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Affinity Determination of Monoclonal Antibodies (mAbs) Using Enzyme- Linked Immunosorbent Assay (ELISA); A Protocol

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ABSTRACT

Monoclonal antibodies (mAbs) have changed diagnostics and therapy due to their high specificity and affinity to the target antigens (Ags). Accurately measuring the affinity of mAbs is critical to understanding their binding properties. It represents the strength of binding between an antibody (Ab) and its target Ag and enables decision making in the development and optimization of these antibodies to improve their efficacy in diagnostics and therapy. Various methods such as the equilibrium dissociation constant, ELISA, surface plasmon resonance (SPR), and microarray-based platforms can be used to determine mAb affinity. The non-competitive ELISA is simple and available method for many laboratories, based on the law of mass action that compares the OD50 of three sigmoidal curves of serial Ab dilutions on plates coated with different Ag concentrations to determine the binding strength between a mAb and its Ag. This protocol provides a step-by-step guide to determining mAb affinity using modified ELISA and enables researchers to make informed decisions about the development and application of mAbs in their respective fields.

INTRODUCTION

Monoclonal antibodies (mAbs) have revolutionized the fields of diagnostics and therapy due to their high specificity and affinity to the target antigens (Ags) (1). Accurate measurement of the affinity of mAbs is critical to fully understand their binding properties and optimizes their use in various tests (2). Affinity is a critical parameter in the characterization of mAbs as it represents the strength of binding between an Ab and its target Ag. It can enable researchers to optimize their use in various applications, such as targeted drug delivery, immunohistochemistry and immunoassays (3). Accurate determination of mAb affinity enables informed decision making in the development and optimization of the Ab and ultimately improves its efficacy in diagnostics and therapy. In addition, it helps to select the most appropriate mAb for a given target and ensures reproducibility, reliability and comparability of results between different experiments and laboratories.

The affinity of a mAb can be measured using a variety of approaches, including equilibrium dissociation constant (KD) measurement, non-competitive enzyme-linked immunosorbent assay (ELISA), surface plasmon resonance (SPR) and microarray-based label-free assay platforms (4). The affinity of an antibody is quantified by the KD, with lower KD values corresponding to higher affinity. The oblique-incidence reflectivity difference (OI-RD) and Biacore are two techniques that can be used (4). In addition, non-competitive ELISA has been used to calculate the

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affinity of a mAb in a simple and reliable way based on the law of mass action (5). Another method to determine affinity is SPR. SPR is a phenomenon that occurs when polarized light strikes a metal layer on an environmental surface with different refractive indices (6). An alternative method is to quantify the affinity constants of mAbs to targets using a label-free microarray-based test platform (7). SPR technology, while powerful, has some limitations. These limitations include challenges in discriminating between specific and non-specific interactions on the sensor surface, the need for elaborate washing to remove non- specifically bound material, difficulty in detecting low molecular weight compounds, limited sensor area leading to diminished capacity, and the requirement for stable conditions during measurements. Additionally, SPR is affected by environmental noise such as temperature changes and nonspecific binding, which can obscure the binding signal and hinder the limit of detection. Despite these challenges, SPR remains a valuable technology for real- time, sensitive, and quantitative analysis of molecular interactions under controlled conditions.

The non- competitive ELISA method for affinity measurement compares the OD50 of three sigmoidal curves of serial Ab dilutions on plates coated with different Ag concentrations to determine the binding strength between an Ab and its immobilized Ag. In this technique, serial dilutions of both the Ag (coated on the plate) and the Ab are used to measure the binding of an Ab with an Ag by ELISA (5).

Using this protocol, researchers can precisely measure the affinity of mAbs, which improves their understanding of mAbs binding kinetics and contributes to the development of more effective diagnostics and therapeutics tools. This method of affinity determination is fast, reliable and easy to perform. But the ELISA method also has limitations, including the variation among different runs and also sampling and pipetting by the subject or by other individuals. The technique modified by Beatty et al. (8) used the law of mass action to calculate the affinity constant of mAbs. In addition, the Beatty procedure was more modified by Haj Ghasemi et al. (9) which was the base of presented protocol for Ab affinity determination according to sigmoidal dose- response curves. In this protocol, step-by-step modified ELISA was described to determine the affinity of an anti-PD-L1 mAb (clone 1D6, generated in our lab) to recombinant PD-L1 protein Ag using binding assays. There are a lot of ELISA based protocols for determination of Ab affinity calculation. However, application of those methods needs to run complex steps that most of times could be confusing for junior researchers. Thus, the current protocol provides researchers with a comprehensive guide to accurate and reliable affinity determination, enabling them to make informed decisions about the development and application of mAb in their respective fields. The protocol includes experimental setups for the binding assay, interpretation of data analysis and troubleshooting tips.

