KAMIYA BIOMEDICAL COMPANY

Human MCP-1 ELISA

**For the quantitative determination of MCP-1
in human cell culture supernatant, serum, plasma and amniotic fluid**

Cat. No. KT-020

For research use only, not for use in diagnostic procedures.

PRODUCT INFORMATION

Human MCP-1 ELISA

Cat. No. KT-020

PRODUCT

The **K-ASSAY®** Human MCP-1 ELISA is an enzyme-linked immunosorbent assay for the quantitative determination of MCP-1 in human cell culture supernatant, serum, plasma and amniotic fluid. For research use only. Not for diagnostic or therapeutic procedures.

DESCRIPTION

Chemokines (chemoattractant cytokines) represent a superfamily of small secreted proteins that function as intercellular messengers to control migration and activation of leukocytes involved in inflammatory reactions and immunity. Furthermore, chemokines are important mediators of many pathologies such as allergic responses, chronic inflammatory and autoimmune diseases, tumor growth and hematopoietic development.

The CC-chemokine monocyte chemoattractant protein 1 (MCP-1), also known as monocyte chemotactic and activating factor (MCAF) was characterized as a monocyte-specific chemoattractant that was later shown to attract also T lymphocytes and NK cells.

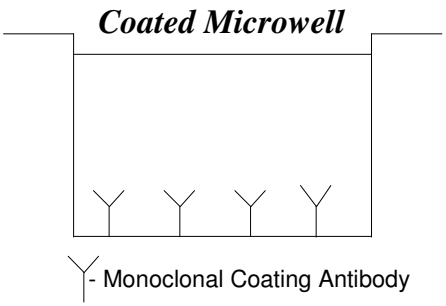
The human mature MCP-1 protein consists of 76 amino acids, derived by cleavage of a hydrophobic signal peptide from the 99 aa precursor protein. MCP-1 is mainly expressed by macrophages in response to a wide range of cytokines such as IL-6, TNF-α and IL-1β, but can, upon stimulation, also be produced by a variety of cells and tissues, such as fibroblasts, endothelial cells or certain tumor cells.

Because of its target cell specificity, MCP-1 was postulated to play a pathogenic role in a variety of diseases characterized by mononuclear cell infiltration, including atherosclerosis, rheumatoid arthritis and allergic responses. Elevated levels of MCP-1 have also been found in connection with osseus inflammation and Alzheimer's disease (AD) as well as Myocardial Ischemia and viral infections.

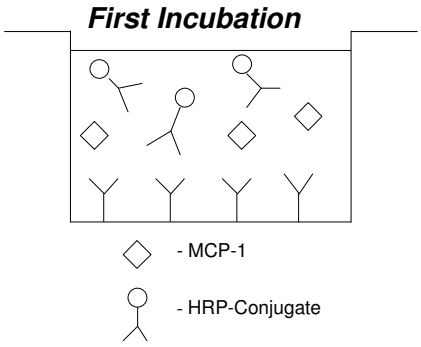
In acute and chronic-active multiple sclerosis (MS) lesions immunoreactivity for MCP-1 was increased whereas MCP-1 was found to be significantly reduced in cerebrospinal fluids (CSF) and chronic lesions of patients with MS. In basophils, MCP-1 is highly effective as a stimulus of histamine release but has only weak chemotactic activity. Additionally it has been shown to chemoattract CD4+ and CD8+ T lymphocytes, and expression of the MCP-1 chemokine may affect HIV infection via signaling through the CCR2 receptor.

PRINCIPLE

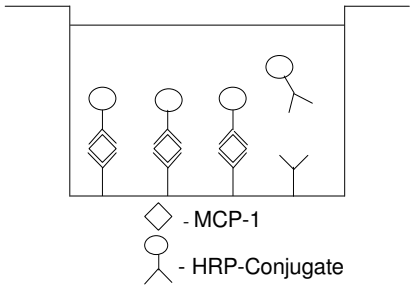
An anti-human MCP-1 coating antibody is adsorbed onto microwells.



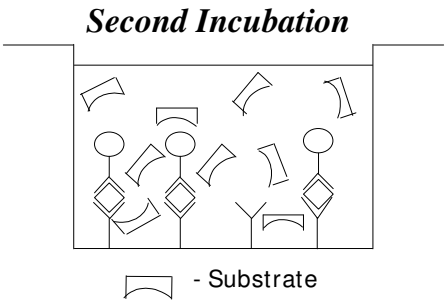
Human MCP-1 present in the sample or calibrator binds to antibodies adsorbed to the microwells and the HRP-conjugated anti-human MCP-1 antibody is added and binds to human MCP-1 captured by the first antibody.

