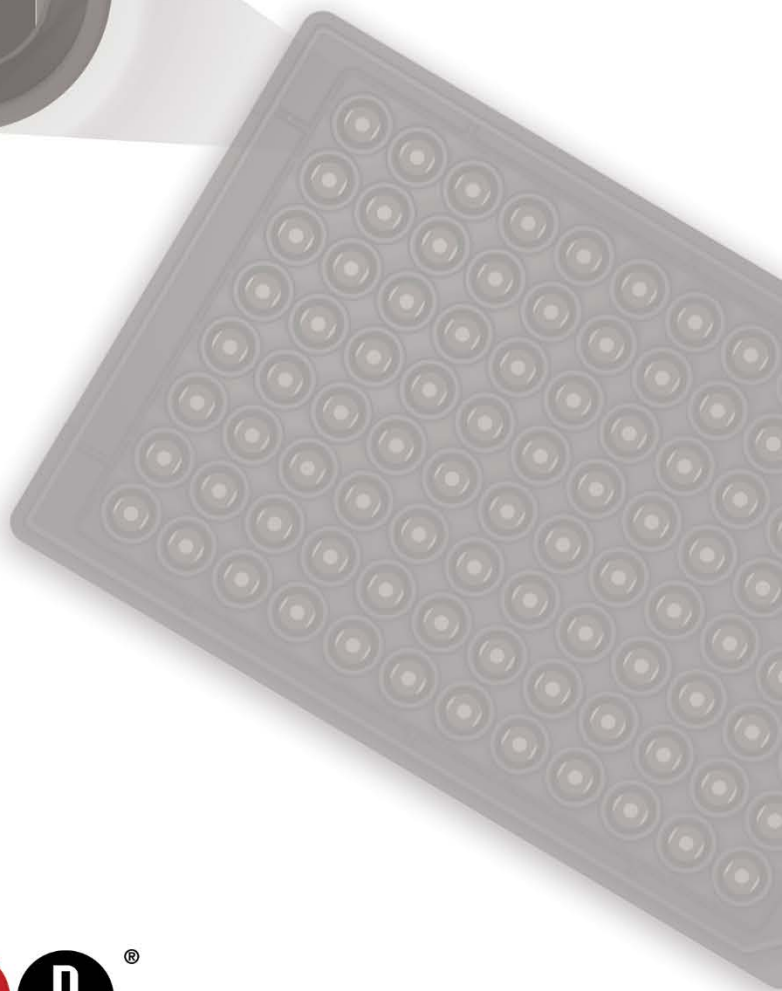


MSD[®] MULTI-ARRAY Assay System

GLP-1 Active Kit

V-PLEX[®]

SECTOR[™] Assay Kits
QuickPlex Ultra[™] Assay Kits



MSD Metabolic Assays

GLP-1 Active Kit

For use with human, NHP, mouse, rat, and canine plasma and cell culture supernatants.

This package insert must be read in its entirety before using this product.

FOR RESEARCH USE ONLY.

NOT FOR USE IN DIAGNOSTIC PROCEDURES.

Meso Scale Discovery

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Introduction

MSD offers V-PLEX® assays for customers who require unsurpassed performance and quality. V-PLEX products are developed under rigorous design control and are analytically validated according to fit-for-purpose principles in accordance with MSD's Quality Management System. They offer exceptional sensitivity, simple protocols, reproducible results, and lot-to-lot consistency. In addition to the analytical validation, robustness of the assay protocol is assessed during development along with the stability of the assay components and kits. V-PLEX assays are available in both single-assay and multiplex formats.

The V-PLEX assay menu is organized by panels. Grouping the assays into panels by species, analytical compatibility, clinical range, and expected use, ensures optimal and consistent performance from each assay while still providing the benefits and efficiencies of multiplexing. V-PLEX panels are provided in MSD's MULTI-SPOT® 96-well plate format. The composition of each panel and the location of each assay (i.e., its spot within the well) are maintained from lot to lot. Most individual V-PLEX assays are provided on MSD's single-spot, 96-well plates.

Glucagon like peptide-1 (GLP-1) is a peptide hormone that belongs to the incretin (enhances the meal-stimulated release of insulin) family. It is synthesized by the enteroendocrine L-cells in the gut (ileum / colon) and neurons of the brain stem / hypothalamus. GLP-1 is formed by the post-translational processing of proglucagon (160 amino acids) by prohormone convertase 1/3 (PC-1/3). The circulating bioactive forms GLP-1 (7–36) amide (MW – 3.29 kD) and GLP-1 (7–37) (MW – 3.35 kD) are further generated from GLP-1(1–37). The primary amino acid sequence of GLP-1 is conserved among the mammalian species, i.e. human, mouse, rat, monkey, canine, etc.¹ Inter-species differences with respect to the dominant molecular forms of GLP-1 have been documented.^{5,9} In humans and mice, the majority of the active circulating form is the 7-36 amide.²

Both of these active forms of GLP-1 are rapidly degraded by dipeptidyl peptidase-4 (DPP-IV) to their corresponding inactive forms GLP-1 (9–36) amide and GLP-1 (9–37) following their release from the intestine (half-life 1-5 minutes).¹⁻⁵ This makes it critical that blood samples are collected in the presence of a DPP-IV inhibitor as well as broad spectrum protease inhibitors such as aprotinin when evaluating the active forms of GLP-1.⁶⁻⁸ More recently, neutral endopeptidase NEP 24.11 (also known as neprilysin, CD10, and CALA antigen) has been identified to be involved in GLP-1 degradation. The major degradation product derived from NEP 24.11 activity is a nonapeptide GLP-1 (28–36) amide. NEP 24.11 is predominantly a membrane-associated enzyme that is expressed in the central nervous system, hepatocytes, bile canaliculi, and other organs^{10,11}.

Actions of GLP-1 are mediated by a specific receptor (GLP-1R) which belongs to the G protein coupled receptor (GPCR) family.¹⁻² GLP-1R is expressed in the pancreas, stomach, GI tract, brain, heart, kidney, and lung. However, the expression of the GLP-1R in other tissues such as liver, muscle, and fat cells is controversial.¹²

GLP-1 plays an important role in glucose homeostasis via several mechanisms. Plasma concentration of active GLP-1 rises quickly following ingestion of food (carbohydrates, fat, and protein all stimulate GLP-1 secretion). In the pancreas, GLP-1 stimulates the secretion of insulin by β cells and suppresses secretion of glucagon by α cells in a glucose-dependent manner. In addition to the "incretin effect," it protects β -cell function, i.e. it stimulates proliferation and inhibits apoptosis of pancreatic β cells.¹⁻³ Actions of GLP-1 also lead to delayed gastric and small bowel emptying, resulting in delayed nutrient absorption. Sustained GLP-1R signaling reduces appetite, leading to reduced food intake and weight loss.¹⁻³

