U-PLEX® Human α-Synuclein Kit

U-PLEX Kit
1-Plate Pack K151WKK-1
5-Plate Pack K151WKK-2
25-Plate Pack K151WKK-4

U-PLEX Plus Kit
1-Plate Pack K151WKP-1
5-Plate Pack K151WKP-2
25-Plate Pack K151WKP-4

www.mesoscale.com
MSD Neurodegenerative Disease Assays

U-PLEX Human $\alpha$-Synuclein Kit

For use with serum, plasma, cerebral spinal fluid, and saliva.

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

FOR RESEARCH USE ONLY.

NOT FOR USE IN DIAGNOSTIC PROCEDURES.
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Introduction

Alpha-synuclein is a 140 amino acid protein abundantly expressed in the nervous system and genetically linked to Parkinson’s disease (PD). It is thought to maintain synaptic integrity and normal cellular homeostasis through synaptic vesicle recycling and neurotransmitter release modulation. While native $\alpha$-synuclein is unfolded, it has a propensity to form toxic soluble oligomers (i.e., protofibrils) that ultimately aggregate into insoluble fibrils termed Lewy bodies (LBs). Aberrant $\alpha$-synuclein pathology is prevalent among neurological samples from patients with $\alpha$-synuclein-related conditions, commonly referred to as “synucleinopathies.” These disorders include PD, dementia with LBs (DLB), and multiple-system atrophy (MSA). $\alpha$-synuclein has been detected in several biological matrices, such as CSF, serum, plasma, and whole blood. Biomarkers that can effectively detect early or pre-symptomatic disease and distinguish PD incidence from other neurodegenerative conditions are of high interest. The U-PLEX Human $\alpha$-Synuclein Kit has been validated for the measurement of $\alpha$-synuclein in human serum, plasma (EDTA, heparin, and citrate), CSF, and saliva. The assay is also suitable for measurement of $\alpha$-synuclein in whole blood.

The U-PLEX Human $\alpha$-Synuclein Kit has been developed according to “Fit for Purpose” principles and is consistent with guidance from the Clinical and Laboratory Standards Institute (www.clsi.org/). The assay kit has undergone a comprehensive validation process, which involved the production and testing of three individual kit lots. Thorough characterization of assay components, combined with comprehensive quality control testing of each lot, assures that the assays will meet the demands of international clinical studies.

Principle of the Assay

The U-PLEX Human $\alpha$-Synuclein Kit is supplied on MSD GOLD™ Small Spot Streptavidin Plates (Figure 1). These plates provide high sensitivity, consistent performance, and excellent inter- and intra-lot uniformity.

The assay is supplied with a biotinylated capture antibody that binds to streptavidin on the plate surface. Analytes in the sample bind to the capture reagent; detection antibodies conjugated with electrochemiluminescent labels (MSD GOLD SULFO-TAG™) bind to the analytes to complete the sandwich immunoassay. Once the sandwich immunoassay is complete, the plate is loaded 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 (which is proportional to the amount of analyte present in the sample) and provides a quantitative measure of each analyte in the sample.

Figure 1: U-PLEX human $\alpha$-synuclein assay on a MSD GOLD Small Spot Streptavidin Plate
## Reagents Supplied

Table 1. Reagents supplied with the U-PLEX and U-PLEX Plus Human α-Synuclein Kits

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Storage</th>
<th>Catalog #</th>
<th>Size</th>
<th>Quantity Supplied</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSD GOLD 96-Well Small Spot Streptavidin SECTOR® Plate</td>
<td>2–8°C</td>
<td>L45SA-1</td>
<td>1-spot</td>
<td>1 plate, 5 plates, 25 plates</td>
<td>96-well plate, foil sealed, with desiccant.</td>
</tr>
<tr>
<td>Biotin Anti-hu α-Synuclein (50X)</td>
<td>2–8°C</td>
<td>C21WK-2</td>
<td>75 µL</td>
<td>1</td>
<td>Biotinylated capture antibody.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C21WK-3</td>
<td>375 µL</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>SULFO-TAG Anti-hu α-Synuclein (50X) (^1)</td>
<td>2–8°C</td>
<td>D21WK-2</td>
<td>75 µL</td>
<td>1</td>
<td>SULFO-TAG–conjugated detection antibody.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>D21WK-3</td>
<td>375 µL</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>α-Synuclein Calibrator</td>
<td>≤-70°C</td>
<td>C01WK-2</td>
<td>30 µL</td>
<td>1 vial, 5 vials, 25 vials</td>
<td>Recombinant human protein in diluent. Analyte concentration is provided in the lot-specific certificate of analysis (COA).</td>
</tr>
<tr>
<td>Diluent 49</td>
<td>≤-10°C</td>
<td>R50AM-1</td>
<td>20 mL</td>
<td>1 bottle</td>
<td>Diluent for samples, controls, and calibrator; contains blockers and preservatives.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R50AM-2</td>
<td>100 mL</td>
<td>1 bottle, 5 bottles</td>
<td></td>
</tr>
<tr>
<td>Read Buffer T (4X)</td>
<td>RT</td>
<td>R92TC-3</td>
<td>50 mL</td>
<td>1 bottle, 1 bottle, 5 bottles</td>
<td>Buffer to catalyze the electrochemiluminescence (ECL) reaction. Dilute to 1X and use at room temperature.</td>
</tr>
</tbody>
</table>

