MSD® U-PLEX Platform

U-PLEX® Human α-Synuclein Kit

Catalog Numbers

	U-PLEX Kit	U-PLEX Plus Kit
1-Plate Pack	K151WKK-1	K151WKP-1
5-Plate Pack	K151WKK-2	K151WKP-2
25-Plate Pack	K151WKK-4	K151WKP-4

MSD Neurodegenerative Disease Assays

U-PLEX Human α-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

Traditional immunoassays, such as ELISAs, provide assay choice but sacrifice sensitivity and read time. The MSD U-PLEX platform combines high sensitivity and a rapid read time (less than 2 minutes) with the flexibility to easily design and build custom assays and efficiently transition from singleplex to multiplex assays. Singleplex assays, using U-PLEX Antibody Sets with MSD GOLD™ Small Spot Streptavidin Plates, have high sensitivity, provide up to 5 logs of linear dynamic range, and use minimal sample volume.

Principle of the Assay

The U-PLEX Human α-Synuclein Kit is supplied on MSD GOLD Small Spot Streptavidin Plates (Figure 1). The reagents (Table 1; Table 2) provide high sensitivity, consistent performance, and excellent inter- and intralot 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; a detection antibody conjugated with electrochemiluminescent labels (MSD GOLD SULFO-TAG™) binds to the analyte 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 α-Synuclein in the sample.

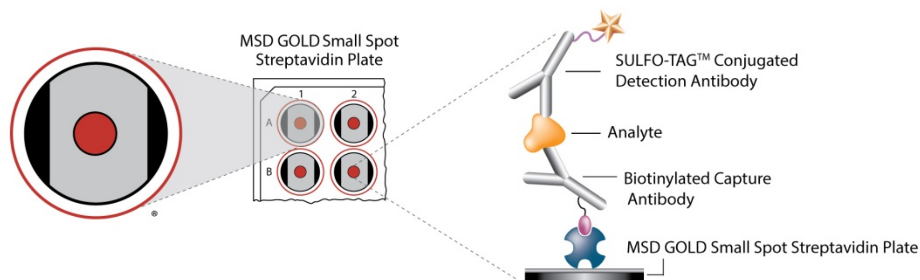

Figure 1: U-PLEX Human α-Synuclein assay on an MSD GOLD Small Spot Streptavidin Plate

α-Synuclein

Alpha-Synuclein is a 140 amino acid protein abundantly expressed in the nervous system and genetically linked to Parkinson's disease (PD).[1-5] It is thought to maintain synaptic integrity and normal cellular homeostasis through synaptic vesicle recycling and neurotransmitter release modulation.[1] When native α-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 α-Synuclein pathology is prevalent among neurological samples from patients with α-Synuclein-related conditions, commonly referred to as "synucleinopathies." These disorders include PD, dementia with LBs (DLB), and multiple-system atrophy (MSA).[6] Alpha-Synuclein has been detected in several biological matrices, such as CSF, serum, plasma, and whole blood.[2,7] Biomarkers that can effectively detect early or presymptomatic disease and distinguish PD incidence from other neurodegenerative conditions are of high interest. The U-PLEX Human α-Synuclein Kit has been validated for the measurement of α-Synuclein in human serum, plasma (EDTA, heparin, and citrate), CSF, and saliva. The assay is also suitable for the measurement of α-Synuclein in whole blood.

The U-PLEX Human α-Synuclein Kit has been developed according to "Fit for Purpose" principles [8] 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.

