Version 01-10/25

User's Manual

Pembrolizumab ELISA Kit

REF **DEIAZ0010**

Σ **96T**

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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.

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PRODUCT INFORMATION

Intended Use

The Pembrolizumab ELISA kit is a validated tool for whole Pembrolizumab and its biosimilar quantification in biological matrices for drug research and development. The kit can be also used for the detection of single chain variable fragment (scFv) of Pembrolizumab.

Principles of Testing

Pembrolizumab ELISA Kit is a classic bridging ELISA assay with a pair of anti-idiotypic monoclonal capture and detection antibodies. When standards or samples are added to the capture plate, the Pembrolizumab in the sample can be captured on the plate coated with the Pembrolizumab capture antibody. Then the Biotin Anti-Pembrolizumab Antibody is added to interact with the Pembrolizumab bound on the plate. Streptavidin-HRP (Streptavidin-Horseradish Peroxidase conjugate) is added to interact with the Biotin Anti-Pembrolizumab Antibody. After the washing steps, TMB Solution is added, resulting in the formation of blue color. The reaction is stopped by adding Stop Solution. Application of the Stop Solution results in the color changing from blue to yellow. The intensity of the color can be read at 450 nm and 630 nm by a microplate reader.

The quantity of Pembrolizumab in the sample is precisely quantified against a Pembrolizumab standard curve.

Reagents And Materials Provided

1. Capture Plate: 1 plate
2. Standard Stock: 1 vial (50 µL)
3. Sample Dilution Buffer: 1 bottle (60 mL)
4. Biotin Anti-Pembrolizumab Antibody: 1 bottle (12 mL)
5. Streptavidin-HRP: 1 bottle (12 mL)
6. TMB Solution: 1 bottle (12 mL)
7. 20× Wash Solution: 1 bottle (60 mL)
8. Stop Solution: 1 bottle (6 mL)
9. Plate Sealer: 2 pieces

Note: l Capture Plate: 96 well microplates (8 wells x 12 strips); 12 strips are configured in plate; plate is sealed in a foil pouch with a desiccant.

l Standard Stock contains 100 µg/mL of Pembrolizumab.

Materials Required But Not Supplied

1. Fresh matrix (normal serum or plasma from human or cynomolgus monkey)
2. Microplate reader capable of measurement at 450 nm with the correction wavelength set at 630 nm
3. Data analysis and graphing software. It is recommended to use software which is capable of generating a four-parameter logistic (4-PL) curve-fit

4. Automated microplate washer
5. Deionized or distilled water
6. Graduated cylinder
7. Plastic container
8. Tubes to aliquot and dilute samples
9. 10 µL, 200 µL, and 1000 µL precision pipettes and pipette tips
10. Multichannel pipettes
11. Disposable reagent reservoir
12. Absorbent paper
13. Lboratory timer
14. Refrigerator
15. Centrifuge
16. 25 ± 2 °C and 37± 2 °C incubator
17. Rotary shaker

Storage

The unopened kit is stable for at least 12 months from the date of manufacture at 2°C to 8°C, and the opened kit is stable for up to 1 month from the date of opening at 2°C to 8°C.

Specimen Collection And Preparation

1. The handling and storage information provided here is intended to be used as a general guideline. Sample stability has not been evaluated. When samples need to be stored for a long time, users need to evaluate the stability of the samples. It is the responsibility of the individual laboratory to use all available references and/or its own studies when establishing alternate stability criteria that meet their needs.
2. Store specimens at -20°C or lower if not tested immediately. Avoid repeated freeze-thaw cycles.

Reagent Preparation

All reagents must be equilibrated to room temperature before use (20°C-25°C). All samples and reagents should be vortexed before use. Store all reagents back in refrigerator promptly after use.

1. **1× Wash Solution:** Dilute the 20× Wash Solution with deionized or distilled water with a volume ratio of 1:19. For example, dilute 40 mL of 20× Wash Solution with 760 mL of deionized or distilled water to make 800 mL of 1× Wash Solution. Store the solution at 2°C to 8°C when not in use.

Note: If any precipitate is found in the 20× Wash Solution, incubate the bottle in a water bath (up to 50°C) with occasional mixing until all the precipitate is dissolved.

2. **Calibration Standard Preparation:** Calibration standards should be prepared with a fresh matrix to generate eight Pembrolizumab concentrations: fresh matrix (NC), 2.5 (AP), 5, 10, 20, 40, 80, and 160 ng/mL. Preparation of a whole set of standards is recommended as table 1. S-TOK1 preparation is described below as an example.