## U-PLEX Plus Human α-Synuclein Kit: Additional Components

Table 2. Additional components provided with the U-PLEX Plus Human α-Synuclein Kit

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Storage</th>
<th>Catalog #</th>
<th>Size</th>
<th>Quantity Supplied</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human α-Synuclein Control 1*</td>
<td>≤-70°C</td>
<td>-</td>
<td>40 µL</td>
<td>1 vial, 5 vials, 25 vials</td>
<td>Human α-synuclein recombinant protein spiked in diluent. The concentration of the controls is provided in the lot-specific COA.</td>
</tr>
<tr>
<td>Human α-Synuclein Control 2*</td>
<td>≤-70°C</td>
<td>-</td>
<td>40 µL</td>
<td>1 vial, 5 vials, 25 vials</td>
<td></td>
</tr>
<tr>
<td>Human α-Synuclein Control 3*</td>
<td>≤-70°C</td>
<td>-</td>
<td>40 µL</td>
<td>1 vial, 5 vials, 25 vials</td>
<td></td>
</tr>
<tr>
<td>Wash Buffer (20X)</td>
<td>RT</td>
<td>R61AA-1</td>
<td>100 mL</td>
<td>1 bottle, 1 bottle, 5 bottles</td>
<td>20-fold concentrated phosphate buffered solution with surfactant.</td>
</tr>
<tr>
<td>Plate Seals</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3, 15, 75</td>
<td>Adhesive seals for sealing plates during incubations.</td>
</tr>
</tbody>
</table>

\(^*\)Provided as components in the Human α-Synuclein Control Pack (Catalog #C41WK-1)

\(^1\)SULFO-TAG conjugated detection antibodies should be stored in the dark.
Additional Materials and Equipment

- Appropriately sized tubes for reagent preparation
- Polypropylene microcentrifuge tubes for preparing dilutions
- Liquid handling equipment suitable for 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
- Phosphate-buffered saline (PBS) plus 0.05% Tween-20 (PBS-T) for plate washing or MSD Wash Buffer (20X, 100 mL, Catalog # R61AA-1)
  - MSD Wash Buffer (Catalog # R61AA-1) is sufficient for washing four plates manually or for washing two plates with an automated plate washer.
  - If the MSD Wash Buffer is purchased separately, prepare a 1X working solution. For one plate, combine 15 mL of MSD Wash Buffer (20X) with 285 mL of deionized water.
- Adhesive plate seals
- Deionized water
- Vortex mixer

Optional Materials

- Human α-Synuclein Control Pack (Catalog #C41WK-1). Controls are included in the U-PLEX Plus Human α-Synuclein Kit.

Safety

Use safe laboratory practices: wear gloves, safety glasses, and lab coats when handling assay 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

- Do not mix or substitute reagents from different sources or different kit lots. 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 22-25°C water bath. Thaw other reagents on wet ice and use as directed without delay.
- Prepare Calibrator Standards, controls, and samples in polypropylene microcentrifuge tubes. Use a fresh pipette tip for each dilution, and mix by vortexing after each dilution.
- Do not touch the pipette tip on the bottom of the wells when pipetting into the MSD plate.
- Avoid prolonged exposure of the 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 during all pipetting steps, as they may lead to variable results. Bubbles introduced when adding Read Buffer T may interfere with signal detection.
- Use reverse pipetting when necessary to avoid the introduction of bubbles. For empty wells, pipette to the bottom corner.
- 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.
- If an incubation step needs to be extended, leave the sample or detection antibody solution in the plate to keep the plate from drying out.
- Remove the plate seal prior to reading the plate.
- Make sure that the Read Buffer T is at room temperature when added to a plate.
- Do not shake the plate after adding Read Buffer T.
- To improve inter-plate precision, keep time intervals consistent between adding Read Buffer T and reading the plate. Unless otherwise directed, read the plate as soon as possible after adding Read Buffer T.
- If the sample 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) with an adhesive plate seal while executing the assay protocol to avoid contaminating unused wells. Remove all plate seals before reading the plate. Partially used plates may be sealed in the original foil pouch with desiccant and stored up to 30 days at 2–8°C. You may adjust volumes proportionally when preparing reagents.
Reagent Preparation

Bring all diluents and buffers to room temperature.