MATERIALS and METHODS

1. NaCl (Merck, Cat: 1.06404.1000)
2. KCl (Merck, Cat: 1.04936)
3. $Na_2HPO_4.2H_2O$ (Merck, Cat: 1.06580.0100)
4. KH_2PO_4 (Sigma, Cat: P3786-100G)
5. Skimmed milk (Thermo scientific, Cat: 1153630500)
6. ELISA microplate Maxibinding (SPL, Cat: 38096)
7. HRP-sheep anti-mouse IgG (Sina biotech, Cat: SB-029951)
8. $NaHCO_3$ (Merck, Cat: 1.06329)
9. Na_2CO_3 (Merck, Cat: 1.06392)
10. Tween-20 (Thermo scientific, Cat: 655205-250ML)
11. HCl (Merck, Cat: 1.00317.2500)
12. Tetramethylbenzidine (TMB) (Pishtazteb, Cat: 9308)
13. Anti-PD-L1 mAb (clone 1D6, Generated in our lab)
14. Recombinant PD-L1 protein (Sinobiological, Cat: 10084-H08H)

Equipment

1. Incubator (Memmert, Germany)
2. pH meter (Metrohm, Switzerland)
3. ELISA reader (Anthos, USA)
4. Multi-channel sampler (Brand, Germany)
5. Sampler 0.5-10 µl (Eppendorf, Germany)
6. Sampler 10-100 µl (Eppendorf, Germany)
7. Sampler 100-1000 µl (Eppendorf, Germany)

Procedure

A. Buffer preparation

1. Coating buffer (sodium bicarbonate buffer; 0.1 Molar with pH = 9.2-10.6)

1.05 grams of $NaHCO_3$ and 9.74 grams of Na_2CO_3 were dissolved in 800 ml of distilled water and after adjusting its pH to 9.5; its volume was reached to 1000 ml with distilled water.

2. Phosphate buffered solution (PBS), pH=7.2-7.4

8 grams of NaCl, 0.2 grams of KCl, 1.44 grams of $Na_2HPO_4.2H_2O$ and 0.245 grams of KH_2PO_4 were dissolved in 900 ml of distilled water and after adjusting its pH to 7.2; the volume was increased to 1000 ml with distilled water.

3. Washing buffer (PBS-Tween20 0.05%)

0.5 ml of Tween 20 was dissolved in 999.5 ml of 1X PBS buffer.

4. Blocking buffer

0.3 grams of skimmed milk powder were dissolved in 10 ml of wash buffer.

5. Stopping solution

20 ml of HCl was dissolved in 220 ml of distilled water.

B. Affinity constant determination

1. ELISA procedure

a. The first layer: serial dilutions of selected Ag concentrations were prepared in coating buffer and coated as 50 µl/well of three strips for each concentration in an ELISA plate. In the next step, the plate was incubated overnight at 4 °C.

Note: Incubation for Ags could be performed at for 2 hours at 37 °C.

Example: The serial dilutions of recombinant PD-L1 protein for three concentrations (500, 250, and 125 ng/ml) in the coating buffer were added into each well (dilution factor, 1:2) (Figure 1).

b. Washing: ELISA plate wells were washed 3 times for 2 minutes each time with 300 µl of wash buffer.

c. Blocking: 100 µl of blocking buffer was added into the wells and then it was incubated for 1 hour at 37 °C.

d. Washing: ELISA plate wells were washed 3 times for 2 minutes each time with 300 µl of wash buffer.

e. Second layer: the mAb with 8 serial dilutions was prepared in blocking buffer and added into the wells (50 µl/well). Then the plate was incubated for 1 hour at 37 °C.

