Assessment of Monoclonal Antibody/Fc Receptor Interactions Using Octet:

Establishment of a Toolbox Panel for Characterization of Therapeutic mAbs and mAb Biosimilars

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Introduction

- **Assessment of biopharmaceutical therapeutic molecules, or their associated derivative biosimilar molecules, requires an orthogonal analytical approach.**
  - No one particular analytical technique can fully define the physical and biological characteristics.
  - Combination of physicochemical and biological analyses
    - Physicochemical analyses
      - Mass spectrometry,
      - HPLC (SEC, IEX, HIC, etc),
      - SDS-electrophoresis (gel or capillary)
      - Isoelectric focusing (gel or capillary)
      - Glycoanalyses
    - Biological analyses
      - Cell-based bioassay
      - ELISA
      - Binding assays
        - Surface plasmon resonance
        - Bio-layer interferometry).
  - Complete and integrated understanding of the biotherapeutic molecule's form and function.

Antibody Binding Properties and Functions

- **IgG monoclonal antibody (mAb) biotherapeutics,**
  - Assays needed both for MOA characterization/definition as well as release (CMC)
  - Multiple functions for one molecule
    - Interactions between “fab” complementarity determining region (CDR) and the target
      - Specific biological function (neutralization, inhibition, etc)
    - Interactions between the Fc region with all of the potential human IgG-Fc-binding cell-surface receptors.
      - Low affinity CD16a and CD16b receptors (FcγRIIIA and FcγRIIIB, respectively),
        - Responsible for the majority of effector function — antibody-dependent cellular cytotoxicity, or ADCC — associated with mAb therapeutics (chiefly IgG1)
      - Low affinity receptors, CD32a and CD32b/c (FcγRIIA and FcγRIIB/C, respectively)
        - Associated with antibody-dependent cellular phagocytosis, or ADCP
      - High affinity receptor CD64 (FcγRI)
        - Responsible for mediating multiple immune responses .
      - ‘Neonatal’ FcRN,
        - Great impact on the in vivo half-life of a therapeutic mAb .

Analyses of mAb Structure and Function

- **There are many alterations to the mAb that can impact its activities**
  - Amino acid substitution(s)
  - Oxidation (met)
  - Glycosylation
  - Etc
- **Biological impact of the changes may be assessed with functional assays**
  - Loss of inhibition (bioassay or ELISA)
  - Loss of effector function (bioassay)
- **Exact changes may be identified via physicochemical means**
- **However, measurements of binding, either CDR/Target and/or Fc/FcR provide crucial understanding of the underlying changes or consistency of mAb biotherapeutics**

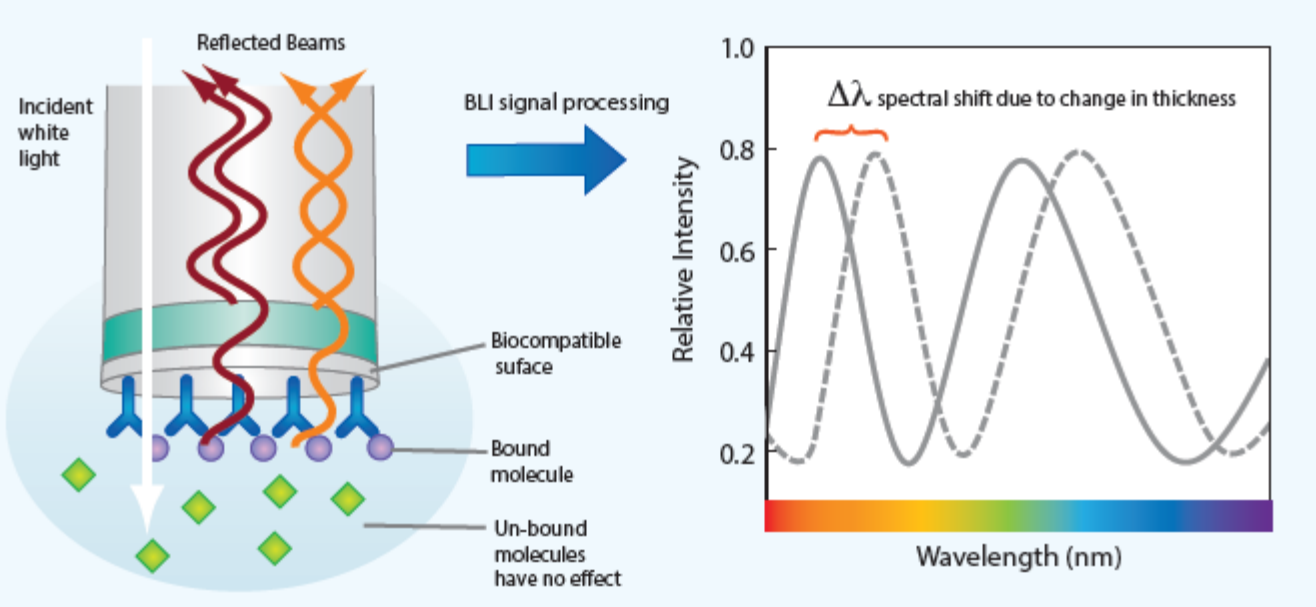
Driver Behind Development of an FcR Binding Panel