GLP-1 is also produced in the CNS, and peripheral GLP-1 has been shown to cross the blood brain barrier. In the brain, GLP-1R is expressed in regions that control glucose homeostasis, gut motility, food intake, and cardiovascular function. It has been reported that GLP-1R signaling exerts neuroprotective and neurotrophic effects, with possibilities for the treatment of neurodegenerative diseases such as Alzheimer's disease.³

Protective effects of GLP-1R agonists on the myocardium and the vascular endothelium have been demonstrated. Recent studies indicate that inflammatory stimuli such as the presence of LPS increase GLP-1 secretion and GLP-1, in turn, modulates inflammation in multiple sites, including the heart and blood vessels. Preliminary results of several cardiovascular studies suggest that some GLP-1R agonists significantly reduce the risk of major cardiovascular complications.¹³⁻¹⁴ Benefits of GLP-1 therapy in rodent models of diabetic nephropathy and acute kidney injury have been demonstrated. Protection is thought to be mediated by inhibition of renal inflammation and oxidative stress.¹⁵ The therapeutic potential of the GLP-1 pathway is thus not limited to type 2 diabetes mellitus (T2DM) and is now being explored in neurodegenerative, cardiovascular, and renal diseases.^{13,15,16-18}

Although the inactive GLP-1 (9–36) amide form exhibits very weak incretin effect through the GLP-1R, it suppresses hepatic glucose production, exerts cardioprotective actions, and reduces oxidative stress in vascular tissues. These insulin-like effects of GLP-1 (9–36) amide suggest that it has a role in the modulation of mitochondrial functions by mechanisms independent of the GLP-1 receptor. A dual receptor hypothesis for GLP-1 has thus been proposed. Following removal of the two amino acids by DPP-IV, the GLP-1 (9–36) amide is translocated to the cytoplasm via a receptor where it is further degraded by endopeptidases into smaller peptides of 5–7 amino acids. These smaller peptides then modulate metabolic processes (oxidative phosphorylation involving fatty acid and glucose metabolism, energy expenditure) and apoptotic pathways within the mitochondria^{10,11}.

Principle of the Assay

MESO SCALE DISCOVERY® metabolic assays provide a rapid and convenient method for measuring the levels of protein targets within a single, small-volume sample. The V-PLEX GLP-1 Active assay is a single analyte sandwich immunoassay. MSD provides a Small Spot plate pre-coated with the capture antibody (Figure 1). The user sequentially adds the calibrator / sample and a solution containing the detection antibody conjugated with electrochemiluminescent (ECL) labels (MSD SULFO-TAG™) over the course of two incubation periods separated by wash steps. The active form of GLP-1, (7–36) amide, in the calibrator or sample binds to the capture antibody immobilized on the working electrode surface and the detection antibody completes the sandwich. The user then adds the MSD read buffer that creates the appropriate chemical environment for the ECL reaction and loads the plate into an MSD instrument where a voltage applied to the plate electrodes causes the captured labels to emit light. The instrument measures the intensity of emitted light to provide a quantitative measure of analytes in the sample. V-PLEX assay kits have been validated according to the principles outlined in “Fit-for-Purpose Method Development and Validation for Successful Biomarker Measurement” by J. W. Lee, et al.¹⁹

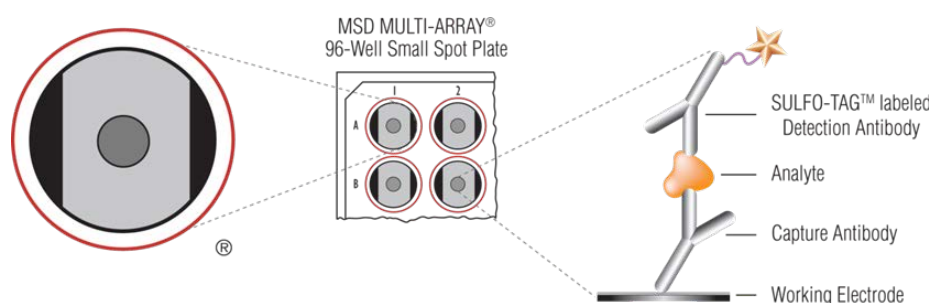


Figure 1. Small Spot plate diagram showing placement of analyte capture antibody.



Figure 2. Schematic of the antibody recognition sites for the V-PLEX GLP-1 Active assay on GLP-1 protein amino acids.

Materials and Equipment

Kit Components

V-PLEX GLP-1 Active assay is available as a Small Spot singleplex kit. V-PLEX Plus kits include additional items (controls, wash buffer, and plate seals). See below for details.

Reagents Supplied With All Kits

Table 1. Reagents that are supplied with V-PLEX and V-PLEX Plus Kits

Reagent	Storage	Catalog #	Size	Quantity Supplied			Description
				1-Plate Kit	5-Plate Kit	25-Plate Kit	
GLP-1 (active) 96-well Small Spot SECTOR Plate	2–8 °C	L45030A-1	1-spot	1	5	25	96-well plate, foil sealed, with desiccant.
GLP-1 (active) 96-well QuickPlex Ultra Plate	2–8 °C	L4B030A-1	1-spot	1	5	25	
GLP-1 (active) Antibody (50X)	2–8 °C	D2030-2	75 µL	1 vial	-	-	SULFO-TAG conjugated antibody
		D2030-3	375 µL	-	1 vial	5 vials	
GLP-1 (active) Calibrator	2–8 °C	C0266-1	1 vial	1 vial	5 vials	25 vials	Lyophilized synthetic GLP-1 (7–36) amide peptide. GLP-1 peptide concentration is provided in the lot-specific certificate of analysis.
Diluent 13	≤-10 °C	R56BB-4	10 mL	1 bottle	-	-	Diluent for samples and calibrator
		R56BB-3	50 mL	-	1 bottle	5 bottles	
Diluent 12	≤-10 °C	R50JA-4	10 mL	1 bottle	-	-	Diluent for detection antibody
		R50JA-3	50 mL	-	1 bottle	5 bottles	
Aprotinin, 200,000 KIU/mL (200X)	2–8 °C	R93AD-1	50 µL	1 bottle	-	-	200X concentrated solution
		R93AD-2	250 µL	-	1 bottle	5 bottles	
MSD GOLD™ Read Buffer A*	RT	R92TG-3	18 mL	1 bottle	-	-	Buffer to catalyze the electro-chemiluminescence reaction on SECTOR plates.
		R92TG-4	90 mL	-	1 bottle	5 bottles	
MSD GOLD Read Buffer B	RT	R60AM-1	18 mL	1 bottle	-	-	Buffer to catalyze the electro-chemiluminescence reaction on QuickPlex Ultra plates.
		R60AM-2	90 mL	-	1 bottle	5 bottles	

* Previously named as MSD GOLD Read Buffer

V-PLEX Plus Kits: Additional Components

Table 2. Additional components that are supplied with V-PLEX Plus Kits

Reagents	Storage	Catalog #	Size	Quantity Supplied			Description
				1-Plate Kit	5-Plate Kit	25-Plate Kit	
GLP-1 (active) Control 1*	2–8 °C	C4266-1	1 vial	1 vial	5 vials	25 vials	Controls lyophilized in a non-human matrix, spiked with amidated GLP-1 (7–36) peptide. The concentrations of the controls are provided in the lot-specific certificate of analysis.
GLP-1 (active) Control 2*	2–8 °C	C4266-1	1 vial	1 vial	5 vials	25 vials	
GLP-1 (active) Control 3*	2–8 °C	C4266-1	1 vial	1 vial	5 vials	25 vials	
Wash Buffer (20X)	RT	R61AA-1	100 mL	1 bottle	1 bottle	5 bottles	20-fold concentrated phosphate buffered solution with surfactant.
Plate Seals	-	-	-	3	15	75	Adhesive seals for sealing plates during incubations.

