Published in final edited form as:

J Immunol Methods. 2017 March ; 442: 49–53. doi:10.1016/j.jim.2016.12.004.

Development and Validation of a Cell-Based Fluorescent Method for Measuring Antibody Affinity

Xin Yu¹, Charles N. Pegram¹, Darell D. Bigner¹, and Vidyakshmi Chandramohan¹

¹Department of Pathology, Duke University Medical Center, Durham, North Carolina, USA

Abstract

Monoclonal antibodies have become essential tools for diagnostic and therapeutic purposes. Antibody affinity is one of the critical factors influencing the therapeutic success of tumor targeting antibodies. Therefore, developing an accurate and reliable method for determining antibody affinity is crucial. In this study, we describe a fluorescent cell-based immunosorbent assay that can accurately measure antibody affinity (K_D) in the nanomolar range. This method involves the addition of fluorescently labelled antibodies to antigen-positive and antigen-negative cell lines fixed on 96-well plates. The fluorescent signals from nonspecific binding to negative control cell lines is subtracted from the specific binding to the antigen-positive cell lines. The K_D values obtained by this method were comparable with values obtained by the flow cytometry and radioactive (I^{125}) scatchard assay. Our results demonstrate that this modified cell-based fluorescent method allows for a convenient and efficient identification of therapeutically relevant leads.

Keywords

antibody affinity; fluorescent cell-based assay; scatchard; flow cytometry

1. Introduction

Monoclonal antibodies (mAbs) have emerged as novel therapeutics for the treatment of cancer and chronic diseases [1, 2]. To function as an efficacious and safe therapeutic agent mAbs are required to display optimum affinity for the target antigen. Therefore, determining antibody affinity with an accurate and reliable method is crucial, especially when applying that method within a therapeutic context. The affinity of an antibody binding to the antigen is expressed as the dissociation equilibrium constant K (K_D), which is determined by the ratio of dissociation to association rate constants (k_{off}/k_{on}). Currently, most common techniques employed to assess K_D are radioimmunoassay, surface plasmon resonance (SPR), flow cytometry, enzyme-linked immunosorbent assays (ELISA), and kinetic exclusion assays (KinExA) [3].

Corresponding author: Vidyakshmi Chandramohan: vidyalakshmi.chandramohan@duke.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

To be successful therapeutic candidates mAbs need to recognize native epitopes on target antigens. Cell-based fluorescent assay (e.g. cell-based ELISA) offers a rapid and sensitive way to measure antibody affinity utilizing a monolayer of cells adhering to a 96-well plate to comprise the solid phase of a conventional assay, in which the protein of interest is immobilized [4]. Advantages of a cell-based fluorescent assay include its use of intact cells expressing target antigens in their native conformation, excluding the need to purify target antigen, high throughput analysis using 96-well plates, conservation of costly reagents due to the small assay volume, and relative inexpensiveness of using a plate reader. Despite these advantages no attempt has been made yet to utilize a direct fluorescent method for assessing antibody affinity and validate it by comparison to other established methods.

In this paper, we describe the development of a simple and rapid fluorescent cell-based method that allows for efficient and accurate measurement of antibody equilibrium dissociation constant (K_D) in the nanomolar range using a standard fluorescence microplate reader. We validated the K_D values obtained by this method with those obtained by the I^{125} scatchard assay and flow cytometry. We also demonstrated that competitive binding assays can be performed using fluorescent cell-based assay.

2. Materials and Methods

2.1 Antibodies

Development, production, and purification of the Mel-14 monoclonal antibody targeting human chondroitin sulfate proteoglycan 4 (CSPG4), the D2C7 monoclonal antibody targeting human wild-type epidermal growth factor receptor (EGFRwt) and human mutant EGFR variant III (EGFRvIII), and the NZ-1 monoclonal antibody targeting human podoplanin have been previously described [5-7]. The purity of Mel-14 (mouse IgG2a), D2C7 (mouse IgG1), and NZ-1 (Rat IgG2a) were determined to be greater than 90% by SDS-PAGE (data not shown).