**Important:** Upon first thaw, aliquot Diluent 49 into suitable volumes before refreezing. Diluent 49 is stable through four freeze-thaw cycles.

Allow the plates to equilibrate in their pouches at room temperature (at least 30 minutes).

**Prepare Capture Antibody Solution**

MSD provides the capture antibody as a 50X stock solution. The working solution is 1X. Once prepared, the capture antibody solution should be added to the plate within 1 hour.

For one plate, combine:

- 60 µL of Biotin Anti-hu α-Synuclein Antibody (50X)
- 2,940 µL of Diluent 49

**Prepare Calibrator Dilutions**

MSD supplies calibrator for the U-PLEX Human α-Synuclein Kit at a concentration that is 20-fold higher than the recommended highest standard.

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

1) Thaw the stock calibrator on wet ice for at least 30 minutes and keep on ice.

   **Note:** Stock calibrator should be stored at ≤-70°C. After the initial thaw, stock calibrator may be refrozen and thawed up to 3 additional times.

2) Prepare the highest calibrator by adding 15 µL of stock calibrator to 285 µL of Diluent 49. Mix well.

3) Prepare the next calibrator by transferring 50 µL of the highest calibrator to 150 µL of Diluent 49. Mix well. Repeat 4-fold serial dilutions 5 additional times to generate 7 calibrators. Diluted calibrator may be stored on wet ice for up to 1 hour prior to using in the assay.

4) Use Diluent 49 as the zero calibrator.

For the lot-specific concentration of the calibrator, refer to the COA supplied with the product. You can also find a copy of the COA at www.mesoscale.com.
Sample Collection and Handling

Below are general guidelines for human sample collection, storage, and handling. If possible, use published guidelines. Evaluate sample stability under the selected method as needed.

CSF

Sample collection methods and pre-analytical conditions may cause variability in measured analyte levels. MSD recommends reviewing current literature and protocols for collection and handling of CSF samples, e.g., the Parkinson’s Progression Markers Initiative (PPMI) Biospecimen Collection, Processing, and Shipment Manual.

Saliva

Samples should be acquired using passive collection technique to avoid spitting. Other considerations include fasting and/or mouth-rinsing immediately prior to collection, as well as the inclusion of protease inhibitors to prevent digestive enzymes from breaking down target proteins.

Serum and Plasma

Under ideal conditions, blood collection should be performed using standard venipuncture technique with vacutainers. Plasma prepared in heparin tubes commonly displays additional clotting following thawing of the sample. Remove clots and all solid material by centrifugation. Avoid multiple freeze-thaw cycles for serum and plasma samples.

Dilute Samples

Dilute samples with Diluent 49. MSD recommends a minimum 8-fold sample dilution for human CSF, serum, and plasma samples, and a minimum 5-fold dilution from human saliva. However, depending on the sample set under investigation, you may need to use a higher dilution factor. For example, to dilute 8-fold, add 20 µL of sample to 140 µL of Diluent 49. Additional diluent can be purchased at www.mesoscale.com.

For validation studies, the frozen samples were thawed on ice prior to dilution. The samples were vortexed to ensure homogenous distribution of proteins and matrix within the sample before dilution.

Prepare Controls

Three levels of controls are available in the Human α-Synuclein Control Pack (Catalog # C41WK-1) or included as components of the U-PLEX Plus Human α-Synuclein Kit. Thaw the controls on wet ice for at least 30 minutes. Mix well by vortexing, then dilute controls 8-fold in Diluent 49. Diluted controls are stable at room temperature for up to 1 hour. The material is intended for one time use; however, the undiluted controls can tolerate up to four freeze-thaw cycles.

Prepare Detection Antibody Solution

MSD provides detection antibody as a 50X stock solution. The working solution is 1X. Once prepared, the detection antibody solution should be added to the plate within 1 hour.

For one plate, combine:

- 60 µL of SULFO-TAG Anti-hu α-Synuclein Antibody (50X)
- 2,940 µL of Diluent 49
Prepare Wash Buffer
MSD provides Wash Buffer as a 20X stock solution in the U-PLEX Plus kit. The working solution is 1X. PBS + 0.05% Tween-20 (PBS-T) can be used instead.
For one plate, combine:
- 15 mL of MSD Wash Buffer (20X)
- 285 mL of deionized water

Prepare Read Buffer
MSD provides Read Buffer T as a 4X stock solution. The working solution is 1X.
For one plate, combine:
- 5 mL of Read Buffer T (4X)
- 15 mL of deionized water
You may keep excess diluted read buffer in a tightly sealed container at room temperature for up to one month.

