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BIG IN TINY SCIENCE

In partnership with

Innova Biosciences

B.I.T.S.

fMLP ELISA kit
BFMLPV1

Catalogue Number: BFMLPV1

For the quantitative determination of fMLP concentration in urine samples.

For research use only.
Not for use in clinical diagnostic procedures.

These instructions must be read and understood prior to use.

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INTRODUCTION

Bacteria derived N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) is a 437.6Da tri-peptide that is recognised by cells of the innate immune system to elicit an inflammatory response characterised by chemotaxis, phagocytosis and generation of reactive oxygen intermediates.

fMLP acts through formyl peptide receptors (FPR), a family of membrane bound heterodimeric G-protein coupled receptors (GPCR) located on phagocytic cells of the innate immune system[1]. fMLP is a potent chemoattractant and also an activator of phagocytosis and cytokine production in monocytes and neutrophils.

Mammalian mitochondria are also a source of formylated peptides that activate the human immune system in a similar manner[2 3], and potentially have a role in the diagnosis of non-infectious disease[4].

PRINCIPLES OF THE ASSAY

This assay is a quantitative competitive enzyme immunoassay. An ovalbumin-fMLP conjugate has been pre-coated onto a 96 well microplate. Standards and samples are pipetted into the well, followed immediately by the addition of an enzyme-linked polyclonal antibody specific for fMLP. Following a wash to remove unbound fMLP-antibody reagent, a substrate solution is added to the wells. Colour intensity reduces in proportion to the amount of fMLP-antibody reagent displaced. The intensity of the colour is measured with a plate reader.

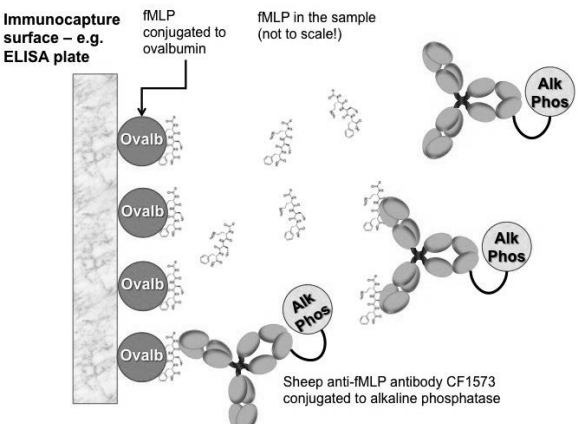

Figure 1. Schematic of assay

LIMITATIONS OF THE ASSAY AND TECHNICAL HINTS

- For research use only. Not for use in clinical diagnostic procedures.
- Use suitable protective clothing and gloves when handling patient samples and test components, and while performing the assay. Never pipette by mouth or allow reagents or urine samples to come into contact with skin.
- This test kit contains components of animal origin.
- The test is designed for single use only.
- Optimal results will be obtained by strict adherence to this protocol.
- Reagents must be added carefully to maintain precision and accuracy.
- Performing the assay outside the prescribed time and temperature ranges may produce invalid results. Assays not falling within the established time and temperature ranges must be repeated.
- The components in this kit have been quality control tested as a master batch. Do not mix or substitute components from different lot numbers.
  Do not mix with components from other manufacturers.
- Care should be exercised to protect the reagents in this kit from contamination. Do not use if there is evidence of microbial contamination. Biological contamination of dispensing equipment, containers or reagents can lead to false results.
- Avoid foaming or bubbles when mixing or reconstituting components
- Avoid cross contamination of samples or reagents by changing pipette tips between sample, standard and reagent additions.
- Keep storage boxes dry.
- Do not use plate if foil pouch is punctured or damaged.
- If sample results fall outside the dynamic range of the assay, dilute the samples and repeat assay.
- The kit should not be used beyond the expiration date on the kit label.
- Testing materials must be disposed of in accordance with local regulation.
- Recycle outer packaging if possible.

