

6. Microplate washing is important. Improperly washed wells will give erroneous results. It is recommended to use a multichannel pipette or an automatic microplate washing system. Do not allow the wells to dry between incubations. Do not scratch coated wells during rinsing and aspiration. Rinse and fill all reagents with care. While rinsing, check that all wells are filled precisely with Wash Buffer, and that there are no residues in the wells.
7. Humidity affects the coated wells/tubes. Do not open the pouch until it reaches room temperature. Unused wells/tubes should be returned immediately to the resealed pouch including the desiccant.

Preparation of Component

Dilute/ dissolve	Component	with	Diluent	Relation	Remarks	Storage	Stability
10 mL	Wash Buffer*	Up to 200 mL	Bidist. water	1:20	Warm up at 37°C to dissolve crystals. Mix vigorously.	2-8 °C	3 w

*. Prepare Wash Buffer before starting assay procedure.

Test Procedure

1	Pipette 100µl of Assay Buffer non-exceptionally into each of the wells to be used.
2	QUALITATIVE ELISA TEST FORMAT Pipette 10 µL of ready-to use Negative Control, Reactive Control, and Samples into the respective wells of microtiter plate. <u>Wells</u> A1: Negative Control B1: Negative Control C1: Reactive Control D1 and on.: Sample (Serum/Plasma)
3	Cover the plate with adhesive film. Briefly mix contents by gently shaking the plate. Incubate 60 min at room temperature (18-25°C).
4	Remove adhesive film. Discard incubation solution. Wash plate 3 times each with 300 µL of diluted Wash Buffer. Remove excess solution by tapping the inverted plate on a paper towel.
5	Pipette 100 µL of ready-to use Peroxidase Conjugate into each well.
6	Cover the plate with adhesive film. Incubate 60 min at room temperature (18- 25°C).
7	Remove adhesive film. Discard incubation solution. Wash plate 3 times each with 300 µL of diluted Wash Buffer. Remove excess solution by tapping the inverted plate on a paper towel.
8	Pipette 100 µL of TMB Substrate Solution into each well.
9	Incubate 20 min (without adhesive foil.) at room temperature (18-25°C) in the dark.
10	Stop the substrate reaction by adding 100 µL of Stop Solution into each well. Briefly mix contents by gently shaking the plate. Color changes from blue to yellow
11	Measure optical density with a photometer at 450/650 nm within 30 min after pipetting of the Stop Solution.

Interpretation Of Results

For the run to be valid, the OD₄₅₀ nm of Positive Control should be ≥ 1.00 and the OD_{450/650} nm of each Negative Control should be <0.200 . If not, improper technique or reagent deterioration may be suspected and the run should be repeated.

The results are evaluated by a cut-off value which is estimated by multiplying the mean OD_{450/650} nm of the negative controls by 3.

E.g.;

If "Sample OD_{450/650} the mean OD₄₅₀ /650 of Negative Controls" is ≥ 3 , the sample is POSITIVE If "Sample OD_{450/650} the mean OD_{450/650} of Negative Controls" is <3 , the sample is NEGATIVE

REFERENCES

1. Eigentler TK, Hassel JC, Berking C, Aberle J, Bachmann O, Grünwald V, Kähler KC, Loquai C, Reinmuth N, Steins M, Zimmer L, Sendl A, Gutzmer R. Diagnosis, monitoring and management of immune-related adverse drug reactions of anti-PD-1 antibody therapy. *Cancer Treat Rev.* 2016 Apr;45:7-18. doi: 10.1016/j.ctrv.2016.02.003. Epub 2016 Feb 18.
2. Zhu Z, Liu W, Gotlieb V. The rapidly evolving therapies for advanced melanoma--Towards immunotherapy, molecular targeted therapy, and beyond. *Crit Rev Oncol Hematol.* 2016 Mar;99:91-9. doi: 10.1016/j.critrevonc.2015.12.002. Epub 2015 Dec 10.
3. Márquez-Rodas I, Cerezuela P, Soria A, Berrocal A, Riso A, González-Cao M, Martín-Algarra S. Immune checkpoint inhibitors: therapeutic advances in melanoma. *Ann Transl Med.* 2015 Oct;3(18):267. doi: 10.3978/j.issn.2305-5839.2015.10.27.
4. Sgambato A, Casaluze F, Sacco PC, Palazzolo G, Maione P, Rossi A, Ciardiello F, Gridelli C. Anti PD-1 and PDL-1 Immunotherapy in the Treatment of Advanced Non- Small Cell Lung Cancer (NSCLC): A Review on Toxicity Profile and its Management. *Curr Drug Saf.* 2016;11(1):62-8.
5. Weber JS, D'Angelo SP, Minor D, Hodi FS, Gutzmer R, Neyns B, Hoeller C, Khushalani NI, Miller WH Jr, Lao CD, Linette GP, Thomas L, Lorigan P, Grossmann KF, Hassel JC, Maio M, Sznol M, Ascierto PA, Mohr P, Chmielowski B, Bryce A, Svane IM, Grob JJ, Krackhardt AM, Horak C, Lambert A, Yang AS, Larkin J. Nivolumab versus chemotherapy in patients with advanced melanoma who progressed after anti-CTLA-4 treatment (CheckMate 037): a randomised, controlled, open-label, phase 3 trial. *Lancet Oncol.* 2015 Apr;16(4):375-84. doi: 10.1016/S1470-2045(15)70076-8. Epub 2015 Mar 18.
6. Topalian SL, Hodi FS, Brahmer JR, Gettinger SN, Smith DC, McDermott DF, Powderly JD, Carvajal RD, Sosman JA, Atkins MB, Leming PD, Spigel DR, Antonia SJ, Horn L, Drake CG, Pardoll DM, Chen L, Sharfman WH, Anders RA, Taube JM, McMiller TL, Xu H, Korman AJ, Jure-Kunkel M, Agrawal S, McDonald D, Kollia GD, Gupta A, Wigginton JM, Sznol M. Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. *N Engl J Med.* 2012 Jun 28;366(26):2443-54. doi: 10.1056/NEJMoa1200690. Epub 2012 Jun 2.

7. Brahmer JR, Drake CG, Wollner I, Powderly JD, Picus J, Sharfman WH, Stankevich E, Pons A, Salay TM, McMiller TL, Gilson MM, Wang C, Selby M, Taube JM, Anders R, Chen L, Korman AJ, Pardoll DM, Lowy I, Topalian SL. Phase I study of single-agent anti-programmed death-1 (MDX-1106) in refractory solid tumors: safety, clinical activity, pharmacodynamics, and immunologic correlates. *J Clin Oncol*. 2010 Jul 1;28(19):3167-75. doi: 10.1200/JCO.2009.26.7609. Epub 2010 Jun 1.

8. Nivolumab, National Cancer Institute.

<http://www.cancer.gov/about-cancer/treatment/drugs/nivolumab>