KRISHGEN BioSystems
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KRIBIOLISA® Tirzepatide (MOUNJARO™) ELISA

REF : KOD1027

Ver.2.3

RUO

Enzyme Immunoassay for the Quantitative Determination of Tirzepatide in Human Serum and Plasma

RUO	**For Research Use Only**	**REF**	**Catalog Number**
	**Store At**	**LOT**	**Batch Code**
	**Manufactured By**		**Biological Risk**
	**Expiry Date**		**Consult Operating Instructions**

For Research Use Only. Purchase does not include or carry the right to resell or transfer this product either as a stand-alone product or as a component of another product. Any use of this product other than the permitted use without the express written authorization of Krishgen Biosystems Private Limited is strictly prohibited.

REF KOD1027 Σ 96 tests

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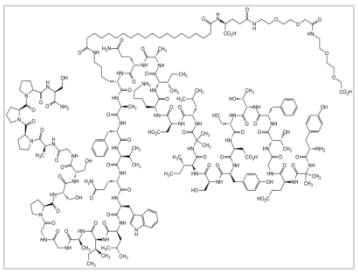
Introduction:

Tirzepatide is a dual GIP and GLP-1 receptor agonist used for the treatment of type II diabetes in adults as an adjunct to diet and exercise. Tirzepatide comprises a 39 amino acid linear synthetic peptide conjugated to a C20 fatty diacid moiety. Tirzepatide was approved by the FDA on May 13, 2022, under the brand name MOUNJARO by the FDA for the treatment of adults with type 2 diabetes, making it the first and only GIP and GLP-1 receptor agonist.



Later, it was approved under a different brand name ZEPBOUND on November 8, 2023, for the chronic weight management in adults with obesity or overweight with at least one weight-related condition. On September 15, 2022, Tirzepatide was also approved by the European Commission.

Intended Use:

The KRIBIOLISA® Tirzepatide (MOUNJARO™) ELISA kit is used for the quantitative determination of Tirzepatide in Human Serum and Plasma.

Principle:

The Tirzepatide ELISA is a competitive immunoassay for the determination of Tirzepatide. It is known that Tirzepatide binds strongly to Glp1-R antigen. Hence using this principle the assay has been developed. A varying concentration of unlabeled standard or sample and constant concentration of Tirzepatide:HRP conjugate will bind in sequence to the antigen coated on the microplate. Upon washing, unbound Tirzepatide:HRP Conjugate will be removed. Bound Tirzepatide:HRP complex will produce a soluble blue colored product after the addition of 3,3',5,5' Tetra Methyl Benzidine (TMB) Substrate. The enzyme reaction is stopped by dispensing of stop solution into the wells. The optical density (OD) of the solution at 450 nm is inversely proportional to the amount of bound Tirzepatide present in the standards or samples.

Materials Provided:

Part	Description	Qty
GLP1R protein Coated Microtiter Plate	96 well polystyrene microplate (12 strips of 8 wells) coated with GLP1R protein Coated Microtiter Plate	1 x 96 wells
Tirzepatide Standard	Tirzepatide in Buffered protein base with preservative Thiomersal < 0.01% standard (lyophilized, concentration - 4,000 ng/ml)	2 vials
Tirzepatide:HRP Conjugate concentrated	Tirzepatide HRP conjugate (concentrated 1 mg/ml)	1 vial
Detection Diluent	Buffered protein base with protein stabilizer and preservatives 0.02% Methylisothiazolinone and 0.02% bromonitrodioxane.	12 ml
(1X) Sample Diluent	Buffered protein base with preservative Thiomersal < 0.01%	2 x 50 ml
(1X) Standard Diluent	Buffered protein base with preservative Thiomersal < 0.01% and 1:1000 dilution human serum	10 ml
(20X) Wash Buffer	20-fold concentrated solution of buffered surfactant with preservative Thiomersal < 0.01%. May turn yellow over time.	25 ml
TMB Substrate	Stabilized Chromogen	12 ml
Stop Solution	0.73M Phosphoric Acid	12 ml
Instruction Manual		1 no

Materials to be provided by the End-User:

1. Microplate Reader able to measure absorbance at 450 nm.
2. Adjustable pipettes to measure volumes ranging from 50 ul to 1000 ul.
3. Deionized (DI) water.
4. Wash bottle or automated microplate washer.
5. Graph paper or software for data analysis.