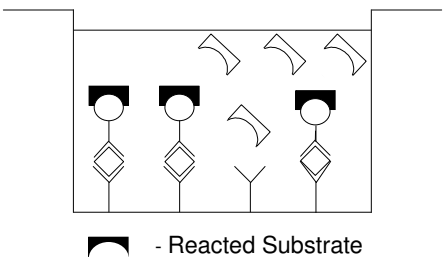


Following incubation unbound HRP-conjugated anti-human MCP-1 is removed during a wash step, and substrate solution reactive with HRP is added to the wells.



A colored product is formed in proportion to the amount of human MCP-1 present in the sample or calibrator. The reaction is terminated by addition of acid and absorbance is measured at 450 nm. A calibration curve is prepared from 7 human MCP-1 calibrator dilutions and human MCP-1 concentration determined.





COMPONENTS

1 aluminium pouch with **a Microwell Plate coated** with monoclonal antibody to human MCP-1

1 vial (70 µL) **HRP-Conjugate** anti-human MCP-1 monoclonal antibody

2 vials human MCP-1 **Calibrator** lyophilized, 2 ng/mL upon reconstitution

1 vial **Control high,** lyophilized

1 vial **Control low,** lyophilized

1 bottle (50 mL) **Wash Buffer Concentrate 20X** (PBS with 1% Tween 20)

1 vial (5 mL) **Assay Buffer Concentrate 20X** (PBS with 1% Tween 20 and protein stabilizer)

1 vial (15 mL) **Substrate Solution** (tetramethyl-benzidine)

1 vial (15 mL) **Stop Solution** (1M Phosphoric acid)

2 adhesive **Plate Covers**

Materials or equipment required but not provided

- 5 mL and 10 mL graduated pipettes
- 5 µL to 1,000 µL adjustable single channel micropipettes with disposable tips
- 50 µL to 300 µL adjustable multichannel micropipette with disposable tips
- Multichannel micropipette reservoir
- Beakers, flasks, cylinders necessary for preparation of reagents
- Device for delivery of wash solution (multichannel wash bottle or automatic wash system)
- Microwell strip reader capable of reading at 450 nm (620 nm as optional reference wave length)
- Glass-distilled or de-ionized water
- Statistical calculator with program to perform linear regression analysis.

SPECIMEN COLLECTION

Cell culture supernatant, serum, plasma (EDTA, heparin) and amniotic fluid were tested with this assay. Other biological samples might be suitable for use in the assay. Remove serum or plasma from the clot or red cells as soon as possible after clotting and separation.

Pay attention to a possible “Hook Effect” due to high sample concentrations.

Samples containing a visible precipitate must be clarified prior to use in the assay. Do not use grossly hemolyzed or lipemic specimens.

Samples should be aliquoted and must be stored frozen at -20°C to avoid loss of bioactive human MCP-1. If samples are to be run within 24 hours, they may be stored at 4°C. Avoid repeated freeze-thaw cycles. Prior to assay, the frozen sample should be brought to room temperature slowly and mixed gently.

STORAGE

Store kit reagents at 4°C except controls. Store lyophilized controls at -20°C. Immediately after use remaining reagents should be returned to cold storage (4°C), or to -20°C, respectively. Expiry of the kit and reagents is stated on labels.
Expiry of the kit components can only be guaranteed if the components are stored properly, and if, in case of repeated use of one component, the reagent is not contaminated by the first handling.

Freeze-Thaw Stability

Aliquots of serum samples (spiked or unspiked) were stored at -20℃ and thawed 5 times, and the human MCP-1 levels determined. There was no significant loss of human MCP-1 immunoreactivity detected by repeated freezing and thawing.

Storage Stability

Aliquots of serum samples (spiked or unspiked) were stored at -20℃, 4℃, room temperature (RT) and at 37℃, and the human MCP-1 level determined after 24 h. There was no significant loss of human MCP-1 immunoreactivity detected during storage under above conditions.