Prepare MSD Plate
MSD GOLD 96-Well Small Spot Streptavidin SECTOR plates are exposed to a proprietary stabilizing treatment to ensure the integrity and stability of the immobilized reagents. Plates may be used as delivered; no additional preparation (e.g., pre-wetting) is required.
Assay Protocol

Note: Follow Reagent Preparation before beginning this assay protocol.

STEP 1: Coat plate
- Add 25 µL of the prepared capture antibody solution to each well.
- Seal the plate with an adhesive plate seal and incubate at room temperature with shaking for 1 hour.
  Note: You may prepare calibrators, samples, and detection antibody during incubation.

STEP 2: Wash, Add Detection Antibody Solution and Sample
- Wash the plate 3 times with at least 150 µL/well of Wash Buffer.
- Add 25 µL of detection antibody solution to each well.
- Add 25 µL of diluted sample or calibrator per well. Seal the plate with an adhesive plate seal and incubate at room temperature with shaking for 2 hours.
  Note: You may prepare diluted read buffer during incubation.

STEP 3: Wash and Read
- Wash the plate 3 times with at least 150 µL/well of Wash Buffer.
- Add 150 µL of 1X Read Buffer T to each well. Read plate on the MSD instrument immediately.

Alternate Protocols
The suggestion below may be useful as an alternate protocol.

Extended Sample Incubation: If required, the detection antibody solution and the sample can be incubated on the plate overnight (at 2-8 °C, with shaking) without adverse effects on performance. However, this protocol was not used to validate the assay.
Validation

U-PLEX validated assays are developed under rigorous design control and are fully validated according to fit-for-purpose principles in accordance with MSD’s Quality Management System. U-PLEX validated 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 U-PLEX validated assay, 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.

- **Development**
  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, diluent-based controls, and matrix-based controls across multiple runs and multiple lots. 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 5%, and inter-run CVs are typically below 15%. Rigorous management of inter-lot reagent consistency and calibrator production results in typical inter-lot CVs below 15%. Validation lots are compared using controls and at least 20 samples in various sample matrices. Samples are well correlated with an inter-lot bias typically below 20%.

- **Matrix Effects and Samples**
  Matrix effects from human CSF, saliva, serum, and plasma are measured as part of development and validation. Dilution linearity and spike recovery studies are performed on individual samples, as well as pooled samples, to assess variability of results due to matrix effects. A recommended sample dilution factor is provided in the protocol.

- **Specificity**
  The specificity of the assay is analyzed by evaluating the ability of the assay to detect closely related proteins in the synuclein family, β- and γ-synuclein. The calibrator concentrations used for cross-reactivity analysis were chosen to ensure that the specific signal is greater than 15,000 counts.
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. For example, the stability of diluted calibrator is assessed in real time over a 4-hour period. Assay component (calibrator, control, diluent) stability is also assessed through freeze-thaw testing. The validation program includes a real-time stability study with scheduled performance evaluations of complete kits for up to 36 months from date of manufacture.

Representative data from the 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. This assay has a wide dynamic range (4 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 U-PLEX Human α-Synuclein Kit were collected over four months of testing by five operators (133 runs in total). Calibration curve accuracy and precision were assessed for three kit lots. Representative data from one lot are presented below. Calibration curves for each lot are presented in the lot-specific COA.

![Typical calibration curve for the U-PLEX human α-synuclein assay. Data is shown in the table at right.](image)

<table>
<thead>
<tr>
<th>Conc. (pg/mL)</th>
<th>Average Signal</th>
<th>%CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>10,000</td>
<td>370,227</td>
<td>4.6</td>
</tr>
<tr>
<td>2,500</td>
<td>85,449</td>
<td>4.3</td>
</tr>
<tr>
<td>625</td>
<td>19,778</td>
<td>3.9</td>
</tr>
<tr>
<td>156</td>
<td>4,634</td>
<td>3.0</td>
</tr>
<tr>
<td>39.1</td>
<td>1,142</td>
<td>2.5</td>
</tr>
<tr>
<td>9.77</td>
<td>337</td>
<td>2.5</td>
</tr>
<tr>
<td>2.44</td>
<td>151</td>
<td>3.3</td>
</tr>
<tr>
<td>0</td>
<td>95</td>
<td>6.7</td>
</tr>
</tbody>
</table>

Table 3. LLOD, LLOQ, and ULOQ for the U-PLEX Human α-Synuclein Kit

<table>
<thead>
<tr>
<th>Median LLOD (pg/mL)</th>
<th>LLOD Range (pg/mL)</th>
<th>LLOQ (pg/mL)</th>
<th>ULOQ (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.900</td>
<td>0.464–1.68</td>
<td>8.00</td>
<td>6,800</td>
</tr>
</tbody>
</table>

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 57 runs, across three independent 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 U-PLEX Human α-Synuclein Kit

\( ^2 \) See the kit-specific COA for standard curve concentrations, specifications, and quality control data.
Precision

Controls were made by spiking calibrator into diluent at three levels. Analyte levels were measured by two operators using a minimum of two replicates on 77 runs over five months. Results are shown below. Controls were designed to span the quantitative range of the assay, as displayed on a representative calibrator curve (left). At least 97% of all runs fell within 20% of the inter-lot average concentration for each control (right). Horizontal lines represent guardbands of 20% around the average. 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 assay, all are \( \leq 10\% \) (bottom).