6. Tubes to prepare standard/sample dilutions.
7. Timer.
8. Absorbent paper.
9. Incubator

Handling/Storage:

1. It is advisable to aliquot and store the Tirzepatide:HRP Conjugate concentrated at -20°C upon receipt. Rest of the kit components should be stored at 2-8°C. Immediately discard any excess Working Tirzepatide:HRP Conjugate after running your assay.
2. All the reagents and wash solutions should be used within 12 months from manufacturing date.
3. Before using, bring all components to room temperature (18-25ºC). Upon assay completion ensure all components of the kit are returned to appropriate storage conditions.
4. The Substrate is light-sensitive and should be protected from direct sunlight or UV sources.

Health Hazard Warnings:

1. Reagents that contain preservatives may be harmful if ingested, inhaled or absorbed through the skin.
2. For Research Use Only.

Sample Preparation and Storage:

Blood is taken by venipuncture. Serum is separated after clotting by centrifugation. Plasma can be used, too. Lipaemic, hemolytic or contaminated samples should not be run. Repeated freezing and thawing should be avoided. If samples are to be used for several assays, initially aliquot samples and keep at - 20°C.

Preparation Before Use:

Allow samples to reach room temperature prior to assay. Take care to agitate patient samples gently in order to ensure homogeneity.

Test Sample preparation - Samples have to be diluted 1:1000 (v/v), e.g. for 1:1000 (1 ul sample + 999 ul sample diluent) prior to assay. The samples may be kept at 2-8°C for up to three days. Long-term storage requires -20°C.

Reagent Preparation (all reagents should be diluted immediately prior to use):

1. Label any aliquots made with the kit Lot No and Expiration date and store it at appropriate conditions mentioned.
2. Bring all reagents to Room Temperature before use.
3. To make **(1X) Wash Buffer**; dilute **25 ml of 20X Wash Buffer in 475 ml of DI water**.
4. **Standards Preparation**: Reconstitute the concentrated Standard lyophilized vial with 1 ml of (1X) Standard Diluent to obtain 4,000 ng/ml. Keep the vial for 15 mins with gentle agitation and then run the assay procedure. Use the (1X) Standard Diluent as the zero standard. Below table shows the calculation for the standard range.

Standard Concentration (ng/ml)	Standard No.	Dilution Particulars
4000 ng/ml	Reconstituted standard	Lyophilized Standard + 1 ml of Standard Diluent (1X)
4000 ng/ml	Standard No.8	Reconstituted Standard only
2000 ng/ml	Standard No.7	500 ul Standard No.8 + 500 ul Standard Diluent (1X)
1000 ng/ml	Standard No.6	500 ul Standard No.7 + 500 ul Standard Diluent (1X)
500 ng/ml	Standard No.5	500 ul Standard No.6 + 500 ul Standard Diluent (1X)
250 ng/ml	Standard No.4	500 ul Standard No.5 + 500 ul Standard Diluent (1X)
125 ng/ml	Standard No.3	500 ul Standard No.4 + 500 ul Standard Diluent (1X)
62.5 ng/ml	Standard No.2	500 ul Standard No.3 + 500 ul Standard Diluent (1X)
31.3 ng/ml	Standard No.1	500 ul Standard No.2 + 500 ul Standard Diluent (1X)
0 ng/ml	Standard No.0	On Standard Diluent (1X)

Mix each tube thoroughly before the next transfer. Use the standards for experiment within one hour of preparation of standard. Discard standard after use.