PRECAUTIONS FOR USE

- All reagents should be considered as potentially hazardous. We therefore recommend that this product is handled only by those persons who have trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice. Wear suitable protective clothing such as laboratory overalls, safety glasses and gloves. Care should be taken to avoid contact with skin or eyes. In the case of contact with skin or eyes wash immediately with water. See material safety data sheet(s) and/or safety statement(s) for specific advice.
- Reagents are intended for research use only and are not for use in diagnostic or therapeutic procedures.
- Do not mix or substitute reagents with those from other lots or other sources.
- Do not use kit reagents beyond expiration date on label.
- Do not expose kit reagents to strong light during storage or incubation.
- Do not pipette by mouth.
- Do not eat or smoke in areas where kit reagents or samples are handled.
- Avoid contact of skin or mucous membranes with kit reagents or specimens.
- Rubber or disposable latex gloves should be worn while handling kit reagents or specimens.
- Avoid contact of substrate solution with oxidizing agents and metal.
- Avoid splashing or generation of aerosols.
- In order to avoid microbial contamination or cross-contamination of reagents or specimens which may invalidate the test, use disposable pipette tips and/or pipettes.
- Use clean, dedicated reagent trays for dispensing the conjugate and substrate reagent.
- Exposure to acids will inactivate the conjugate.
- Glass-distilled or de-ionized water must be used for reagent preparation.
- Substrate solution must be at room temperature prior to use.
- Decontaminate and dispose specimens and all potentially contaminated materials as they could contain infectious agents. The preferred method of decontamination is autoclaving for a minimum of 1 hour at 121.5℃.
- Liquid wastes not containing acid and neutralized waste may be mixed with sodium hypochlorite in volumes such that the final mixture contains 1.0% sodium hypochlorite. Allow 30 minutes for effective decontamination. Liquid waste containing acid must be neutralized prior to the addition of sodium hypochlorite.

LIMITATIONS

- Since exact conditions may vary from assay to assay, a calibration curve must be established for every run.
- Bacterial or fungal contamination of either screen samples or reagents or cross-contamination between reagents may cause erroneous results.

- Disposable pipette tips, flasks or glassware are preferred, reusable glassware must be washed and thoroughly rinsed of all detergents before use.

- Improper or insufficient washing at any stage of the procedure will result in either false positive or false negative results. Empty wells completely before dispensing fresh wash solution, fill with Wash Buffer as indicated for each wash cycle and do not allow wells to sit uncovered or dry for extended periods.

- The use of radioimmunotherapy has significantly increased the number of patients with human anti-mouse IgG antibodies (HAMA). HAMA may interfere with assays utilizing murine monoclonal antibodies leading to both false positive and false negative results. Serum samples containing antibodies to murine immunoglobulins can still be analysed in such assays when murine immunoglobulins (serum, ascitic fluid, or monoclonal antibodies of irrelevant specificity) are added to the sample.

SPECIFICITY

The assay detects both natural and recombinant human MCP-1. The cross reactivity of circulating factors of the immune system was evaluated by spiking these proteins at physiologically relevant concentrations into a human MCP-1 positive serum. There was no cross reactivity detected, namely not with MCP-3.

EXPECTED VALUES

A panel of 40 sera samples from randomly selected apparently healthy donors (males and females) was tested for human MCP-1. The detected human MCP-1 levels ranged between 74 and 760 pg/mL.

PREPARATION OF REAGENTS

Buffer Concentrates should be brought to room temperature and should be diluted before starting the test procedure. If crystals have formed in the **Buffer Concentrates, warm them gently until they have completely dissolved.**

Wash Buffer (1X)

Pour entire contents (50 mL) of the **Wash Buffer Concentrate (20X)** into a clean 1,000 mL graduated cylinder. Bring to final volume of 1,000 mL with glass-distilled or de-ionized water. Mix gently to avoid foaming.

Transfer to a clean wash bottle and store at 2° to 25°C. Please note that the Wash Buffer (1X) is stable for 30 days. Wash Buffer (1X) may be prepared as needed according to the following table:

Number of Strips	Wash Buffer Concentrate (20X) (mL)	Distilled Water (mL)
1 - 6	25	475
1 - 12	50	950

Assay Buffer (1X)

Pour the entire contents (5 mL) of **Assay Buffer Concentrate (20X)** into a clean 100 mL graduated cylinder. Bring to final volume of 100 mL with distilled water. Mix gently to avoid foaming. Store at 4°C. Please note that the Assay Buffer (1X) is stable for 30 days. Assay Buffer (1X) may also be prepared as needed according to the following table:

Number of Strips	Assay Buffer Concentrate (20X) (mL)	Distilled Water (mL)
1 - 6	2.5	47.5
1 - 12	5.0	95.0

HRP-Conjugate

Please note that the HRP-Conjugate should be used within 30 minutes after dilution.

