI Calprotectin Fecal Sample Collection Kit (KT-843):

1. If the Epitope Diagnostics Calprotectin Fecal Sample Collection Kit (Cat. KT-843) is used, there is no sample preparation required.

3. Assay Procedure

1. Place a sufficient number of microwell strips (30439) in a holder to run calibrators (30571 - 30577), controls (30878 - 30880), and extracted samples in duplicate.

2. Test Configuration

Row	Strip 1	Strip 2	Strip 3
A	Calibrator Level 1	Calibrator Level 5	Control 2
B	Calibrator Level 1	Calibrator Level 5	Control 2
C	Calibrator Level 2	Calibrator Level 6	Control 3
D	Calibrator Level 2	Calibrator Level 6	Control 3
E	Calibrator Level 3	Calibrator Level 7	SAMPLE 1
F	Calibrator Level 3	Calibrator Level 7	SAMPLE 1
G	Calibrator Level 4	Control 1	SAMPLE 2
H	Calibrator Level 4	Control 1	SAMPLE 2

3. Add **50 µL** of assay buffer (30485) into the designated microwells. Mix by gently tapping the plate.
4. Add **50 µL** of calibrators (30571 - 30577), controls (30878 - 30880), and extracted samples into the designated microwells. Mix by gently tapping the plate.
5. Cover the plate with one plate sealer and aluminum foil. Incubate at **room temperature (20-25 ºC)** with **shaking at 400 to 450 rpm** for **60 minutes.**
6. Remove the plate sealer. Aspirate the contents of each microwell. Wash each well **5 times** by dispensing **350 µL** of diluted wash solution (10010) into each well, then completely aspirating the contents. Alternatively, an automated microplate washer can be used.
7. Prepare the antibody working solution by 1:21 fold dilution of the tracer antibody (30662) with the diluent (30458). For each strip, it is required to mix 1 mL of the tracer antibody diluent with 50 µL of the tracer antibody in a clean test tube. *Note: This antibody working solution should be freshly prepared.*
8. Add **100 µL** of antibody working solution to each microwell.
9. Cover the plate with one plate sealer and aluminum foil. Incubate at **room temperature (20-25 ºC)** with **shaking at 400 to 450 rpm** for **45 minutes.**
10. Remove the plate sealer. Aspirate the contents of each microwell. Wash each well **5 times** by dispensing **350 µL** of diluted wash solution (10010) into each well, then completely aspirating the contents. Alternatively, an automated microplate washer can be used
11. Add **100 µL** of substrate (10020) into each microwell. Mix by gently tapping the plate.
12. Cover the plate with one plate sealer and aluminum foil. Incubate at **room temperature (20-25 ºC)** for **12 minutes.**
13. Remove the aluminum foil and plate sealer.
14. Read the absorbance at **620 nm** (optional wavelengths from **595** to **650** nm depending on available filters) **immediately** with a microplate reader.
15. Immediately add **100 µL** of Stop Solution (30559) into each of the wells. Mix by gently tapping the plate.
16. Read the absorbance at **450/620 nm** or **450/650 nm** within **10 minutes** with a microplate reader.

PROCEDURAL NOTES

1. It is recommended that all standards, controls and unknown samples be assayed in duplicate. The average absorbance reading of each duplicate should be used for data reduction and the calculation of results.
2. Keep light-sensitive reagents in the original amber bottles.
3. Store any unused antibody-coated strips in the foil zipper bag with desiccant to protect from moisture.
4. Careful technique and use of properly calibrated pipetting devices are necessary to ensure reproducibility of the test.
5. Incubation times or temperatures other than those stated in this insert may affect the results.
6. An orbital mixer with a larger orbit radius (e.g. > 1 cm) may be used at speeds of 150 to 200 rpm.
7. Avoid air bubbles in the microwell as this could result in lower binding efficiency and higher CV% of duplicate reading.
8. All reagents should be mixed gently and thoroughly prior to use. Avoid foaming.
9. If adapting this assay to automated ELISA system such as DS-2, a procedural validation is necessary if there is any modification of the assay procedure.

