Version 19-04/24

User's Manual

Interleukin-6 ELISA kit

REF **DEIA-XY56**

Σ **96T**

RUO

This product is for research use only and is not intended for diagnostic use.

For illustrative purposes only. To perform the assay the instructions for use provided with the kit have to be used.

Creative Diagnostics

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PRODUCT INFORMATION

Intended Use

The IL-6 ELISA Kit is a sandwich immunoassay specifically designed and validated for the quantitative measurement of salivary IL-6. It is not intended for diagnostic use. This assay kit was designed and optimized for salivary research use in humans. CD has not validated this kit for serum, plasma or saliva samples from any other species.

Please read the complete kit insert before performing this assay. Failure to follow kit procedure and recommendations for saliva collection and sample handling may result in unreliable values.

General Description

Interleukin-6 (IL-6) is a pleiotropic cytokine involved in a multitude of inflammatory responses with roles in immune regulation and pathologic conditions including both acute and chronic inflammatory diseases. IL-6 initiates and up-regulates inflammation, triggers the release of acute phase proteins, regulates inflammatory response, attracts immune cells to sites of injury or infection and stimulates coagulation. Salivary levels have varying correlations to serum levels depending on the research applications.

Principles of Testing

This is a sandwich ELISA kit. IL-6 in standards and samples binds to the antibody binding sites on a microtitre plate. After incubation, unbound components are washed away. Biotin conjugated to goat antibodies to human IL-6 are added and attach to the bound IL-6. After incubation, unbound components are washed away. Streptavidin conjugated to horseradish peroxidase (HRP) is added and binds to the biotin conjugated to the goat antibodies. Bound Streptavidin-HRP is measured by the reaction of the horseradish peroxidase enzyme to the substrate tetramethylbenzidine (TMB). This reaction produces a blue color. A yellow color is formed after stopping the reaction with an acidic solution. The optical density is read on a standard plate reader at 450 nm. The amount of Streptavidin-HRP detected is proportional to the amount of IL-6 present in the sample.

Reagents And Materials Provided

1. Microtitre Plate, Coated with mouse anti-IL-6 monoclonal antibodies, 1/96 well

2. IL-6 Standard, 100 pg/mL, in a buffered solution with stabilizer protein. Serially dilute before use according to Reagent Preparation. Contains: IL-6, buffer, preservative. 1 vial / 1.5 mL

3. IL-6 Controls, High, Low, in a buffered solution with stabilizer protein. Contains: IL-6, buffer, preservative. 2 vials / 1 mL each

4. IL-6 Antibody Conjugate, Concentrate. Dilute before use with IL-6 Assay Diluent. Contains: anti-IL-6 conjugated to biotin, preservative. 1 vial / 50 µL

5. Streptavidin-HRP, Concentrate. Dilute before use with IL-6 Assay Diluent. Contains: Streptavidin conjugated to HRP, preservative. 1 vial / 200 µL.

6. IL-6 Sample Diluent, Contains: Trizma buffer with protein stabilizer, preservative.1 bottle / 15 mL

7. IL-6 Assay Diluent, Contains: buffer with protein stabilizer, preservative. 1 bottle / 30 mL

8. Wash Buffer Concentrate (10×), Dilute before use according to Reagent Preparation. Contains: phosphate buffer, detergent, preservative. 1 bottle / 100 mL.

9. TMB Substrate Solution, Non-toxic, ready to use. 1 bottle / 25 mL

10. Stop Solution, 1 bottle / 12.5 mL

Materials Required But Not Supplied

1. Precision pipette to deliver 10 µL to 300 µL
2. Precision multichannel pipette to deliver 50 µL and 100 µL
3. Vortex
4. Plate rotator with 0.08-0.17 inch orbit capable of 500 rpm
5. Plate reader with 450 nm and 620 to 630 reference filters
6. Computer software for data reduction
7. Deionized water
8. Reagent reservoirs
9. Two disposable polypropylene tubes to hold at least 12 mL
10. Small disposable polypropylene tubes for dilution of standard and samples
11. Pipette tips
12. Serological pipette to deliver up to 12 mL
13. Centrifuge capable of 1500 x g

Storage

All unopened components of this kit are stable at 2-8°C until the kit's expiration date

Specimen Collection And Preparation

Specimen Collection:

Avoid sample collection within 60 minutes after eating a major meal or within 12 hours after consuming alcohol. Acidic or high sugar foods can compromise assay performance by lowering sample pH and influencing bacterial growth. To minimize these factors, rinse mouth thoroughly with water 10 minutes before sample is collected.