Note: NC is Negative Control. AP is Anchor Point. The Anchor Point is used to improve the fit of the curve.

3. **S-TOK1 Preparation:** Vortex and Centrifuge Standard Stock for several seconds. Dilute Standard Stock with a fresh matrix with a volume ratio of 1:50. For example, add 5 µL of Standard Stock to 245 µL of fresh matrix and mix it well to make 250 µL of >S-TOK1.

Table 1. Recommended standard preparation

Standard ID	Dilution Factor	Source	Source Volume (µL)	Matrix Volume (µL)	Final Volume (µL)	Final Conc. (ng/mL)
S-TOK1	50	Standard Stock (H1-10)	5	245	250	2,000
Std1	12.5	S-TOK1	8	92	100	160
Std2	2	Std1	30	30	60	80
Std3	2	Std2	30	30	60	40
Std4	2	Std3	30	30	60	20
Std5	2	Std4	30	30	60	10
Std6	2	Std5	30	30	60	5
AP	2	Std6	30	30	60	2.5
NC	0	/	/	60	60	/

4. **Quality Control Preparation:** QCs should be prepared with fresh matrix to generate five Pembrolizumab concentrations: 15 (LQC), 30 (MQC), 120 (HQC), 5 (LLOQ), and 160 (ULOQ) ng/mL. Preparation of a whole set of standards is recommended as table 2. Q-TOK1 preparation is described below as an example.

Note: QC is quality control. LQC is low quality control. MQC is medium quality control. HQC is high quality control. LLOQ is lower limit of quantification. ULOQ is upper limit of quantification.

5. **Q-TOK1 preparation:** Vortex and Centrifuge Standard Stock for several seconds. Dilute Standard Stock with a fresh matrix with a volume ratio of 1:50. For example, add 5 µL of Standard Stock to 245 µL of fresh matrix and mix it well to make 250 µL of Q-TOK1.

Table 2. Recommended quality control preparation

QC ID	Dilution Factor	Source	Source Volume (µL)	Matrix Volume (µL)	Final Volume (µL)	Final Conc. (ng/mL)
Q-TOK1	50	Standard Stock (H1-10)	5	245	250	2,000
ULOQ	12.5	Q-TOK1	8	92	100	160
HQC	1.33	ULOQ	60	20	80	120
MQC	4	HQC	15	45	60	30
LQC	2	MQC	20	20	40	15
LLOQ	3	LQC	10	20	30	5

6. **Capture Plate Preparation**

1. It is recommended that all standards, quality controls, and samples be prepared in duplicate at least. Table 3 is an example for setup of Pembrolizumab standards and samples.

2. Count the strips according to the number of test samples and install the strips. Make sure the strips are tightly snapped into the plate frame.

3. Leave the unused strips in the foil pouch and store at 2°C to 8°C. The strips must be stored in the closed foil pouch to prevent moisture from damaging the Capture Plate.

Table 3. Setup of standards, quality controls and samples on Capture Plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	Std1	Std1	ULOQ	ULOQ	S4	S4	S12	S12	S20	S20	S28	S28
B	Std2	Std2	HQC	HQC	S5	S5	S13	S13	S21	S21	S29	S29
C	Std3	Std3	MQC	MQC	S6	S6	S14	S14	S22	S22	S30	S30
D	Std4	Std4	LQC	LQC	S7	S7	S15	S15	S23	S23	S31	S31
E	Std5	Std5	LLOQ	LLOQ	S8	S8	S16	S16	S24	S24	S32	S32
F	Std6	Std6	S1	S1	S9	S9	S17	S17	S25	S25	S33	S33
G	AP	AP	S2	S2	S10	S10	S18	S18	S26	S26	S34	S34
H	NC	NC	S3	S3	S11	S11	S19	S19	S27	S27	S35	S35

S: Sample number

Assay Procedure

Standards and Samples Incubation

1. Dilute standards, QCs and samples with Sample Dilution Buffer with a volume ratio of 1:100.

Note: Both standards and QCs are working solutions that have been diluted in matrix, see VIII. PROTOCOL.Reagent Preparation for step details.

2. Add 100 µL of the diluted standard solutions, controls and samples to the corresponding wells in the Capture Plate. Place the plate on a rotary shaker for 30 -60 seconds for mixture.
3. Cover the plate with Plate Sealer and incubate at 37°C for 60 minutes.
4. Remove the Plate Sealer and wash the plate with 260 µL of 1× Wash Solution four times.
5. Tap the inverted plate onto absorbent paper to remove residual liquid in the wells after the wash steps.