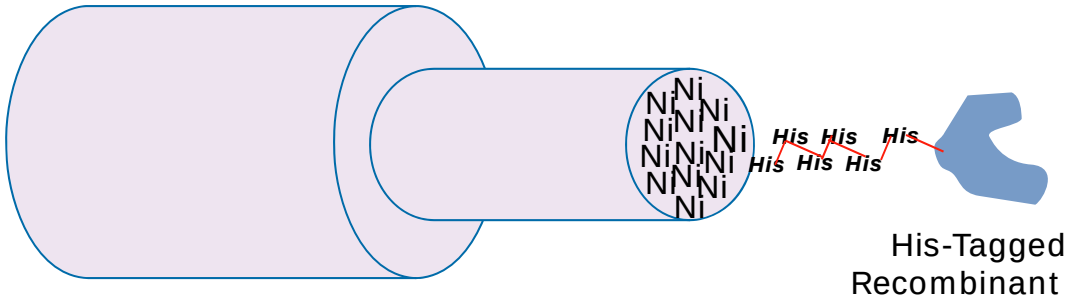
- **Important to test therapeutic mAb for binding with the complete panel of human IgG-Fc-binding receptors for characterization of original therapeutics and/or testing of biosimilar mAbs .**
  - If the mAb is an effector mAb, then it is necessary to show consistency in binding to the high and low affinity receptors CD64, CD32 and CD16.
  - If the mAb is a neutralizing moiety only (no effector function), one might then expect minimal binding, if any, to the Fcγ receptors.
  - However, all antibodies will bind in some fashion to the Fcγ receptors.
  - More importantly, all antibodies will bind to FcRN, which despite the name 'neonatal' is present in adult bone marrow-derived cells, as well as other tissue.
    - When an antibody binds to the FcRN, the Ab/FcRN complex is endocytosed and ends up in an endosomal vesicle with a low pH.
    - In the low pH environment of the endosome, the FcRN is in its high affinity state and as such retains a very tight hold on the antibody, essentially sequestering it from the blood stream.
    - When the endosome recycles back to the cell surface, the Ab/FcRN complex is exposed to neutral pH serum/plasma, and the FcRN shifts to its low affinity state, allowing the Ab to detach .
    - It is in this way that the FcRN is thought to play an important role in the PK of mAb therapeutics .

Toolbox Panel Approach

- **We (Catalent Pharma Solutions, Large Molecule Analytical Chemistry, Kansas City, Mo), set out to develop essentially a toolbox approach for assessing mAb/Fc receptor binding kinetics.**
  - The intent was to eventually use the analytical panel both for characterization of biosimilar molecules and comparison to originator molecules, as well as for CMC-related release and stability testing.
  - Use of SPR was one possible approach
  - We chose to proceed with the more robust, user friendly and more economical method of BLI, with an OctetRed96™.
  - A kinetics binding panel was developed on the Octet RED96 platform with recombinant
    - CD16a (FcγRIIIA), CD16b (FcγRIIIB),
    - CD32a (FcγRIIA), CD32b/c (FcγRIIB/C),
    - CD64 (FcγRI)
    - FcRN.