Collect whole saliva by unstimulated passive drool. Donors may tilt the head forward, allowing the saliva to pool on the floor of the mouth, then pass the saliva through the SalivaBio Collection Aid (SCA) into a polypropylene vial.

Samples visibly contaminated with blood should be recollected. Samples may be screened for possible blood contamination using our Blood Contamination EIA Kit. Do not use dipsticks, which result in false positive values due to salivary enzymes. Record the time and date of specimen collection.

Sample Handling and Preparation:

After collection, it is important to keep samples cold in order to avoid bacterial growth in the specimen. Refrigerate sample within 30 minutes, and freeze at or below -20°C within 4 hours of collection. (Samples may be stored at -20°C for up to 6 months.)

Do not add sodium azide to saliva samples as a preservative, as it may cause interference in the immunoassay.

On day of assay, thaw the saliva samples completely, vortex, and centrifuge at 1500 x g for 15 minutes. Freezing saliva samples will precipitate mucins. Centrifuging removes mucins and other particulate matter which may interfere with antibody binding and affect results. Samples should be at room temperature before making dilutions. Pipette clear sample into appropriate dilution tubes. Re-freeze saliva samples as soon as possible after running assay. Re-centrifuge saliva samples each time that they are thawed. Avoid multiple freeze-thaw cycles. Saliva samples must be diluted for this assay. See Procedure for details.

Reagent Preparation

1. Bring all reagents to room temperature and mix before use. A minimum of 1.5 hours is recommended for the 12 mL of IL-6 Assay Diluent used in Steps 8 and 11 to come to room temperature.
2. Bring Microtitre Plate to room temperature before use. It is important to keep the foil pouch with the plate strips closed until warmed to room temperature, as humidity may have an effect on the coated wells.
3. Prepare 1X wash buffer by diluting Wash Buffer Concentrate (10X) 10-fold with roomtemperature deionized water (100 mL of Wash Buffer Concentrate (10X) to 900 mL of deionized water). Dilute only enough for current day's use and discard any leftover reagent. (If precipitate has formed in the concentrated wash buffer, it may be heated to 40°C for 15 minutes. Cool to room temperature before use in assay.)
4. Prepare serial dilutions of the IL-6 Standard as follows:

   Label six polypropylene microcentrifuge tubes or other small tubes 2 through 7.

   Pipette 300 µL of IL-6 Assay Diluent into tubes 2 through 7.

   Serially dilute the standard 2X by adding 300 µL of the 100 pg/mL standard (tube 1) to tube 2. Mix well.

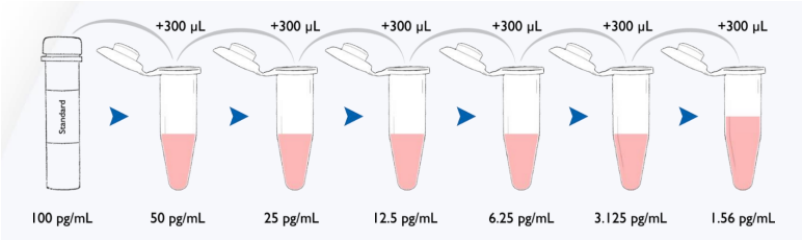
   After changing pipette tips, remove 300 µL from tube 2 to tube 3. Mix well.

   Continue for tubes 4, 5, 6 and 7.

   The final concentrations of standards for tubes 1 through 7 are, respectively, 100pg/mL, 50 pg/mL, 25 pg/mL, 12.5 pg/mL, 6.25 pg/mL, 3.125 pg/mL, and 1.56pg/mL.

   IL-6 Assay Diluent is used for the Zero Standard.

   (IL-6 molecular weight is 21-29 kDa, depending on post-translational modifications, 1 pg/mL = 3.4 to 4.7×10^{-5} pM.)