Reagents Supplied

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

Reagent/Product	Storage	Catalog No.	Size	Quantity Supplied			Description
				1-Plate Pack	5-Plate Pack	25-Plate Pack	
MSD GOLD 96-Well Small Spot Streptavidin SECTOR™ Plate	2–8 °C	L45SA-1	1-spot	1 plate	5 plates	25 plates	96-well plate, foil sealed, with desiccant
Biotin Anti-hu α-Synuclein (50X)	≤–70 °C	C21WK-2	75 µL	1	—	—	Biotinylated capture antibody
		C21WK-3	375 µL	—	1	5	
SULFO-TAG Anti-hu α-Synuclein (50X)	≤–70 °C	D21WK-2	75 µL	1	—	—	SULFO-TAG-conjugated detection antibody
		D21WK-3	375 µL	—	1	5	
α-Synuclein Calibrator	≤–70 °C	C01WK-2	30 µL	1 vial	5 vials	25 vials	Recombinant human protein in diluent; analyte concentration provided in the lot-specific certificate of analysis (COA)
Diluent 49	≤–10 °C	R50AM-1	20 mL	1 bottle	—	—	Diluent for samples, controls, and calibrator; contains blockers and preservatives
		R50AM-2	100 mL	—	1 bottle	5 bottles	
Read Buffer T (4X)	RT	R92TC-3	50 mL	1 bottle	1 bottle	5 bottles	Buffer to catalyze the electrochemiluminescence (ECL) reaction; diluted to 1X and used at room temperature

Dash (—) = not applicable
RT = room temperature

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

Reagent/Product	Storage	Catalog No.	Size	Quantity Supplied			Description
				1-Plate Pack	5-Plate Pack	25-Plate Pack	
Human α-Synuclein Control 1*	≤–70 °C	—	40 µL	1 vial	5 vials	25 vials	Human α-Synuclein recombinant protein spiked in diluent; the concentration of the controls provided in the lot-specific COA
Human α-Synuclein Control 2*	≤–70 °C	—	40 µL	1 vial	5 vials	25 vials	
Human α-Synuclein Control 3*	≤–70 °C	—	40 µL	1 vial	5 vials	25 vials	
Wash Buffer (20X)	RT	R61AA-1	100 mL	1 bottle	1 bottle	5 bottles	20-fold concentrated phosphate buffered solution with surfactant
Plate Seals	—	—	—	3	15	75	Adhesive seals for sealing plates during incubations

*Provided as components in the Human α-Synuclein Control Pack (catalog number C41WK-1)
Dash (—) = not applicable
RT = room temperature

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 number R61AA-1)
  - MSD Wash Buffer (catalog number 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 number 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 following 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 the www.mesoscale.com® website.

Best Practices

- Bring frozen diluent to room temperature in a 22-25 °C water bath.
  - If you are running multiple assays, avoid cross-contamination between antibodies by following the techniques below:
  - Open one capture antibody vial at a time. Close the cap after use.
  - Use filtered pipette tips.
  - Use a fresh pipette tip after each reagent addition.
- Prepare Calibrator Standards 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 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 in 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 before reading the plate.
- Make sure that the Read Buffer is at room temperature when added to a plate.
- Do not shake the plate after adding Read Buffer.
- To improve interplate precision, keep time intervals consistent between adding Read Buffer and reading the plate. Unless otherwise directed, read the plate as soon as possible after adding Read Buffer.
- 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 to avoid contaminating unused wells. Remove all seals before reading. The uncoated wells of a partially used plate 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.
- Some assays are temperature sensitive. U-PLEX assays were characterized at 20–26 °C. Assays run above or below that range may be negatively affected.

Reagent Preparation

Bring all diluents, reagents, and buffers to room temperature.

Important: Upon the 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 frozen 50X stock solution. Thaw the vial on wet ice. The working solution is 1X. Once prepared, the capture antibody solution should be added to the plate within 1 hour. Unused antibody stock can be freeze-thawed up to 4 times. Alternatively, 75 µL aliquots can be frozen at ≤–70 °C.

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.

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.

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.
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 before using in the assay.
4. Use Diluent 49 as the zero calibrator.

Consult Figure 2. 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.

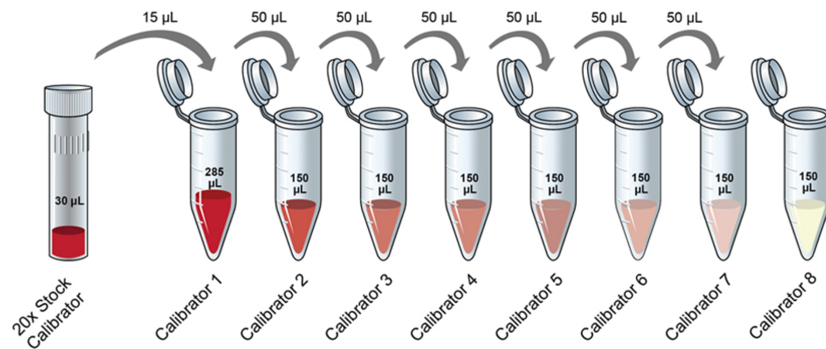

Figure 2. Dilution schema for calibrator standards for the U-PLEX Human α-Synuclein Kit