2.2 Cell lines

H350 is a human melanoma cell line expressing CSPG4 maintained in our laboratory. The human embryonic kidney cells, HEK293 (ATCC, Manassas, VA), which lacks expression of CSPG4, and murine Swiss 3T3 fibroblast cell line, NR6 (kindly provided by Dr. Harvey Herschman, University of California, Los Angeles, CA), which lacks expression of murine or human EGFRwt, EGFRvIII, or human podoplanin, were used as negative controls. NR6EGFRwt is the murine NR6 cell line transfected with human EGFRwt [8]. All cells were cultured in an incubator at 37°C, 5% CO₂, and passaged at confluence with Accutase Cell Detachment Solution (BD Biosciences, San Jose, CA). HEK293 cells were cultured in DMEM media supplemented with 10% heat-inactivated fetal calf serum (FBS) (Thermo Fisher Scientific, Waltham, MA). All other cell lines were grown in 1× MEM ZINC Option media with 10% FBS (Thermo Fisher Scientific).

2.3 Dissociation of xenograft

D-341 MED, medulloblastoma xenograft tissue was obtained from mice under sterile conditions from the Duke animal facility, and prepared for cell culture in a laminar flow

hood using sterile techniques. The tumor was finely minced and digested with 100ug Liberase (Roche, Indianapolis, IN) at 37°C for 20 min. The dissociated cells were filtered through a 70-µm cell strainer (BD Biosciences), and red blood cells were removed with ACK Lysing Buffer (Thermo Fisher Scientific). The tumor cells were then washed with phosphate-buffered saline, (1× PBS, pH 7.4), and treated with Ficoll-Paque Plus (GE Healthcare Bio-Sciences, Pittsburgh, PA) to remove residual red blood cells, debris, and dead cells. The cells were finally washed twice with 1× MEM Zinc Option media before they were transferred to tissue culture-treated flasks. The cells were cultured and passaged until sufficient numbers were obtained.

2.4 Labeling of antibodies

Mel-14, D2C7, and NZ-1 antibodies were directly conjugated with Alexa Fluor 488 (AF488) fluorescent dye using the amine-reactive agent tetrafluorophenyl esters (Thermo Fisher Scientific) according to the manufacturer's protocol. Briefly, AF488 dye was added to the purified antibody at a 10:1 molar ratio and incubated in 0.1 M Na₂HCO₃, pH 8.2 buffer for 1 h at room temperature. The excess dye was removed by overnight dialysis using Slide-A-Lyzer cassettes (Thermo Fisher Scientific). Protein concentrations were calculated from measurements of absorbance at 280 nm and 494 nm obtained using Nanodrop 1000 (Thermo Fisher Scientific). The fluorophore to protein ratio was calculated to be for 4.6 for Mel-14-AF488, 4.0 for NZ-1-AF488, and 1.2 for D2C7-AF488.

2.5 Affinity measurement by flow cytometry

To test antigen specificity, flow cytometry analysis was performed by incubating 66.7nM of Mel-14-AF488, NZ-1-AF488, and D2C7-AF488 antibodies, or the corresponding AF488 labeled isotype control antibodies with both the antigen-positive and antigen-negative cell lines. To measure antibody affinity by flow cytometry, the cells were first prepared as described below. 3×10⁵ cells (H350, HEK293, NR6, NR6EGFRwt, or D-341 MED) were resuspended in 500 ul of 1× PBS containing 5% FBS (5%FBS-PBS). Serially diluted Mel-14-AF488, D2C7-AF488, and NZ-1-AF488 antibodies were added to their corresponding antigen-positive cell lines at concentrations ranging from 100 pM to 500 nM. Cells were incubated for 45min at 4°C, washed 3 times with 1×PBS, and analyzed on a Becton Dickinson FACSCalibur instrument (BD Biosciences). Mean fluorescent intensity values were obtained from the histograms and were used to plot the binding curves. A standard approach of nonlinear regression using the one-site binding hyperbola available in Graphpad Prism 5 (Graphpad Software, La Jolla, CA), was used to fit the curves, and K_D values were calculated by the program.

2.6 Radioactive scatchard affinity analysis

Mel-14, D2C7, and NZ-1 antibodies were conjugated with I¹²⁵ as previously described [9]. Then, antigen- positive cells (H350 for Mel-14, NR6EGFRwt for D2C7, and D-341 MED for NZ-1) and antigen-negative cells (HEK293 for Mel-14, NR6 for D2C7, and NZ-1) were seeded in 24-well plates at 2×10⁵ cells/well in triplicates, and cultured overnight. The next day, the cells were fixed in 0.25% glutaraldehyde (Sigma, St. Louis, MO) for 5 min at room temperature, washed three times with incubation buffer (115 mM PO₄ [KH₂PO₄ + K₂HPO₄], 0.05% BSA, 0.05% gelatin), and blocked with the same buffer at room