Assay Procedure

Notes:

1. This kit uses break-apart microtitre strips. You may run less than a full plate. Unused wells must be stored at 2-8°C in the foil pouch with desiccant and used in the frame provided.
2. Avoid microbial contamination of opened reagents. Salimetrics recommends using opened reagents within one month. Store all reagents at 2-8°C.
3. The quantity of reagent provided with a single kit is sufficient for two partial runs. The volumes of wash buffer, antibody conjugate and Streptavidin-HRP prepared for assays using less than a full plate should be scaled down accordingly, keeping the same dilution ratio.
4. Do not mix components from different lots of kits.
5. We recommend saving all reagents until data analysis has confirmed a successful run to facilitate troubleshooting if necessary.
6. Prior to sample addition, please label each strip to assure plate orientation and sample order when data is acquired on plate reader.
7. To ensure highest quality assay results, pipetting of samples and reagents must be done as quickly as possible (without interruption) across the plate. Ideally, the process should be completed within 20 minutes or less.
8. When using a multichannel pipette to add reagents, always follow the same sequence when adding all reagents so that the incubation time is the same for all wells.
9. When running multiple plates, or multiple sets of strips, a standard curve must be run with each individual plate and/or set of strips.
10. The temperature of the laboratory may affect assays. Salimetrics' kits have been validated at 68-74°F (20-23.3°C). Higher or lower temperatures may affect OD values.
11. Routine calibration of pipettes and other equipment is critical for the best possible assay performance.
12. When mixing plates during assay procedures, avoid speeds that spill the contents of the wells.

Procedure:

Step 1: Read and prepare reagents according to the Reagent Preparation section before beginning assay. Determine your plate layout. Here is a suggested layout. (Standards, controls, and saliva samples should be assayed in duplicate.)

	1	2	3	4	5	6	7	8	9	10	11	12
A	100 Std	100 Std	Ctrl-H	Ctrl -H								
B	50 Std	50 Std	Ctrl-L	Ctrl -L								
C	25 Std	25 Std	SMP-1	SMP-1								
D	12.5 Std	12.5 Std	SMP-2	SMP-2								
E	6.25 Std	6.25 Std	SMP-3	SMP-3								
F	3.125 Std	3.125 Std	SMP-4	SMP-4								
G	1.56 Std	1.56 Std	SMP-5	SMP-5								
H	0 Std	0 Std	SMP-6	SMP-6								

Step 2: Keep the desired number of strips in the strip holder and place the remaining strips back in the foil pouch. Reseal the foil pouch with unused wells and desiccant. Store at 2-8°C.

Step 3: Pipette 12 mL of IL-6 Assay Diluent into each of two different disposable tubes. (Scale down

proportionally if not using a full plate). Set aside for Step 8 and Step 11.

Step 4: Dilute saliva samples 5× in IL-6 Sample Diluent using 60 µL saliva to 240 µL IL-6 Sample Diluent. Do not dilute samples in IL-6 Assay Diluent.

Step 5:

a. Pipette 100 µL of standards, controls, and diluted saliva samples into appropriate wells.

b. Pipette 100 µL of IL-6 Assay Diluent into 2 wells to serve as the Zero Standard.

Step 6: Place adhesive cover provided over plate. Mix plate on a plate rotator continuously at 500 rpm for 1 hour at room temperature.

Step 7: Wash the plate 4 times with 1X wash buffer. A plate washer is recommended. However, washing may be done by gently squirting wash buffer into each well with a squirt bottle, or by pipetting 300 µL of wash buffer into each well and then discarding the liquid over a sink. After each wash, the plate should be thoroughly blotted on paper towels before turning upright. If using a plate washer, blotting is still recommended after the last wash.

Step 8: Dilute the antibody conjugate 1:500 by adding 24 µL of the antibody conjugate to the 12 mL of IL-6 Assay Diluent. (Scale down proportionally if not using the entire plate.) Antibody conjugate tube may be centrifuged for a few minutes to bring the liquid down to the tube bottom. Immediately mix the diluted antibody conjugate solution and add 100 µL to each well using a multichannel pipette.

Step 9: Place a new adhesive cover (provided) over plate. Mix plate on a plate rotator continuously at 500 rpm for 2 hours at room temperature.

Step 10: Repeat wash procedure from Step 7.

Step 11: Dilute the Streptavidin-HRP 1:100 by adding 120 µL of the Streptavidin-HRP to the 12 mL of IL-6 Assay Diluent.(Scale down proportionally if not using the entire plate.) Streptavidin-HRP tube may be centrifuged for a few minutes to bring the liquid down to the tube bottom. Immediately mix the diluted Streptavidin-HRP solution and add 100 µL to each well using a multichannel pipette.

Step 12: Mix plate on a plate rotator continuously at 500 rpm for 20 minutes at room temperature.

Step 13: Repeat wash procedure from Step 7.