5. **Working Tirzepatide:HRP Conjugate – Refer to the Reagent Preparation sheet attached with the IFU and COA (enclosed in the kit).**

Procedural Notes:

1. In order to achieve good assay reproducibility and sensitivity, proper washing of the plates to remove excess un-reacted reagents is essential.
2. Avoid assay of Samples containing Sodium Azide (NaN_3), as it could destroy the HRP activity of the conjugate resulting in under-estimation of the antibodies.
3. It is recommended that all Standards and Samples be assayed in duplicates or triplicates.
4. Maintain a repetitive timing sequence from well to well for all the steps to ensure that the incubation timings are same for each well.
5. If the Substrate has a distinct blue color prior to use it may have been contaminated and use of such substrate can lead to compromisation of the sensitivity of the assay.
6. The plates should be read within 30 minutes after adding the Stop Solution.
7. Make a work list in order to identify the location of Standards and Samples.
8. Making serial dilution in the wells directly is not permitted.
9. Prepare the Standard within 15 minutes prior to running the assay.
10. Please carefully dilute Standards according to the instruction, and avoid foaming. To minimize imprecision caused by pipetting, use small volumes and ensure that pipettes are calibrated.
11. If crystals have formed in the Wash Solution (20X) concentrate, warm to room temperature and mix gently until the crystals are completely dissolved.
12. Contaminated water or container for reagent preparation will influence the detection results.

Assay Procedure:

1. It is strongly recommended that all Standards and Samples be run in duplicates or triplicates. A standard curve is required for each assay.

2. Pipette out **100 ul of Standards and samples** to the respective wells.

3. Add **100 ul working Tirzepatide:HRP Conjugate** to each well mix it.

4. Cover the plate and incubate for 90 mins at 37°C.

5. Aspirate and wash plate 4 times with **Wash Buffer (1X)** and blot residual buffer by firmly tapping plate upside down on absorbent paper. Wipe of any liquid from the bottom outside of the microtiter wells as any residue can interfere in the reading step.

6. Add **100 ul** of **TMB Substrate** in each well.

7. Incubate the plate at RT for 30 minutes in dark. DO NOT SHAKE or else it may result in higher backgrounds and worse precision. Positive wells should turn bluish in color.

8. Pipette out **100 ul** of **Stop Solution**. Wells should turn from blue to yellow in color.

9. Read the absorbance at 450 nm with a microplate within 10-15 minutes after addition of Stop solution.

Calculation of Results:

Determine the Mean Absorbance for each set of duplicate or triplicate Standards and Samples. Using Semi-Log graph paper, plot the average value (absorbance 450 nm) of each standard on the Y-axis versus the corresponding concentration of the standards on the X-axis. Draw the best fit curve through the standard points.

To determine the unknown Tirzepatide concentrations, find the unknown's Mean Absorbance value on the Y-axis and draw a horizontal line to the standard curve.

At the point of intersection, draw a vertical line to the X-axis and read the Tirzepatide Concentration.

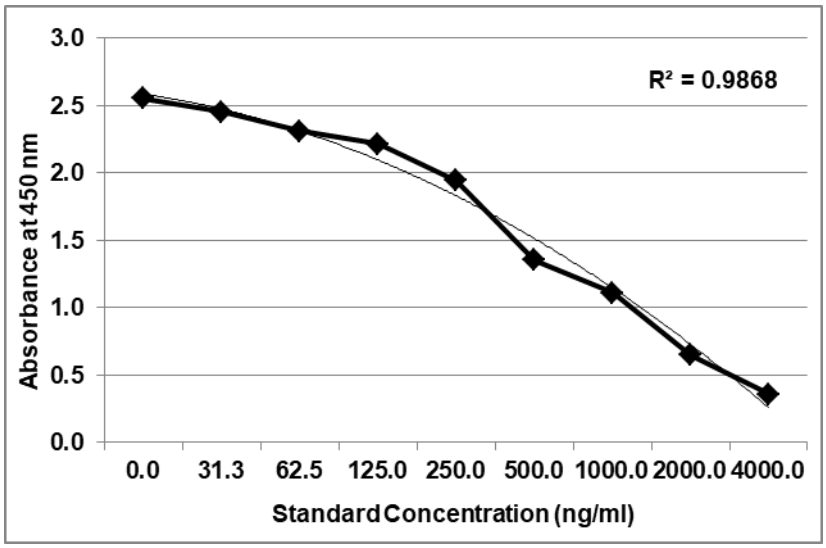
If samples were diluted, multiply by the appropriate dilution factor. Software which is able to generate a cubic spline curve-fit or 4PL (2nd order) is best recommended for automated results.

