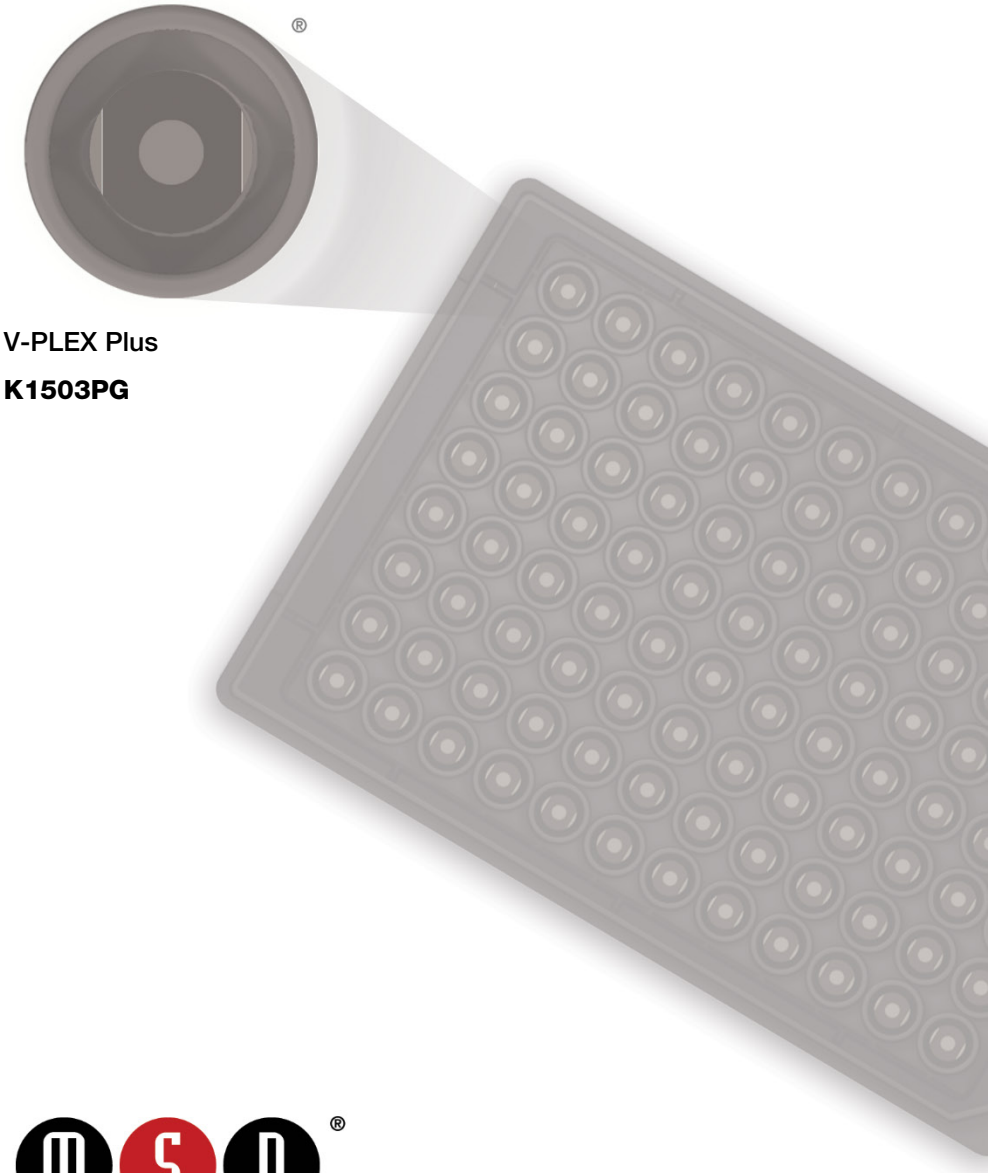
MSD® MULTI-ARRAY Assay System

GLP-1 Total Kit

V-PLEX®



	V-PLEX®	V-PLEX Plus
GLP-1 Total	**K1503PD**	**K1503PG**

MSD®

MSD Metabolic Assays

GLP-1 Total 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 GLP-1 (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 blood vessels and the heart. 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 have been demonstrated in rodent models of diabetic nephropathy and acute kidney injury. 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 (T2D) 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