*Provided as components in GLP-1 (active) Control Pack (catalog # C4266-1)

Additional Materials and Equipment

- ☐ Appropriately sized tubes for reagent preparation
- ☐ Polypropylene microcentrifuge tubes for preparing dilutions
- ☐ Liquid handling equipment for desired throughput, capable of dispensing 10 to 150 μL /well into a 96-well microtiter plate
- ☐ Plate washing equipment: automated plate washer or multichannel pipette
- ☐ Microtiter plate shaker (rotary) capable of shaking at 500–1,000 rpm
- ☐ MSD Wash Buffer catalog # R61AA-1 (included in V-PLEX Plus kits) or Phosphate-buffered saline (PBS) plus 0.05% Tween-20 for plate washing
- ☐ Adhesive plate seals (3 per plate included in V-PLEX Plus kits)
- ☐ Deionized water
- ☐ DPP-IV Inhibitor – Not provided. Formulate and store as recommended by the manufacturer.
- ☐ Vortex mixer

Optional Materials and Equipment

- ☐ GLP-1 (active) Control Pack, available for separate purchase from MSD, catalog # C4266-1 (included in V-PLEX Plus kits)
- ☐ Centrifuge (for sample preparation)
- ☐ Blocker A Kit (R93AA-2; 250 mL)

Safety

Use safe laboratory practices and wear gloves, safety glasses, and lab coats when handling kit components. Handle and dispose of all hazardous samples properly in accordance with local, state, and federal guidelines.

Additional product-specific safety information is available in the safety data sheet (SDS), which can be obtained from MSD Customer Service or at www.mesoscale.com.

Protocol

Best Practices

- Mixing or substituting reagents from different sources or different kit lots is not recommended. Lot information is provided in the lot-specific COA.
- Assay incubation steps should be performed between 20-26 °C to achieve the most consistent signals between runs.
- Bring frozen diluent to room temperature in a 24 °C water bath. Thaw other reagents on wet ice and use as directed without delay.
- Prepare calibrators, samples, and controls in polypropylene microcentrifuge tubes; use a fresh pipette tip for each dilution; vortex after each dilution before proceeding.
- Avoid prolonged exposure of detection antibody (stock or diluted) to light. During the antibody incubation step, plates do not need to be shielded from light except for direct sunlight.
- Avoid bubbles in wells at all pipetting steps. Bubbles may lead to variable results; bubbles introduced when adding read buffer may interfere with signal detection.
- Do not touch the pipette tip on the bottom of the wells when pipetting into the MSD plate.
- Use reverse pipetting when necessary to avoid introduction of bubbles. For empty wells, pipette to the bottom corner.
- Plate shaking should be vigorous, with a rotary motion between 500 and 1,000 rpm. Binding reactions may reach equilibrium sooner if you use shaking at the middle of this range (~700 rpm) or above.
- When using an automated plate washer, rotate the plate 180 degrees between wash steps to improve assay precision.
- Gently tap the plate on a paper towel to remove residual fluid after washing.
- Read buffer should be at room temperature when added to the plate.
- Keep time intervals consistent between adding read buffer and reading the plate to improve inter-plate precision. Unless otherwise directed, read plate as soon as practical after adding read buffer.
- Do not shake the plate after adding read buffer.
- If an incubation step needs to be extended, avoid letting the plate dry out by keeping sample or detection antibody solution in the plate.
- Remove the plate seals prior to reading the plate.
- If assay results are above the top of the calibration curve, dilute the samples and repeat the assay.
- When running a partial plate, seal the unused sectors (see sector map in instrument and software manuals) to avoid contaminating unused wells. Remove all seals before reading. Partially used plates may be sealed and stored up to 30 days at 2–8 °C in the original foil pouch with desiccant. You may adjust volumes proportionally when preparing reagents.

Reagent Preparation

Bring all reagents to room temperature.

Important: If required, upon first thaw, aliquot Diluent 13 and Diluent 12 into suitable sizes before refreezing.

Prepare Metabolic Assay Working Solution

This is used for preparing the calibrator, controls, and diluting the samples.

For one plate, combine the following in a 15-mL tube:

- ☐ 6,965 μ L of Diluent 13
- ☐ 35 μ L of Aprotinin

Note: Addition of a final concentration of 0.1 mM DPP-IV inhibitor (not provided in kit) in the Metabolic Assay Working Solution is strongly recommended. DPP-IV inhibitor aids in limiting enzymatic action of DPP-IV present in serum/plasma and provides the most accurate measurement of active GLP-1. If DPP-IV inhibitor is added, adjust the volume of Diluent 13 accordingly.

Important: Aprotinin and DPP-IV inhibitor should be added prior to use. The Metabolic Assay Working Solution should be kept on ice. Do not freeze the Metabolic Assay Working Solution for later use.

Prepare Calibrator Dilutions

MSD supplies a lyophilized calibrator that yields the recommended highest calibrator concentration when reconstituted in 1,000 μ L of Metabolic Assay Working Solution. Keep reconstituted calibrator and calibrator solutions on wet ice until use.

To prepare 7 calibrator solutions plus a zero calibrator for up to 4 replicates:

- 1) Prepare the highest calibrator (Calibrator 1) by adding 1,000 μ L of Metabolic Assay Working Solution to the lyophilized calibrator vial. After reconstituting, invert at least 3 times (do not vortex). Let the reconstituted solution equilibrate at room temperature for 15-30 minutes and then vortex briefly using short pulses.
- 2) Prepare the next calibrator by transferring 100 μ L of the highest calibrator to 300 μ L of Metabolic Assay Working Solution. Mix well by vortexing. Repeat 4-fold serial dilutions 5 additional times to generate 7 calibrators.
- 3) Use Metabolic Assay Working Solution as the zero calibrator.

Calibrator	GLP-1 Active concentration		Dilution Factor
	pM	pg/mL	
1	243	801	NA
2	60.8	200	4
3	15.2	50.1	4
4	3.80	12.5	4
5	0.949	3.13	4
6	0.237	0.783	4
7	0.059	0.196	4
8	0	0	NA

Note: Reconstituted calibrator is not stable when stored at 2–8 °C; however, it may be stored frozen at \leq -70 °C and is stable through three freeze–thaw cycles. For the lot-specific concentration of each calibrator in the blend, refer to the COA supplied with the kit. You can also find a copy of the COA at www.mesoscale.com.