Note:
It is recommended to repeat the assay at a different dilution factor in the following cases:
- If the sample absorbance value is below the first standard.
- If the absorbance value is equivalent or higher than the 4000 ng/ml standard.

Typical Data (representative only)

Standard Concentration (ng/ml)	Mean Absorbance	Interpolated Concentration	% Interpolated Concentration against Actual Concentration
0.0	2.555	--	--
31.3	2.451	31.1	99.5
62.5	2.313	72.1	115.3
125.0	2.213	109.9	87.9
250.0	1.942	221.0	88.4
500.0	1.353	609.7	121.9
1000.0	1.110	888.0	88.8
2000.0	0.649	1986.9	99.3
4000.0	0.356	4194.5	104.9

Typical Graph (representative only)



Quality Control:

It is recommended that for each laboratory assay appropriate quality control samples in each run to be used to ensure that all reagents and procedures are correct.

Performance Characteristics:

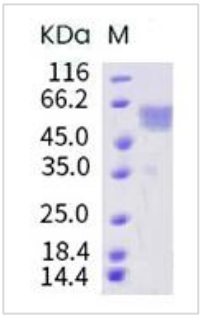
Please note that this validation is performed in our laboratory and will not necessarily be duplicated in your laboratory. This data has been generated to enable the user to get a preview of the assay and the

characteristics of the kit and is generic in nature. We recommend that the user performs at the minimum; the spike and recovery assay and the dilutional linearity assay to assure quality results.

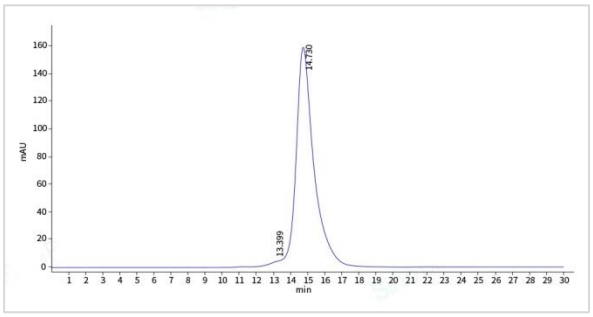
For a more comprehensive validation, the user may run the protocols as suggested by us herein below to develop the parameters for quality control to be used with the kit.

Standard Range:
31.3 ng/ml - 4000 ng/ml.

Specificity:
The kit uses a recombinant GLP1r protein expressed in HEK293 as capture antigen. The protein construct is a DNA sequence encoding the human GLP1R (NP_002053.3) (Met1-Tyr145) expressed with the Fc region of human IgG1 at the C-terminus.



Gel Image of the Coat/Capture Protein



Chromatogram of the Coat/Capture Protein

The competition between the standard and the detection conjugate uses a synthetically derived peptide as a biosimilar to Tirzepatide. Detailed specifications of the standard (synthetic Tirzepatide) used in the kit are -
Formula: C225H348N48O68
MW:4813.5
Sequence:H-Tyr-{Aib}-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Tyr-Ser-Ile-{Aib}-Leu-Asp-Lys-Ile-Ala-Gln-{diacid-gamma-Glu-(AEEA)2-Lys}-Ala-Phe-Val-Gln-Trp-Leu-Ile-Ala-Gly-Gly-Pro-Ser-Ser-Gly-Ala-Pro-Pro-Pro-Ser-NH2.
Purity: ≥98% (HPLC)

Sensitivity:
The minimum detectable dose of Tirzepatide is 30 ng/ml.

Precision:
Intra-Assay Precision: 3 samples with low, middle and high level human Tirzepatide were tested 20 times on one plate, respectively.
Inter-Assay Precision: 3 samples with low, middle and high level human Tirzepatide were tested on 3 different plates, 8 replicates in each plate.