Basic Strategy For FcR Panel Development





Nickel-coated Biosensor Tip

His-Tagged Recombinant Fc Receptor

Full Panel:

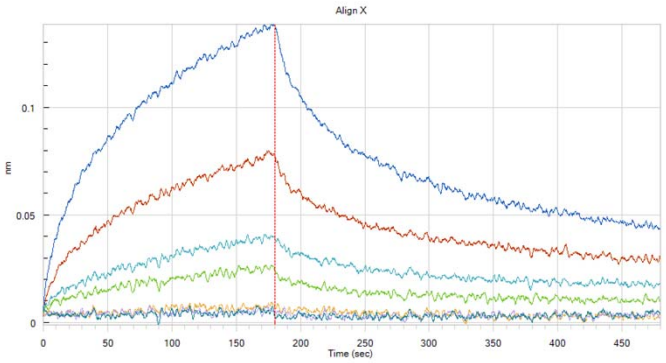
His-tagged (Commercially Available)

huFcγRI
huFcγRIIA
huFcγRIIB/C
huFcγRIIIA
huFcγRIIIB
raFcRN

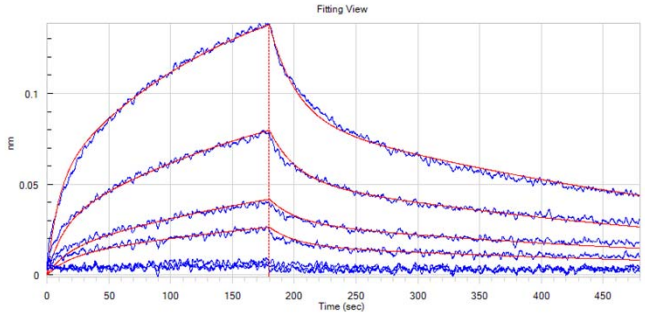
Case #1: FcR K_D Values for an Effector IgG_1 First Attempt at Establishing Fc Receptor Panel

- **mAb with known effector activity**
- **Goal: to characterize/measure the K_D for Fc binding to each of the Fc Receptors**
- **Optimized load conditions for each receptor type**
- **Tested multiple association/dissociation conditions for each mAb/FcR interaction**
- **Project was cancelled before anything beyond optimizing reactions for the initial mAb/FcR K_D determinations**

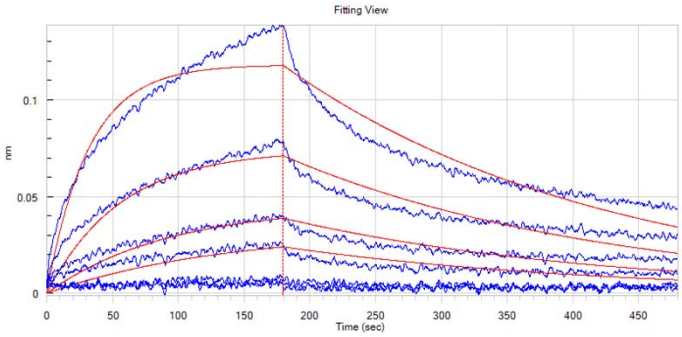
Case #1: FcR K_D Values for an Effector IgG_1 First Attempt at Establishing Fc Receptor Panel



2:1 Global Fitting Model



1:1 Global Fitting Model (not as pretty, but statistically more accurate)