INTERPRETION OF RESULTS

1. It is recommended to use a point-to-point or 4-parameter standard curve fitting.
2. Calculate the average absorbance for each pair of duplicate test results.
3. Subtract the average absorbance of the level 1 standard (0 ng/mL) from the average absorbance of all other readings to obtain corrected absorbance.
4. The standard curve is generated by the corrected absorbance of all standard levels on the ordinate against the standard concentration on the abscissa using point-to-point or log-log paper. Appropriate computer assisted data reduction programs may also be used for the calculation of results.
5. The fecal human calprotectin concentrations for the controls and the patient samples are read directly from the standard curve using their respective corrected absorbance.
6. The use of the two absorbance wavelength at A 620 nm and A450/620 nm allows for two ways to calculate sample results. It is recommended to get sample results by using the primary standard curve at A 450/620 nm for samples with value below standard level 5. For samples with calprotectin value above standard level 5, it is recommended to use the secondary standard curve at A 620 nm.

LIMITATIONS OF THE PROCEDURE

1. A strong positive of fecal calprotectin is likely to indicate a more significant clinical pathological condition of a patient. However, a low positive of fecal calprotectin does not indicate a lesser possibility of inflammation.
2. A normal fecal calprotectin level does not rule out the presence of any gastrointestinal diseases such as IBD.
3. For sample values reading greater than the highest standard, it is recommended to re-assay samples with dilution (i.e. 1:10 or 1:100 with Extraction Buffer).
4. Water deionized with polyester resins may inactivate the horseradish peroxidase enzyme.

QUALITY CONTROL

To assure the validity of the results each assay should include adequate controls.

EXPECTED VALUES

Stool samples from normal healthy adults with age of 24 – 58 were collected and measured with this ELISA. The recommended **normal cut-off** for fecal Calprotectin concentration by using this ELISA and

sample collection system is **120 ng/mL or 43.2 µg/g directly read from assay standard curve**. We strongly recommend that each clinical laboratory to establish its own normal cut-off level by measuring normal stool samples with this ELISA and sample collection system.

Please be aware that patients with recent diarrhea would give a much higher level of fecal Calprotectin. Consuming spicy food or alcohol may also cause intestinal irritation resulting in an abnormal fecal Calprotectin level.

Calprotectin ng/mL X 0.36 = Calprotectin µg/g

Calprotectin µg/g X 2.78 = Calprotectin ng/mL

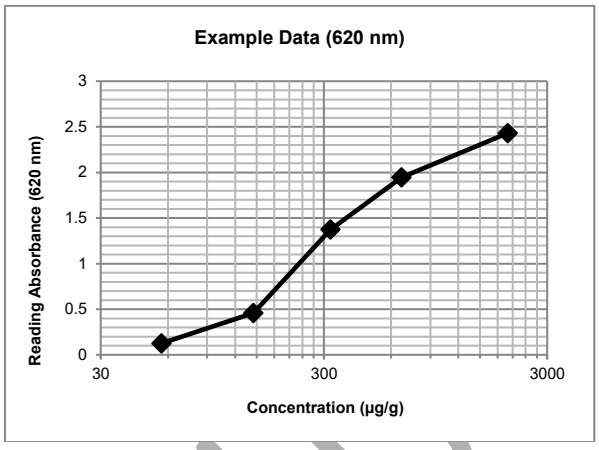
Note: Please program ELISA reader by selecting assay standards concentration either in "µg/g" or "ng/mL to avoid manual calculation!

