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# Development and Characterization of GLP-1 Total and Active V-PLEX® Assays


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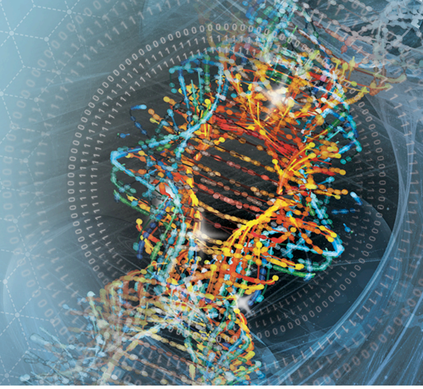
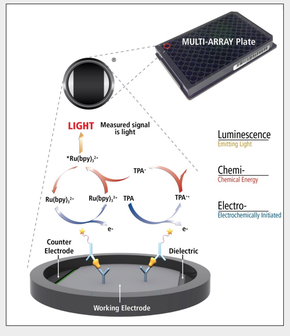


## PURPOSE

Glucagon like peptide-1 (GLP-1), an incretin hormone, is a major target of interest for researchers studying metabolic, neurologic, and cardiovascular disorders. After post-translational processing of proglucagon, the GLP-1 peptide is secreted in its bioactive form, which binds a specific receptor (GLP-1R) to stimulate insulin release. Once in circulation, however, the peptide is rapidly cleaved by proteases (e.g. DPP-IV), yielding several other metabolites that account for the majority of measurable GLP-1. As a result, both the intact bioactive form of GLP-1 and its cleavage products provide important information about initial peptide secretion, protease activity, and stability of the circulating bioactive peptide. One of the major challenges faced by researchers studying GLP-1 is the ability to reliably measure multiple forms of GLP-1 at potentially very low endogenous levels. Sensitivity and required sample volume are especially important in rodent models where sample may be extremely limited. To address these needs, we have developed and validated GLP-1 Total and GLP-1 Active (7-36 amide) assays for human, NHP, mouse, rat, and canine donors on MSD's analytically validated V-PLEX platform. The combined utility of GLP-1 Total and Active assays provides researchers the ability to monitor changes to Total GLP-1 secretion (inclusive of the 1-36 amide, 7-36 amide, and 9-36 amide peptides) and specifically to the Active form (7-36 amide peptide) across multiple species. Endogenous levels of these analytes can be measured and monitored throughout the metabolic process with as little as 25 µL of plasma sample.

## METHODS

MSD's electrochemiluminescence detection technology uses SULFO-TAG™ labels that emit light upon electrochemical stimulation initiated at the electrode surfaces of MULTI-ARRAY® and MULTI-SPOT® microplates.




### ELECTROCHEMILUMINESCENCE TECHNOLOGY

- Minimal non-specific background and strong responses to analyte yield high signal-to-background ratios.
- The stimulation mechanism (electricity) is decoupled from the response (light signal), minimizing matrix interference.
- Only labels bound near the electrode surface are excited, enabling non-washed assays.
- Labels are stable, non-radioactive, and directly conjugated to biological molecules.
- Emission at ~620 nm eliminates problems with color quenching.
- Multiple rounds of label excitation and emission enhance light levels and improve sensitivity.
- Carbon electrode surface has 10X greater binding capacity than polystyrene wells.
- Surface coatings can be customized.

Sensitive and specific assays for the small GLP-1 peptides were developed through epitope mapping and antibody selection, recursive rounds of antibody engineering to enhance sensitivity, and extensive diluent optimization to eliminate matrix effects. Analytical validation performed across multiple lots included development of calibrator curves anchored to established reference standards and testing for dynamic range, matrix tolerance, and limits of quantitation (LOQ). Specificity was tested against common metabolic markers, proglucagon fragments, and multiple GLP-1R agonists. Assay robustness was confirmed at varying temperatures, sample/detector incubation times, and other common use-case scenarios including the presence of interfering agents.