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 preanalytical conditions may cause variability in measured analyte levels.[4,9,10]. 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 a passive collection technique to avoid spitting. Other considerations include fasting and/or mouth rinsing immediately before 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 before 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 number 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 frozen 50X stock solution. Thaw the vial on wet ice. The working solution is 1X. Once prepared, the detection antibody solution should be added to the plate within 1 hour. Unused antibody stock can be freeze/thawed up to 4 times. Alternatively, 75 µL aliquots can be frozen at ≤–70 °C.

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 PBS-T.

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., prewetting) 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 the plate on the MSD instrument immediately.

Alternate Protocol

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 in 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[5] 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. Before 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 quantitation 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 quantitation (ULOQ) and the lower limit of quantitation (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). Intrarun CVs are typically below 5%, and interrun CVs are typically below 15%. Rigorous management of interlot reagent consistency and calibrator production results in typical interlot CVs below 15%. Validation lots are compared using controls and at least 20 samples in various sample matrices. Samples are well correlated, with an interlot 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 the 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 4 hours. 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 the 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 backfitting to the calibration curve. This assay has a wide dynamic range (4 logs), which allows accurate quantitation 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 quantitation 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 (Figure 3A; Figure 3B). Calibration curves for each lot are presented in the lot-specific COA.

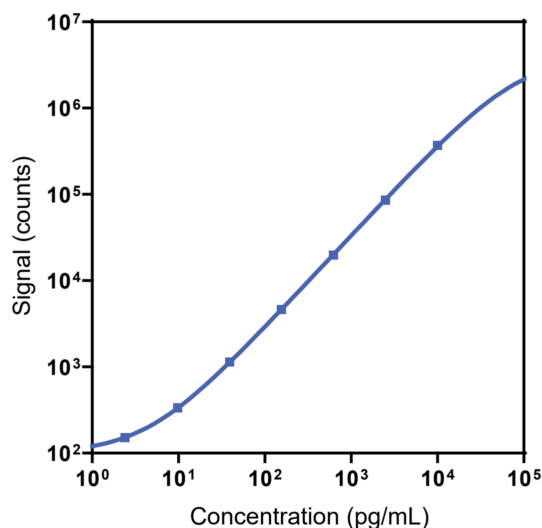

Figure 3A. Typical calibration curve for the U-PLEX Human α-Synuclein assay. Supporting data is shown in Figure 3B (see kit-specific COA for standard curve concentrations, specifications, and quality control data).

Concentration (pg/mL)	α-Synuclein	
	Average Signal	%CV
10,000	370,227	4.6
2,500	85,449	4.3
625	19,778	3.9
156	4,634	3.0
39.1	1,142	2.5
9.77	337	3.2
2.44	151	3.3
0	95	6.7

Figure 3B. Supporting data for Figure 3A, average signal, and %CV according to concentration.

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 (Table 3).

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. Median LLOD, LLOD, LLOQ, and ULOQ values for the U-PLEX Human α-Synuclein Kit

Median LLOD (pg/mL)	LLOD Range (pg/mL)	LLOQ (pg/mL)	ULOQ (pg/mL)
0.900	0.464–1.68	8.00	6,800

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 (Figure 4). At least 97% of all runs fell within 20% of the interlot average concentration for each control (Figure 5). Horizontal lines represent guard bands 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 interday runs, for this assay, all are greater than or equal to 10%. See Table 4.