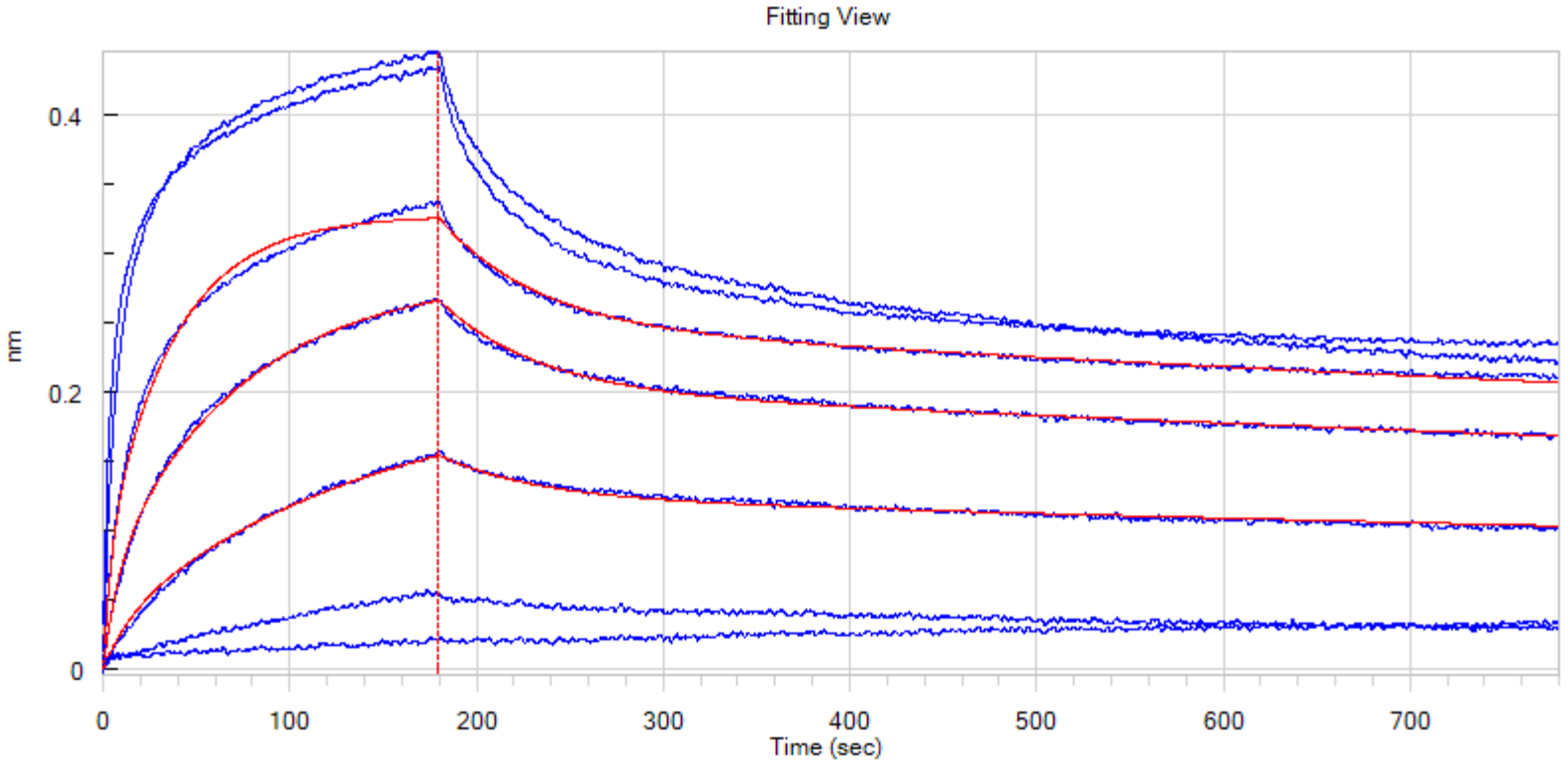


Case #1: FcR K_D Values for an Effector IgG_1 First Attempt at Establishing Fc Receptor Panel

FcR Type	CD nomenclature	Octet-determined mean K_D values (from 5-6 different conditions) (M)	Averaged K_D Error	Averaged K_D Error (% of K_D)	Octet-determined K_D value variability (n = 5 - 6) (%CV)
FcγRI	CD64	2.2×10^{-9}	8.3×10^{-11}	4%	18%
FcγRIIA	CD32a	1.4×10^{-7}	3.6×10^{-9}	3%	11%
FcγRIIB/C	CD32b/c	2.4×10^{-7}	1.4×10^{-8}	6%	37%
FcγRIIIA	CD16a	1.8×10^{-7}	4.7×10^{-9}	3%	30%
FcγRIIIB	CD16b	1.0×10^{-7}	1.4×10^{-9}	1%	23%
FcRN (@pH7.4) **FcRN (@pH 6.0)**	NA	2.1×10^{-7} 1.6×10^{-9}	5.1×10^{-9} 4.3×10^{-11}	2% 3%	53% 34%

Case #1: FcR K_D Values for an Effector IgG_1 First Attempt at Establishing Fc Receptor Panel

Analysis of FcRN Binding, High Affinity (Acidic pH) Condition



Keep this in mind for later

Case #2: Recent Project using Fc Receptor Panel

- **IgG_1 mAb with no known effector activity**
  - MOA: Neutralization of a soluble factor
- **Goal: to characterize/measure the K_D for Fc binding to each of the Fc Receptors**
- **Establish assay for eventual use as part of release and stability testing**
- **Started with receptor load conditions for each receptor type based upon optimization from prior project**
  - Required minimal reoptimization
  - Primarily around selection of buffer with minimal non-specific signal
- **Tested multiple association/dissociation conditions for each mAb/FcR interaction**
  - This did require reoptimization (load time and dissociation time)
- Final execution done with 1 reference standard and 2 lots of drug substance
  - Each sample run as independent duplicates