The B.I.T.S. fMLP ELISA has been rigorously validated with human urine samples. fMLP in different animal species is identical to fMLP in humans so there is no basis for designating fMLP antibodies or assay kits as specific for any particular species. However, this B.I.T.S. ELISA has been optimised (in terms of assay range, specificity and interference) for the assay of fMLP in human urine. For use with other sample types, the user should either confirm that the sample characteristics are equivalent to human urine, or validate the kit performance with the sample type they intend to analyse.

MATERIALS PROVIDED

DESCRIPTION	PART #	SUPPLIED
fMLP Microtitre plate-96 well polystyrene microtitre plate (12 strips of 8 wells) coated with an ovalbumin-fMLP conjugate.	FV1001	1 plate
Alk-Phos conjugate - 80µL/vial of polyclonal antibody against fMLP conjugated to alkaline phosphatase with preservatives.	FV1003	1 vial
fMLP standard - 10µg/vial of fMLP lyophilized.	FV1002	1 vial
10X Sample diluent concentrate -7mL/vial	GC002	1 vial
20X Wash buffer concentrate - 25mL/vial	GC001	2 vials
pNPP substrate - 12mL/vial	GC003	1 vial
Plate sealers	GC004	2

STORAGE

Store the kit at 4°C immediately upon receipt, apart from the Alk-phos conjugate, which should be stored at -20°C.

UNOPENED KIT	Alk-Phos conjugate	-20°C
	Rest of the components	Store at 4°C. Do not use past expiration date.
OPENED RECONSTITUTED REAGENTS	Microtitre plate	Return unused strips to the foil pouch containing the desiccant pack, reseal along entire edge of zip-seal. May be stored for up to 1 month at 4°C.*
	Alk-Phos conjugate	Discard dilutions after use, stock can be stored at -20°C for up to 1 month.*
	fMLP standard	Discard dilutions after use, stock can be stored at -20°C for up to 1 month.*
	Sample diluent	Store at 4°C for up to 1 month.*
	Wash buffer	Store at 4°C for up to 1 month.*
	pNPP substrate	Store at 4°C for up to 1 month.*

* Provided this is within the expiration date of the kit

OTHER SUPPLIES REQUIRED

- Microtitre plate reader capable of measuring absorbance at 405nm.
- Pipettes and pipette tips to deliver 1µL to 1mL volumes.
- Deionized or distilled water.
- Wash bottle, or automated microplate washer.
- Horizontal orbital shaker capable of maintaining a speed of 500 +/- 50rpm.
- 100mL and 1000mL graduated cylinder.
- Polypropylene tubes for dilution.
- Computer and computer software for data analysis.

SAMPLE COLLECTION AND STORAGE

Collect urine mid-stream (not the first sample of the day) directly into a sterile container. Assay immediately or aliquot and store at -20°C or below. Avoid repeated freeze-thaw cycles.

SAMPLE PREPARATION

Use polypropylene tubes.
A 5-fold dilution of urine samples into sample diluent is recommended.
A suggested dilution is 30µL +120µL sample diluent.

REAGENT PREPARATION

Bring all reagents to room temperature (18-25°C) before use.

Prepare fresh reagents immediately prior to use. If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved

Wash buffer – Dilute 25mL of wash buffer concentrate (20X) into 475mL deionized or distilled water to prepare 500mL of wash buffer. Mix gently and thoroughly.

Sample diluent – Dilute 5mL of sample diluent concentrate (10X) into 45mL deionized or distilled water to prepare 50mL of sample diluent. Mix gently and thoroughly.

Alk-phos conjugate – The stock antibody must be diluted with 1X sample diluent. Calculate the required amount of 1X Alk-phos conjugate solution to use in the assay procedure according to how many wells you wish to use and the following calculation:

NUMBER OF STRIPS	NUMBER OF WELLS	TOTAL VOLUME OF 1X ALK-PHOS CONJUGATE (µL)	CONJUGATE (µL)	DILUENT(µL)
4	32	2500	25	2475
6	48	3500	35	3465
8	64	4500	45	4455
10	80	5500	55	5445
12	96	6500	65	6435

fMLP standard –
Reconstitute the fMLP standard with 200µL of sample diluent provided and mix thoroughly with gentle agitation for a minimum of 10 minutes prior to making dilutions.

