GLUCAGON-LIKE PEPTIDE-1 (TOTAL) RIA KIT
125 TUBES (Cat. # GLP1T-36HK)

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I. INTENDED USE

This kit is for the quantitative determination of all forms of Glucagon-Like Peptide-1[i.e. GLP-1(7-36) amide, GLP-1(7-37), GLP-1(9-36) amide, GLP-1(9-37), GLP-1(1-36) amide and GLP-1(1-37)] in plasma and other biological media. The GLP-1 sequence is highly conserved between the species with no sequence variation occurring at all in mammals. The antibody used in this assay binds specifically to the C-terminal portion of GLP-1, both amidated and non-amidated forms. Both the standards and tracer are prepared with GLP-1 (7-36) amide. ***This kit is for research purposes only.***

II. PRINCIPLES OF PROCEDURE

In radioimmunoassay, a fixed concentration of labeled tracer antigen is incubated with a constant dilution of antiserum such that the concentration of antigen binding sites on the antibody is limited, for example, only 50% of the total tracer concentration may be bound by antibody. If unlabeled antigen (including any cross reacting substance) is added to this system, there is competition between labeled tracer and unlabeled antigen for the limited and constant number of binding sites on the antibody. Thus, the amount of tracer bound to antibody will decrease as the concentration of unlabeled antigen increases. This can be measured after separating antibody-bound from free tracer and counting one or the other, or both fractions. A standard curve is set up with increasing concentrations of standard unlabeled antigen and from this curve the amount of antigen in unknown samples can be calculated. Thus, the four basic necessities for a radioimmunoassay system are: a primary antiserum to the antigen to be measured, the availability of a radioactive labeled form of the antigen, a method whereby antibody-bound tracer can be separated from the unbound tracer, and finally, an instrument to count radioactivity.

III. REAGENTS SUPPLIED

Each kit is sufficient to run 125 tubes and contains the following reagents:

A. GLP-1 Assay Buffer
0.05M Phosphosaline, pH 6.8 containing proprietary protease inhibitors, Tween 20, 0.08% Sodium Azide, and 1% RIA grade BSA
Quantity: 25 mL/vial
Preparation: **Ready to use**

B. GLP-1 (Total) Antibody
Rabbit anti-GLP-1 antibody diluted in Assay Buffer
Quantity: 13 mL/vial
Preparation: **Ready to use**

C. ^{125} I-GLP-1
^{125} I-GLP-1(7-36) Amide Tracer, HPLC purified (specific activity 636 µCi/µg)
Lyophilized for stability. Freshly iodinated tracer contains <1.5 µCi (<56 kBq) calibrated to the 1st Monday of each month.
Quantity: 13.5 mL/vial upon hydration
Preparation: Contents lyophilized. Hydrate with 13.5 mL of Assay Buffer on day of use. Allow to sit at room temperature for 30 minutes, with occasional mixing prior to addition. Store hydrated tracer frozen at ≤ -20°C.

III. REAGENTS SUPPLIED (continued)

D. GLP-1 Standards
GLP-1 (7-36) amide in Assay Buffer at the following concentrations: 10, 20, 50, 100, 200, 500, 1000 pM.
Quantity: 1 mL/vial
Preparation: **Ready to use**

E. Quality Controls 1 & 2
Various peptides including GLP-1 (7-36) amide in buffer
Quantity: 2 mL/vial
Preparation: **Ready to use**

F. Precipitating Reagent
Goat anti rabbit IgG serum, 3% PEG in 0.05M Phosphosaline, 0.025M EDTA, 0.08% Sodium Azide, 0.05% Triton X-100
Quantity: 130 mL/vial
Preparation: **Ready to use; chill to 4°C**

G. Rabbit Carrier
Assay Buffer containing Normal Rabbit IgG as a carrier
Quantity: 2 mL/vial
Preparation: **Ready to use**

H. Sample Hydrating Solution
Proprietary mixture of protease inhibitors
Quantity: 30 mL/vial
Preparation: **Ready to use; chill to 4°C**

IV. STORAGE AND STABILITY

Freeze all reagents at ≤ -20°C upon receipt and refreeze after use. Avoid multiple (>5) freeze/thaw cycles, prepare aliquots if necessary. Refer to date on bottle for expiration when stored at ≤ -20°C. Do not mix reagents from different kits unless they have the same lot number.

V. REAGENT PRECAUTIONS

A. Radioactive Materials

This radioactive material may be received, acquired, possessed and used only by research personnel or clinical laboratories for in vitro research tests not involving internal or external administration of the material, or the radiation therefrom, to human beings or animals. Its receipt, acquisition, possession, use and transfer are subject to the regulations of the U. S. Nuclear Regulatory Commission or of a State with which the Commission has entered into an agreement for the exercise of regulatory authority.