Case #2: FcR K_D Values for a Neutralizing IgG1

FcγRI (CD64)

Reference Standard						Drug Substance #1						Drug Substance #2					
	KD	Error	% Error	X^2	R^2		KD	Error	% Error	X^2	R^2		KD	Error	% Error	X^2	R^2
Rep1	2.102E-09	1.466E-10	7.0%	0.451	0.999	Rep1	9.676E-09	2.076E-10	2.1%	1.283	0.991	Rep1	9.267E-09	1.925E-10	2.1%	1.136	0.994
Rep2	3.991E-09	2.082E-10	5.2%	0.667	0.997	Rep2	8.176E-09	1.685E-10	2.1%	13.477	0.991	Rep2	8.081E-09	1.693E-10	2.1%	1.488	0.992
Ave	3.047E-09	1.801E-10				Ave	8.926E-09	1.891E-10				Ave	8.674E-09	1.813E-10			
RD	1.336E-09					RD	1.061E-09					RD	8.386E-10				
%RD	44%					%RD	12%		% of RS K_D	293%		%RD	10%		% of RS K_D	285%	

FcγRIIA (CD32a)

Reference Standard						Drug Substance #1						Drug Substance #2					
	KD	Error	% Error	X^2	R^2		KD	Error	% Error	X^2	R^2		KD	Error	% Error	X^2	R^2
Rep1	3.572E-08	1.078E-09	3.0%	0.025	0.999	Rep1	4.768E-08	1.320E-09	2.8%	0.028	0.999	Rep1	4.951E-08	1.643E-09	3.3%	0.036	0.998
Rep2	NA	NA	NA	NA	NA	Rep2	4.704E-08	1.365E-09	2.9%	0.025	0.999	Rep2	4.036E-08	1.810E-09	4.5%	0.047	0.996
Ave	NA	NA				Ave	4.736E-08	1.343E-09				Ave	4.494E-08	1.729E-09			
RD	NA					RD	4.525E-10					RD	6.470E-09				
%RD	NA					%RD	1%		% of RS K_D	133%		%RD	14%		% of RS K_D	126%	

FcγRIIB/C (CD32b/c)

Reference Standard						Drug Substance #1						Drug Substance #2					
	KD	Error	% Error	X^2	R^2		KD	Error	% Error	X^2	R^2		KD	Error	% Error	X^2	R^2
Rep1	6.319E-08	2.651E-09	4.2%	0.074	0.994	Rep1	8.908E-08	3.937E-09	4.4%	0.068	0.993	Rep1	8.565E-08	4.610E-09	5.4%	0.071	0.986
Rep2	5.586E-08	2.743E-09	4.9%	0.056	0.993	Rep2	8.676E-08	4.631E-09	5.3%	0.075	0.990	Rep2	7.210E-08	4.053E-09	5.6%	0.096	0.981
Ave	5.953E-08	2.697E-09				Ave	8.792E-08	4.298E-09				Ave	7.888E-08	4.340E-09			
RD	5.183E-09					RD	1.640E-09					RD	9.581E-09				
%RD	9%					%RD	2%		% of RS K_D	148%		%RD	12%		% of RS K_D	133%	

Case #2: FcR K_D Values for a Neutralizing IgG1

FcγRIIIA (CD16a)

Reference Standard						Drug Substance #1						Drug Substance #2					
	KD	Error	% Error	X^2	R^2		KD	Error	% Error	X^2	R^2		KD	Error	% Error	X^2	R^2
Rep1	1.746E-07	1.542E-08	8.8%	0.105	0.979	Rep1	6.056E-07	3.300E-08	5.4%	0.122	0.979	Rep1	3.842E-07	2.316E-08	6.0%	0.098	0.979
Rep2	2.145E-07	1.316E-08	6.1%	0.049	0.957	Rep2	4.191E-07	2.252E-08	5.4%	0.122	0.973	Rep2	2.314E-07	1.743E-08	7.5%	0.250	0.989
Ave	1.946E-07	1.433E-08				Ave	5.124E-07	2.825E-08				Ave	3.078E-07	2.050E-08			
RD	2.821E-08					RD	1.319E-07					RD	1.080E-07				
%RD	15%					%RD	26%		% of RS K_D	263%		%RD	35%		% of RS K_D	158%	