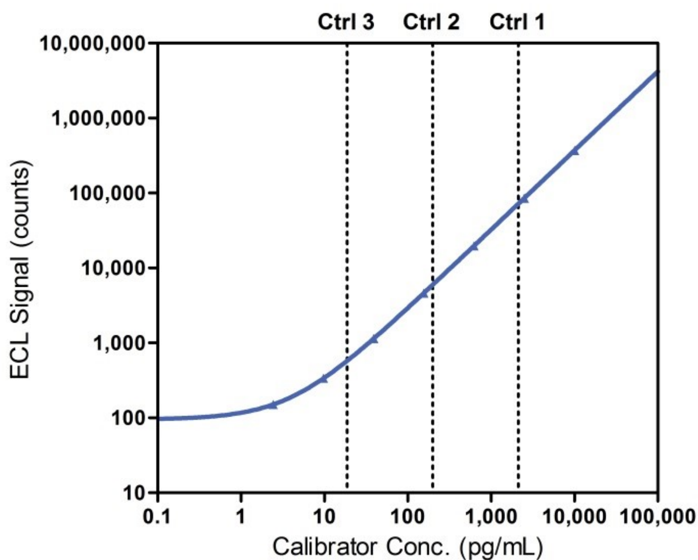

Figure 4. *Control levels span the quantitative range of the assay.*

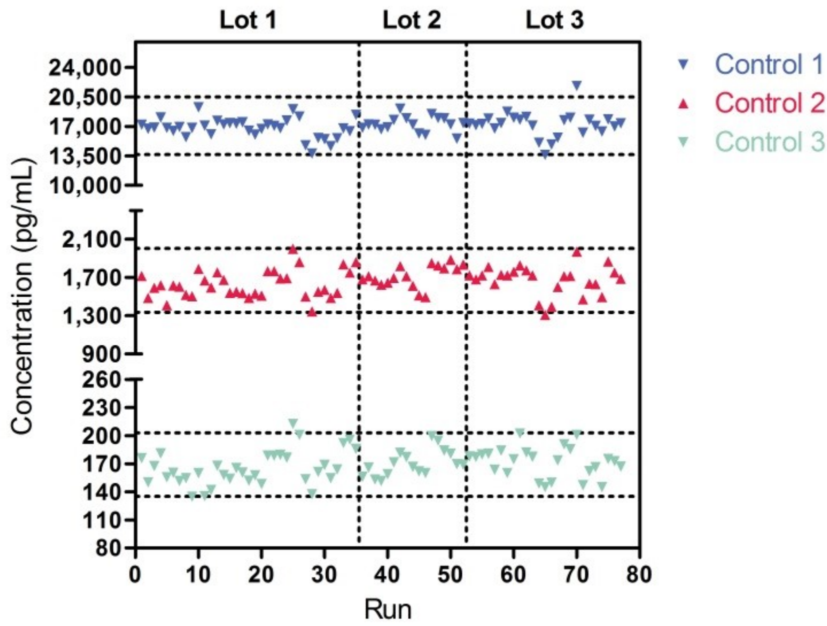

Figure 5. Interlot precision of the U-PLEX Human α-Synuclein Kit.

Table 4. %CVs for the controls in the U-PLEX Human α-Synuclein Kit

Control	Average Concentration (pg/mL)	Average Intrarun %CV	Interrun %CV	Interlot %CV
Control 1	17,022	3.2	7.3	1.5
Control 2	1,661	3.2	8.7	2.7
Control 3	168	3.6	10.0	2.1

Average intrarun %CV is the average %CV of the control replicates within an individual run.
Interrun %CV is the variability of controls across 77 runs.
Interlot %CV is the variability of controls across three kit lots.

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 in Table 5. Intra- and interday run CVs are <16% for all of the matrix-based controls.

Table 5. Intra- and interday run %CVs for matrix-based controls

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

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 (Table 6) is based on samples within the quantitative range of the assay.

$$\%\,recovery = \frac{measured\ concentration}{expected\ concentration} \times 100$$

Table 6. Percent recovery at various dilutions in different sample types

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

N/A = not available

Spike Recovery

Spike recovery measurements of different sample types were evaluated throughout the quantitative range of the assays (Table 7). 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 percent recovery for each sample type is reported along with %CV and percent recovery range.

$$\% \, recovery = \frac{measured \ concentration}{expected \ concentration} \times 100$$

Table 7. Spike recovery measurements of sample types evaluated in the U-PLEX Human α-Synuclein Kit

Sample Type	Average % Recovery	%CV	% Recovery Range
CSF (*n* = 5)	105	4.1	99–112
Saliva (*n* = 10)	110	6.1	99-124
Serum (*n* = 5)	112	6.3	99-127
EDTA Plasma (*n* = 5)	100	6.5	93-119
Heparin Plasma (*n* = 5)	105	3.5	101-113
Citrate Plasma (*n* = 5)	107	7.2	98-122