Make a 1:100 dilution of the concentrated **HRP-Conjugate** solution with Assay Buffer (1X) in a clean plastic test tube as needed according to the following table:

Number of Strips	HRP-Conjugate (mL)	Assay Buffer (1X) (mL)
1 - 6	0.03	2.97
1 - 12	0.06	5.94

Human MCP-1 Calibrator

Reconstitute **human MCP-1 calibrator** by addition of distilled water. Reconstitution volume is stated on the label of the calibrator vial. Swirl or mix gently to insure complete and homogeneous solubilization (concentration of reconstituted calibrator = 2 ng/mL). Allow the calibrator to reconstitute for 10-30 minutes. Mix well prior to making dilutions. After usage remaining calibrator cannot be stored and has to be discarded. **Calibrator dilutions** can be prepared directly on the microwell plate or alternatively in tubes.

External Calibrator Dilution

Label 7 tubes, one for each calibrator point.
C1, C2, C3, C4, C5, C6, C7
Then prepare 1:2 serial dilutions for the calibration curve as follows: Pipette 225 µL of Assay Buffer (1X) into each tube.
Pipette 225 µL of reconstituted calibrator (concentration of calibrator = 2 ng/mL) into the first tube, labeled C1, and mix (concentration of calibrator 1 = 1 ng/mL).
Pipette 225 µL of this dilution into the second tube, labeled C2, and mix thoroughly before the next transfer.
Repeat serial dilutions 5 more times thus creating the points of the calibration curve.
Assay Buffer (1X) serves as blank.

Controls

Reconstitute by adding 150 µL distilled water to lyophilized **controls** (10-30 minutes). Further treat the controls like your samples in the assay. For control range please refer to vial label. Store reconstituted controls aliquoted at -20°C. Avoid repeated freeze and thaw cycles.

TEST PROTOCOL

a. Determine the number of microwell strips required to test the desired number of samples plus appropriate number of wells needed for running blanks and calibrators. Each sample, calibrator, blank and optional control sample should be assayed in duplicate. Remove extra microwell strips from holder and store in foil bag with the desiccant provided at 4°C sealed tightly.

b. Wash the microwell strips twice with approximately 400 µL **Wash Buffer** per well with thorough aspiration of microwell contents between washes. Allow the Wash Buffer to sit in the wells for about **10 – 15 seconds** before aspiration. Take care not to scratch the surface of the microwells.
After the last wash, empty wells and tap microwell strips on absorbent pad or paper towel to remove excess Wash Buffer. Use the microwell strips immediately after washing. Alternatively microwell strips can be placed upside down on a wet absorbent paper for not longer than 15 minutes. **Do not allow wells to dry.**

c. **Calibrator dilution on the microwell plate** (Alternatively the calibrator dilution can be prepared in tubes): Add 100 µL of Assay Buffer (1X) in duplicate to all **calibrator wells**. Pipette 100 µL of prepared **Calibrator** (see Preparation of Reagents, concentration = 2,000 pg/mL) in duplicate into well A1 and A2. Mix the contents of wells A1 and A2 by repeated aspiration and ejection (concentration of calibrator 1, C1 = 1,000 pg/mL), and transfer 100 µL to wells B1 and B2, respectively. Take care not to scratch the inner surface of the microwells. Continue this procedure 5 times, creating two rows of human MCP-1 calibrator dilutions ranging from 1,000 to 15.6 pg/mL. Discard 100 µL of the contents from the last microwells (G1, G2) used.