Step 14: Add 100 µL of TMB Substrate Solution to each well with a multichannel pipette.

Step 15: Mix on a plate rotator for 5 minutes at 500 rpm and incubate the plate in the dark (covered) at room temperature for an additional 15 minutes.

Step 16: Add 50 µL of Stop Solution with a multichannel pipette.

Step 17:

a. Mix on a plate rotator for 3 minutes at 500 rpm. If green color remains, continue mixing until green color turns to yellow. Be sure all wells have turned yellow. Caution: Spillage may occur if mixing speed exceeds 600 rpm.

b. Wipe off bottom of plate with a water-moistened, lint-free cloth and wipe dry.

c. Read in a plate reader at 450 nm. Read plate within 10 minutes of adding Stop Solution. (For best results, a secondary filter correction at 620 to 630 nm is recommended.)

Quality Control

The CD High and Low IL-6 Controls should be run with each assay. The control ranges established at CD are to be used as a guide. Each laboratory should establish its own range. Variations between laboratories may be caused by differences in techniques and instrumentation.

Calculation

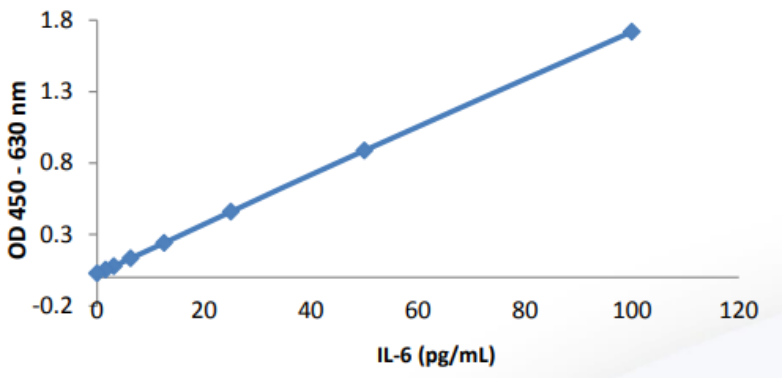
1. Compute the average optical density (OD) for all duplicate wells.
2. Determine the concentrations of the controls and saliva samples by interpolation using data reduction software. We recommend using a 4-parameter non-linear regression curve fit.
3. Multiply the calculated concentrations of the saliva samples by the dilution factor of 5 to obtain final IL-6 concentrations in pg/mL.
4. Samples (diluted 5×) with IL-6 values greater than 100 pg/mL (or >500 pg/mL after multiplying by the dilution factor of 5) should be diluted further with IL-6 Sample Diluent and rerun for accurate results. If a dilution of the sample is used, multiply the results by the additional dilution factor. **A new Standard Curve must be run with each full or partial plate.**

Typical Standard Curve

The results shown below are for illustration only and should not be used to calculate results from another assay.

Well	Standard	Average OD	IL-6 (pg/mL)
A1,A2	S1	1.719	100.0
B1,B2	S2	0.888	50.0
C1,C2	S3	0.459	25.0
D1,D2	S4	0.240	12.5
E1,E2	S5	0.133	6.25
F1,F2	S6	0.078	3.125
G1,G2	S7	0.053	1.56
H1,H2	Zero	0.028	0.0

Example: IL-6 4-Parameter Curve Fit



Performance Characteristics

Salivary IL-6 Example Ranges*

Group	N	Median (pg/mL)	Min (pg/mL)	Max (pg/mL)
Adult Passive Drool	19	1.27	0	5.37

***To be used as a guide only. Each laboratory should establish its own range.**

IL-6 levels obtained using the CD Salivary IL-6 (Interleukin-6) Enzyme Immunoassay Kit, on saliva from normal healthy subjects, may be very low or not detectable. CD recommends using pilot studies in the population(s) of interest to determine if baseline measures will be in the readable range. This kit was developed and validated using a 1:5 dilution of saliva. The sample diluent contains a factor necessary for this IL-6 assay to work properly.

Drift

Drift was determined by individually pipetting 96 wells of one IL-6 concentration across the plate and determining the CV of the optical densities for all wells. The CV resulting from this calculation was 2.7%.

Precision

The intra-assay precision was determined from the mean of 20 replicates each.

Saliva Sample	N	Mean (pg/mL)	Standard Deviation (pg/mL)	Coefficient of Variation (%)
1	20	323	8.4	3
2	20	63	1.5	2
3	20	67	1.7	3
4	20	9	0.5	6
5	20	4	0.4	10

The inter-assay precision was determined from the mean of average duplicates for 10 separate runs.