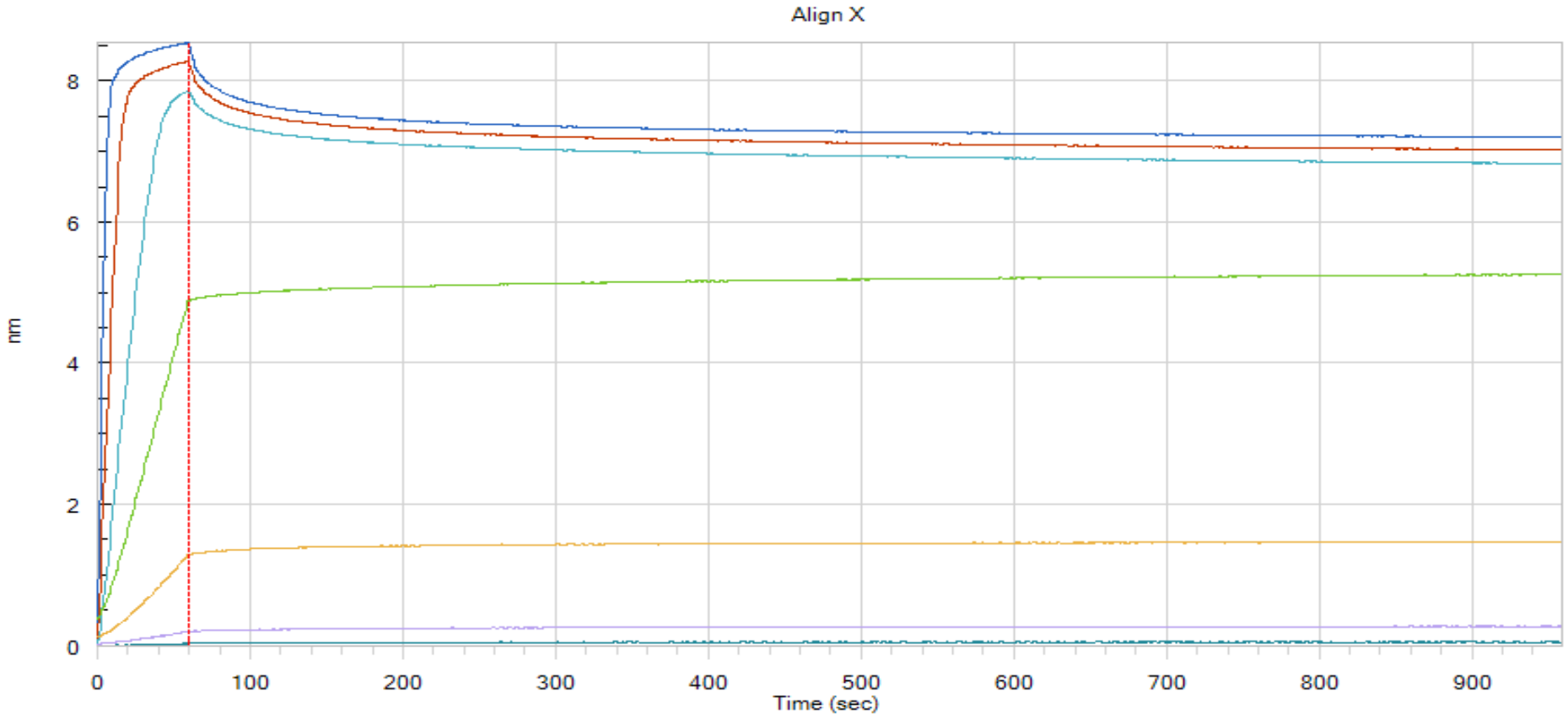
FcγRIIIB (CD16b)