Specificity

The assay specifically recognizes α-Synuclein. Cross-reactivity to β- and γ-Synuclein was evaluated through titration of these proteins in the assay. Nonspecific binding of β- and γ-Synuclein is less than 0.05% across all validation lots (Table 8).

$$\% \, nonspecificity = \frac{nonspecific \; signal}{specific \; signal} \times 100$$

Table 8. Nonspecific binding of β- and γ-Synuclein in the U-PLEX Human α-Synuclein Kit

α-Synuclein		**β**-Synuclein		**γ**-Synuclein	
Conc. (pg/mL)	ECL Signal Counts	Conc. (pg/mL)	ECL Signal Counts	Conc. (pg/mL)	ECL Signal Counts
10,800	550,365	10,000	62	10,000	196
2,700	101,208	2,500	66	2,500	95
675	20,650	625	62	625	71
169	5,283	156	66	156	64
42.2	1,247	39.1	60	39.1	75
10.5	393	9.77	65	9.77	74
2.64	158	2.44	61	2.44	65
0.0	72	0.0	66	0.0	69

Stability

The stock calibrator, controls, antibodies, and diluent were tested for freeze-thaw stability. Results (not shown) demonstrated that each component could undergo four freeze-thaw cycles without significantly affecting the performance of the assay. Stock calibrator, antibodies, and controls must be stored frozen at ≤–70 ºC. The validation study included a real-time stability study with scheduled performance evaluations of complete kits for up to 36 months from the 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 (Table 9). 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 α-Synuclein Kit

Sample Type	Statistic	α-Synuclein
CSF (*n* = 20)	Median (pg/mL)	263
	Range (pg/mL)	141–396
	% Detected	100
Saliva (*n* = 32)	Median (pg/mL)	136
	Range (pg/mL)	54–14,875
	% Detected	91
Serum (*n* = 20)	Median (pg/mL)	7,698
	Range (pg/mL)	2,525–20,332
	% Detected	100
EDTA Plasma (*n* = 20)	Median (pg/mL)	19,424
	Range (pg/mL)	9,934–48,940
	% Detected	95
Heparin Plasma (*n* = 20)	Median (pg/mL)	8,856
	Range (pg/mL)	3,783–18,649
	% Detected	100
Citrate Plasma (*n* = 20)	Median (pg/mL)	3,558
	Range (pg/mL)	1,601–15,688
	% Detected	95

Assay Components

Calibrators

The assay calibrator uses recombinant human α-Synuclein (residues 1-140) expressed in E. coli.

Antibodies

Table 10. Antibody source species and generation

Analyte	Source Species		Assay Generation
	MSD Capture Antibody	MSD Detection Antibody	
α-Synuclein	Rabbit Monoclonal	Mouse Monoclonal	A

Summary Protocol

U-PLEX Human α-Synuclein Kit

MSD provides this summary protocol for your convenience.

Please read the entire detailed protocol before performing the U-PLEX Human α-Synuclein assay.

Sample and Reagent Preparation

- ❑ Thaw frozen reagents (calibrators, antibodies, 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 the 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 the plate 3 times with at least 150 µL/well of Wash Buffer.
- ❑ Add 150 µL/well of 1X Read Buffer T.

Frequently Asked Questions

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

The validated U-PLEX Human α-Synuclein Kit (catalog number K151WKK-2) was directly compared to the previously developed Human α-Synuclein Kit (catalog number K151TGD-2). The assays achieve similar sensitivity, as well as quantitation of controls, human CSF, and serum samples. The correlation between sample quantitation on the two assays was very strong ($R^2 = 0.97$). 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 before 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 number R50AM-1 and catalog number R50AM-2) can be purchased at www.mesoscale.com.

Can samples be used 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 dilutions. In addition, changing the dilution factor can risk moving samples out of the quantitative 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 because 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 K_2EDTA plasma, sodium heparin plasma, and sodium citrate plasma were tested in validation work. MSD does not recommend one form over another; however, differences in quantitation 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, because this may adversely affect performance.

Should plates be prewashed before use?