MSD 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 Total 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 side-viewing detection antibody conjugated with electrochemiluminescent (ECL) labels (MSD SULFO-TAG™) over the course of two incubation periods separated by wash steps. GLP-1, in the calibrator (inactive form) or sample (inactive and active forms), binds to the GLP-1 C-terminal specific 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]

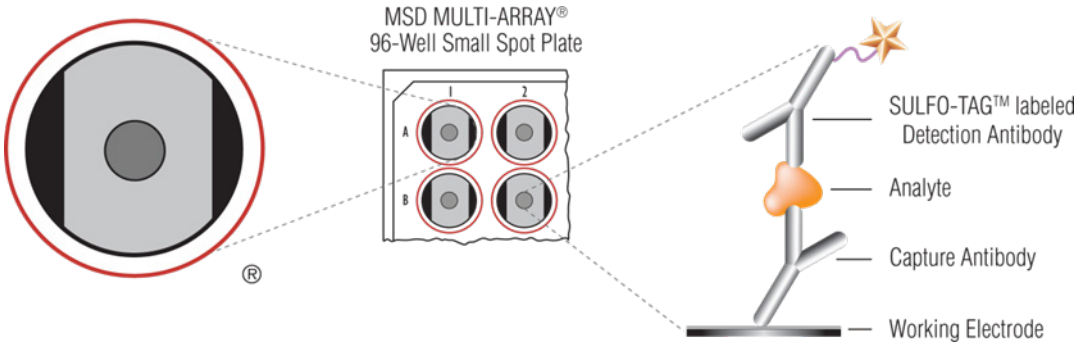

Figure 1. Small Spot plate diagram showing placement of analyte capture antibodies.

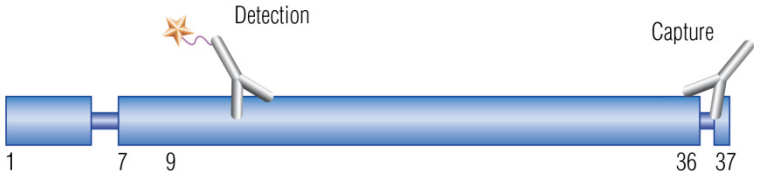

Figure 2. Schematic of the antibody recognition sites for the V-PLEX GLP-1 Total assay on GLP-1 protein amino assays.

Kit Components

V-PLEX GLP-1 Total 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 (total) 96-well Small Spot SECTOR® Plate	2–8 °C	L4503PA-1	Small Spot	1	5	25	96-well plate, foil sealed, with desiccant.
GLP-1 (total) Antibody (50X)	2–8 °C	D203P-2	75 µL	1			SULFO-TAG conjugated antibody
		D203P-3	375 µL		1	5	
GLP-1 (total) Calibrator	2–8 °C	C003P-2	1 vial	1 vial	5 vials	25 vials	Lyophilized synthetic GLP-1 (9-36) amide peptide. GLP-1 peptide concentration is provided in the lot-specific certificate of analysis (COA).
Diluent 12	≤-10 °C	R50JA-4	10 mL	1 bottle			Diluent for samples and calibrator; contains protein, blockers, and preservatives.
		R50JA-3	50 mL		1 bottle	5 bottles	
Diluent 11	≤-10 °C	R55BA-4	5 mL	1 bottle			Diluent for detection antibody; contains protein, blockers, and preservatives.
		R55BA-3	50 mL		1 bottle	5 bottles	
MSD GOLD™ Read Buffer A*	RT	R92TG-3	18 mL	1 bottle			Buffer to catalyze the electro-chemiluminescence reaction.
		R92TG-4	90 mL		1 bottle	5 bottles	