Average intra-run %CV is the average %CV of the control replicates within an individual run.

Inter-run %CV is the variability of controls across 77 runs.

Inter-lot %CV is the variability of controls across three kit lots.

![Figure 4. Control levels span the quantitative range of the assay](image1)

![Figure 5. Inter-lot precision of the U-PLEX Human \( \alpha \)-Synuclein Kit](image2)

<table>
<thead>
<tr>
<th>Control</th>
<th>Average Conc. (pg/mL)</th>
<th>Average Intra-run %CV</th>
<th>Inter-run %CV</th>
<th>Inter-lot %CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 1</td>
<td>17,022</td>
<td>3.2</td>
<td>7.3</td>
<td>1.5</td>
</tr>
<tr>
<td>Control 2</td>
<td>1,661</td>
<td>3.2</td>
<td>8.7</td>
<td>2.7</td>
</tr>
<tr>
<td>Control 3</td>
<td>168</td>
<td>3.6</td>
<td>10.0</td>
<td>2.1</td>
</tr>
</tbody>
</table>
In addition, controls were prepared from pools of patient samples from several of the validated matrices. Native analyte levels were measured by at least two operators, using a minimum of two replicates, on at least 14 runs, over two months. Results are shown below. Intra- and inter-day run CVs are <16% for all of the matrix-based controls.

**Table 5. Intra- and inter-day run %CVs for matrix-based controls**

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Control</th>
<th>Runs</th>
<th>Average Conc. (pg/mL)</th>
<th>Average Intra-run %CV</th>
<th>Inter-run %CV</th>
<th>Inter-lot %CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSF</td>
<td>Control 1</td>
<td>46</td>
<td>912</td>
<td>2.8</td>
<td>13.9</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>Control 2</td>
<td>46</td>
<td>399</td>
<td>2.7</td>
<td>12.3</td>
<td>4.9</td>
</tr>
<tr>
<td></td>
<td>Control 3</td>
<td>46</td>
<td>188</td>
<td>3.0</td>
<td>15.1</td>
<td>0.9</td>
</tr>
<tr>
<td>Serum</td>
<td>Control 1</td>
<td>18</td>
<td>11,334</td>
<td>3.9</td>
<td>10.1</td>
<td>11.6</td>
</tr>
<tr>
<td></td>
<td>Control 2</td>
<td>18</td>
<td>6,823</td>
<td>3.5</td>
<td>11.0</td>
<td>9.8</td>
</tr>
<tr>
<td></td>
<td>Control 3</td>
<td>18</td>
<td>3,946</td>
<td>3.3</td>
<td>7.8</td>
<td>6.9</td>
</tr>
<tr>
<td>Citrate Plasma</td>
<td>Control 1</td>
<td>14</td>
<td>16,870</td>
<td>3.4</td>
<td>12.6</td>
<td>12.6</td>
</tr>
<tr>
<td></td>
<td>Control 2</td>
<td>14</td>
<td>3,584</td>
<td>3.0</td>
<td>10.1</td>
<td>8.5</td>
</tr>
<tr>
<td></td>
<td>Control 3</td>
<td>14</td>
<td>1,578</td>
<td>3.5</td>
<td>8.9</td>
<td>6.3</td>
</tr>
<tr>
<td>EDTA Plasma</td>
<td>Control 1</td>
<td>20</td>
<td>37,234</td>
<td>3.8</td>
<td>9.1</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>Control 2</td>
<td>20</td>
<td>17,856</td>
<td>5.1</td>
<td>9.3</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>Control 3</td>
<td>20</td>
<td>10,701</td>
<td>4.4</td>
<td>11.9</td>
<td>1.7</td>
</tr>
<tr>
<td>Heparin Plasma</td>
<td>Control 1</td>
<td>19</td>
<td>13,593</td>
<td>3.2</td>
<td>10.3</td>
<td>14.5</td>
</tr>
<tr>
<td></td>
<td>Control 2</td>
<td>19</td>
<td>7,582</td>
<td>3.1</td>
<td>12.0</td>
<td>11.7</td>
</tr>
<tr>
<td></td>
<td>Control 3</td>
<td>19</td>
<td>4,508</td>
<td>3.8</td>
<td>10.1</td>
<td>8.5</td>
</tr>
</tbody>
</table>
Dilution Linearity