This reconstitution produces a stock solution of 50µg/mL.

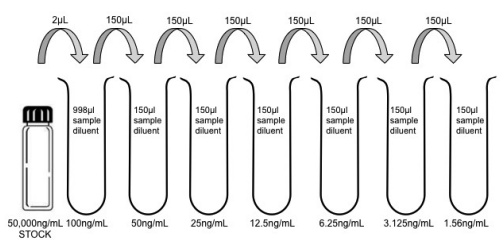

Figure 2. Schematic of standard dilutions

Use polypropylene tubes throughout. Pipette 2µL of stock standard into 998µL sample diluent to produce top standard of 100ng/mL. Pipette 150µL of sample diluent into each tube and use the top standard to produce a dilution series (below). Mix each tube thoroughly before each transfer. The sample diluent serves as the zero standard (0ng/mL).

STANDARD #	VOLUME TO DILUTE (µL)	VOLUME DILUENT (µL)	TOTAL VOLUME (µL)	STARTING CONC. (ng/mL)	FINAL CONC. * (ng/mL)
1	2	998	1000	100	50
2	150	150	300	50	25
3	150	150	300	25	12.5
4	150	150	300	12.5	6.25
5	150	150	300	6.25	3.125
6	150	150	300	3.125	1.56
7	150	150	300	1.56	0.78
8	-	300	300	0	0

* final concentration after a further 1 in 2 dilution with Alk-Phos conjugate

ASSAY PROCEDURE

Equilibrate all materials and prepared reagents to room temperature (18-25°C) prior to use. It is recommended that all standards, controls and samples are tested in duplicate.

1. Prepare all reagents, working standards, and samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame and return them immediately to the foil pouch containing the desiccant and reseal.
3. Wash plate four times with 300µL per well of 1X wash buffer manually or three times with 300µL of 1X wash buffer if using a plate washer. Tap the microplate 4-5 times on absorbent paper towel to completely remove the liquid.
4. Add 50µL of fMLP standard or diluted sample per well followed by 50µL Alk-Phos conjugate per well. Cover the plate with a plate sealer and incubate for one hour at room temperature on a shaker. Start the timer after the last sample addition.
5. Wash plate as previously described.
6. Add 100µL pNPP substrate solution to each well and incubate for 40-50 minutes to allow yellow colour to develop. Protect from light.
7. Read the absorbance on a microplate reader at a wavelength of 405nm.

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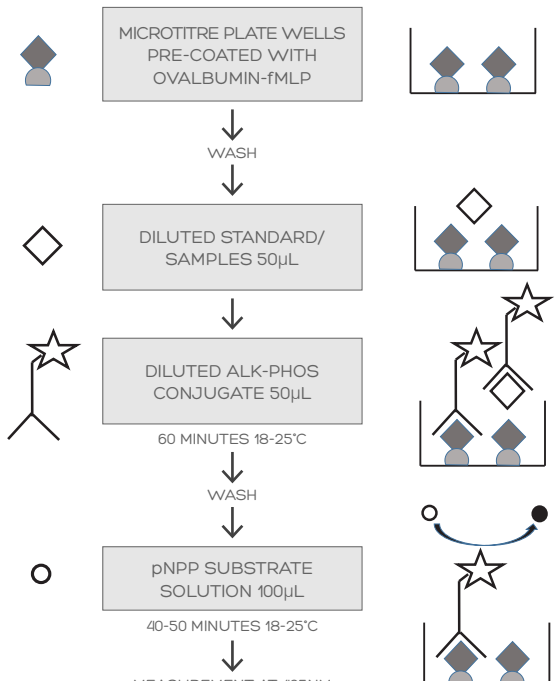
ASSAY PROCEDURE SUMMARY