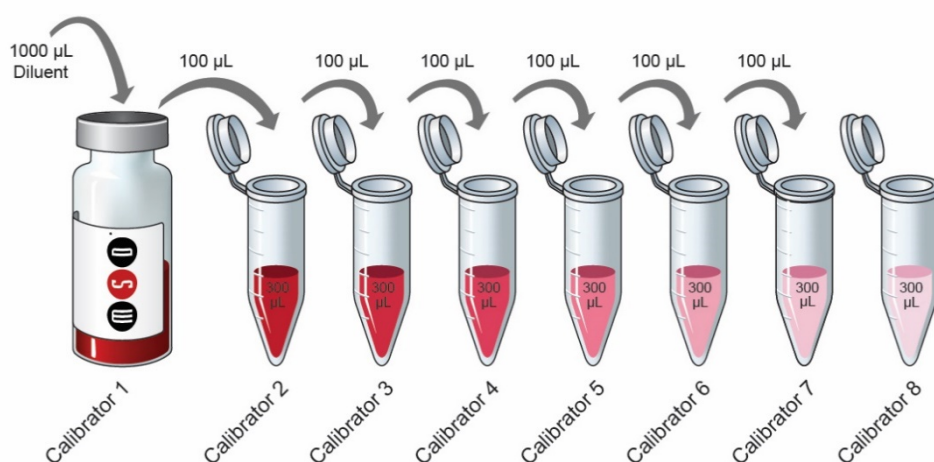


Figure 3. Dilution schema for preparation of Calibrator Standards.

Sample Collection and Handling

Below are general guidelines for human, NHP, mouse, rat, and canine sample collection, storage, and handling for metabolic markers including the active forms of GLP-1. If other methods are used, evaluate sample stability under the selected method as needed.

The assay requires 25 µL/well of the sample. An adequate volume of each sample should be prepared depending upon desired number of replicates.

Human, NHP, Rat, and Canine: Samples should be collected using BD P800 Blood Collection and Preservation System which contains DPP-IV and other protease inhibitor cocktails (Product Number 366420 or 366421). Alternative collection method described below with K2EDTA tubes can also be used.

Mouse: K2EDTA collection tubes are recommended, but additional inhibitors should be added at the time of collection (see below).

Non-P800 collection method: Collect blood in BD Vacutainer EDTA Tubes (Product Number 367841 or 366643). **Immediately** add DPP-IV inhibitor (0.1 mM final concentration, not provided with the kit) and aprotinin (1,000 KIU/mL final concentration) and mix to avoid cleavage/degradation of GLP-1.

For BD tubes, process as follows²⁰;

- In a swing out rotor centrifuge, spin the blood collection tubes as follows;
 - For 2 mL tubes - 10 minutes at 1,000 x g (4–8 °C).
 - For 8.5 and 10 mL tubes - 20 minutes at 1,300 x g (4–8 °C).
- Use the plasma immediately or the samples can be stored at 2–8 °C if used within 3 hours. For future use, aliquot the plasma and freeze in suitably-sized aliquots at ≤-70 °C.

For samples other than plasma: **Immediately** add DPP-IV inhibitor (0.1 mM final concentration, not provided with the kit) and aprotinin (1,000 KIU/mL final concentration) and use immediately or freeze at ≤-70 °C.

Samples with hemolysis or significant lipemia may hinder accurate measurements.

Repeated freezing and thawing of samples is not recommended. After thawing, centrifuge samples at 2,000g for 3 minutes to remove particulates prior to using in the assay. If the samples are clear and no particulates are visible, you may not need to centrifuge. Hold on wet ice or 4–8 °C until processed and used in the assay.

Dilute Samples

Dilute samples with Metabolic Assay Working Solution. For plasma from human and all other species MSD recommends a minimum 2-fold dilution. For example, when running samples in duplicate, add 75 µL of sample to 75 µL of Metabolic Assay Working Solution. We recommend running at least two replicates per sample. You may conserve sample volume by using a higher dilution when possible. Tissue culture supernatants may require additional dilution based on stimulation and analyte concentrations in the sample. Additional diluent can be purchased at www.mesoscale.com.

Prepare Controls

Three levels of single-analyte lyophilized controls are available for separate purchase from MSD in the GLP-1 (active) Control Pack, catalog # C4266-1. (Controls are included only in V-PLEX Plus kits.)

Reconstitute the lyophilized controls in 250 µL of Metabolic Assay Working Solution. Do not invert or vortex the vials. Wait for a minimum of 15-30 minutes at room temperature before diluting controls 2-fold in Metabolic Assay Working Solution. Vortex briefly using short pulses. Reconstituted controls may be stored frozen at ≤-70 °C and are stable through three freeze-thaw cycles. For the lot-specific concentration of each analyte in the control pack, refer to the supplied COA. You can also find a copy of the COA at www.mesoscale.com.

Prepare Detection Antibody Solution

MSD provides the detection antibody as a 50X stock solution. The working solution is 1X. Prepare the detection antibody solution immediately prior to use.

For one plate, add 60 µL of the supplied detection antibody to 2,940 µL of Diluent 12.

Prepare Wash Buffer

MSD provides 100 mL of Wash Buffer as a 20X stock solution in the V-PLEX Plus kit. Dilute the stock solution to 1X before use. PBS + 0.05% Tween-20 can be used instead.

For one plate, combine:

- ☐ 15 mL of MSD Wash Buffer (20X)
- ☐ 285 mL of deionized water

1X MSD Wash Buffer can be stored at room temperature for up to two weeks.

MSD GOLD Read Buffer A: SECTOR Plates Only

MSD provides MSD GOLD Read Buffer A ready for use. Do not dilute.

Important: Unlike Read Buffer T, which is provided at a 4X concentration, MSD GOLD Read Buffer A is provided at the working concentration of the assay. Dilution of MSD GOLD Read Buffer A may compromise the results of this assay.

MSD GOLD Read Buffer B: QuickPlex Ultra Plates Only

MSD provides MSD GOLD Read Buffer B ready for use. Do not dilute.

Note: Unlike Read Buffer T, which is provided at 4X concentration, MSD GOLD Read Buffer B is provided at the working concentration for the assay. Diluting MSD GOLD Read Buffer B may compromise assay results.

Prepare MSD Plate

MSD V-PLEX plates are pre-coated with capture antibodies (Figure 1) and exposed to a proprietary stabilizing treatment to ensure the integrity and stability of the immobilized antibodies. Pre-wash plates before use as recommended on the assay protocol.

Assay Protocol

Note: Follow **Reagent Preparation** before beginning this assay protocol.

STEP 1: Wash and Add Sample

- ☐ Wash the plate 3 times with 150 μ L/well of 1X MSD Wash Buffer.
- ☐ Prepare the Metabolic Assay Working Solution
- ☐ Add 50 μ L of prepared samples, calibrators or controls per well. Seal the plate with an adhesive plate seal and incubate at room temperature with shaking for 2 hours.