## RESULTS

We developed assays for Total and Active GLP-1 that are highly specific and demonstrate little interference from other circulating proglucagon fragments or components in plasma. These assays have over a three-log dynamic range and sub-picomolar sensitivity with lower limits of quantitation (LLOQ) at 0.18 pM and 0.3 pM for the GLP-1 Total and Active assays, respectively. These highly sensitive assays allow for accurate quantitation even in populations with suppressed GLP-1 secretion, such as fasting, diabetic, or heart disease groups. Accuracy and precision were confirmed by testing three levels of controls across multiple lots and all controls had concentration %CVs (replicates) of less than 5% with recovery of 80-120%. Matrix compatibility was verified through dilution linearity and spike recovery studies in plasma treated with protease inhibitors from multiple species owing to high cross-species homology for GLP-1 peptides. Spiked human plasma samples typically recovered between 80-120% of the expected value. Further, specificity experiments confirmed that the Total GLP-1 assay was able to measure the 1-36 amide, 7-36 amide, and 9-36 amide GLP-1 peptide fragments, and that the Active GLP-1 assay was specific for the 7-36 amide peptide. Cross-reactivity with unintended targets was less than 2.0% when evaluated against other related analytes, with the exception of the GLP-1 (7-37) fragment and liraglutide, an acylated GLP-1 agonist therapeutic with sequence homology to GLP-1 (7-36). When the GLP-1 Total and Active assays are used in combination, excellent resolution of circulating levels of both bioactive and metabolized GLP-1 can be discerned, highlighting postprandial variation in multiple species; results from testing human samples are shown in Figure 2.

## CALIBRATION CURVES AND LIMITS OF DETECTION

The figure below demonstrates typical calibration curves for the analytes in the V-PLEX GLP-1 Total and GLP-1 Active Kits. Data below include upper limit of quantitation (ULOQ), lower limit of quantitation (LLOQ), and ranges for the lower limits of detection (LLOD, n=3 runs each from a different kit lot). Calibrators were reconstituted and diluted serially (4-fold) to generate a 7-point standard curve for each panel.

The LLOD is a calculated concentration corresponding to the average signal 2.5 standard deviations above the background (zero calibrator). The ULOQ and 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.

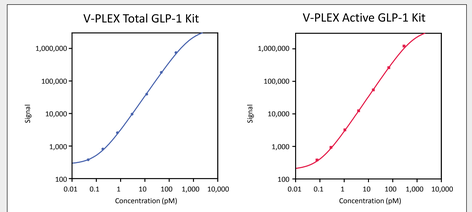



*Figure 1. Typical calibration curves for the V-PLEX GLP-1 Total and V-PLEX GLP-1 Active Kits.*

| | Median LLOD (pM) | LLOD Range (pM) | LLOQ (pM) | ULOQ (pM) |
|---|---|---|---|---|
| V-PLEX GLP-1 Total Kit | 0.017 | 0.013 – 0.035 | 0.180 | 120 |
| V-PLEX GLP-1 Active Kit | 0.020 | 0.010 – 0.020 | 0.300 | 120 |

## SPECIFICITY

To assess the specificity of each assay, both V-PLEX GLP-1 Total and GLP-1 Active Kits were tested for nonspecific binding to the following GLP-1 metabolites and other general metabolic targets. Cross-reactivity at or below 0.02% is reported as not detected (ND).

*Although weakly cross-reactive, Liraglutide and GLP-1 have nearly identical sequences and have the same homology at the N-terminus (7 cleavage site). GLP-1 concentrations may be suppressed when Liraglutide concentrations are higher than 8 nM for the V-PLEX GLP-1 Total Kit and 50 pM for the V-PLEX GLP-1 Active Kit.