To assess linearity, normal human CSF, serum, EDTA plasma, heparin plasma, and citrate plasma, were diluted 4-fold, 8-fold, 16-fold, and 32-fold before testing. Normal human saliva was tested at 2.5-fold, 5-fold, 10-fold, and 20-fold dilutions. Percent recovery at each dilution level was normalized to the dilution-adjusted, recommended concentration (5-fold for saliva, 8-fold for all other matrices). The average percent recovery shown below is based on samples within the quantitative range of the assay.

\[
% \text{ Recovery} = \frac{\text{measured concentration}}{\text{expected concentration}} \times 100
\]

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Fold Dilution</th>
<th>Average % Recovery</th>
<th>% Recovery Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSF (N=5)</td>
<td>4</td>
<td>97</td>
<td>92–103</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>100</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>101</td>
<td>96–108</td>
</tr>
<tr>
<td></td>
<td>32</td>
<td>105</td>
<td>95–114</td>
</tr>
<tr>
<td>Saliva (N=5)</td>
<td>2.5</td>
<td>104</td>
<td>102–105</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>100</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>94</td>
<td>83–101</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>93</td>
<td>N/A</td>
</tr>
<tr>
<td>Serum (N=5)</td>
<td>4</td>
<td>103</td>
<td>98–106</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>100</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>99</td>
<td>97–103</td>
</tr>
<tr>
<td></td>
<td>32</td>
<td>106</td>
<td>104–109</td>
</tr>
<tr>
<td>EDTA Plasma (N=5)</td>
<td>4</td>
<td>90</td>
<td>81–106</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>100</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>87</td>
<td>83–94</td>
</tr>
<tr>
<td></td>
<td>32</td>
<td>87</td>
<td>78–96</td>
</tr>
<tr>
<td>Heparin Plasma (N=5)</td>
<td>4</td>
<td>103</td>
<td>94–108</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>100</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>99</td>
<td>98–100</td>
</tr>
<tr>
<td></td>
<td>32</td>
<td>99</td>
<td>98–100</td>
</tr>
<tr>
<td>Citrate Plasma (N=5)</td>
<td>4</td>
<td>100</td>
<td>100-101</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>100</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>102</td>
<td>99-103</td>
</tr>
<tr>
<td></td>
<td>32</td>
<td>111</td>
<td>108-113</td>
</tr>
</tbody>
</table>
Spike Recovery

Spike recovery measurements of different sample types were evaluated throughout the quantitative range of the assays. Tested matrices included normal human CSF, saliva, serum, EDTA plasma, heparin plasma, and citrate plasma. Samples were spiked with recombinant α-synuclein protein at three levels (high, mid, and low) then diluted per the recommended sample dilution factor (5-fold for saliva, 8-fold for all other matrices). The average % recovery for each sample type is reported along with %CV and % recovery range.

\[
\% \ Recovery = \frac{\text{measured concentration}}{\text{expected concentration}} \times 100
\]

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Average % Recovery</th>
<th>%CV</th>
<th>% Recovery Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSF (N=5)</td>
<td>105</td>
<td>4.1</td>
<td>99–112</td>
</tr>
<tr>
<td>Saliva (N=10)</td>
<td>110</td>
<td>6.1</td>
<td>99–124</td>
</tr>
<tr>
<td>Serum (N=5)</td>
<td>112</td>
<td>6.3</td>
<td>99–127</td>
</tr>
<tr>
<td>EDTA Plasma (N=5)</td>
<td>100</td>
<td>6.5</td>
<td>93–119</td>
</tr>
<tr>
<td>Heparin Plasma (N=5)</td>
<td>105</td>
<td>3.5</td>
<td>101–113</td>
</tr>
<tr>
<td>Citrate Plasma (N=5)</td>
<td>107</td>
<td>7.2</td>
<td>98–122</td>
</tr>
</tbody>
</table>

Specificity

The assay specifically recognizes α-Synuclein. Cross-reactivity to β- and γ-synuclein was evaluated through titration of these proteins in the assay. Non-specific binding of β- and γ-synuclein is less than 0.05% across all validation lots.

\[
\% \ Non specificity = \frac{\text{non specific signal}}{\text{specific signal}} \times 100
\]

<table>
<thead>
<tr>
<th>α-Synuclein</th>
<th>β-Synuclein</th>
<th>γ-Synuclein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conc. (pg/mL)</td>
<td>ECL Signal Counts</td>
<td>Conc. (pg/mL)</td>
</tr>
<tr>
<td>10,800</td>
<td>550,365</td>
<td>10,000</td>
</tr>
<tr>
<td>2,700</td>
<td>101,208</td>
<td>2,500</td>
</tr>
<tr>
<td>675</td>
<td>20,650</td>
<td>625</td>
</tr>
<tr>
<td>169</td>
<td>5,283</td>
<td>156</td>
</tr>
<tr>
<td>42.2</td>
<td>1,247</td>
<td>39.1</td>
</tr>
<tr>
<td>10.5</td>
<td>393</td>
<td>9.77</td>
</tr>
<tr>
<td>2.64</td>
<td>158</td>
<td>2.44</td>
</tr>
<tr>
<td>0.0</td>
<td>72</td>
<td>0.0</td>
</tr>
</tbody>
</table>
Stability