Example: The serial dilutions of anti-PD-L1 mAb (2000, 1000, 500, 250, 125, 62.5, 31.25, and 15 ng/ml) in blocking buffer were added into each coated well (dilution factor, 1:2) (Figure. 1).

f. Washing: ELISA plate wells were washed 3 times for 2 minutes each time with 300 µl of wash buffer.

g. Third layer: Secondary Ab (HRP conjugated-sheep anti-mouse IgG) was prepared with appropriate dilution in blocking buffer and added into the wells (50 µl/well) (1:16000 according to manufacture). In the next step, the plate was incubated for one hour at 37 °C.

h. Washing: ELISA plate wells were washed 3 times for 2 minutes each time with 300 µl of wash buffer.

i. Substrate addition and reading: Following the final washing step, the reaction was revealed with TMB (50 µl/well). After the appropriate time passed (10-15 minutes), the reactions were stopped with stop solution and the optical density (OD) was measured by ELISA Reader at 450 nm and 620 nm as a reference.

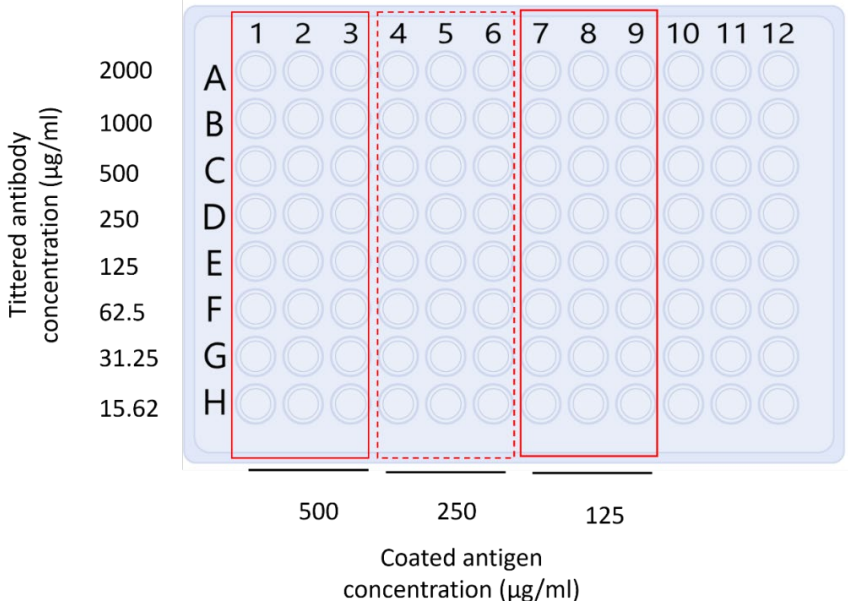

FIGURE 1. Schematic representation of antigen and antibody cross titration.

Tips and Troubleshooting:

1) To determine the appropriate concentrations of Ag and Ab, ELISA for titration of Ag and Ab should be performed prior to the final ELISA for affinity determination as described below (Table.1 and Figure. 2).

Table 1. OD information about determination of appropriate concentrations of antigen (recombinant PD-L1 protein) and anti-PD-L1 mAb titration

	PD-L1 (ng/ml)		
1D6 (ng/ml)	500	250	125
1000	2.334	1.521	0.394
250	1.433	0.974	0.262
62	0.721	0.352	0.174

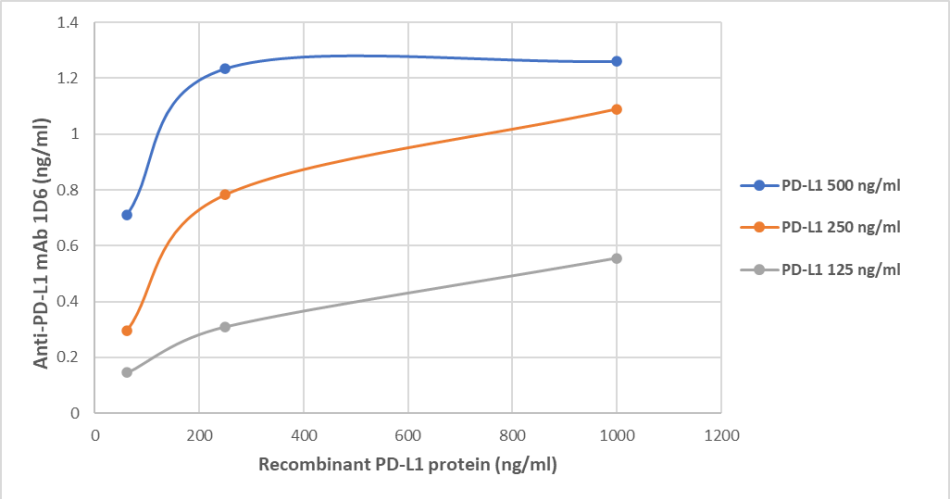

FIGURE 2. Preliminary Chart related to anti-PD-L1 mAb (clone 1D6) affinity determination.