| | | V-PLEX GLP-1 Total Kit | V-PLEX GLP-1 Active Kit |
|---|---|---|---|
| | GLP-1 Fragment | % Cross-reactivity | |
| | GLP-1 (1-36) amide | 87 | 0.09 |
| | GLP-1 (7-36) amide | 87 | 100 |
| | GLP-1 (9-36) amide | 100 | 0.01 |
| | GLP-1 (7-37) | 16 | 38 |
| | Metabolite/Hormone | % Cross-reactivity | |
| GLP-1 Receptor Agonists | Liraglutide* | 0.03 | 0.03 |
| | Exendin | ND | ND |
| Proglucagon Fragments | Major Proglucagon Fragment (MPGF) | 1.72 | ND |
| | Glucagon | ND | ND |
| | Glicentin | ND | ND |
| | GLP-2 | ND | ND |
| | Oxyntomodulin | ND | ND |
| | Miniglucagon | ND | ND |
| General Metabolic Analytes | Insulin | ND | ND |
| | Leptin | ND | ND |
| | PYY | ND | ND |
| | Resistin | ND | ND |
| | GIP | ND | ND |
| | Pancreatic Polypeptide (PP) | ND | ND |
| | Active Ghrelin | ND | ND |
| | Mouse Leptin | ND | ND |
| | Rat Leptin | ND | ND |
| | Canine Leptin | ND | not tested |

## ACCURACY AND PRECISION

Quality control samples were prepared by spiking calibrator into non-human serum matrix at three levels (high, mid, and low) within the quantitative linear range of the assay. The controls were measured using a minimum of three replicates tested over multiple days and multiple operators for a total of at least 36 runs. The accuracy of control determinations fell within 20% of the expected concentration with precision of less than 20% CV in the majority of runs.

Accuracy is defined as the average measured control concentration for a given lot divided by the expected control concentration. The accuracy shown is an average of three replicates on a single plate. Runs were conducted using four kit lots by at least three different operators.

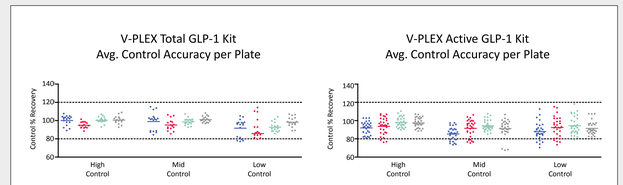



*Figure 4. Control accuracy across multiple kit lots. Kit 1 (blue), Kit 2 (red), Kit 3 (green), Kit 4 (gray).*

Precisions are defined as follows:

- Intra-run precision is the %CV of the control replicates within an individual run across at least 36 runs (4 kit lots).
- Inter-run precision is the %CV from control concentrations measured on a given kit (n=15).
- Inter-lot precision (shown as %CV) is the variability of controls across 4 kit lots (total of at least 36 runs).

| Control Precision | | | | | |
|---|---|---|---|---|---|
| Kit | Control | Avg. Conc. (pM) | Intra-Run % CV | Inter-Run % CV | Inter-Lot % CV |
| V-PLEX GLP-1 Total Kit | 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 |
| V-PLEX GLP-1 Active Kit | 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 |

## MATRIX PERFORMANCE

### SAMPLE TESTING

To assess the performance of the V-PLEX assays, human samples of P800-collected EDTA plasma from a commercial source were diluted 2-fold and tested on the V-PLEX GLP-1 Total (blue) and V-PLEX GLP-1 Active Kits (red); see Figure 2, at right. The results are in agreement with the expected ratios of Active and Total GLP-1 reported in the literature. Owing to the sequence homology of GLP-1 across mammalian species, several common animal models were assayed for Active GLP-1 and Total GLP-1. Results from testing mouse, non-human primate (NHP), rat, and canine samples are analogous to that on human samples (data not shown).

### POST-MEAL CHANGES IN CIRCULATING GLP-1

Post-meal samples from apparently healthy individuals were tested for the Total (blue) and Active (red) GLP-1 levels. Samples were collected from multiple individuals at different time points. The dilution-adjusted concentrations (pM) for each sample are displayed in Figure 3 (at right). Dotted line indicates the in-sample LLOQ, which is two times the in-well LLOQ. Results are consistent with the expected post-prandial degradation of GLP-1 in normal donors.