CALCULATIONS

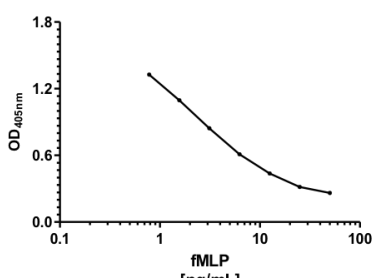
Calculate the mean value of the readings for each standard and sample. To generate a standard curve, plot the graph using the standard concentrations on the x-axis and the corresponding mean 405nm absorbance on the y-axis. The best-fit line can be determined by regression analysis using 4-parameter logistic curve fit.

Determine the unknown sample concentration from the standard curve and multiply the value by the dilution factor 10 (final dilution factor after a further 1 in 2 dilution with Alk-Phos conjugate on the plate).

TYPICAL STANDARD CURVE

Data provided for demonstration purposes only.

A typical standard curve is shown below with the standards starting at 50ng/mL. The standard curve can be used to determine unknown levels of fMLP in urine samples using a 4-parameter fit.



ng/mL	AVERAGE (Abs 405nm)
0	1.768
0.78125	1.327
1.5625	1.096
3.125	0.844
6.25	0.610
12.5	0.437
25	0.316
50	0.262

SENSITIVITY

The lowest level of detection (LLOD) of fMLP is typically 0.15ng/mL. The LLOD was determined by subtracting two standard deviations from the mean optical density value of twenty zero standard replicates and calculating the corresponding concentration.

PRECISION

Intra-assay precision Four samples of known concentration were tested twenty-four times on one plate using a nested layout. This was repeated four times.

SAMPLE	HIGH	MED	LOW	NEG
n	96	96	96	96
Mean	0.24	0.39	1.04	1.72
SD	0.01	0.01	0.03	0.05
CV	4.3	3.8	2.9	2.7

Inter-assay precision Eight samples of known concentration were tested twelve times on three different batches of plates by three different operators.

SAMPLE	BATCH 1	BATCH 2	BATCH 3
CV	<7 %	<4%	<8%

RECOVERY

The recovery of fMLP spiked into ten urine samples was evaluated.

Standard added value: 125ng/mL

Recovery %: 85-112.

Average recovery %: 102

LINEARITY

To assess the linearity of the assay, eight samples spiked with high concentrations of fMLP (75ng/mL) were diluted to produce samples with values within the dynamic range of the assay.

URINE DILUTION	AVERAGE % EXPECTED VALUE
1:5	100
1:10	98.1
1:20	96.8
1:40	97.8

SPECIFICITY

The specificity of the assay was evaluated by measuring the degree of cross-reactivity to various related compounds. The amount of antibody that was bound at each concentration was calculated and the approximate percentage of cross-reactivity was calculated from the amount of compound that produced a signal equivalent to 50%.

CROSS REACTANT	% CROSS REACTIVITY
fMLP	100%
fMLA*	12%
fML	3.75%
N-f-norleucine	<1.5%
MLF	0%
LF	0%
fM	0%
fMAF	0%
Ac-MLF	0%

*fMLA is not a naturally occurring peptide and so is not expected to interfere with the analysis of physiological fluids.