2) To determine the appropriate titration of Ab concentration, a fixed concentration of the Ag (500 ng/ml) was coated in the wells. In this case, the results of titration of Ab were compared and the titer where the OD started to decrease considered as the saturation point. It may be necessary to repeat the ELISA with different Ab concentrations to reach the optimal titer.
3) In the next step, a titration of the Ag concentration should be carried out. For this purpose, saturation point of Ab concentration (1000 ng/ml obtained from previous step) was applied to different Ag concentrations (500, 250, 125 ng/ml).
   Note: Our data showed that three selected Ag concentrations had a parallel line without any overlap. If there is overlap among the graphs, two Ag concentrations higher and three concentrations lower than the highest concentration were selected for the ELISA and the Ab titration is performed with these concentrations.
4) The Ab titration should be performed again according to the titers selected in step 2 with the three Ag concentrations selected in step 3. The special feature of the selection of these three concentrations is that the graphs do not overlap at a dilution factor of 2 and the graph has a sigmoid shape.
5) The selection of three concentrations is sufficient to determine the affinity. Two or one concentration points should not be selected; otherwise the value of the test will be lost.
6) If you choose a higher number of concentrations (>3 points) for the Ag, the sensitivity of the test will increase.
7) After the affinity has been determined for each concentration, the average of the three concentrations is finally calculated and given as the final affinity.
8) The conditions of Ag coating have a considerable influence on the functional affinity constants. Coating must increase by a factor of two as soon as the Ag concentration is doubled for the mathematical equation used in the calculations to be correct. A complete sigmoidal curve of mAb was obtained by selecting serial dilutions of Ag and Ab.

2. Affinity constant calculation

A sigmoidal curves of the OD versus (y axis) the logarithm of the Ab concentration (x axis) in each well shows how much Ab is bound to the Ag on the plate. For the calculation of affinity, the Ab concentration that gives 50% of the highest absorbance value for a given Ag coating concentration is chosen. The equation, for plates coated with two amounts of Ag, one ([Ag']) being half the amount of the other ([Ag]), is an estimate of the affinity constant of the Ag-Ab interaction according to Beatty et al. (8). It is based only on the Ab concentration at OD50 of ([Ab'] t and ([Ab] t)(t refers to every Ag concentrations) Equation (1) can be generalized to obtain equation (2) when the concentration of [Ag] ([Ag] = n [Ag']) is varied, one concentration being half that of the other (e.g. Ag = 2Ag'= 4Ag''= 8 Ag'''), according to et al. (9):

Equation (1): K_{aff}=1/2(2[Ab']t - [Ab]t)

Equation (2): K_{aff}= (n-1)/2(n[Ab']t - [Ab]t)

n = [Ag] / [Ag']

Note:

The mean of three calculations was the final affinity constant measured in this study. The final functional affinity constant was calculated twice using the mAb concentration at OD50

of a specific sigmoid curve selected for calculation based on the above criteria.

Example: Affinity determination related to a produced anti-PD-L1 mAb is presented in the tables 2, 3 and figure 3.

Table 2. OD information about titration of Ag (recombinant PD-L1 protein) and anti-PD-L1 mAb.

mAb (ng/ml)	PD-L1 (500 ng/ml)	PD-L1 (250 ng/ml)	PD-L1 (125 ng/ml)
2000	2.1	1.784	1.366
1000	2.052	1.652	1.07
500	1.798	1.436	0.922
250	1.695	1.313	0.726
125	1.584	0.972	0.598
62.5	1.276	0.646	0.374
31.25	0.937	0.581	0.356
15.625	0.593	0.417	0.285

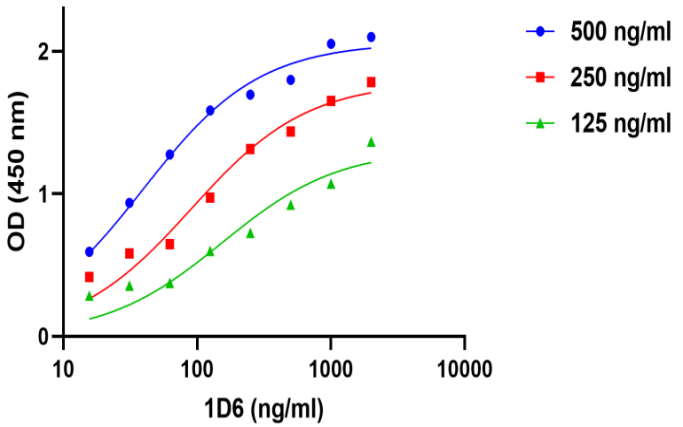

FIGURE 3. Chart related to anti-PD-L1 mAb (clone 1D6) affinity determination.