EXAMPLE DATA

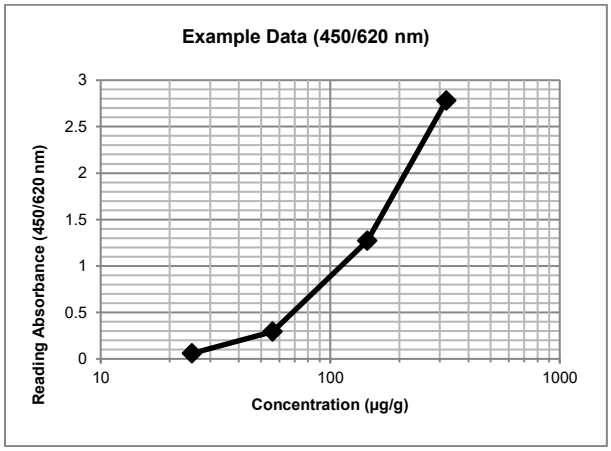
A typical absorbance data and the resulting standard curves from are represented.

Note: This curve should not be used in lieu of calibrator curve run with each assay.

Well ID	Reading Absorbance (620 nm)			Concentration µg/g (ng/mL)
	Readings	Average	Corrected	
Calibrator Level 1: 0 µg/g (0 ng/mL)	0.043	0.042	0.000	
	0.041			
Calibrator Level 3: 56.2 µg/g (156 ng/mL)	0.132	0.126	0.084	
	0.120			
Calibrator Level 4: 145 µg/g (403 ng/mL)	0.494	0.457	0.415	
	0.420			
Calibrator Level 5: 321 µg/g (892 ng/mL)	1.368	1.374	1.332	
	1.380			
Calibrator Level 6: 669 µg/g (1860 ng/mL)	1.945	1.948	1.906	
	1.950			
Calibrator Level 7: 2000 µg/g (5560 ng/mL)	2.415	2.432	2.390	
	2.448			
Control 2	1.145	1.147	1.105	266.3 (740)
	1.149			
Control 3	1.778	1.779	1.737	423 (1176)
	1.778			



Well ID	Reading Absorbance (450/620 nm)			Concentration µg/g (ng/mL)
	Readings	Average	Corrected	
Calibrator Level 1: 0 µg/g (0 ng/mL)	0.026	0.027	0.000	
	0.027			
Calibrator Level 2: 25 µg/g (69.5 ng/mL)	0.061	0.059	0.032	
	0.058			
Calibrator Level 3: 56.2 µg/g (156 ng/mL)	0.305	0.292	0.265	
	0.279			
Calibrator Level 4: 145 µg/g (403 ng/mL)	1.388	1.272	1.245	
	1.155			
Calibrator Level 5: 321 µg/g (892 ng/mL)	2.760	2.781	2.754	
	2.802			
Control 1	0.148	0.134	0.107	36.1 (100)
	0.121			
Control 2	2.601	2.607	2.580	291.4 (810)
	2.614			



PERFORMANCE CHARACTERISTICS

Sensitivity

The analytical sensitivity (LLOD) of the human calprotectin ELISA as determined by the 95% confidence limit on 12 duplicate determination of zero standard is approximately 2.5 ng/mL. A LLOQ was determined by dilution of assay standards and it is about 5 ng/mL.

Hook Effect

This assay has showed that it did not have any high dose "hook" for calprotectin level up to 40,000 ng/mL in extraction buffer.

Reproducibility and Precision

The intra-assay precision was validated by measuring three sample extracts in a single assay with 12 replicate determinations. The inter-assay precision was validated by measuring two samples in duplicate in 4 individual assays. The results are as follows:

	Intra-Assay			Inter-Assay	
Sample	**1**	**2**	**3**	**1**	**2**
Mean (ng/mL)	5.74	26.59	54.70	21.64	70.31
CV (%)	2.9	3.5	2.5	8.6	2.0

The precision of inter-sample collection was performed by collecting five specimens from one bowel movement. These grouped samples are measured in an assay according to the assay procedure. The results of Calprotectin concentration in the value of ng/mL indicate that there are very satisfactory agreements of the five samples collected from one bowel movement.