CV (%) = SD/mean X 100
Intra-Assay: CV<15%
Inter-Assay: CV<18%

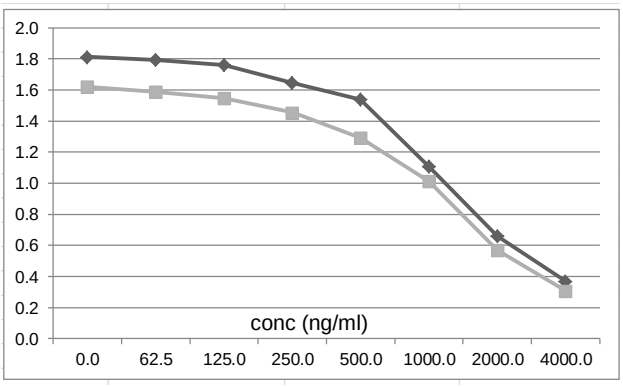
Inter-Operator: CV<15%

Standard (ng/ml)	Mean Absorbance	Mean Absorbance	%CV
0.0	2.486	2.515	0.84
31.3	2.355	2.425	2.09
62.5	2.212	2.287	2.36
125.0	2.016	1.986	1.05
250.0	1.686	1.755	2.82
500.0	1.225	1.290	3.66
1000.0	0.744	0.713	3.08
2000.0	0.428	0.501	11.15
4000.0	0.224	0.267	12.54

Comparison and Recovery of Standard used in the kit with *commercially sourced Tirzepatide Injection*

Three runs were conducted using the commercially sourced Tirzepatide Injection (Mounjaro) and the standards were prepared using the kit dilution buffer to estimate the similarity in terms of %CV for both the standards diluted and interpolated concentration to establish the confidence in the assay.

Standards (ng/ml)	Kit Standard (Tirzepatide)	Tirzepatide Injection	%CV
0.0	1.812	1.621	7.88
62.5	1.792	1.588	8.55
125.0	1.759	1.546	9.11
250.0	1.646	1.455	8.74
500.0	1.539	1.294	12.24
1000.0	1.107	1.013	6.24
2000.0	0.659	0.571	10.10
4000.0	0.370	0.308	12.83

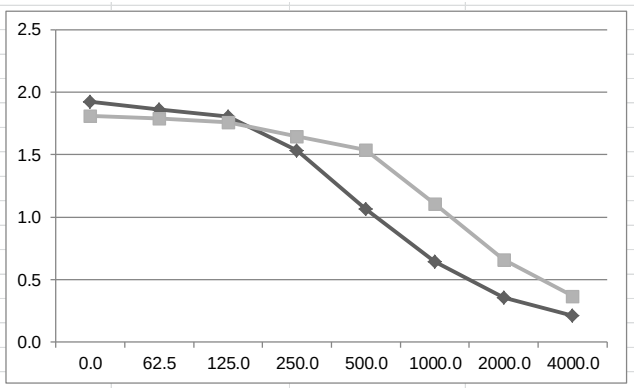


Conclusion: The kit showed good recovery using the commercially sourced injection against the internal standards of the kit.

Comparison and Recovery of Standard used in the kit with *commercially sourced Tirzepatide Injection biosimilar*

Three runs were conducted using the commercially sourced Tirzepatide Injection (biosimilar) and the standards were prepared using the kit dilution buffer to estimate the similarity in terms of %CV for both the standards diluted and interpolated concentration to establish the confidence in the assay

Standards (ng/ml)	Kit Standard (Tirzepatide)	Tirzepatide Injection	%CV
0.0	1.925	1.812	4.27
62.5	1.860	1.792	2.64
125.0	1.806	1.759	1.84
250.0	1.535	1.646	4.95
500.0	1.068	1.539	25.59
1000.0	0.644	1.107	37.38
2000.0	0.358	0.659	41.85
4000.0	0.213	0.370	38.20



Conclusion: The kit demonstrated showed good linearity of the standard curve despite high variance using the commercially sourced biosimilar injection against the internal standards of the kit.