Table 3. Calculations related to determining affinity of anti-PD-L1 mAb (clone 1D6).

	PD-L1 (500 ng/ml)	PD-L1 (250 ng/ml)	PD-L1 (125 ng/ml)
$OD_{max}/2$	1.05	0.892	0.683
AB (ng/ml)	35	114.7	189
AB/150 (Molarity) (nM)	0.23	0.76	1.26
(n×[AB"]-[AB])	1.296	1.755333333	4.806666667
2×(n×[AB"]-[AB])	2.592	3.510666667	9.613333333
(n-1)/2×(n×[AB"]-[AB])	0.385802469	0.284846183	0.312066574
[(n-1)/2× (n× [Ab "]-[Ab])] $\times 10^9$	385802469.1	284846183.1	312066574.2
k_{aff} (nM=1/M^{-1})	2.59E-09	3.51E-09	3.20E-09
Average k_{aff} (M^{-1})		3.10E-09	

CONCLUSION

Affinity determination is one of the most important characteristics of mAbs. Because it demonstrates how strongly mAb binds to antigen in various assays. There are various ways to determine this character and in this protocol article, the method of determining affinity with the help of ELISA test is explained. ELISA is a simple and accessible method in comparison to other methods; however, it is preferable to employ SPR if it is available. It would have been better to calculate the affinity with the SPR method and then compare the results with this method, but due to the limitations of the SPR, it was not possible to compare these methods. Because of the way the ELISA test is designed to identify analytes, variations may be seen in subsequent runs by the subject or by other individuals. To minimize this limitation's inaccuracy as much as feasible, the test is conducted in triplicate. Additionally, the first layer of this test employed the purchased recombinant PD-L1 protein; nevertheless, in the future, another PD-L1 protein purchased from another company might be utilized to ascertain the affinity, in which case the affinity for the recently acquired antigen might alter.

REFERENCES

1. Kinumi T, Saikusa K, Kato M, Kojima R, Igarashi C, Noda N, et al. Characterization and Value Assignment of a Monoclonal Antibody Reference Material, NMIJ RM 6208a, AIST-MAB. Front Mol Biosci. 2022;9:842041.

2. Bee C, Abdiche YN, Pons J, Rajpal A. Determining the binding affinity of therapeutic monoclonal antibodies towards their native unpurified antigens in human serum. PLoS One. 2013;8(11):e80501.

3. Ranawakage DC, Takada T, Kamachi Y. HiBiT-qIP, HiBiT-based quantitative immunoprecipitation, facilitates the determination of antibody affinity under immunoprecipitation conditions. Sci Rep. 2019;9(1):6895.

4. Method for determining the affinity [Available from: https://www.perplexity.ai/search/Method-for-determining-0BF1sQm5TSWJJxx4.7MBvQ?s=u.

5. Raghava GP, Agrewala JN. Method for determining the affinity of monoclonal antibody using non-competitive ELISA: a computer program. J Immunoassay. 1994;15(2):115-28.

6. Ahn H, Song H, Choi JR, Kim K. A Localized Surface Plasmon Resonance Sensor Using Double-Metal-Complex Nanostructures and a Review of Recent Approaches. Sensors (Basel). 2017;18(1):98.

7. Landry JP, Ke Y, Yu GL, Zhu XD. Measuring affinity constants of 1450 monoclonal antibodies to peptide targets with a microarray-based label-free assay platform. J Immunol Methods. 2015;417:86-96.

8. Beatty JD, Beatty BG, Vlahos WG. Measurement of monoclonal antibody affinity by non-competitive enzyme immunoassay. J Immunol Methods. 1987;100(1-2):173-9.

9. HAJI GF, SABOUR YA, Shokri F. Measurement of affinity constant of anti-human IgG monoclonal antibodies by an Elisa-based method. 2004.