Note: Washing the plate prior to sample addition is an optional step that may provide greater uniformity of results for certain assays. Analytical parameters, including limits of quantification, recovery of controls, and sample quantification, are not affected by washing the plate prior to sample addition.

Blocking step: A pre-assay, blocking step may be incorporated if consistency with older protocols is required. Prepare Blocker A according to the instructions provided in the Blocker A Kit. Dispense 150 μ L of Blocker A solution into each well. Seal the plate with an adhesive plate seal and incubate for 1 hour with shaking at room temperature, then proceed to step 1 above.

STEP 2: Wash and Add Detection Antibody Solution

- ☐ Wash the plate 3 times with at least 150 μ L/well of 1X MSD Wash Buffer.
- ☐ Add 25 μ L of detection antibody solution to each well. Seal the plate with an adhesive plate seal and incubate at room temperature with shaking for 2 hours.

STEP 3: Wash and Read

- ☐ Wash the plate 3 times with at least 150 μ L/well of 1X MSD Wash Buffer.
- ☐ Add 150 μ L of read buffer to each well. Analyze the plate on an MSD instrument. Incubation in read buffer is not required before reading the plate.

Alternate Protocols

The suggestions below may be useful as alternate protocols; however, not all were tested using multiple kit lots.

- **Alternate Protocol 1, Extended Sample Incubation:** Incubating samples overnight at 2–8 °C may improve sensitivity for some assays. See **Appendix A**.
- **Alternate Protocol 2, Reduced Wash:** You may simplify the protocol by eliminating one of the wash steps. After incubating diluted sample, calibrator, or control, add detection antibody solution to the plate without decanting or washing the plate. See **Appendix A** for assay performance using this protocol.
- **Alternate Protocol 3, Dilute-in-Plate:** To limit sample handling, you may dilute samples and controls in the plate. For 2-fold dilution, add 25 μ L of assay diluent to each sample/control well, and then add 25 μ L of neat control or sample. Calibrators should not be diluted in the plate; add 50 μ L of each calibrator directly into empty wells. Tests conducted according to this alternate protocol produced results that were similar to the recommended protocol (data not shown).

Assay Characteristics

Validation

MSD's V-PLEX products are analytically validated following fit-for-purpose principles¹⁸ and MSD design control procedures. V-PLEX assay components go through an extensive critical reagents program to ensure that the reagents are controlled and well characterized. Prior to the release of each V-PLEX panel, at least three independent kit lots are produced. Using results from multiple runs (typically greater than 50) and multiple operators, these lots are used to establish production specifications for sensitivity, specificity, accuracy, and precision. The COA provided with each kit outlines the kit release specifications for sensitivity, specificity, accuracy, and precision.

➤ **Dynamic Range**

Calibration curve concentrations for the assay are optimized for a maximum dynamic range while maintaining enough calibration points near the bottom of the curve to ensure a proper fit for accurate quantification of samples that require high sensitivity.

➤ **Sensitivity**

The lower limit of detection (LLOD) is a calculated concentration corresponding to the average signal 2.5 standard deviations above the background (zero calibrator). The LLOD is calculated using results from multiple plates for each lot, and the median and range of calculated LLODs for a representative kit lot are reported in this product insert. The upper limit of quantification (ULOQ) and lower limit of quantification (LLOQ) are established for each lot by measuring multiple levels near the expected LLOQ and ULOQ levels. The final LLOQ and ULOQ specifications for the product are established after assessment of all validation lots.

➤ **Accuracy and Precision**

Accuracy and precision are evaluated by measuring calibrators and matrix-based validation samples or controls across multiple runs and multiple lots. For most assays, the results of control measurements fall within 20% of the expected concentration for each run. Precision is reported as the coefficient of variation (CV). Intra-run CVs are typically below 7%, and inter-run CVs are typically below 25%. Rigorous management of inter-lot reagent consistency and calibrator production results in typical inter-lot CVs below 10%. Validation lots are compared using controls and at least 30 samples in various sample matrices. Samples are well correlated with an inter-lot bias typically below 10%.

➤ **Matrix Effects and Samples**

Matrix effects from plasma, and cell culture media are measured as part of development and validation. Dilution linearity and spike recovery studies are performed on individual samples to assess variability of results due to matrix effects. The sample dilution suggested in the protocol gives an appropriate dilution factor for all assays in the multiplex. In addition to the matrices listed above, peripheral blood mononuclear cells (PBMCs) and/or cell lines that have been stimulated to generate elevated levels of analytes are tested.

➤ **Specificity**

The specificity of both capture and detection antibodies is measured during assay development. For the GLP-1 (active) antibody, specificity was assessed by testing closely related forms of GLP-1, products of the proglucagon protein, and other related cytokines.

➤ **Assay Robustness and Stability**

The robustness of the assay protocol is assessed by examining the boundaries of the selected incubation times and evaluating the stability of assay components during the experiment and the stability of reconstituted lyophilized components during storage. Assay component (calibrator, antibody, control) stability was assessed through freeze–thaw testing and accelerated stability studies. The validation program includes a real-time stability study with scheduled performance evaluations of complete kits for up to 54 months from date of manufacture.

Representative data from the verification and validation studies are presented in the following sections. The calibration curve and measured limits of detection for each lot can be found in the lot-specific COA that is included with each kit and available for download at www.mesoscale.com.

Analysis of Results

The calibration curves used to calculate analyte concentrations were established by fitting the signals from the calibrators to a 4-parameter logistic (or sigmoidal dose-response) model with a $1/Y^2$ weighting. The weighting function provides a better fit of data over a wide dynamic range, particularly at the low end of the calibration curve. Analyte concentrations were determined from the ECL signals by back-fitting to the calibration curve. These assays have a wide dynamic range (>3 logs), which allows accurate quantification of samples without the need for multiple dilutions or repeated testing. The calculations to establish calibration curves and determine concentrations were carried out using MSD's DISCOVERY WORKBENCH® and Methodical Mind® analysis software. Best quantification of unknown samples will be achieved by generating a calibration curve for each plate using a minimum of two replicates at each calibrator level.

Typical Data

Data from the V-PLEX GLP-1 Active Kit were collected over one week of testing by five operators (20 runs, 60 plates in total). Calibration curve accuracy and precision were assessed for two kit lots. Representative data from one lot are presented below. Appendix A compares data from alternate protocols 1, 2, and 3 to the reference protocol.

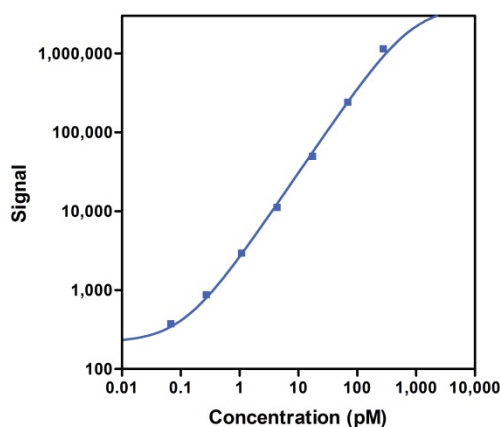


Figure 4. Typical calibration curve for the V-PLEX GLP-1 Active Kit.