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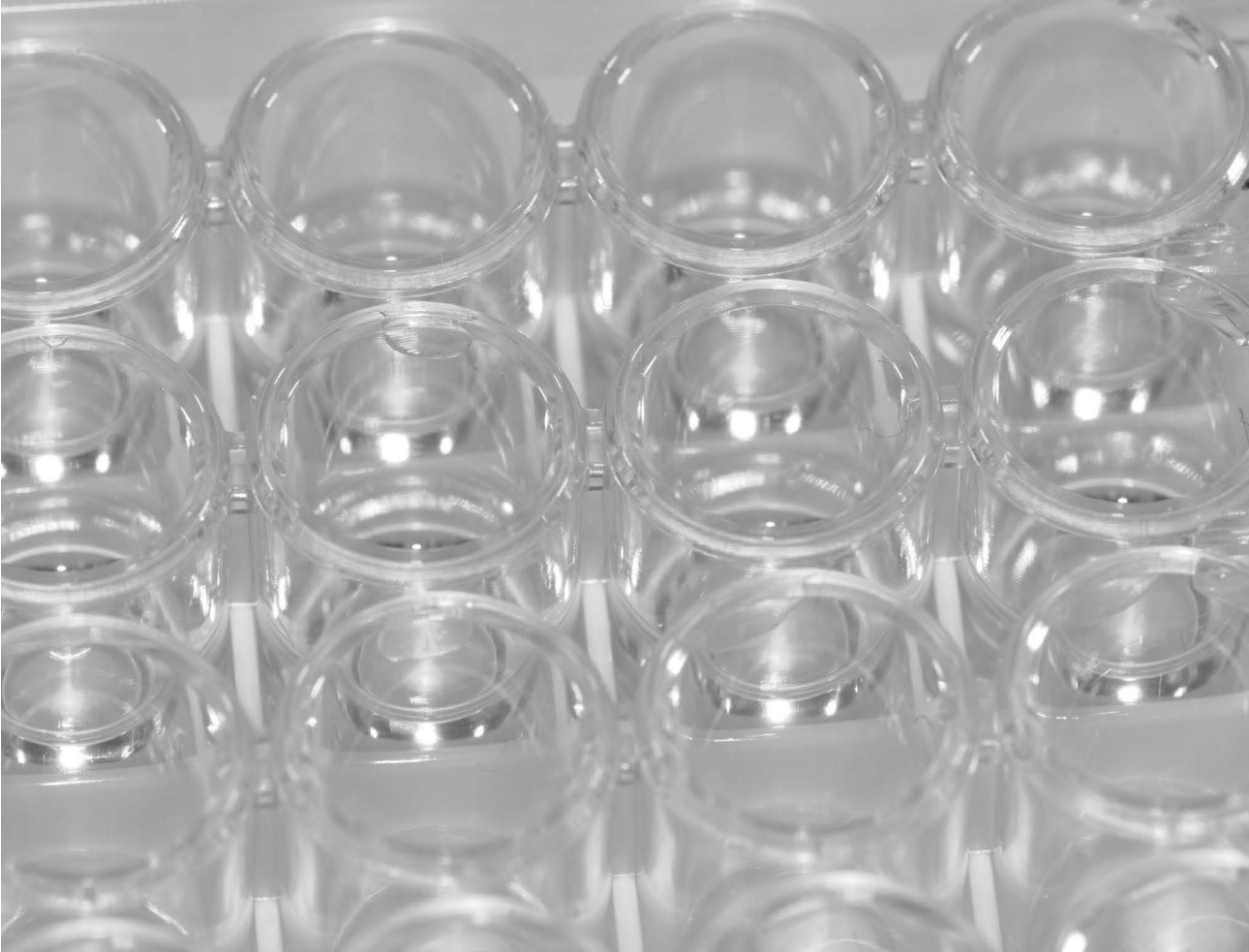
NOTES

NOTES

PLATE LAYOUT

	1	2	3	4	5	6	7	8	9	10	11	12
A	○	○	○	○	○	○	○	○	○	○	○	○
B	○	○	○	○	○	○	○	○	○	○	○	○
C	○	○	○	○	○	○	○	○	○	○	○	○
D	○	○	○	○	○	○	○	○	○	○	○	○
E	○	○	○	○	○	○	○	○	○	○	○	○
F	○	○	○	○	○	○	○	○	○	○	○	○
G	○	○	○	○	○	○	○	○	○	○	○	○
H	○	○	○	○	○	○	○	○	○	○	○	○



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