In case of an **external calibrator dilution**, pipette 100 µL of these calibrator dilutions (C1 – C7) in the calibrator wells.

d. Add 100 µL of **Assay Buffer (1X)** in duplicate to the **blank wells**.

e. Add 80 µL of **Assay Buffer (1X)** to the **sample wells**.

f. Add 20 µL of each **sample** in duplicate to the **sample wells**.

g. Prepare **HRP-Conjugate** (see Preparation of Reagents).

h. Add 50 µL of **HRP-Conjugate** to all wells.

i. Cover with an adhesive film and incubate at room temperature (18 to 25°C) for 2 hours, if available on a microplate shaker set at 400 rpm.

j. Remove adhesive film and empty wells. **Wash** microwell strips 3 times according to point b. of the test protocol. Proceed immediately to the next step.

k. Pipette 100 µL of **TMB Substrate Solution** to all wells.

l. Incubate the microwell strips at room temperature (18° to 25°C) for about 10 min. Avoid direct exposure to intense light.
The color development on the plate should be monitored and the substrate reaction stopped (see next point of this protocol) before positive wells are no longer properly recordable. Determination of the ideal time period for color development has to be done individually for each assay.
It is recommended to add the stop solution when the highest calibrator has developed a dark blue color.
Alternatively the color development can be monitored by the ELISA reader at 620 nm. The substrate reaction should be stopped as soon as Calibrator 1 has reached an OD of 0.9 – 0.95.

m. Stop the enzyme reaction by quickly pipetting 100 µL of **Stop Solution** into each well. It is important that the Stop Solution is spread quickly and uniformly throughout the microwells to completely inactivate the enzyme. Results must be read immediately after the Stop Solution is added or within one hour if the microwell strips are stored at 4°C in the dark.

n. Read absorbance of each microwell on a spectro-photometer using 450 nm as the primary wave length (optionally 620 nm as the reference wave length; 610 nm to 650 nm is acceptable). Blank the plate reader according to the manufacturer's instructions by using the blank wells. Determine the absorbance of both the samples and the calibrators.

Note: In case of incubation without shaking the obtained O.D. values may be lower than indicated below. Nevertheless the results are still valid.

PERFORMANCE CHARACTERISTICS

Sensitivity

The limit of detection of human MCP-1 defined as the analyte concentration resulting in an absorbance significantly higher than that of the dilution medium (mean plus 2 standard deviations) was determined to be 2.3 pg/mL (mean of 6 independent assays).

Reproducibility

a. Intra-assay

Reproducibility within the assay was evaluated in 3 independent experiments. Each assay was carried out with 6 replicates of 8 serum samples containing different concentrations of human MCP-1. 2 calibration curves were run on each plate. Data below show the mean human MCP-1 concentration and the coefficient of variation for each sample. The calculated overall intra-assay coefficient of variation was 4.7%.

Sample	Experiment	Mean Human MCP-1 Concentration (pg/mL)	Coefficient of Variation (%)
1	1	1,074	3.2
	2	965	4.3
	3	923	3.6
2	1	256	9.7
	2	233	9.1
	3	205	7.3
3	1	393	1.7
	2	354	8.3
	3	357	2.8
4	1	1,194	3.2
	2	1,177	7.6
	3	1,037	2.6
5	1	118	4.3
	2	129	2.8
	3	137	1.6
6	1	562	4.6
	2	630	8.7
	3	494	0.9

7	1	949	6.5
	2	1,112	9.1
	3	910	1.4
8	1	131	2.8
	2	137	4.7
	3	120	2.6

Inter-assay

Assay to assay reproducibility within one laboratory was evaluated in 3 independent experiments. Each assay was carried out with 6 replicates of 8 serum samples containing different concentrations of human MCP-1. 2 calibration curves were run on each plate. Data below show the mean human MCP-1 concentration and the coefficient of variation calculated on 18 determinations of each sample. The calculated overall inter-assay coefficient of variation was 8.7%.

Sample	Mean Human MCP-1 Concentration (pg/mL)	Coefficient of Variation (%)
1	987	7.9
2	231	11.1
3	368	5.9
4	1,136	7.6
5	128	7.5
6	562	12.1
7	991	10.8
8	129	6.8

Spike Recovery

The spike recovery was evaluated by spiking 4 levels of human MCP-1 into serum. Recoveries were determined in 3 independent experiments with 6 replicates each. The amount of endogenous human MCP-1 in unspiked serum was subtracted from the spike values. The recoveries ranged from 84% to 98% with an overall mean recovery of 92%.

Dilution Parallelism

4 serum samples with different levels of human MCP-1 were analysed at serial 2 fold dilutions with 4 replicates each. The recovery ranged from 93% to 117% with an overall recovery of 105%.