* Previoulsy 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 (total) Control 1*	2–8 °C	C403P-1	1 vial	1 vial	5 vials	25 vials	Controls lyophilized in a non-matrix based diluent, spiked with amidated GLP-1 (9-36) peptide. The concentrations of the controls are provided in the lot-specific COA.
GLP-1 (total) Control 2*	2–8 °C	C403P-1	1 vial	1 vial	5 vials	25 vials	
GLP-1 (total) Control 3*	2–8 °C	C403P-1	1 vial	1 vial	5 vials	25 vials	
Wash Buffer (20X)	RT	R61AA-1	100 mL				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 (total) Control Pack (catalog # C403P-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 kit) 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
- ❑ Vortex mixer

Optional Materials and Equipment

- ❑ GLP-1 (total) Control Pack, available for separate purchase from MSD, catalog # C403P-1 (included in V-PLEX Plus kit)
- ❑ Centrifuge (for sample preparation)

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.

Best Practices

- Mixing and 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 MSD GOLD Read Buffer A 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.
- MSD GOLD Read Buffer A 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 the MSD GOLD Read Buffer A.
- 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 12 and Diluent 11 into suitable sizes before refreezing.

Prepare Calibrator Dilutions

MSD supplies a lyophilized calibrator that yields the recommended highest calibrator concentration (Calibrator 1) when reconstituted in 1,000 µL of Diluent 12. 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 µL of Diluent 12 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 µL of the highest calibrator to 300 µL of Diluent 12. Mix well by vortexing. Repeat 4-fold serial dilutions 5 additional times to generate 7 calibrators.
3) Use Diluent 12 as the zero calibrator.

Calibrator	GLP-1 (9-36) amide concentration		Dilution Factor
	(pM)	(pg/mL)	
1	180	556.0	NA
2	45.0	139.0	4
3	11.3	34.8	4
4	2.81	8.69	4
5	0.703	2.17	4
6	0.176	0.543	4
7	0.044	0.136	4
8	0	0	NA

Note: Reconstituted calibrator is not stable when stored at 2–8 °C; however, it may be stored frozen at ≤-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.

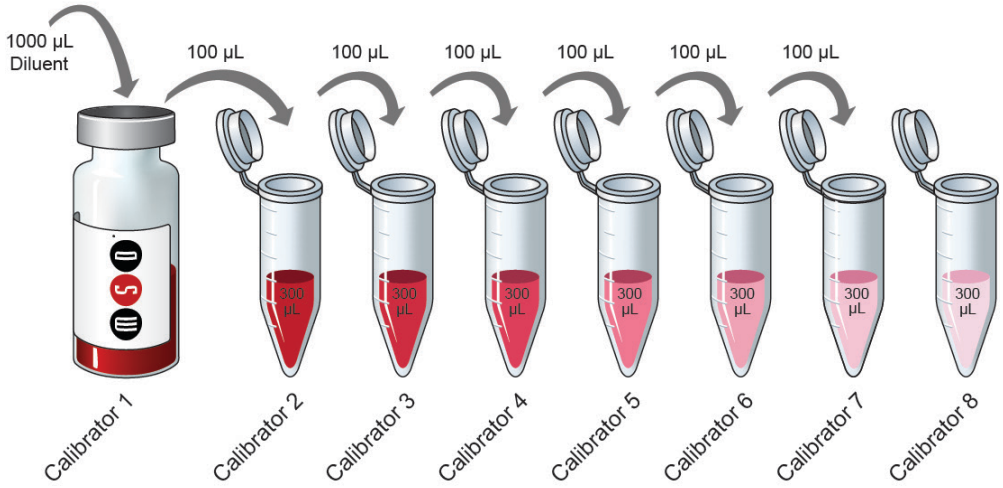

***Figure 3**. Dilution schema for preparation of Calibrator Standards.*

Sample Collection and Handling

Below are general guidelines for human, NHP, mouse, rat, and canine sample collection, storage, and handling for metabolic markers including 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, not provided with the kit) and mix to avoid cleavage/degradation of GLP-1.

For BD tubes, process as follows[20];

- 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 serum and 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 2,000 x g 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 at 4–8 °C until processed and used in the assay.