Donor	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	CV%
A	57.0	65.0	59.2	56.2	49.8	9.5
B	60.4	55.3	58.8	71.7	81.1	16.3
C	72.3	69.3	51.5	65.7	65.6	12.3

Linearity

One sample was diluted with assay buffer and tested. The results are as follows:

Sample	Observed (ng/mL)	Expected (ng/mL)	Recovery (%)
Sample 1	195.84	-	-
50%	87.88	97.92	89.7
25%	46.58	48.96	95.1
12.5%	24.53	24.48	100.2
6.25%	13.77	12.24	112.5

Spike Recovery

Three fecal extracts and three assay standards were spiked together in various volume combinations and tested. The results are as follows:

Samples	Observed (ng/mL)	Expected (ng/mL)	Recovery (%)
Calibrator Level 1	61.9	67.1	92.2
Calibrator Level 2	85.7	89.3	104.2
Calibrator Level 3	248.0	256.9	96.5

WARRANTY

This product is warranted to perform as described in its labeling and literature when used in accordance with all instructions. Epitope Diagnostics, Inc. DISCLAIMS ANY IMPLIED WARRANTY OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE, and in no event shall Epitope Diagnostics, Inc. be liable for consequential damages. Replacement of the product or refund of the purchase price is the exclusive remedy for the purchaser. This warranty gives you specific legal rights and you may have other rights, which vary from state to state.

REFERENCES

1. Tibble et al. A simple method for assessing intestinal inflammation in Crohn's disease. Gut.2000;47:506-513
2. Costa F et al. Calprotectin is a stronger predictive marker of relapse in ulcerative colitis than in Crohn's disease. Gut. 2005;54:364-8.

TECHNICAL ASSISTANCE AND CUSTOMER SERVICE

For technical assistance or place an order, please contact Epitope Diagnostics, Inc. at (858) 693-7877 or fax to (858) 693-7678.

This product is developed and manufactured by

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Please visit our website at www.epitopediagnostics.com to learn more about our products and services.

EC	REP

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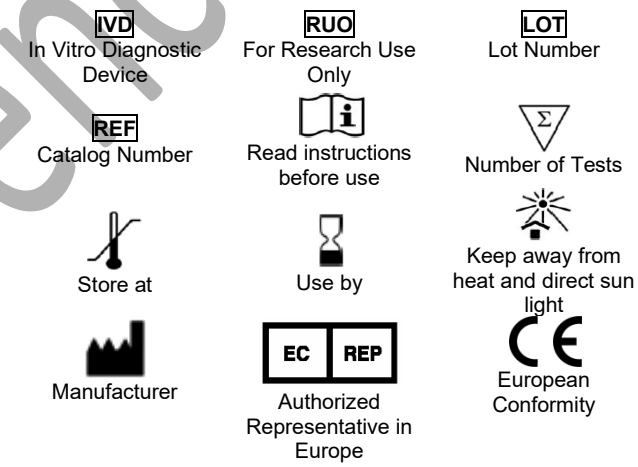
GLOSSARY OF SYMBOLS (EN 980/ISO 15223)



SHORT ASSAY PROCEDURE

1. Add **50 µL** of assay buffer into the designated microwells.
2. Add **50 µL** of calibrators, controls, and extracted samples into the designated microwells.
3. Mix, cover, and incubate at **room temperature (20-25 ºC)** with **shaking 400 rpm – 450 rpm** for **60 minutes**.
4. Wash each well five times.
5. Add **100 µL** of antibody working solution.
6. Mix, cover, and incubate at **room temperature (20-25 ºC)** with **shaking 400 rpm – 450 rpm** for **45 minutes**.
7. Wash each well five times.
8. Add **100 µL** of substrate to each well.
9. Cover and incubate at **room temperature (20-25 ºC)** for **12 minutes**.
10. Read the absorbance at **620 nm**.
11. Add **100 µL** of the stop solution to each well.
12. Read the absorbance at **450/620 nm** or **450/650 nm.**