The following are suggested general rules for the safe use of radioactive material. The customer's Radiation Safety Officer (RSO) is ultimately responsible for the safe handling and use of radioactive material.

1. Wear appropriate personal devices at all times while in areas where radioactive materials are used or stored.

2. Wear laboratory coats, disposable gloves and other protective clothing at all times.

3. Monitor hands, shoes, clothing and immediate area surrounding the work station for contamination after each procedure and before leaving the area.

4. Do not eat, drink or smoke in any area where radioactive materials are stored or used.

5. Never pipette radioactive material by mouth.

6. Dispose of radioactive waste in accordance with NRC rules and regulations.

7. Avoid contaminating objects such as telephones, light switches, doorknobs, etc.

8. Use absorbent pads for containing and easy disposing of small amounts of contamination.

9. Wipe up all spills immediately and thoroughly and dispose of the contaminated materials as radioactive waste. Inform RSO.

B. Sodium Azide

Sodium Azide has been added to all reagents as a preservative at a concentration of 0.08%. Although it is at a minimum concentration, Sodium Azide may react with lead and copper plumbing to form highly explosive metal azides. On disposal, flush with large volume of water to prevent azide build up.

VI. MATERIALS REQUIRED BUT NOT PROVIDED

1. Borosilicate glass tubes, 12 x 75 mm. (NOTE: Polypropylene or polystyrene tubes may not be used as GLP-1 sticks to these tubes.)
2. 100 µl pipette with disposable tips
3. 10 µl, 100 µl & 1.0 mL repeating dispenser
4. Refrigerated centrifuge with swinging bucket capable of developing 2,000 - 3,000 xg. (Use of fixed-angle buckets is not recommended.)
5. Absorbent paper
6. Vortex mixer
7. Refrigerator
8. Gamma Counter
9. Centrifugal Vacuum Evaporator (e.g. Savant Speed Vac) or nitrogen gas dry-down apparatus
10. Microcentrifuge Tubes
11. 95% Ethyl Alcohol
12. Deionized Water
13. Ice Bath
14. Microcentrifuge
15. Rubber Stoppers with 18 gauge Needles inserted

VII. SPECIMEN COLLECTION AND STORAGE

1. For plasma collection, collect blood in ice-cooled Vacutainer® EDTA-plasma tubes. Centrifuge immediately at 1000 xg for 10 minutes in refrigerated centrifuge or place tubes on ice and centrifuge within one hour.

2. Care must be taken when using heparin as an anticoagulant, since an excess will provide falsely high values[5]. Use no more than 10 IU heparin per mL of blood collected.

3. Specimens can be stored at 4°C if they will be tested within 3 hours of collection. For longer storage, specimens should be stored at ≤ -70°C. Avoid multiple (>3) freeze/thaw cycles. Aliquot samples before freezing if necessary.

4. Avoid using samples with gross hemolysis or lipemia.

VIII. ASSAY PROCEDURE

For optimal results, accurate pipetting and adherence to the protocol are recommended.

Sample Extraction Procedure

Note: Due to the low circulating levels of GLP-1 in plasma, a minimum of 600 µl plasma sample is required for duplicate assay tubes (see Flow Chart).
Keep all assay and extraction reagents (except alcohol) at 4°C before use. Samples must be thawed in a manner to maintain plasma in a cold (4°C) environment at all times. During extraction procedures, samples may be stored in an ice water bath or at 4°C for not longer than 3 hours.

SAMPLE EXTRACTION WITH ALCOHOL

Note: Samples are typically extracted and assayed in duplicate although other multiples may be used. Singlets may be used if sample volume is very limited, although this is not recommended.

1. Label microfuge tubes (1.5 mL size) and arrange in an ice bath. Add 1.1 mL of 95% ethyl alcohol in each tube.
2. Add 300µl plasma sample to each tube. Do not extract less than 300 µl sample volume. Cap tubes tightly, invert tubes and vortex well immediately after the plasma sample has been added.
3. Incubate tubes in an ice bath for 30 minutes.
4. Invert tubes to mix, then centrifuge at 10,000 rpm for 10 minutes in a microfuge.
5. Decant supernatants into glass assay tubes (Borosilicate glass 12x75mm). Stopper the tubes with a rubber stopper with an 18 gauge needle inserted.
6. Place tubes into a speed vac® (centrifugal vacuum evaporator) for dry down. Dry the tubes for 2 hours on medium heat (45°C) then switch to 6 hours at ambient temperature (total of 8 hours). Check all tubes for complete dryness. Remove dried tubes, continue drying partially dried tubes in 30-minute increments until complete. Other methods to dry plasma extract may be used (i.e. nitrogen stream without heat); however, these methods must be validated prior to routine use.
7. Rehydrate samples with 300 µl of **cold** Sample Hydrating Solution. Incubate 30 minutes on ice or at 4°C. Vortex gently until sample has dissolved into solution (Note: most samples will exhibit some turbidity). The samples are now ready to be assayed directly in these tubes.