Dilute Samples

Dilute samples with Diluent 12. 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 Diluent 12. 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 (total) Control Pack, catalog # C403P-1. (Controls are included only in V-PLEX Plus kits.)

Reconstitute the lyophilized controls in 250 µL of Diluent 12. Do not invert or vortex the vials. Wait for a minimum of 15-30 minutes at room temperature before diluting controls 2-fold in Diluent 12. Vortex briefly using short pulses. Reconstituted controls may be stored frozen at ≤-70 °C and are stable through six 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 1 plate, add 60 µL of the supplied detection antibody to 2,940 µL of Diluent 11.

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

Read Buffer

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.

Prepare MSD Plate

MSD plates are pre-coated with capture antibody (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 in the assay protocol.

Protocol

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

STEP 1: Wash and Add Sample

- ❑ Wash the plate 3 times with 150 µL/well of 1X MSD Wash Buffer.
- ❑ Add 50 µL of prepared samples, calibrators, or controls per well. Seal the plate with an adhesive plate seal and incubate at room temperature with shaking (700 rpm) 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.

STEP 2: Wash and Add Detection Antibody Solution

- ❑ Wash the plate 3 times with at least 150 µL/well of 1X MSD Wash Buffer.
- ❑ Add 25 µL of detection antibody solution to each well. Seal the plate with an adhesive plate seal and incubate at room temperature with shaking (700 rpm) for 2 hours.

STEP 3: Wash and Read

- ❑ Wash the plate 3 times with at least 150 µL/well of 1X MSD Wash Buffer.
- ❑ Add 150 µL of MSD GOLD Read Buffer A* to each well. Analyze the plate on an MSD instrument. Incubation in Read Buffer is not required before reading the plate.

  ***Important:** Unlike Read Buffer T which is provided at a 4X concentration, MSD GOLD Read Buffer A should not be diluted. Dilution of MSD GOLD Read Buffer A may compromise the results of this assay.

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:** For tissue culture samples, 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 µL of assay diluent to each sample/control well, and then add 25 µL of neat control or sample. Calibrators should not be diluted in the plate; add 50 µL of each calibrator directly into empty wells. See **Appendix A** for assay performance using this protocol.

Validation

MSD's V-PLEX products are 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 (total) 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. For example, the stability of reconstituted calibrator is assessed in real time over a 30-day period. 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 the MSD DISCOVERY WORKBENCH® 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 Total Kit were collected by 3 operators (36 runs in total). Calibration curve accuracy and precision were assessed for two kit lots. Representative data from one lot are presented below.

Data from alternative protocols are shown in the Appendix. Appendix A compares data from alternative protocols 1, 2, and 3 to the reference protocol. The calibration curves and control recovery were comparable among the protocols. Calibration curves for each lot are presented in the lot-specific COA.

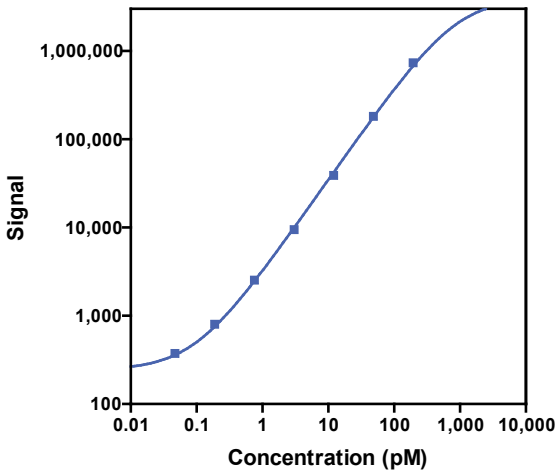

Figure 4. Typical calibration curves for the V-PLEX GLP-1 Total Kit.

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 3 runs, one run from each of 3 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 Total Kit

	Median LLOD (pM)	LLOD Range (pM)	LLOQ (pM)	ULOQ (pM)
GLP-1 (total)	0.017	0.013 – 0.035	0.180	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 3 operators using a minimum of three replicates on 36 runs over 3 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 36 runs (three kit lots, 12 runs each).