Detection Antibody Incubation

6. Add 100 µL of Biotin Anti-Pembrolizumab Antibody to all the testing wells.
7. Cover the plate with Plate Sealer and incubate at 37°C for 30 minutes.
8. Remove the Plate Sealer and wash the plate with 260 µL of 1× Wash Solution four times.
9. Tap the inverted plate onto absorbent paper to remove residual liquid in the wells after the wash steps.

Enzyme Conjugate Incubation

10. Add 100 µL of Streptavidin-HRP to all the testing wells.
11. Cover the Plate with Plate Sealer and incubate at 37°C for 10 minutes.
12. Remove the Plate Sealer and wash the plate with 260 µL of 1× Wash Solution four times.
13. Tap the inverted plate onto absorbent paper to remove residual liquid in the wells after the wash steps.

Absorbance Measurement and Calculation

14. Add 100 µL of TMB Solution to each well and incubate the plate in the dark at 25°C for 15 minutes (start timing after the addition of TMB Solution to the first well) .

Note: TMB incubation time could extend to 20 minutes based on test signals.

15. Add 50 µL of Stop Solution to each well to stop the reaction.
16. Read the absorbance in the microplate reader at 450 nm against 630 nm as a reference filter.
17. Plot the standard curve with the Pembrolizumab concentration (ng/mL) on the x-axis and the corresponding mean absorbance value on the y-axis.
18. Using a 4- or 5-parameter logistic curve fitting program, calculate the best-fitting linear line through the points of the standard curve.

ASSAY PROCEDURE SUMMARY

• Prepare 1× Wash Solution and Capture Plate.

• Dilute Standard Stock with fresh matrix to generate calibration standards and QCs

• Dilute the test samples and a set of standards and QCs with Sample Dilution Buffer.

• Add 100 µL of the diluted standard solutions, controls and samples to the corresponding wells. Incubate the plate at 37°C for 60 minutes.

• Wash the plate with 260 µL of 1× Wash Solution per well four times.

• Add 100 µL of the Biotin Anti-Pembrolizumab Antibody to the well and incubate at 37°C for 30 minutes.

• Wash the plate with 260 µL of 1× Wash Solution per well four times.

• Add 100 µL of the Streptavidin-HRP and incubate at 37°C for 10 minutes.

• Wash the plate with 260 µL of 1× Wash Solution per well four times.

• Add 100 µL of TMB Solution and incubate the plate in dark at 25°C for 15 minutes.

• Add 50 µL of Stop Solution to each well to stop the reaction.

• Read the plate immediately.

Performance Characteristics

LLOQ 5 ng/mL

ULOQ 160 ng/mL

Intra-assay CV ≤10%

Inter-assay CV ≤15%

Minimum required dilution (MRD) 1:100 selected by cynomolgus monkey plasma

Specificity No cross-reactivity at 1600 ng/mL of Human IgG4

Hook effect Not observed at 15000 ng/mL of Pembrolizumab

Precision

Pembrolizumab Quality Controls at five concentrations (ULOQ of 160 ng/mL, HQC of 120 ng/mL, MQC of 30 ng/mL, LQC of 15 ng/mL and LLOQ of 5 ng/mL) were measured for intra-and inter-assay accuracy.

Quality Control	PBZ (ng/mL)	Intra-assay (n=3)			Inter-assay (n=9)		
		Measured PBZ (ng/mL)	CV %	Accuracy %	Measured PBZ (ng/mL)	CV %	Accuracy %
ULOQ	160	153.67	1.99	96.05	150.93	2.97	94.33
HQC	120	109.94	2.62	91.62	110.99	4.21	92.49
MQC	30	28.39	6.53	94.64	28.20	5.28	94.01
LQC	15	14.04	5.84	93.57	14.34	6.72	95.60
LLOQ	5	4.32	8.19	86.44	4.73	7.91	94.64

Detection Range

5 ng/mL-160 ng/mL

Specificity

Pembrolizumab QC samples at two concentrations (ULOQ of 160 ng/mL and LLOQ of 5 ng/mL) were spiked with different amounts of human IgG4 (160 and 1600 ng/mL). The test result demonstrated that the high concentration of human IgG4 did not interfere with the detection of Pembrolizumab.