Sensitivity

The LLOD is a calculated concentration corresponding to the signal 2.5 standard deviations above the background (zero calibrator). The LLOD shown below was calculated based on three runs from three kit lots.

The ULOQ is the highest concentration at which the CV of the calculated concentration is <20% and the recovery of each analyte is within 80% to 120% of the known value.

The LLOQ is the lowest concentration at which the CV of the calculated concentration is <20% and the recovery of each analyte is within 80% to 120% of the known value.

The quantitative range of the assay lies between the LLOQ and ULOQ.

The LLOQ and ULOQ are verified for each kit lot and the results are provided in the lot-specific COA that is included with each kit and available at www.mesoscale.com.

Table 3. LLOD, LLOQ, and ULOQ for the V-PLEX GLP-1 Active Kit

	Median LLOD (pM)	LLOD Range (pM)	LLOQ (pM)	ULOQ (pM)
GLP-1 (active)	0.020	0.010–0.020	0.300	120

Precision

Controls were made by spiking calibrator into non-human matrix at three levels within the quantitative range of the assay. Analyte levels were measured by five operators using a minimum of three replicates on 60 plates (12 plates per operator) over five days. Results are shown below. While a typical specification for precision is a concentration CV of less than 20% for controls on both intra- and inter-day runs, for this panel, the data shows most assays are below 10%.

Average intra-run %CV is the average %CV of the control replicates within an individual run across 60 runs (four kit lots).

Inter-run %CV is the variability of controls across 15 runs within a single kit lot.

Inter-lot %CV is the variability of controls across four kit lots (total of 60 runs, 15 runs per kit lot).

Table 4. Intra-run and inter-run %CVs for the V-PLEX GLP-1 Active Kit

	Control	Average Conc. (pM)	Average Intra-run %CV	Inter-run %CV	Inter-lot %CV
GLP-1 (active)	Control 1	126	2.7	4.7	2.8
	Control 2	30.5	3.1	4.9	4.0
	Control 3	3.35	2.8	11.8	2.5

Dilution Linearity

To assess linearity, normal human plasma (collected in P800 tubes) from a commercial source, as well as cell culture media, were spiked with GLP-1 (active) Calibrator and diluted 2-fold, 4-fold, 8-fold, and 16-fold, before testing. Percent recovery at each dilution level was normalized to the 2-fold dilution-adjusted concentration. The average percent recovery is based on samples within the quantitative range of the assay.

$$\% \text{ Recovery} = \frac{\text{measured concentration}}{\text{expected concentration}} * 100$$

Table 5. GLP-1 (7-36) amide percent recovery at various dilutions in P800 EDTA plasma and cell culture media samples. Data from human, mouse, rat, canine, NHP are shown in the following tables. Mouse samples were tested using EDTA plasma spiked with aprotinin and DPP-IV inhibitor (P800 tubes were not used for mouse samples).

Sample Type	Fold Dilution	Average % Recovery	% Recovery Range
Human P800 EDTA plasma (N=18)	4	90	79–99
	8	94	85–101
	16	95	87–100
NHP P800 EDTA plasma (N = 10)	4	101	91–110
	8	101	90–113
	16	102	83–118
Mouse Citrate plasma (N = 10)	4	104	95–114
	8	104	90–115
	16	104	88–122
Mouse K2 EDTA plasma (N = 10)	4	98	91–105
	8	100	90–107
	16	100	91–111
Rat P800 EDTA plasma (N = 10)	4	100	77–113
	8	95	70–115
	16	89	75–107

Sample Type	Fold Dilution	Average % Recovery	% Recovery Range
Canine P800 EDTA plasma (N = 10)	4	95	89–100
	8	95	86–101
	16	95	89–107
Krebs Ringer	4	99	NA
	8	100	NA
	16	85	NA
RPMI (10% FBS, 1% Pen-Strep, 2mM Glu)	4	92	NA
	8	90	NA
	16	92	NA
DMEM (10% FBS, 1% Pen-Strep)	4	86	NA
	8	85	NA
	16	85	NA
DMEM (2.5% FBS, 15% HS)	4	97	NA
	8	100	NA
	16	93	NA

Spike Recovery

Spike recovery measurements were evaluated for different sample types throughout the quantitative range of the assays. Multiple individual human and pooled mouse, rat, canine, and non-human primate samples) were obtained from a commercial source. These samples, along with cell culture media, were spiked with GLP-1 (active) Calibrator at three levels (high, mid, and low) then diluted 2-fold. The average % recovery for each sample type is reported along with %CV and % recovery range.

$$\% \text{ Recovery} = \frac{\text{measured concentration}}{\text{expected concentration}} * 100$$

Table 6. Average percent recovery at various GLP-1 (active) levels in P800 EDTA plasma (human, rat, canine, NHP), and cell culture media samples. Mouse samples were tested using EDTA plasma spiked with aprotinin and DPP-IV inhibitor (P800 tubes were not used for mouse samples).

Human P800 Plasma (N=18)			NHP P800 Plasma (N=10)			Mouse Citrate Plasma (N=10)		
Average % Recovery	%CV	% Recovery Range	Average % Recovery	%CV	% Recovery Range	Average % Recovery	%CV	% Recovery Range
90	10.0	78–106	94	5.1	84–102	76	9.9	66–87

Mouse K2 EDTA Plasma (N=10)			Rat P800 Plasma (N=10)			Canine K2 EDTA Plasma (N=10)		
Average % Recovery	%CV	% Recovery Range	Average % Recovery	%CV	% Recovery Range	Average % Recovery	%CV	% Recovery Range
78	3.8	73–83	97	4.3	91–103	94	5.1	84–102

Krebs-Ringer			RPMI (10% FBS, 1% Pen-Strep, 2mM Glu)			DMEM (10% FBS, 1% Pen-Strep)			DMEM (2.5% FBS, 15% HS)		
Average % Recovery	%CV	% Recovery Range	Average % Recovery	%CV	% Recovery Range	Average % Recovery	%CV	% Recovery Range	Average % Recovery	%CV	% Recovery Range
95	NA	NA	97	NA	NA	93	NA	NA	96	NA	NA

Specificity

To assess specificity, the assay was tested for nonspecific binding to the following GLP-1 metabolites and hormones. All analytes were tested at 1,000 pg/mL except for GLP-1 (7–37), which was tested at 10 pg/mL to more accurately represent the levels of the glycine-extended form of active GLP-1 in circulation. Nonspecific binding was less than 0.5% for all assays in the kit.

$$\% \text{ Nonspecificity} = \frac{\text{nonspecific signal}}{\text{specific signal}} * 100$$