Inter-run %CV is the variability of controls across a 3 plate run within a single kit lot.

Inter-lot %CV is the variability of controls across 3 kit lots (12 runs for each kit lot, total of 36 runs).

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

	Control	Average Conc. (pM)	Average Intra-run %CV	Inter-run %CV	Inter-lot %CV
GLP-1 (total)	Control 1	95.2	2.6	2.3	3.4
	Control 2	18.1	3.3	2.4	2.5
	Control 3	2.73	3.6	3.1	3.9

Dilution Linearity

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

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

Table 5. *GLP-1 (9-36) 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 table. Mouse samples were EDTA plasma spiked with aprotinin & the DPP-IV inhibitor at the time of collection (P800 tubes were not used for mouse samples).*

Sample Type	Fold Dilution	Average % Recovery	% Recovery Range
Human P800 EDTA plasma (N=18)	4	87	76–93
	8	81	71–91
	16	79	68–89
NHP P800 EDTA plasma (N=10)	4	77	72–80
	8	68	64–75
	16	69	63–79
Mouse K2 EDTA plasma (N=10)	4	97	90–101
	8	96	92–99
	16	97	93–102
Rat P800 EDTA plasma (N=10)	4	82	73–88
	8	80	74–83
	16	78	71–84
Canine P800 EDTA plasma (N=10)	4	96	86–110
	8	98	82–127
	16	103	81–138
RPMI (10% FBS, 1% Pen-Strep, 2mM Glu)	4	96	NA
	8	86	NA
	16	83	NA
DMEM (10% FBS, 1% Pen-Strep)	4	86	NA
	8	84	NA
	16	83	NA
DMEM (2.5% FBS, 15% HS)	4	91	NA
	8	85	NA
	16	87	NA

Spike Recovery

Spike recovery measurements were evaluated for different sample types throughout the quantitative range of the assays. Mouse K2 EDTA plasma samples and plasma samples collected in P800 tubes from human, rat, canine, and NHP were obtained from a commercial source. These samples, along with cell culture media, were spiked with GLP-1 (inactive) 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.

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

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

Sample Type	Average % Recovery	%CV	% Recovery Range
Human P800 EDTA plasma (N=18)	107	6.3	99–118
NHP P800 EDTA plasma (N=10)	118	3.5	111–125
Mouse K2 EDTA plasma (N=10)	98	2.5	94–103
Rat P800 EDTA plasma (N=10)	102	1.7	99–105
Canine P800 EDTA plasma (N=10)	89	12.2	66–98
RPMI (10% FBS, 1% Pen-Strep, 2mM Glu)	116	NA	NA
DMEM (10% FBS, 1% Pen-Strep)	114	NA	NA
DMEM (2.5% FBS, 15% HS)	100	NA	NA

Specificity

To assess specificity, the assay was tested for nonspecific binding to the following GLP-1 metabolites and other general metabolic targets. All analytes were tested at **1,000 pg/mL**.

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

Table 7. Assay cross-reactivity to selected human GLP-1 metabolites and hormones (unless otherwise indicated).

	Metabolite/Hormone	Conc (pg/mL)	Signal (counts)	% Cross-reactivity
GLP-1 Receptor Agonists	Liraglutide*	1,000	484	0.03
	Exenatide	1,000	264	ND**
Proglucagon Metabolites	Major Proglucagon Fragment (MPGF)	1,000	5,553	1.72
	Glucagon	1,000	247	ND
	Glicentin	1,000	307	ND
	GLP-2	1,000	234	ND
	Oxyntomodulin	1,000	287	ND
	Miniglucagon	1,000	234	ND
General Metabolic Analytes	Insulin	1,000	385	ND
	Leptin	1,000	386	ND
	PYY	1,000	341	ND
	Resistin	1,000	362	ND
	GIP	1,000	375	ND
	PPY	1,000	442	ND
	Active Ghrelin	1,000	227	ND
	Mouse Leptin	1,000	374	ND
	Rat Leptin	1,000	380	ND
	Canine Leptin	1,000	218	ND

*Note: Liraglutide interferes with the assay at concentrations of ≥ 8 nM.