Assay Method

Notes:

1. Borosilicate glass tubes (12 x 75 mm) are required for this procedure.
2. Refer to the Assay Procedure Flow Chart for a suggested arrangement of tubes. Quality Control (QC) samples should be included in every assay prior to sample analysis and at the end of all assays.

VIII. ASSAY PROCEDURE

Day One

1. Pipette 400 µL of Assay Buffer in the Total tubes (1-2) and Non-Specific Binding (NSB) tubes (3-4). Pipette 300 µL of Assay Buffer in the Reference (Bo) tubes (5-6). Pipette 200 µL of Assay Buffer into the Standard Curve and QC tubes (7-24).

2. Pipette 100 µL of Standards and Quality Controls (Low & High) in duplicate to tubes 7-24.

3. Organize hydrated sample tubes. Pipette 300 µL of each sample in duplicate.

4. Pipette 100 µL of GLP-1 antibody to all tubes except Total Count tubes (1-2) and NSB tubes (3-4).

5. Vortex, cover, and incubate overnight (20-24 hours) at 4°C.

Day Two

6. Hydrate the ^{125}I-GLP-1 tracer with 13.5 mL of Assay Buffer. Gently mix. Pipette 100 µL of ^{125}I-GLP-1 to all tubes. Freeze any unused tracer for future use.

7. Vortex, cover and incubate overnight (22-24 hours) at 4°C.

Day Three

8. Add 10 µL of Carrier IgG to all tubes except total count tubes (1-2).

9. Add 1.0 mL of **cold** Precipitating Reagent to all tubes except Total Count tubes (1-2).

10. Vortex and incubate 20 minutes at 4°C.

11. Centrifuge, at 4°C, for 20 minutes at 2,000-3,000 xg. Note: If less than 2,000xg is used, the time of centrifugation must be increased to obtain a firm pellet (e.g. 40 minutes). Multiple centrifuge runs within an assay must be consistent. Conversion of rpm to xg:
    $xg = (1.12 \times 10^{-5})(r)(rpm)^2$
    r = radial distance in cm (from axis of rotation to the bottom of the tube)
    rpm = revolutions per minute

12. Immediately decant supernatant from all centrifuged tubes except Total Count tubes (1-2). Drain tubes for 5-60 seconds (be consistent between racks), blot excess liquid from lip of tubes and count pellet using the gamma counter according to the manufacturer's instructions.

Assay Procedure Flow Chart

Day One					Day Two		Day Three		
Set-up	Step 1	Step 2&3	Step 4	Step 5	Step 6	Step 7	Step 8	Step 9	Steps 10, 11, & 12
Tube Number	Add Buffer	Add Standard/QC Sample	Add GLP-1 (Total) Antibody	Vortex, Cover, and Incubate 20-24 hrs at 4°C	Add I-125 GLP-1 Tracer	Vortex, Cover and Incubate 22-24 hrs at 4°C	Add Carrier IgG	Add Precipitating Reagent	Incubate 20 min. at 4°C, Centrifuge 20 min. at 4°C, Decant and Count
1,2	400 µl	-	-		100 µl		-	-	
3,4	400 µl	-	-		100 µl		10µl	1.0 mL	
5,6	300 µl	-	100 µl		100 µl		10µl	1.0 mL	
7,8	200 µl	100 µl of 10 pM	100 µl		100 µl		10µl	1.0 mL	
9,10	200 µl	100 µl of 20 pM	100 µl		100 µl		10µl	1.0 mL	
11,12	200 µl	100 µl of 50 pM	100 µl		100 µl		10µl	1.0 mL	
13,14	200 µl	100 µl of 100 pM	100 µl		100 µl		10µl	1.0 mL	
15,16	200 µl	100 µl of 200 pM	100 µl		100 µl		10µl	1.0 mL	
17,18	200 µl	100 µl of 500 pM	100 µl		100 µl		10µl	1.0 mL	
19,20	200 µl	100 µl of 1000 pM	100 µl		100 µl		10µl	1.0 mL	
21,22	200 µl	100 µl of QC low	100 µl		100 µl		10µl	1.0 mL	
23,24	200 µl	100 µl of QC high	100 µl		100 µl		10µl	1.0 mL	
25-n	-	300 µl of unknown	100 µl		100 µl		10µl	1.0 mL	

IX. CALCULATIONS

A. Explanation

The calculations for GLP-1 (Total) can be automatically performed by most gamma counters possessing data reduction capabilities or by independent treatment of the raw data using a commercially available software package. Choose weighted 4-parameter or weighted log/logit for the mathematical treatment of the data.