Sample	Dilution	Human MCP-1 Concentration (pg/mL) Expected Value	Observed Value	% Recovery of Exp. Value
1	1:5	--	3,320	--
	1:10	1,660	1,731	104
	1:20	830	901	109
	1:40	415	441	106
2	1:5	--	2,983	--
	1:10	1,491	1,503	101
	1:20	746	763	102
	1:40	373	364	98
3	1:5	--	3,287	--
	1:10	1,643	1,811	110
	1:20	822	949	116
	1:40	411	481	117
4	1:5	--	3,521	--
	1:10	1,760	1,787	102
	1:20	880	889	101
	1:40	440	409	93

CALCULATION OF RESULTS

- Calculate the average absorbance values for each set of duplicate calibrators and samples. Duplicates should be within 20 per cent of the mean value.

- Create a calibration curve by plotting the mean absorbance for each calibrator concentration on the ordinate against the human MCP-1 concentration on the abscissa. Draw a best fit curve through the points of the graph (a 5-parameter curve fit is recommended).

- To determine the concentration of circulating human MCP-1 for each sample, first find the mean absorbance value on the ordinate and extend a horizontal line to the calibration curve. At the point of intersection, extend a vertical line to the abscissa and read the corresponding human MCP-1 concentration.

- **If instructions in this protocol have been followed samples have been diluted 1:5 (20 µL + 80 µL Assay Buffer (1X)), the concentration read from the calibration curve must be multiplied by the dilution factor (x 5).**

- **Calculation of samples with a concentration exceeding calibrator 1 will result in incorrect, low human MCP-1 levels (Hook Effect). Such samples require further external predilution according to expected human MCP-1 values with Assay Buffer (1X) in order to precisely quantitate the actual human MCP-1 level.**

- It is suggested that each testing facility establishes a control sample of known human MCP-1 concentration and runs this additional control with each assay. If the values obtained are not within the expected range of the control, the assay results may be invalid.

Typical data using the human MCP-1 ELISA
Measuring wavelength: 450 nm
Reference wavelength: 620 nm

Calibrator	Human MCP-1 Concentration (pg/mL)	O.D. at 450 nm	Mean O.D. at 450 nm	C.V. (%)
1	1,000	2.226	2.292	2.9
	1,000	2.359		
2	500	1.322	1.341	1.4
	500	1.360		
3	250	0.722	0.740	2.4
	250	0.758		
4	125	0.411	0.414	0.8
	125	0.417		
5	62.5	0.216	0.227	5.0
	62.5	0.239		
6	31.3	0.123	0.127	2.9
	31.3	0.131		
7	15.6	0.083	0.084	0.8
	15.6	0.085		
Blank	0	0.022	0.022	0.2
	0	0.022		

The OD values of the calibration curve may vary according to the conditions of assay performance (e.g. operator, pipetting technique, washing technique or temperature effects). Furthermore shelf life of the kit may affect enzymatic activity and thus color intensity. Values measured are still valid.

TEST PROTOCOL SUMMARY

- Determine the number of microwell strips required.
- Wash microwell strips twice with Wash Buffer.
- Calibrator dilution on the microwell plate: Add 100 µL Assay Buffer (1X), in duplicate, to all calibrator wells. Pipette 100 µL prepared calibrator into the first wells and create calibrator dilutions by transferring 100 µL from well to well. Discard 100 µL from the last wells. Alternatively external calibrator dilution in tubes: Pipette 100 µL of these calibrator dilutions in the microwell strips.
- Add 100 µL Assay Buffer (1X), in duplicate, to the blank wells.
- Add 80 µL Assay Buffer (1X) to sample wells.
- Add 20 µL sample in duplicate, to designated sample wells.
- Prepare HRP-Conjugate.
- Add 50 µL HRP-Conjugate to all wells.
- Cover microwell strips and incubate 2 hours at room temperature (18° to 25°C).
- Empty and wash microwell strips 3 times with Wash Buffer.
- Add 100 µL of TMB Substrate Solution to all wells.
- Incubate the microwell strips for about 10 minutes at room temperature (18°C to 25°C).
- Add 100 µL Stop Solution to all wells.
- Blank microwell reader and measure color intensity at 450 nm.

Note: **If instructions in this protocol have been followed samples have been diluted 1:5 (20 µL + 80 µL Assay Buffer (1X)), the concentration read from the calibration curve must be multiplied by the dilution factor (x 5).**

FOR RESEARCH USE ONLY

KAMIYA BIOMEDICAL COMPANY
12779 Gateway Drive, Seattle WA 98168
Tel: (206) 575-8068 Fax: (206) 575-8094
Email: LifeScience@k-assay.com
www.k-assay.com