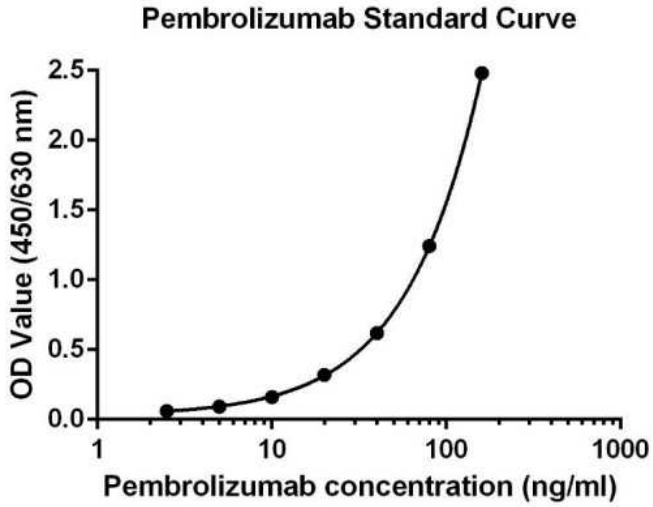
PBZ (ng/mL)	Human IgG4 (ng/mL)	Measured PBZ (ng/mL)	CV%	Accuracy%
160	1,600	136.90	2.04	85.56
160	160	142.48	0.46	89.05
5	1,600	5.69	5.13	113.86
5	160	5.60	0.51	111.92

Linearity

A set of Pembrolizumab (PBZ) calibration standards were freshly prepared and analyzed. Standard curves were constructed using a four or five-parameter logistic curve. The typical dynamic range of the kit is 5-160 ng/mL (0.05-1.60 ng/mL diluted) and its detection limit is 5 ng/mL.

Table . Sample data for stan dard curve

PBZ (ng/mL)	Absorbance (OD 450/630nm)			Measured PBZ (ng/mL)	CV %	Accuracy %
	Duplicate 1	Duplicate 2	Average			
160	2.408	2.559	2.483	160.00	4.36	100.00
80	1.254	1.231	1.242	80.02	1.26	100.03
40	0.651	0.587	0.619	39.88	7.42	99.70
20	0.340	0.300	0.320	20.32	9.18	101.60
10	0.169	0.155	0.162	9.72	7.01	97.20
5	0.092	0.093	0.093	4.91	1.83	98.20
2.5	0.061	0.061	0.061	2.65	0.19	106.00
NC	0.026	0.028	0.027	N/A	N/A	N/A



A set of Pembrolizumab (PBZ) calibration standards from 160 ng/mL to 2.5 ng/mL was then diluted with Sample Dilution Buffer with a volume ratio of 1:100.

Recovery

Selectivity was tested by spiking plasma of ten different samples from cynomolgus monkeys with Pembrolizumab Quality Controls at two concentrations (HQC of 120 ng/mL and LLOQ of 5 ng/mL). The mean accuracy for LLOQ was required to be within 75%-125% of the low spiked concentration in at least 90% of the evaluated matrices. The mean accuracy for HQC was required to be within 80%-120% of the high spiked concentration in at least 100% of the evaluated matrices.

HQC-Selectivity			LLOQ-Selectivity		
Measured PBZ (ng/mL)	CV%	Accuracy%	Measured PBZ (ng/mL)	CV%	Accuracy%
96.64	2.52	80.54	2.51	4.46	50.22
103.05	3.70	85.87	5.29	1.05	105.78
107.10	0.92	89.25	5.21	3.90	104.22
110.28	4.34	91.90	4.92	1.01	98.36
108.26	6.36	90.22	4.97	2.24	99.42
111.30	5.28	92.75	5.09	1.33	101.78
114.19	3.74	95.16	5.84	6.89	116.76
126.40	2.29	105.33	5.47	6.61	109.42
107.82	5.54	89.85	4.79	8.16	95.82
102.95	1.49	85.79	5.06	10.61	101.22

Precautions

1. All reagents containing human material should be handled as potentially infectious. Operators should wear gloves and protective clothing when handling any patient sera or serum based products.
2. Reagents that contain preservatives may be toxic if ingested, inhaled, or spilled on the skin.
3. Avoid contact of skin, eyes, or clothing with Stop Solution or TMB Substrate. Keep the container tightly closed. In case of an accident, please seek medical advice immediately.
4. Do not use the kit if there is any visible damage to the packaging or kit contents.
5. Do not mix components from different batches. Do not mix with components from other manufacturers.
6. Do not use reagents beyond the stated expiry date.
7. All reagents must be equilibrated to room temperature (20°-25°C) before running the assay. Only take an appropriate amount of reagents at once. Do not put unused reagents back into the vials as reagent contaminations may occur.
8. Before opening the Standard Stock, quickly span the vial to ensure that all the liquid has collected at the bottom, and prevent the liquid from splashing when opening the lid.
9. Use only distilled or deionized water and clean glassware.
10. Do not let wells dry during the test, add reagents immediately after completing washing steps.