The stock calibrator, controls, and diluent were tested for freeze-thaw stability. Results (not shown) demonstrated that each component can undergo four freeze-thaw cycles without significantly affecting the performance of the assay. Stock calibrator and controls must be stored frozen at \(-70^\circ\text{C}\). The validation study includes a real-time stability study with scheduled performance evaluations of complete kits for up to 36 months from date of manufacture.

Tested Samples

Normal Samples

Normal human CSF, saliva, serum, EDTA plasma, heparin plasma, and citrate plasma were diluted according to the recommended dilution (5-fold for saliva, 8-fold for all other matrices) and tested. Results for each sample type are reported below. Measured concentrations are corrected for sample dilution. Median and range are calculated from samples with concentrations within the quantitative range of the assay. Percent detected is the percentage of samples with concentrations within the quantitative range of the assay using the recommended sample dilution factor.

Table 9. Normal human samples tested in the U-PLEX Human\(\alpha\)-Synuclein Kit

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Statistic</th>
<th>(\alpha)-Synuclein</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSF (N=20)</td>
<td>Median (pg/mL)</td>
<td>263</td>
</tr>
<tr>
<td></td>
<td>Range (pg/mL)</td>
<td>141–396</td>
</tr>
<tr>
<td></td>
<td>% Detected</td>
<td>100</td>
</tr>
<tr>
<td>Saliva (N=32)</td>
<td>Median (pg/mL)</td>
<td>136</td>
</tr>
<tr>
<td></td>
<td>Range (pg/mL)</td>
<td>54–14,875</td>
</tr>
<tr>
<td></td>
<td>% Detected</td>
<td>91</td>
</tr>
<tr>
<td>Serum (N=20)</td>
<td>Median (pg/mL)</td>
<td>7,698</td>
</tr>
<tr>
<td></td>
<td>Range (pg/mL)</td>
<td>2,525–20,332</td>
</tr>
<tr>
<td></td>
<td>% Detected</td>
<td>100</td>
</tr>
<tr>
<td>EDTA Plasma (N=20)</td>
<td>Median (pg/mL)</td>
<td>19,424</td>
</tr>
<tr>
<td></td>
<td>Range (pg/mL)</td>
<td>9,934–48,940</td>
</tr>
<tr>
<td></td>
<td>% Detected</td>
<td>95</td>
</tr>
<tr>
<td>Heparin Plasma (N=20)</td>
<td>Median (pg/mL)</td>
<td>8,856</td>
</tr>
<tr>
<td></td>
<td>Range (pg/mL)</td>
<td>3,783–18,649</td>
</tr>
<tr>
<td></td>
<td>% Detected</td>
<td>100</td>
</tr>
<tr>
<td>Citrate Plasma (N=20)</td>
<td>Median (pg/mL)</td>
<td>3,558</td>
</tr>
<tr>
<td></td>
<td>Range (pg/mL)</td>
<td>1,601–15,688</td>
</tr>
<tr>
<td></td>
<td>% Detected</td>
<td>95</td>
</tr>
</tbody>
</table>
Assay Components

Calibrators
The assay calibrator uses recombinant human α-synuclein, (residues 1-140), expressed in *E.coli*.

Antibodies

Table 10. Antibody source species

<table>
<thead>
<tr>
<th>Source Species</th>
<th>Analyte</th>
<th>MSD Capture Antibody</th>
<th>MSD Detection Antibody</th>
<th>Assay Generation</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-synuclein</td>
<td></td>
<td>Rabbit Monoclonal</td>
<td>Mouse Monoclonal</td>
<td>A</td>
</tr>
</tbody>
</table>

References

Frequently Asked Questions

How does the performance of this kit compare to your existing products for quantification of human α-synuclein?

The validated U-PLEX Human α-Synuclein Kit (Catalog #K151WKK-2) was directly compared to the previously-developed Human α-Synuclein Kit (Catalog #K151TGD-2). The assays achieve similar sensitivity, as well as quantification of controls, human CSF, and serum samples. The correlation between sample quantification on the two assays was very strong (R²=0.97). The previously-released version was not designed for use with other human matrices.

Is the assay compatible with other human matrices?