**ND = not detected

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

	Peptide	Average % Assay Reactivity	Concentration range tested (pM)
GLP-1 Forms	GLP-1 (1-36) amide	87	0.36–93
	GLP-1 (7-36) amide	87	0.27–69
	GLP-1 (9-36) amide	100	Calibrator
	GLP-1 (7-37)	16	1.8–456

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 ≤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 concentrations are calibrated to a reference calibrator generated at MSD that was assigned using three independent lots of amino acid-analyzed inactive GLP-1 peptide to provide a highly accurate measurement of total GLP-1.

Tested Samples

Normal Samples

Commercially available apparently normal human 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. 40 normal human P800 EDTA Plasma samples and 10 pooled rat, canine, and NHP P800 EDTA Plasma samples and 10 pooled mouse EDTA plasma samples (collected in the presence of DPP-IV inhibitor and protease inhibitor) were tested in the V-PLEX GLP-1 Total Kit.

Sample Type	Statistic	pg/mL*	pM
Human P800 EDTA plasma (N=40)	Median	33.2	10.7
	Range	12.5–113	4.04–36.6
	% of Samples Detected	100	
Mouse K2 EDTA plasma (N=10)	Median	29.3	9.46
	Range	22.8–42.1	7.39–13.6
	% of Samples Detected	100	
NHP P800 EDTA plasma (N=10)	Median	114.2	37
	Range	61.0–174.8	19.7–56.6
	% of Samples Detected	100	
Rat P800 EDTA plasma (N=10)	Median	23.4	7.58
	Range	15.9–27.1	5.2–8.8
	% of Samples Detected	100	
Canine P800 EDTA plasma (N=10)	Median	27.0	8.74
	Range	9.8–72.0	3.2–23.3
	% of Samples Detected	100	

*as calculated for GLP-1 inactive (9-36) amide (FW: 3089.5 Daltons)

% 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 µM), forskolin (10 µM), insulin (2 µM), and sucrose (200 mM) for a given period of time. These cell culture supernatants were tested for the presence of total GLP-1. Total GLP-1 was expressed at levels higher than the detection limit of the assay, so they were diluted 10 fold prior to the assay. The dilution-adjusted concentrations (pM) for each stimulation condition are displayed below.

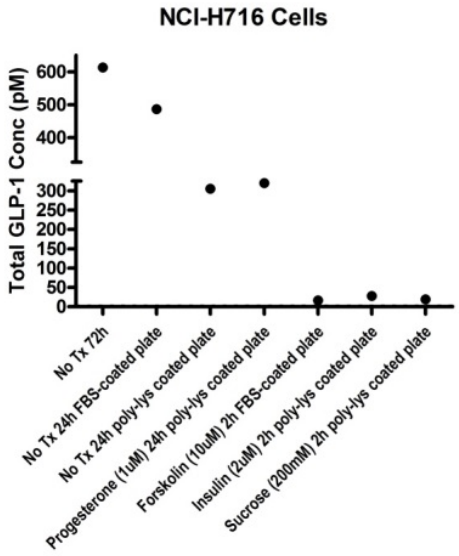

Figure 5. Effect of cell stimulation on total GLP-1 production as measured in the V-PLEX GLP-1 Total Kit.

Post meal changes in circulating GLP-1:

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

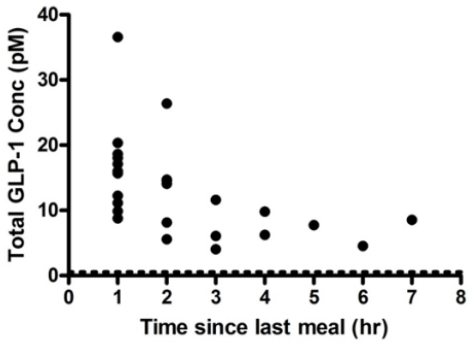

Figure 6. Post-meal levels of total GLP-1 in individuals (black circles), measured using the V-PLEX GLP-1 Total Kit. Dotted line indicates the in-sample LLOQ, which is two times the in-well LLOQ.