MSD does not recommend prewetting or prewashing plates before beginning an assay, as this may adversely affect performance.

Is the α-Synuclein calibrator a 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- or γ-Synuclein.

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Plate Diagram

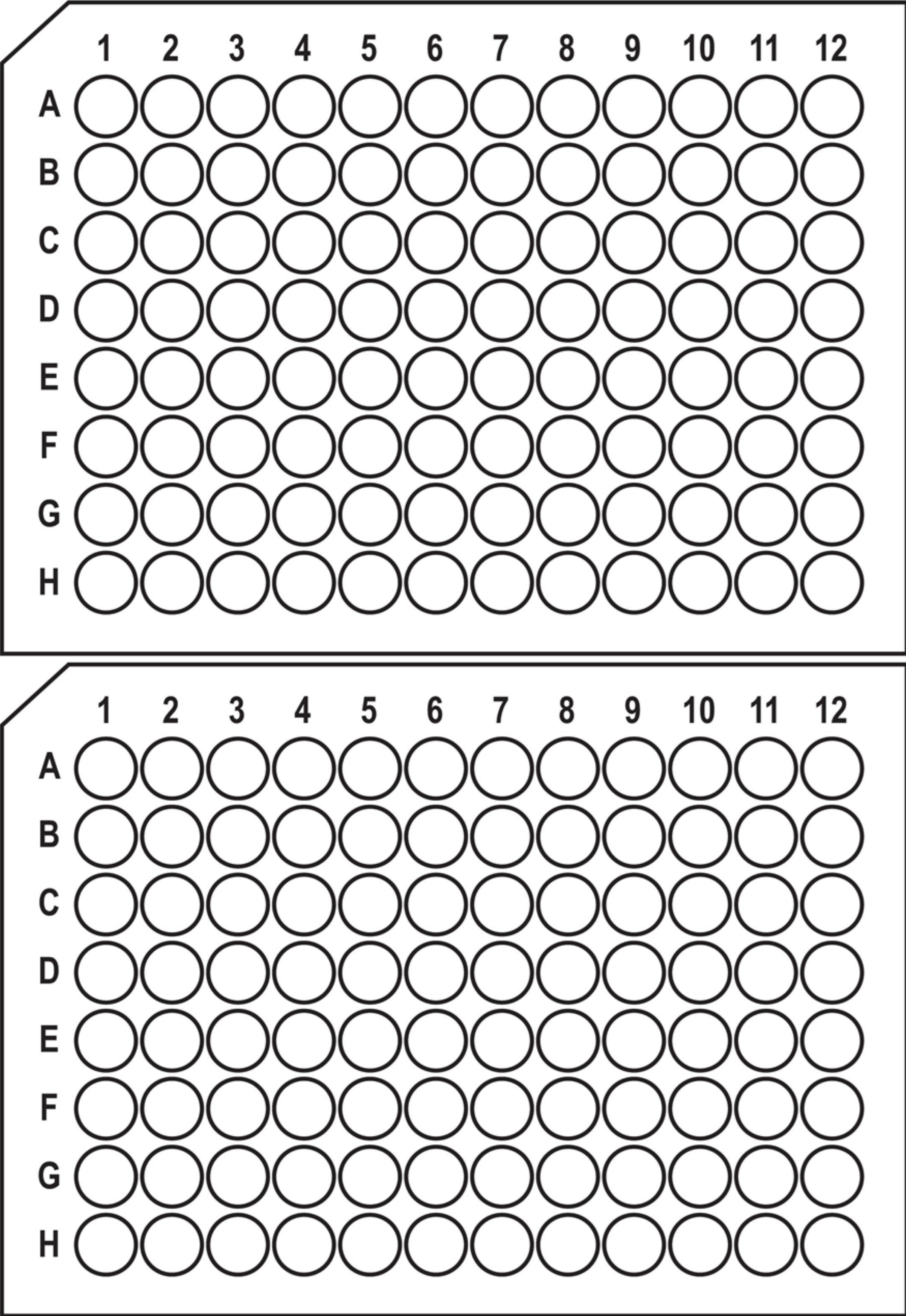

Figure 6. *Plate diagram. A similar plate layout can be created in Excel and easily imported into DISCOVERY WORKBENCH software.*