NOTE: Be certain the procedure used subtracts the NSB counts from each average count, except Total Counts, prior to final data reduction. Due to use of 300µl of extracted sample, computer generated data must be divided by 3 to accommodate for the concentration factor.

B. Manual Calculation

1. Average duplicate counts for Total Count tubes (1-2), NSB tubes (3-4), Total Binding tubes (reference, Bo) (5-6), and all duplicate tubes for standards and samples to the end of the assay.
2. Subtract the average NSB counts from each average count (except for Total Counts). These counts are used in the following calculations.
3. Calculate the percentage of tracer bound [(Total Binding Counts/Total Counts) X 100]. This should be 30-40%.
4. Calculate the percentage of total binding (%B/Bo) for each standard and sample

   %B/Bo = (Sample or Standard/Total Binding) X 100
5. Plot the % B/Bo for each standard on the y-axis and the known concentration of the standard on the x-axis using log-log graph paper.
6. Construct the reference curve by joining the points with a smooth curve.
7. Determine the pM of GLP-1 (Total) in the unknown samples and controls by interpolation of the reference curve.

   NOTE: Due to use of 300 µL of extracted sample, manual or computer generated data must be divided by 3 to accommodate for the concentration factor. When sample volumes assayed differ from 300 µl, an appropriate mathematical adjustment must be made to accommodate for the dilution factor.

X. INTERPRETATION

Acceptance Criteria

1. The run will be considered accepted when all Quality Control Values fall within the calculated Quality Control Range; if any QC's fall outside the control range, review the results with the supervisor.

2. If the difference between duplicate results of a sample is >10% CV, repeat the sample.

3. The limit of sensitivity for the assay is 3 pM (300 µL sample size).

4. The limit of linearity for the assay is 333 pM (300 µL sample size). Any result greater than 333 pM should be repeated on dilution using Assay Buffer as a diluent.

XI. ASSAY CHARACTERISTICS

A. Sensitivity

The lowest level of GLP-1 (Total) that can be detected by this assay is 3 pM when using a 300 µL extracted sample size.

B. Performance

The following parameters of assay performance are expressed as Mean ± Standard Deviation.

ED(80) =	13	±	3	pM
ED(50) =	77	±	12	pM
ED(20) =	423	±	63	pM

C. Specificity

The specificity (also known as selectivity) of an analytical test is its ability to selectively measure the analyte in the presence of other like components in the sample matrix.

GLP-1 (7-36) (Human)	100 %
GLP-1 (9-36) (Human)	100 %
GLP-1 (7-37) (Human)	100 %
GLP-2 (Human)	< 0.01 %
Glucagon (Human)	0.2 %
Exendin	< 0.01 %

XI. ASSAY CHARACTERISTICS (continued)

D. Precision

Within and Between Assay Variation

Sample Number	Mean pM	Within % CV	Between % CV
1	20	22	23
2	53	29	10
3	131	35	21
4	204	38	14

Within and between assay variation was performed on four human plasma samples containing varying concentrations of GLP-1. Data shown are from replicates of ten determinations of each serum in four assays.

E. Recovery

Extraction Recoveries of QC Samples

Sample	Non-extracted Value, pM	Extracted Value pM	% Recovery
QC Low	42	34	82
QC High	437	343	78

Quality Control Low & High samples measured before and after extraction procedure.

Spike and Recovery of GLP-1 (Total) in Human Plasma

Human Plasma Sample	GLP-1 (Total) Added, pM	Observed pM	Expected pM	% Recovery
1	0	16	-	-
2	10	31	26	119
3	50	57	66	86
4	100	92	116	79
1	0	40	-	-
2	10	55	50	110
3	50	90	90	100
4	100	128	140	91
1	0	29	-	-
2	10	38	39	97
3	50	41	79	90
4	100	108	129	94

Varying concentrations of GLP-1 were spiked into four human plasma samples and the GLP-1 content determined by RIA. The percent recovery was calculated on the observed vs. expected.