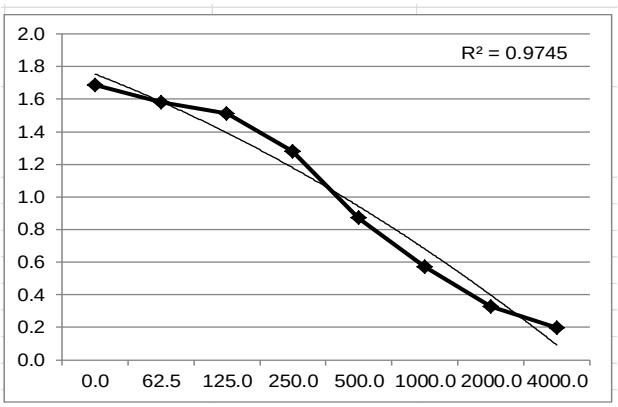
For researchers and clinical companies looking to use the kit for demonstration and estimation of their own developed Tirzepatide injection, it is recommended to run similarity assay using the kit standard. In case the demonstrated variance is beyond the defined quality limits, we would recommend to use your own injection as standards in the kit using our kit diluent. In case the recoveries obtained are not as per the desired results, please connect with us (email: sales1@krishgen.com) to help you optimize the assay using our own differently formulated diluents to best optimize on the kit.

Demonstrated Variability using Different Diluents Formulated on the Assay

We compared two diluents, one being a PBS based diluent buffer with Tween20 and the other a proprietary buffer procured commercially as ELISA diluent buffer to demonstrate the effect of different diluents on the assay.

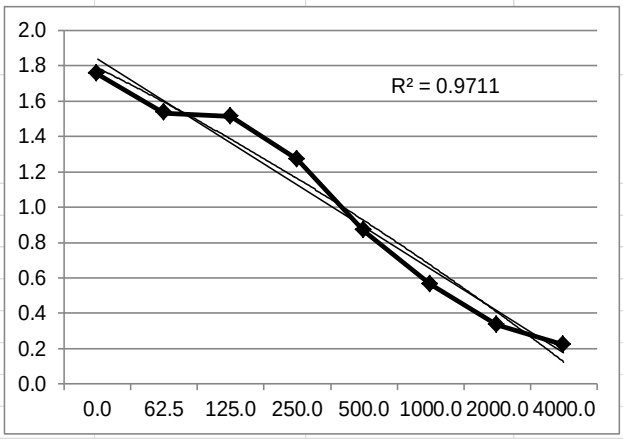
PBS based Buffer (pH: 7.5) used for diluting the Kit Standard:

Standard (ng/ml)	Mean Absorbance	Interpolated Concentration	% Interpolated Concentration against Actual Concentration
0.0	1.686		--
62.5	1.582	77.0	123.1
125.0	1.513	115.1	92.1
250.0	1.281	239.4	95.8
500.0	0.870	533.4	106.7
1000.0	0.571	954.1	95.4
2000.0	0.327	1934.8	96.7
4000.0	0.197	4488.4	112.2



Proprietary Buffer (commercially available, pH: 7.5) used for diluting the Kit Standard:

Standard (ng/ml)	Mean Absorbance	Interpolated Concentration	% Interpolated Concentration against Actual Concentration
0.0	1.754		--
62.5	1.534	99.6	159.4
125.0	1.513	109.5	87.6
250.0	1.269	231.5	92.6
500.0	0.868	526.5	105.3
1000.0	0.565	994.8	99.5
2000.0	0.334	2024.1	101.2
4000.0	0.223	3823.9	95.6



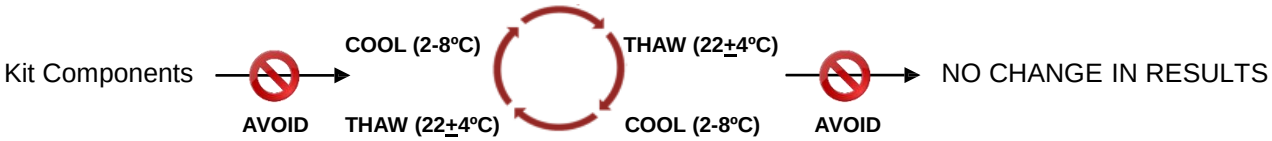
Safety Precautions:

- **This kit is For Research Use only**. Follow the working instructions carefully.
- The expiration dates stated on the kit are to be observed. The same relates to the stability stated for reagents.
- Do not use or mix reagents from different lots.
- Do not use reagents from other manufacturers.
- Avoid time shift during pipetting of reagents.
- All reagents should be kept in the original shipping container.
- Some of the reagents contain small amount of sodium azide (< 0.1 % w/v) as preservative. They must not be swallowed or allowed to come into contact with skin or mucosa.
- Source materials maybe derived from **human body fluids** or organs used in the preparation of this kit were tested and found negative for HBsAg and HIV as well as for HCV antibodies. However, no known test guarantees the absence of such viral agents. Therefore, handle all components and all patient samples as if potentially hazardous.
- Since the kit contains potentially hazardous materials, the following precautions should be observed
  - Do not smoke, eat or drink while handling kit material
  - Always use protective gloves
  - Never pipette material by mouth
  - Wipe up spills promptly, washing the affected surface thoroughly with a decontaminant.
- In any case GLP should be applied with all general and individual regulations to the use of this kit.

SCHEMATIC ASSAY PROCEDURE

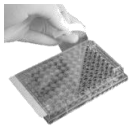
1. Remove all components, 30 minutes before adding into the assay plate.

2. Avoid repeated cool-thaw of the components as there will be a loss of activity and this can affect the results.



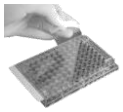
3.  Pipette **100 ul prepared Standard** and **Sample** into respective well.

4.  Pipette **100 ul working Tirzepatide:HRP Conjugate** into each wells mix it.

5. Cover plate  and incubate for  at 37°C.

6.  Aspirate and wash wells **4 times** with **Wash Buffer (1X).**

7.  Pipette **100 ul TMB Substrate** into each well.

8. Cover plate  and incubate for  at Room Temperature.

9.  Pipette **100 ul Stop Solution** into each well.

10. Read absorbance at 450 nm with a  microplate reader within  of stopping reaction.

Typical Example of a Work List

Well #	Contents	Absorbance at 450nm	Mean Absorbance	ng/ml Tirzepatide
1A 2A	zero standard zero standard			
1B 2B	31.3 ng/ml 31.3 ng/ml			
1C 2C	62.5 ng/ml 62.5 ng/ml			
1D 2D	125 ng/ml 125 ng/ml			
1E 2E	250 ng/ml 250 ng/ml			
1F 2F	500 ng/ml 500 ng/ml			
1G 2G	1000 ng/ml 1000 ng/ml			
1H 2H	2000 ng/ml 2000 ng/ml			
3A 4A	4000 ng/ml 4000 ng/ml			
3B 4B	Sample Sample			

LIMITED WARRANTY

Krishgen Biosystems Private Limited does not warrant against damages or defects arising in shipping or handling, or out of accident or improper or abnormal use of the product; against defects in products or components not manufactured by Krishgen Biosystems Private Limited, or against damages resulting from such non-Krishgen Biosystems Private Limited made products or components. Krishgen Biosystems Private Limited passes on to customer the warranty it received (if any) from the maker thereof of such non-Krishgen made products or components. This warranty also does not apply to product to which changes or modifications have been made or attempted by persons other than pursuant to written authorization by Krishgen Biosystems Private Limited.

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KRISHGEN BIOSYSTEMS PRIVATE LIMITED PRIVATE LIMITED| OUR REAGENTS | YOUR RESEARCH |

MOUNJARO is the registered trademark of Eli Lilly and Company.

SYMBOLS KEY

MTP	**GLP1R protein Coated Microtiter Plate (12x8 wells)**
STD	**Tirzepatide Standard, Lyophilized**
HRP CONJ	**Tirzepatide:HRP Conjugate concentrated**
DET DIL	**Detection Diluent**
1X STD DIL	**(1X) Standard Diluent**
1X SAMP DIL	**(1X) Sample Diluent**
20X WASH BUF	**(20X) Wash Buffer**
SUB TMB	**TMB Substrate**
SOLN STOP	**Stop Solution**
	**Consult Instructions for Use**
REF	**Catalog Number**
	**Expiration Date**
	**Storage Temperature**