temperature for 1 h. I^{125} labeled antibodies were serially diluted from 10 pM to 80 nM with the incubation buffer. Diluted antibodies were added to the corresponding triplicate wells plated with their antigen-positive cells (Ag Pos wells) and antigen-negative cells (Ag Neg wells). The same series of antibody dilutions, in the same concentrations as the antibody solutions added to the wells, were prepared and added directly to 2ml screw-cap tubes at 200 μ l/tube in triplicates. These tubes were used as standard samples. The cell plates and the standard sample tubes were incubated at 4°C overnight. The next day, the free radioactive antibodies were removed from the plates. Then the plates were washed four times with the incubation buffer. After that, 500 μ l 2N sodium hydroxide was added to each well, and the plates were incubated overnight at 37°C. The following day, the cells were completely re-suspended in 500 μ l 2N sodium hydroxide and transferred into new 2 ml screw-capped tubes. The test tubes (containing intact cells and cell debris bound to antibodies) as well as standard sample tubes were then placed in a gamma counter. The readout values (counts per min, cpm) were recorded. Equation 1 presented below was used to calculate the average amount of antibodies bound to cells (molar bound) at each antibody concentration. Molar bound values were plotted against the molar input values, to determine the average amounts of antibody added to each well. The curves were fitted with one-site binding hyperbola in Graphpad Prism 5, and K_D values were calculated by the Prism 5 program.

$$\text{Molar bound}(M) = \frac{\sum \text{cpm of } ^3\text{Ag Pos wells} - \sum \text{cpm of } ^3\text{Ag Neg wells}}{\sum \text{cpm of standard samples tubes}} * \text{molar input}$$

(Equation 1)

2.7 Affinity measurement by cell-based fluorescent assay

Antigen-positive and negative cells were seeded in duplicates in flat clear bottom black 96-well plates (Corning, Corning, NY) at 2×10^4 cells/well, and cultured overnight. For each antibody, its antigen-positive and antigen-negative cell line pair were cultured on the same plate to avoid differences between individual 96-well plates. The next day, the cells were fixed in 0.25% glutaraldehyde (Sigma) for 5min at room temperature, and washed three times with $1 \times$ PBST buffer ($1 \times$ PBS, 5% FBS, 0.05% Tween 20). The wells were then blocked with 5% FBS-PBS buffer for 1h at room temperature. Serially diluted Mel-14-AF488, D2C7-AF488, and NZ-1-AF488 antibodies ranging from 40 pM to 400 nM were added to the corresponding antigen-positive and antigen-negative cells. The plates were incubated at 4°C in dark for 1 h, and then washed six times with $1 \times$ PBST buffer. Then 5% FBS-PBS buffer was added to each well at 100 μ l/well. The excitation and emission wavelengths on the Tecan Infinite 200Pro plate reader (Tecan, Morrisville, NC) were set at 490 nm and 525 nm, respectively. Gain, the amplification factor for the photomultiplier tube, was set to optimal so that its value was calculated automatically by the instrument according to the highest signal within the selected wells. Integration time was set to 20 μ s. Relative fluorescence units (RFU) was recorded. RFU for specific binding was calculated using Equation 2 shown below and plotted against molar concentrations of antibody input. The

curves were fitted with sigmoidal dose response algorithm and K_D was calculated by the Prism 5 program.

Specific Binding RFU=(Avg RFU of Ag Pos Wells–Avg RFU of Blank Ag Pos Wells)–(Avg RFU of Ag Neg Wells)

(Equation 2)

2.8 Competitive binding assay using the cell-based fluorescent format

Competitive binding assay was carried out using NR6EGFRwt cells. Ninety-six-well plates containing fixed NR6EGFRwt cells were prepared and blocked as described in section 2.7. In parallel duplicate tubes, 40 nM of D2C7-AF488 solution was incubated with increasing concentrations (2 nM to 1 μ M) of truncated human EGFRwt protein (amino acids 25-645 with a C-terminus polyhistidine tag) (ACROBiosystem, Newark, DE), or with an unlabeled negative control protein (purified truncated CSPG4 protein [amino acids 641-1233 with a C-terminus 6 $\times$ histidine tag]) in 5% FBS-PBS buffer. The mixture (100 μ l/tube) was incubated at 4°C for 1 h. At the end of the incubation period, 90 μ l of the mixture was transferred to the corresponding wells in a 96-well plate. The plates were incubated at 4°C for 1 h, and washed 6 times with the 1 $\times$ PBST buffer. Then 100 μ l 5% FBS-PBS buffer was added to each well. The plates were read by the Tecan plate reader using the same settings as before, and RFU was plotted against the molar concentrations of the EGFRwt protein. The curves were fitted with sigmoidal dose response equation and K_D was calculated by the Prism 5 program.