XI. ASSAY CHARACTERISTICS (continued)

F. Linearity

Effect of Serum Dilution

Sample No.	Volume Sampled	Observed pM	Final pM/mL
1	300 µl	104.3	34.8
	200 µl	31.9	15.9
	100 µl	2.8	2.8
2	300 µl	167.2	55.7
	200 µl	83.5	41.8
	100 µl	17.1	17.1
3	300 µl	47.8	15.9
	200 µl	6.8	3.4
	100 µl	Not Detectable	-

To demonstrate the linearity of recovery of GLP-1 in diluted plasma samples, three different volumes, 100, 200, and 300 µL of Human Plasma were analyzed following the defined assay procedure. Diluted samples of GLP-1 do not demonstrate linearity; therefore, 300 µL plasma sample should be used in the assay.

XI. ASSAY CHARACTERISTICS (continued)

G. Example of Assay Results

This data is presented as an example only and should not be used in lieu of a standard curve prepared with each assay.

Tube #	ID	CPM	Ave CPM	Ave Net CPM	% B/Bo	pM
1	Totals	10809				
2	〃	11386	11098			
3	NSB	314				
4	〃	280	297			
5	Bo	5592				
6	〃	5396	5494	5197		
Standards						
7	10 pM	5106				
8		4975	5040	4743	91.2	
9	20 pM	4697				
10		4644	4671	4374	84.2	
11	50 pM	3445				
12		3557	3501	3204	61.7	
13	100 pM	2677				
14		2649	2663	2366	45.5	
15	200 pM	1816				
16		1736	1776	1479	28.5	
17	500 pM	1251				
18		1144	1198	901	17.3	
19	1000 pM	934				
20		818	876	579	11.1	
Controls/Unknown						
21	QC 1	3904				
22		3697	3801	3504	67.4	40.7
23	QC 2	1261				
24		1214	1238	941	18.1	434.2
25-n	Unknown					

XII. QUALITY CONTROLS

Good Laboratory Practice (GLP) requires that Quality Control (QC) specimens be run with each standard curve to check the assay performance. Two levels of controls are provided for this purpose. These and any other control materials should be assayed repeatedly to establish mean values and acceptable ranges. Each individual laboratory is responsible for defining their system for quality control decisions and is also responsible for making this system a written part of their laboratory manual. The ranges for Quality Control 1 and 2 are provided on the card insert or can be located at the Millipore website www.millipore.com/bmia.

Recommended batch analysis decision using two controls (Westgard Rules).

1. When both controls are within ±2 SD. Decision: Approve batch and release analyte results.

2. When one control is outside ±2 SD and the second control is within ±2 SD.
   Decision: Hold results, check with supervisor. If no obvious source of error is identified by the below mentioned check of systems, the supervisor may decide to release the results.

   Technician check of systems:
   1. Check for calculation errors
   2. Repeat standards and controls
   3. Check reagent solutions
   4. Check instrument

XIII. REPLACEMENT REAGENTS

Reagents	**Cat. #**
^{125}I-GLP-1 (<1.5 µCi, <56 kBq)	9035-HK
GLP-1 Standards (1 mL each)	8035-K
GLP-1 (Total) Antibody (13 mL)	1036-HK
Precipitating Reagent (130 mL)	PR-81HK
Quality Controls 1&2 (2 mL each)	6016-K
GLP-1 (Total) Assay Buffer (25 mL)	AB-GLPHK
Rabbit Carrier (2 mL)	RC-HK
Sample Hydrating Solution (30 mL)	SHS-GLPHK

XIV. ORDERING INFORMATION

A. To place an order:

For USA Customers:

Please provide the following information to our customer service department to expedite your telephone, fax or mail order:

1. Your name, telephone and/or fax number
2. Customer account number
3. Shipping and billing address
4. Purchase order number
5. Catalog number and description of product
6. Quantity and product size
   NOTE: Appropriate license from NRC (or equivalent) must be on file at Millipore before radioactive orders can be shipped.

TELEPHONE ORDERS: Toll Free US (800) MILLIPORE

FAX ORDERS: (636) 441-8050

MAIL ORDERS: Millipore
6 Research Park Drive
St. Charles, Missouri 63304 U.S.A.

For International Customers:
To best serve our international customers, it is Millipore's policy to sell our products only through a network of distributors. To place an order or to obtain additional information about Millipore products, please contact your local distributor.

B. Conditions of Sale
All products are for research or manufacturing use only. They are not intended for use in clinical diagnosis or for administration to humans or animals. All products are intended for *in vitro* use only.

C. Material Safety Data Sheets (MSDS)
Material safety data sheets for Millipore products may be ordered by fax or phone. See section A above for details on ordering.

XV. REFERENCES

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5. Thorell, J.I. *Scand. J. Clin. Lab. Invest*. 31:187, 1973.