Table 7. Assay cross-reactivity to selected GLP-1 metabolites and hormones

	Metabolite/Hormone	Conc (pg/mL)	Signal (counts)	% Cross-reactivity
GLP-1 Receptor Agonists	Liraglutide*	1,000	330	0.03
	Exendin	1,000	121	0.01
Proglucagon Metabolites	Major Proglucagon Fragment (MPGF)	1,000	129	0.01
	Glucagon	1,000	81	0.01
	Glicentin	1,000	117	0.01
	GLP-2	1,000	109	0.01
	Oxyntomodulin	1,000	113	0.01
	Miniglucagon	1,000	158	0.02
General Metabolic Analytes	Insulin	1,000	118	0.01
	Leptin	1,000	116	0.01
	PYY	1,000	146	0.01
	Resistin	1,000	88	0.01
	GIP	1,000	115	0.01
	PPY	1,000	120	0.01
	Active Ghrelin	1,000	117	0.01
	Mouse Leptin	1,000	158	0.02
	Rat Leptin	1,000	165	0.02

***Note:** Liraglutide and GLP-1 have nearly identical sequences and have the same homology at the N-terminus (7-cleavage site), therefore the assay is able to capture both molecules. The detection antibody is not expected to specifically bind to liraglutide, but there may be some weak affinity that leads to elevated apparent levels of active GLP-1 when the concentrations of active GLP-1 are low. When levels of liraglutide are much higher than 50 pM, the active GLP-1 concentrations may be suppressed.

Table 8. Assay reactivity to different forms of GLP-1

	Peptide	Conc (pg/mL)	Signal (counts)	% Cross-reactivity
GLP-1 Forms	GLP-1 (7–37)	10	2,072	38
	GLP-1 (9–36) amide	1,000	142	0.01
	GLP-1 (1–36) amide	1,000	897	0.09

Stability

The reconstituted calibrator, reconstituted controls, and diluents were tested for freeze–thaw stability. Results (not shown) demonstrated that reconstituted calibrator, reconstituted controls, and diluents can go through three freeze–thaw cycles without significantly affecting the performance of the assay. Reconstituted calibrator and controls must be stored frozen at ≤ -70 °C. Partially used MSD plates may be sealed and stored up to 30 days at 2–8 °C in the original foil pouch with desiccant. Results from control measurements changed by $\leq 30\%$ after partially used plates were stored for 30 days. The validation study includes a real-time stability study with scheduled performance evaluations of complete kits for up to 54 months from date of manufacture.

Calibration

There is no GLP-1 reference standard available from NIBSC or other equivalent body. The peptide calibrator concentrations are calibrated against a reference calibrator generated at MSD that was assigned using three independent lots of amino acid-analyzed active GLP-1 peptide to provide a highly accurate measurement of active GLP-1.

Tested Samples

Normal Samples

Commercially available apparently normal human, rat, canine, and NHP P800 collected plasma samples were diluted 2-fold and tested. Results for each sample set are displayed below. Concentrations are corrected for sample dilution. Median and range are calculated from samples with concentrations at or above the LLOD. Percent detected is the percentage of samples with concentrations at or above the LLOD.

Table 9. Normal human P800 EDTA plasma samples, pooled rat, canine, and NHP P800 EDTA plasma samples, and pooled mouse K2 EDTA plasma samples tested in the V-PLEX GLP-1 Active Kit.

Sample Type	Statistic	pg/mL	pM
Human P800 EDTA plasma (N=40)	Median	2.61	0.790
	Range	0.637–40.2	0.019–12.2
	% of Samples Detected	100	
Mouse K2 EDTA plasma (N=10)*	Median	1.53	0.465
	Range	0.969–2.08	0.294–0.632
	% of Samples Detected	100	
NHP P800 EDTA plasma (N=10)	Median	2.36	0.716
	Range	1.79–2.65	0.542–0.803
	% of Samples Detected	100	
Rat P800 EDTA plasma (N=10)	Median	1.28	0.387
	Range	0.778–1.89	0.236–0.572
	% of Samples Detected	100	
Canine P800 EDTA plasma (N=10)	Median	1.15	0.35
	Range	0.340–8.33	0.103–2.53
	% of Samples Detected	100	

*K2EDTA blood collection tubes (10 mL) were treated with 10,000 kIU of aprotinin and 1.0 mmole of diprotin A immediately prior to blood collection.

% Detected = % of samples with concentrations at or above the LLOD

In vitro Stimulated Cells and Post-Meal Plasma Samples

Cell culture supernatants from *in vitro* Stimulated NCI-H716 Cells:

NCI-H716 cells (Human Epithelium (cecum) colorectal adenocarcinoma) were treated with various stimulants, including progesterone (1 μ M), forskolin (10 μ M), insulin (2 μ M), and sucrose (200 mM) for a given period of time. These cell culture supernatants were tested for the presence of active GLP-1 (7–36) amide. Active GLP-1 was expressed at levels higher than the detection limit of the assay, so they were diluted 4 fold prior to the assay. The dilution-adjusted concentrations (pM) for the stimulation conditions are displayed below.

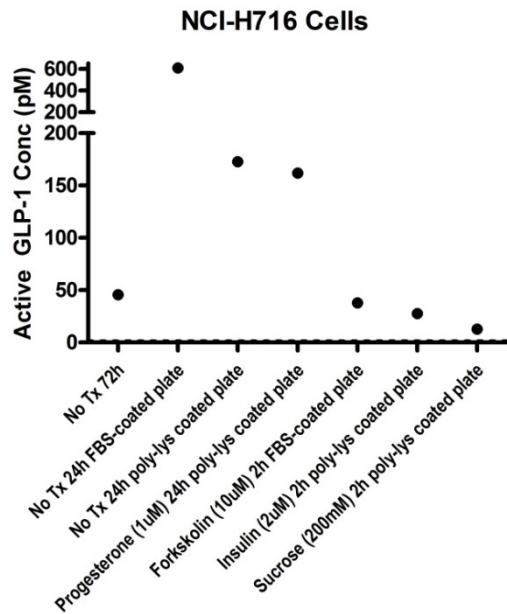


Figure 5. Effect of cell stimulation on active GLP-1 production as measured in the V-PLEX GLP-1 Active Kit.

Post-meal changes in circulating GLP-1:

Post-meal samples from apparently healthy individuals were tested for the active GLP-1 levels. Samples were collected from different individuals at different time points. The dilution-adjusted concentrations (pM) for the samples are displayed below.

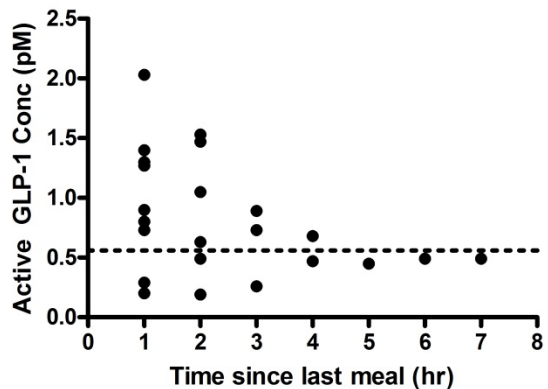


Figure 6. Post-meal levels of active GLP-1 in individuals (black circles), measured using the V-PLEX GLP-1 Active Kit. Dotted line indicates the in-sample LLOQ, which is two times the in-well LLOQ.