### DILUTION LINEARITY AND SPIKE RECOVERY

Plasma samples collected in P800 tubes from human, rat, canine, and NHP along with mouse K2 EDTA plasma samples were obtained from a commercial source. To assess linearity , samples were spiked with Active or Total GLP-1 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 (below, left). To assess recovery, samples were spiked with Active or Total GLP-1 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 (below, right).

| Dilution Linearity | | | V-PLEX GLP-1 Total Kit | | V-PLEX GLP-1 Total Kit | |
|---|---|---|---|---|---|---|
| Species | Sample Type | Fold Dilution | Average % Recovery | % Recovery Range | Average % Recovery | % Recovery Range |
| Human | P800 EDTA plasma (N=18) | 4 | 87 | 76–93 | 90 | 79–99 |
| | | 8 | 81 | 71–91 | 94 | 85–101 |
| | | 16 | 79 | 68–89 | 95 | 87–100 |
| NHP | P800 EDTA plasma (N=10) | 4 | 77 | 72–80 | 101 | 91–110 |
| | | 8 | 68 | 64–75 | 101 | 90–113 |
| | | 16 | 69 | 63–79 | 102 | 83–118 |
| Mouse | K2 EDTA plasma (N=10) | 4 | 97 | 90–101 | 98 | 91–105 |
| | | 8 | 96 | 92–99 | 100 | 90–107 |
| | | 16 | 97 | 93–102 | 100 | 91–111 |
| Rat | P800 EDTA plasma (N=10) | 4 | 82 | 73–88 | 100 | 77–113 |
| | | 8 | 80 | 74–83 | 95 | 70–115 |
| | | 16 | 78 | 71–84 | 89 | 75–107 |
| Canine | P800 EDTA plasma (N=10) | 4 | 96 | 86–110 | 95 | 89–100 |
| | | 8 | 98 | 82–127 | 95 | 86–101 |
| | | 16 | 103 | 81–138 | 95 | 89–107 |

| Spike Recovery | | V-PLEX GLP-1 Total Kit | | V-PLEX GLP-1 Active Kit | |
|---|---|---|---|---|---|
| Species | Sample Type | Average % Recovery | % Recovery Range | Average % Recovery | % Recovery Range |
| Human | P800 EDTA plasma (N=18) | 107 | 99–118 | 90 | 78–106 |
| NHP | P800 EDTA plasma (N =10) | 118 | 111–125 | 94 | 84–102 |
| Mouse | K2 EDTA plasma (N =10) | 98 | 94–103 | 78 | 73–83 |
| Rat | P800 EDTA Plasma (N =10) | 102 | 99–105 | 97 | 91–103 |
| Canine | P800 EDTA plasma (N =10) | 89 | 66–98 | 94 | 84–102 |

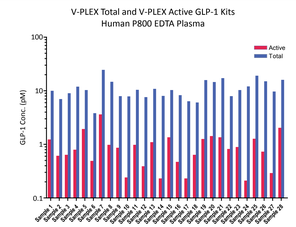



*Figure 2. Twenty-eight human EDTA plasma samples (collected in P800 tubes) were tested on both the V-PLEX GLP-1 Active Kit and V-PLEX GLP-1 Total Kit.*

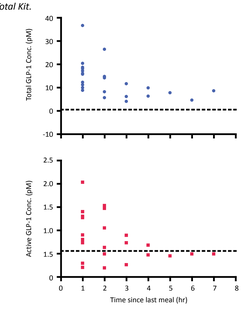



*Figure 3. Twenty-five human EDTA plasma samples (collected in P800 tubes) were collected within 8 hours of a meal. Total and Active GLP-1 levels are consistent with expected post-prandial degradation. In-sample LLOQ (dotted line).*

## CONCLUSIONS

The GLP-1 Total and Active assays are highly specific, sensitive, and validated for use with plasma from multiple species. The multi-lot analytical validation demonstrated consistent assay performance and accurate measurements in relevant matrices, making these assays potentially valuable tools for basic research and pre-clinical studies.

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