Saliva Sample	N	Mean (pg/mL)	Standard Deviation (pg/mL)	Coefficient of Variation (%)
1	20	9	0.7	8
2	20	29	1.8	6
3	20	173	8.7	5
4	20	342	19.1	6
5	20	56	4.3	8

Sensitivity

Analytical Sensitivity: The lower limit of sensitivity was determined by interpolating the mean optical density plus 2 SDs of 10 sets of duplicates at the 0 pg/mL level. The minimal concentration of IL-6 that can be distinguished from 0 is 0.07 pg/mL (0.35 when multiplied by the x5 dilution factor).

Functional Sensitivity: The functional sensitivity was determined by assaying 20 samples at a concentration level resulting in a CV of 20%. The functional sensitivity of the salivary IL-6 ELISA is 2.08 pg/mL (10.4 pg/mL when multiplied by the ×5 dilution factor).

Specificity

The listed factors were found to have no cross reactivity or interference when assayed at 50ng/mL and 100 ng/mL: Human IL-11, Human CNTF, Human G-CSF, Human Sgp130, Human IL-6 sR, Human IL-12, Human LIF, Human LIF R, Human OSF, Mouse IL-6, Mouse IL-11, Mouse IL-12 and Rat CNTF.

Linearity

Two saliva samples were serially diluted with each other proportionately and assayed.

Saliva Sample	Dilution Factor	Expected (pg/mL)	Observed (pg/mL)	Recovery (%)
1			42.31	
	1:9	69.54	67.17	97
	2:8	96.77	94.70	98
	3:7	124.00	122.97	99
	4:6	151.23	147.44	97
	5:5	178.46	153.89	86
	6:4	205.69	184.02	89
	7:3	232.92	210.44	90
	8:2	260.15	240.55	92
	9:1	287.38	272.47	95
2			314.61	

Recovery

Three saliva samples containing different levels of an endogenous IL-6 were spiked with known quantities of IL-6 and assayed.

Saliva Sample	Endogenous (pg/mL)	Added (pg/mL)	Expected (pg/mL)	Observed (pg/mL)	Recovery (%)
1	3.63	402.39	406.02	408.40	101
		83.77	87.40	83.49	96
		32.65	36.28	34.13	94
2	5.79	390.32	396.11	421.29	106
		80.86	86.65	85.70	99
		31.60	37.39	36.79	98
3	17.57	394.44	412.01	416.25	101
		80.00	97.57	93.69	96
		31.60	49.17	46.43	94

Sample Dilution Recovery

Three saliva samples were serially diluted with IL-6 Sample Diluent and assayed.

Saliva Sample	Endogenous (pg/mL)	Dilution Factor	Expected (pg/mL)	Observed (pg/mL)	Recovery (%)
1	336.14	1:2	168.07	170.64	102
		1:4	84.04	87.93	105
		1:8	42.02	45.51	108
2	71.04	1:2	35.52	36.13	102
		1:4	17.76	18.72	105
		1:8	8.88	9.93	112
3	172.74	1:2	86.37	88.87	103
		1:4	43.19	44.25	102
		1:8	21.59	22.65	105

Precautions

Hazardous Ingredients

Liquid Stop Solution is caustic; use with care. We recommend the procedures listed below for all kit reagents.

Handling

Follow good laboratory practices when handling kit reagents. Laboratory coats, gloves, and safety goggles are recommended. Wipe up spills using appropriate absorbent materials while wearing protective clothing. Follow local regulations for disposal.

Emergency Exposure Measures

In case of contact, immediately wash skin or flush eyes with water for 15 minutes. Remove contaminated clothing. If inhaled, remove individual to fresh air. If individual experiences difficulty breathing call a physician.

Limitations

1. Samples (diluted 5X) with IL-6 values greater than 100 pg/mL (or >500 pg/mL after multiplying by the dilution factor of 5) should be diluted further with IL-6 Sample Diluent and rerun for accurate results. If a dilution of the sample is used, multiply the results by the additional dilution factor.
2. See "Specimen Collection" recommendations to ensure proper collection of saliva specimens and to avoid interfering substances.
3. Samples collected with sodium azide are unsuitable for this assay.
4. Any quantitative results indicating abnormal IL-6 levels should be followed by additional testing and evaluation.