Assay Components

Calibrators

The assay calibrator blend uses the following recombinant human proteins:

Table 10. Recombinant human proteins used in the Calibrators

Calibrator	Expression System
GLP-1 (7–36) amide	Synthetic peptide

Antibodies

Table 11. Antibody source species

Analyte	Source Species		Assay Generation
	MSD Capture Antibody	MSD Detection Antibody	
GLP-1 (active)	Mouse Monoclonal	Mouse Monoclonal	A

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Additional Information

Appendix A: Alternate Protocols

Calibration curves below illustrate the relative sensitivity for each assay under **Alternate Protocols**: Reference Protocol (2-hour sample incubation/2 wash steps, blue curve), Alternate Protocol 1 (overnight [O/N] sample incubation, orange curve), Alternate Protocol 2 (single wash, red curve), and Alternate Protocol 3 (in-well dilution, grey curve). Details of the alternate protocols are found on page 14.

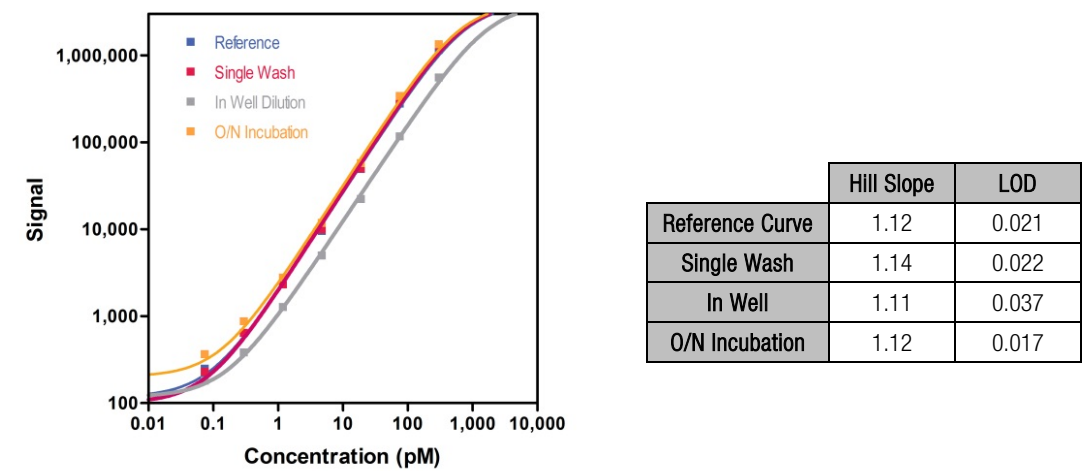


Figure 7. Calibrator curves generated by the indicated protocols. Table shows the hillslope of each curve and the estimated lower limit of detection (LOD) for the assay when performed using each protocol.

Each control (high, mid, and low) was tested under each alternate protocol. The plot below demonstrates for each protocol the recovery of measured concentrations of the controls relative to the expected concentrations, which shows equivalent performance among all the protocols tested.

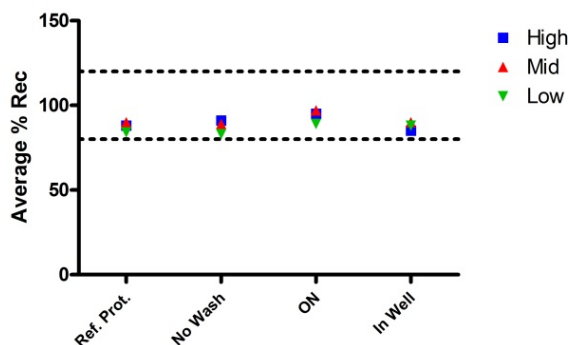


Figure 8. Recovery of measured concentrations of the controls resulting from each protocol relative to the expected concentrations.

Summary Protocol

V-PLEX GLP-1 Active Kit

MSD provides this summary protocol for your convenience.

Please read the entire detailed protocol prior to performing the V-PLEX GLP-1 Active assay.

Sample and Reagent Preparation

- ☐ Bring all reagents to room temperature.
- ☐ Prepare Metabolic Assay Working Solution.
- ☐ Prepare calibration solutions in Metabolic Assay Working Solution using the supplied calibrator:
 - ☐ Reconstitute the lyophilized calibrator blend.
 - ☐ Invert 3 times, equilibrate 15-30 minutes at room temperature.
 - ☐ Vortex briefly using short pulses.
 - ☐ Perform a series of 4-fold dilution steps and prepare a zero calibrator.
- ☐ Dilute the plasma samples and controls 2-fold in Metabolic Assay Working Solution before adding to the plate.
- ☐ Prepare the detection antibody solution by diluting the 50X detection antibody 50-fold in Diluent 12.
- ☐ SECTOR Plates Only – MSD provides MSD GOLD Read Buffer A ready for use. Do not dilute.
- ☐ QuickPlex Ultra Plates Only – MSD provides MSD GOLD Read Buffer B ready for use. Do not dilute.

STEP 1: Wash* and Add Sample

- ☐ Wash the plate 3 times with at least 150 μ L/well of 1X MSD Wash Buffer.
- ☐ Add 50 μ L/well of sample (calibrators, controls, or unknowns).
- ☐ Incubate at room temperature with shaking (700 rpm) for 2 hours.

STEP 2: Wash and Add Detection Antibody Solution

- ☐ Wash the plate 3 times with at least 150 μ L/well of 1X MSD Wash Buffer.
- ☐ Add 25 μ L/well of 1X detection antibody solution.
- ☐ Incubate at room temperature with shaking (700 rpm) for 2 hours.

STEP 3: Wash and Read Plate

- ☐ Wash the plate 3 times with at least 150 μ L/well of 1X MSD Wash Buffer.
- ☐ Add 150 μ L/well of read buffer.
- ☐ Analyze plate on the MSD instrument.

***Note:** Washing the plate prior to sample addition is an optional step that may provide greater uniformity of results for certain assays. Analytical parameters including limits of quantification, recovery of controls, and sample quantification, are not affected by washing the plate prior to sample addition.

Catalog Numbers

Kit Name	V-PLEX			V-PLEX Plus*		
	1-Plate Kit	5-Plate kit	25-Plate Kit	1-Plate Kit	5-Plate Kit	25-Plate Kit
V-PLEX GLP-1 Active SECTOR Assay	K15030D-1	K15030D-2	K15030D-4	K15030G-1	K15030G-2	K15030G-4
V-PLEX GLP-1 Active QuickPlex Ultra Assay	K15030D-21	K15030D-22	K15030D-24	K15030G-21	K15030G-22	K15030G-24

*V-PLEX Plus kits include controls, plate seals, and wash buffer. See **Kit Components** for details.

Plate Diagram