3. Results

3.1 Affinity measurement by flow cytometry, I^{125} scatchard, and cell-based fluorescent assay method

Measurement of antibody affinity by cell-based fluorescent assay requires the use of antigen-positive and antigen-negative cell lines. The NR6EGFRwt cell line expressing EGFRwt, D-341 MED xenograft cells expressing podoplanin, and H350 cell line expressing CSPG4 were chosen as antigen-positive cell lines for D2C7, NZ-1, and Mel-14, respectively. The NR6 (D2C7 and NZ-1) and HEK293 (Mel-14) cells were used as negative controls. A flow cytometry analysis was conducted to validate the binding of the antibodies to the antigen-positive cell lines and antigen-negative cell lines. The results demonstrated strong binding of Mel-14-AF488, NZ-1-AF488, D2C7-AF488 antibodies to the CSPG4 positive H350 cell line, podoplanin positive D-341 MED cells, and EGFRwt positive NR6EGFRwt cell line, respectively (Figure 1B). No binding of Mel-14-AF488, NZ-1-AF488, D2C7-AF488 antibodies to the antigen-negative cell lines HEK293 and NR6 was observed (Figure 1A). Similarly no binding was observed with the isotype control antibodies on all the cell lines tested (Figure 1A and 1B).

The cell binding affinities of Mel-14, D2C7, and NZ-1 mAbs were determined using conventional approaches such as flow cytometry and I^{125} scatchard analysis, as well as by

the newly developed cell-based fluorescent method. The results are summarized in Table 1 and representative plot of cell-based fluorescent assay for Mel-14, NZ-1, and D2C7 are shown in Figure 1C. The K_D value of Mel-14 mAb by flow cytometry, I^{125} scatchard analysis, and cell-based fluorescent method was 0.56 ± 0.06 nM, 1.16 ± 0.76 nM, and 2.18 ± 1.09 nM, respectively (Table 1 and Figure 1C). The K_D value of NZ-1 mAb by flow cytometry, I^{125} scatchard analysis, and cell-based fluorescent assay was 2.41 ± 0.89 nM, 1.78 ± 1.13 nM, and 2.59 ± 1.66 nM, respectively (Table 1 and Figure 1C). The K_D value of D2C7 mAb by flow cytometry, I^{125} scatchard analysis, and cell-based fluorescent assay was 78.74 ± 3.48 nM, 28.91 ± 8.37 nM, and 39.97 ± 5.27 nM, respectively (Table 1 and Figure 1C). For all three antibodies, the K_D values obtained from cell-based fluorescent assay were similar to those obtained by I^{125} scatchard and flow cytometry.

3.2 Competitive binding assay using cell-based fluorescent method

Competitive assays offers advantages such as greater specificity, flexibility, and sensitivity. It can be used to validate the specific antibody binding epitope on the antigen. To investigate whether the cell-based fluorescent assay approach can be utilized for a competitive binding assay, we carried out the competition assay using 40 nM of the D2C7-AF488 antibody incubated with the serially diluted (2 nM-1 μ M), unlabeled EGFRwt protein or a negative control protein. The mixtures were incubated and then transferred to plates fixed with NR6EGFRwt cells to detect the AF488 signal from the D2C7 antibodies. The average K_D for the D2C7 antibody measured using a competitive binding assay in a cell-based fluorescent format is 40.31 ± 8.75 nM (Figure 2), which is very close to the value (39.97 ± 5.27 nM) obtained by the direct binding cell-based fluorescent method (Table 1). Therefore, the cell-based fluorescent method can also be used in competitive binding assays.

4. Discussion

Antibodies with optimal affinity to native target antigens is a critical requirement for the development of antibody based therapeutics. Therefore, it is essential to have reliable and efficient methods for assessing antibody affinity. In this study, we have developed a fluorescent cell-based assay for the measurement of the dissociation equilibrium constant K_D of antibodies. Three mAbs directly labeled with AF488 fluorophore were incubated with their antigen-positive cell lines and antigen-negative cell lines on 96-well plates. The non-specific background binding to antigen-negative cells was subtracted from the binding signal from the antigen-positive cells. We validated this method by comparing the K_D values obtained using this method with the values from the conventional I^{125} scatchard analysis and flow cytometry, and demonstrated that the cell-based fluorescent method provides the same accuracy of measurement as the traditional affinity assays. There was only a 1.38-1.88 fold difference between K_D values obtained by cell-based fluorescent assay and by I^{125} scatchard analysis and a 1.1-3.9 fold difference between the K_D values obtained cell-based fluorescent method and flow cytometry.