Assay Components

Calibrators

The assay calibrator blend uses the following recombinant human proteins:

Table 10. Synthetic human peptide used in the Calibrators

Calibrator	Expression System
GLP-1 (9-36) amide	Synthetic peptide

Antibodies

Table 11. Antibody source species

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

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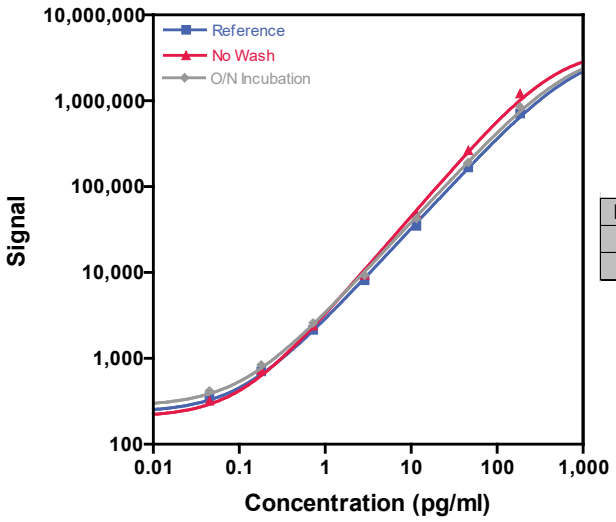
Appendix A

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 (tissue culture: single wash, red curve), and Alternate Protocol 2 (overnight sample incubation, grey curve). Alternate Protocol 3 (in-well dilution) is not shown since the calibrator curve is not changed in this protocol.



	Hill Slope	LLOD
Reference Curve	1.06	0.017
Single Wash	1.13	0.014
O/N Incubation	1.06	0.010

Figure 7. Calibrator curves generated by the indicated protocols. Table shows the hillslope of each curve and the estimated lower limit of detection (LLOD) 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 concentration measured with the reference protocol, which shows equivalent performance among all the protocols tested.

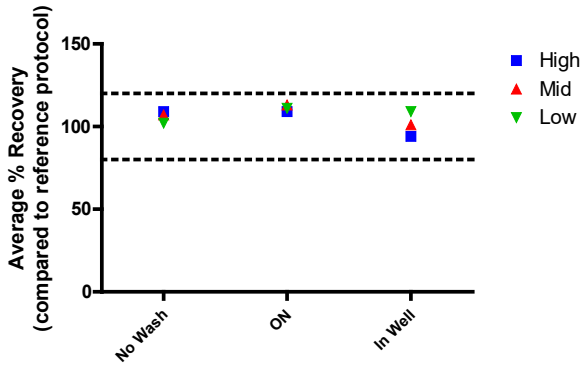


Figure 8. Recovery of measured concentrations of the controls resulting from each protocol relative to the concentrations of controls resulting from the reference protocol.

Summary Protocol

V-PLEX GLP-1 Total Kit

MSD provides this summary protocol for your convenience.
Please read the entire detailed protocol prior to performing the V-PLEX GLP-1 Total Kit.

Sample and Reagent Preparation

- ❑ Bring all reagents to room temperature.
- ❑ Prepare calibration solutions in Diluent 12 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 Diluent 12 before adding to the plate.
- ❑ Prepare the detection antibody solution by diluting the 50X detection antibody 50-fold in Diluent 11.

STEP 1: Wash* and Add Sample

- ❑ Wash the plate 3 times with at least 150 µL/well of 1X MSD Wash Buffer.
- ❑ Add 50 µL/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 µL/well of 1X MSD Wash Buffer.
- ❑ Add 25 µL/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 µL/well of 1X MSD Wash Buffer.
- ❑ Add 150 µL/well of MSD GOLD Read Buffer A.
- ❑ 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 Total	K1503PD-1	K1503PD-2	K1503PD-4	K1503PG-1	K1503PG-2	K1503PG-4

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

Plate Diagram