Yes, the U-PLEX human α-synuclein assay has been tested extensively with human whole blood. Under ideal conditions, blood collection should be performed using standard venipuncture technique with vacutainers. MSD recommends pretreatment of whole blood samples with detergent, e.g. 1% Triton X-100, to lyse cells prior to diluting in assay diluent. Subsequently, a minimum 75,000-fold sample dilution is recommended. Using the assay for whole blood may require additional assay diluent. Additional Diluent 49 (Catalog #R50AM-1 and # R50AM-2) can be purchased at www.mesoscale.com.

Can samples be utilized at different dilutions than what is recommended?

Dilution linearity for all validated matrices is provided in this product insert (see Dilution Linearity). If required, you may run samples at higher or lower dilutions. However, the assay was validated only at the recommended dilution. In addition, changing the dilution factor can risk moving samples out of the quantifiable range for the assay.

Can samples be refrozen and thawed multiple times?

In validation work, human CSF, saliva, whole blood, serum, and plasma were collected and handled as recommended. These samples were stable for up to three freeze-thaw cycles. However, MSD recommends that users avoid freeze-thaw cycles since collection and handling methods may vary between laboratories.

Is there a particular form of human plasma that is recommended for use with the assay?

Human K2 EDTA plasma, sodium heparin plasma, and sodium citrate plasma were tested in validation work. MSD does not recommended one form over another; however, differences in quantification may exist between these sample types.

Can plates be incubated with samples overnight?

If required, the detection antibody solution and the sample can be incubated on the plate overnight (at 2-8°C, with shaking) without adverse effects on performance. However, this protocol was not used to validate the assay.

Can incubation times be reduced or extended?

The performance of the assay is consistent when incubating capture antibody on the plate between 30 minutes and 2 hours. Similarly, the detection antibody solution and the sample can be incubated on the plate from 1.5 to 3 hours without adverse effects. However, only the standard, recommended protocol was used to validate the assay.
Can the detection antibody and sample incubation be run in a two-step format, with separate 1-hour incubations for each component?
MSD does not recommend using a two-step format for the detection antibody and sample incubation, as this may adversely affect performance.

Should plates be pre-washed prior to use?
MSD does not recommend pre-wetting or pre-washing plates before beginning an assay, as this may adversely affect performance.

Is the α-synuclein calibrator native protein?
No, the assay calibrator uses recombinant human α-synuclein, (residues 1-140), expressed in E.coli.

Does the assay detect aggregated α-synuclein proteins?
The validated U-PLEX Human α-Synuclein Kit does not distinguish between monomeric and aggregated forms of α-synuclein.

Does the assay cross-react with other proteins in the synuclein family, e.g. β- or γ-synuclein?
As demonstrated by the validation data in the Specificity section of the product insert, the U-PLEX Human α-Synuclein Kit displays little to no cross-reactivity with β- and γ-synuclein.
Summary Protocol

U-PLEX Human \( \alpha \)-Synuclein Kit

MSD provides this summary protocol for your convenience. Please read the entire detailed protocol prior to performing the U-PLEX human \( \alpha \)-synuclein assay.

Sample and Reagent Preparation

- Thaw frozen reagents (calibrators, controls, and samples) on ice until ready to dilute.
- Thaw Diluent 49 in a water bath (approximately 22-25°C) or equivalent room temperature.
- Prepare the capture antibody solution by diluting stock capture antibody in Diluent 49.
- Bring the plates, Read Buffer, and Wash Buffer to room temperature.

*Note*: The calibration solutions, controls, and detection antibody solution may be prepared during step 1 and used within one hour of preparation.

- Prepare 7 calibration solutions in Diluent 49 using the supplied calibrator:
  - Dilute the stock calibrator by adding 15 µL of stock calibrator to 285 µL of Diluent 49. Mix well.
  - Perform a series of 4-fold dilution steps and prepare a zero calibrator blank.
- Dilute samples (8-fold dilution for human CSF, serum, and plasma; a minimum of 5-fold dilution for saliva) in Diluent 49 before adding to the plate.
- Prepare the detection antibody solution by diluting the stock detection antibody in Diluent 49.
- Prepare 1X Read Buffer T by diluting 4X Read Buffer T 4-fold with deionized water.

**STEP 1: Coat Plate**

- Add 25 µL/well of 1X capture antibody solution.
- Incubate at room temperature with shaking for 1 hour.

**STEP 2: Wash, Add Detection Antibody Solution and Sample**

- Wash plate 3 times with at least 150 µL/well of Wash Buffer.
- Add 25 µL/well of 1X detection antibody solution.
- Add 25 µL/well of sample (calibrators, controls, or diluted samples).
- Incubate at room temperature with shaking for 2 hours.

**STEP 3: Wash and Read Plate**

- Wash plate 3 times with at least 150 µL/well of Wash Buffer.
- Add 150 µL/well of 1X Read Buffer T.
- Analyze plate on MSD instrument.
Plate Diagram