It would be appropriate to perform K_D measurements under equilibrium conditions for antigen-antibody interaction. The incubation time required to reach equilibrium during K_D measurements is dependent upon individual antibody and the corresponding antigen. To

demonstrate the applicability of the cell-based fluorescent assay in situations where extended incubation time is required to reach equilibrium, the binding analysis was performed using Mel-14-AF488, D2C7-AF488, or NZ-1-AF488 antibodies under the same conditions as described previously, with the following incubation times: 45 min, 1 h, or overnight (18 h) at 4°C. For each of the three antibodies, the K_D values calculated from the 45 min, 1 h, and overnight reactions were comparable, indicating that antigen binding of Mel-14, D2C7, and NZ-1 antibodies had reached a state of equilibrium at 45 min (Supp.Figure S1 and Supp.Table S1). This demonstrated that the cell-based fluorescent assay can be utilized under wide range of incubation times required for the antigen-antibody interaction to reach equilibrium.

Cell-based fluorescent method offers distinct advantages as a method of antibody affinity measurement compared to the traditional approaches such as flow cytometry and I^{125} scatchard. First, the cell-based fluorescent assay does not involve the use of radioisotopes and direct labeling of antibodies with fluorophores can be accomplished easily in 1-2 h using commercial kits. Secondly, the cell-based fluorescent assay uses cells expressing target antigen in their native conformation as opposed to purified protein from recombinant sources which might present non-native epitopes. Thirdly, cell-based fluorescent does not require the expression and purification of target antigens which is especially helpful in situations where the antigen is difficult to produce or purify, or where the identity of the antigen is unknown. In this study, CSPG4, the target antigen for the Mel-14 antibody, has a large extracellular domain which is approximately 237kD. Expression and purification of the whole protein (450kD) or the extracellular domain as a recombinant or native protein is relatively difficult due to the higher molecular weight as well as the complexity of having both chondroitin sulfate glycosaminoglycan-modified and unmodified core proteins expressed in the same population of transfected or tumor cells [10]. The fourth advantage of the cell-based fluorescent assay is its high-throughput assay format and efficiency of time and reagents. Unlike flow cytometry, in which only one sample is analyzed during each run, cell-based fluorescent assay is fully compatible with 96-well plates, so multiple samples can be analyzed at the same time. This offers great convenience, especially when characterizing or selecting high-affinity mutant clones from a large antibody library. Cell-based fluorescent assay can be easily performed using a plate reader with fluorescent channels, without the need for expensive apparatuses. Finally, the cell-based fluorescent assay can also be used in competitive binding assays allowing for the possibility of epitope validation studies.

Like all assays, the cell-based fluorescent assay does have certain limitations. First, it might not be suitable for crude samples or samples with complex compositions, since contaminating proteins and antibodies can bind to various receptors on the cell, resulting in a false binding signal. Second, while the number of cells plated in each well can be controlled, it is difficult to quantify the number of receptors on the cells after the fixation step. Third, some receptors are occasionally very sensitive to fixation by glutaraldehyde, and need to be tested after fixation using a positive control antibody to make sure there is no denaturation or reduction of binding affinity. Finally, fluorescent labeling of antibodies might interfere with their ability to bind the target antigens. However, in light of the benefits our cell-based fluorescent assay offers, these setbacks are minor.

In summary, we have developed a simple and efficient fluorescent cell-based method for measuring antibody affinity (K_D). We validated the method by comparing the results with those obtained by I^{125} scatchard assay and flow cytometry. The results obtained by these assays were found to be comparable, demonstrating the potential of this method to be utilized as an antibody affinity measurement assay. The advantages of the cell-based fluorescent assay include the use of whole cells without the need to purify antigens, the ability to differentiate nonspecific and receptor specific cell binding, and a high-throughput format compatible with plate readers. Our cell based fluorescent method allows for convenient and rapid identification of therapeutically relevant leads.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Jenna Lewis for her editorial assistance. This work was supported by the following grants from the National Institutes of Health (NIH) of the United States: P01 – 5P01CA154291 (DDB) and the Outstanding Investigator Award – 1R35CA197264 (DDB).

References

1. Alewine C, Hassan R, Pastan I. Advances in anticancer immunotoxin therapy. *Oncologist*. 2015; 20(2):176–185. [PubMed: 25561510]
2. Modjtahedi H, Ali S, Essapen S. Therapeutic application of monoclonal antibodies in cancer: advances and challenges. *Br Med Bull*. 2012; 104:41–59. [PubMed: 23118261]
3. Salimi-Moosavi H, Rathanaswami P, Rajendran S, Toupikov M, Hill J. Rapid affinity measurement of protein-protein interactions in a microfluidic platform. *Anal Biochem*. 2012; 426(2):134–141. [PubMed: 22542978]
4. Donald D, Smith CBC, Lindsley Herbert B. Optimization of Cellular ELISA for Assay of Surface Antigens on Human Synoviocytes. *BioTechniques*. 1997; 22
5. Carrel S MS, Schmidt-Kessen A, Mach JP. Reactivity spectrum of 30 monoclonal antimelanoma Abs to a panel of 28 melanoma and control cell lines. *Hybridoma*. 1982
6. Zalutsky MR, Boskovitz A, Kuan CT, Pegram CN, Ayriss J, Wikstrand CJ, Buckley AF, Lipp ES, Herndon JE 2nd, McLendon RE, et al. Radioimmunotargeting of malignant glioma by monoclonal antibody D2C7 reactive against both wild-type and variant III mutant epidermal growth factor receptors. *Nucl Med Biol*. 2012; 39(1):23–34. [PubMed: 21958852]
7. Kato Y, Kaneko MK, Kuno A, Uchiyama N, Amano K, Chiba Y, Hasegawa Y, Hirabayashi J, Narimatsu H, Mishima K, et al. Inhibition of tumor cell-induced platelet aggregation using a novel anti-podoplanin antibody reacting with its platelet-aggregation-stimulating domain. *Biochem Biophys Res Commun*. 2006; 349(4):1301–1307. [PubMed: 16979138]
8. Carol J, Wikstrand REM, Friedman Allan H, Bigner Darell. Cell Surface Localization and Density of the Tumor-associated Variant of the Epidermal Growth Factor Receptor, EGFRvIII. *Cancer Research*. 1997
9. Kato Y, Vaidyanathan G, Kaneko MK, Mishima K, Srivastava N, Chandramohan V, Pegram C, Keir ST, Kuan CT, Bigner DD, et al. Evaluation of anti-podoplanin rat monoclonal antibody NZ-1 for targeting malignant gliomas. *Nucl Med Biol*. 2010; 37(7):785–794. [PubMed: 20870153]
10. Price, Matthew A., LECW, Yang, Jianbo, Carlson, Jennifer, Xiang, Bo, Li, Guiyuan, Ferrone, Soldano, Dudek, Arkadiusz Z., Turley, Eva A., McCarthy, James B, 1. CSPG4, a potential therapeutic target, facilitates malignant progression of melanoma. *Pigment Cell Melanoma Res*. 2011; 24(6)

Figure 1. Binding analysis and affinity measurement of Mel-14, NZ-1, and D2C7 antibodies. Binding of Mel-14-AF488, NZ-1-AF488, and D2C7-AF488 antibodies to the corresponding antigen-negative (A) and antigen-positive (B) cell lines. Histogram labels: grey: cells only; orange: AF488 labeled isotype control antibodies; blue: AF488 labeled Mel-14, NZ-1, or D2C7 antibodies. (C) Representative graphs for cell-based fluorescent assay measurement with K_D and R^2 values for Mel-14, NZ-1, and D2C7 mAbs using antigen-positive and antigen-negative cell lines. All experiments were repeated at least three times.

Figure 2. Competitive binding assay of D2C7 mAb using cell-based fluorescent assay format. Representative graph with K_D and R^2 values for D2C7 mAb using EGFRwt or negative control protein is presented. The experiment was repeated at least three times.

Table 1

Summary of affinity measurements of Mel-14, D2C7, and NZ-1 mAbs by flow cytometry, I125-scratchard, and cell-based fluorescent assay.

Antibody	Antigen	Cell Lines		K _D Measurement		
		Ag+	Ag-	FACS (nM)	I125-Scatchard (nM)	Cell-Based Fluor.Assay (nM)
Mel-14	CSPG4	H350	HEK293	0.56±0.06	1.16±0.76	2.18±1.09
NZ-1	podoplanin	D-341 MED	NR6	2.41±0.89	1.78±1.13	2.59±1.66
D2C7	EGFRwt	NR6EGFRwt	NR6	78.74±3.48	28.91±8.37	39.97±5.27