Reference Standard						Drug Substance #1						Drug Substance #2					
	KD	Error	% Error	X^2	R^2		KD	Error	% Error	X^2	R^2		KD	Error	% Error	X^2	R^2
Rep1	1.231E-07	1.210E-08	9.8%	0.050	0.882	Rep1	3.159E-07	1.644E-08	5.2%	0.142	0.986	Rep1	3.936E-07	2.059E-08	5.2%	0.215	0.979
Rep2	9.526E-08	8.266E-09	8.7%	0.028	0.969	Rep2	2.686E-07	1.820E-08	6.8%	0.082	0.977	Rep2	1.964E-07	1.208E-08	6.2%	0.258	0.984
Ave	1.092E-07	1.036E-08				Ave	2.923E-07	1.734E-08				Ave	2.950E-07	1.688E-08			
RD	1.969E-08					RD	3.345E-08					RD	1.394E-07				
%RD	18%					%RD	11%		% of RS K_D	268%		%RD	47%		% of RS K_D	270%	

FcγRN, pH 7.5 (Low Affinity Condition)

Reference Standard						Drug Substance #1						Drug Substance #2					
	KD	Error	% Error	X^2	R^2		KD	Error	% Error	X^2	R^2		KD	Error	% Error	X^2	R^2
Rep1	1.490E-07	1.192E-08	8.0%	0.018	0.984	Rep1	3.963E-07	1.668E-08	4.2%	0.067	0.993	Rep1	3.113E-07	1.446E-08	4.6%	0.137	0.993
Rep2	1.000E-07	8.228E-09	8.2%	0.010	0.980	Rep2	4.665E-07	2.294E-08	4.9%	0.075	0.990	Rep2	2.991E-07	1.397E-08	4.7%	0.135	0.992
Ave	1.245E-07	1.024E-08				Ave	4.314E-07	2.006E-08				Ave	3.052E-07	1.422E-08			
RD	3.465E-08					RD	4.964E-08					RD	8.627E-09				
%RD	28%					%RD	12%		% of RS K_D	347%		%RD	3%		% of RS K_D	245%	

FcRN Binding at Acidic (High Affinity) pH



Summary of mAb/Fc Receptor Binding Panel

- **Results derived for the K_D's of IgG for the different Fc receptors fall in line with expectations.**
- **FcγRII and FcγRIII show low to medium binding kinetics in the 'µM' range (0.01 – 1.0).**
- **FcγRI is high affinity with KD values in the nM range.**
- **FcRN has µM range binding at neutral pH (low affinity condition), but, when measurable, nM range binding at acidic pH (high affinity condition)**
  - The FcRN binding kinetics at the two different pH conditions is consistent with the current understanding of the function of the FcRN receptor, and how it impacts antibody half-life in vivo.
  - Extremely high affinity for some FcRN binding (in acidic conditions) are more problematic to derive an actual K_D value.
- **Replicate analyses of the same sample demonstrate excellent reproducibility/precision.**

Conclusions

- **Successfully demonstrated development and establishment of a platform for characterization of IgG/Fc receptor binding at Catalent**
- **Utilizing full panel of appropriate Fc receptors and easy-to-use Octet instrumentation.**
- **As the immobilized moieties for this panel are the Fc receptors, the analyte of interest (the mAb) is in no way derivatized**
  - Allows objective analysis of the receptor panel with any mAb.
- **With minimal additional optimization, the Fc receptor panel established here can be quickly adapted for any mAb.**
- **In the process of finalizing validation of the Octet RED96 system, and will be further developing and qualifying/validating the Fc receptor panel testing for use not only in characterization, but in cGMP release and stability assessments when appropriate .**

A few other insights

Use of Octet in Determining Biosimilarity

CDR/Target Binding

Batch Number	K_D (M)	K_D Average (M)
1	$4.8x10^{-8}$	$4.7x10^{-8}$
2	$3.5x10^{-8}$	
3	$2.9x10^{-8}$	
4	$2.8x10^{-8}$	
5	$7.5x10^{-8}$	
6	$6.7x10^{-8}$	

K_D determination of originator mAb CDR/Ligand interaction

Biosimilar Candidate Number	K_D (M)
1	$5.9x10^{-8}$
2	$4.6x10^{-8}$
3	$4.8x10^{-8}$

K_D determination of mAb CDR/Ligand interaction for potential biosimilar candidates

Use of Octet in Determining Biosimilarity

Fc/FcRγIIIa Binding

Batch Number	K_D (M)	K_D Average (M)
1	$5.1x10^{-6}$	$4.4x10^{-6}$
2	$4.7x10^{-6}$	
3	$6.2x10^{-6}$	
4	$5.7x10^{-6}$	
5	$2.3x10^{-6}$	
6	$2.3x10^{-6}$	

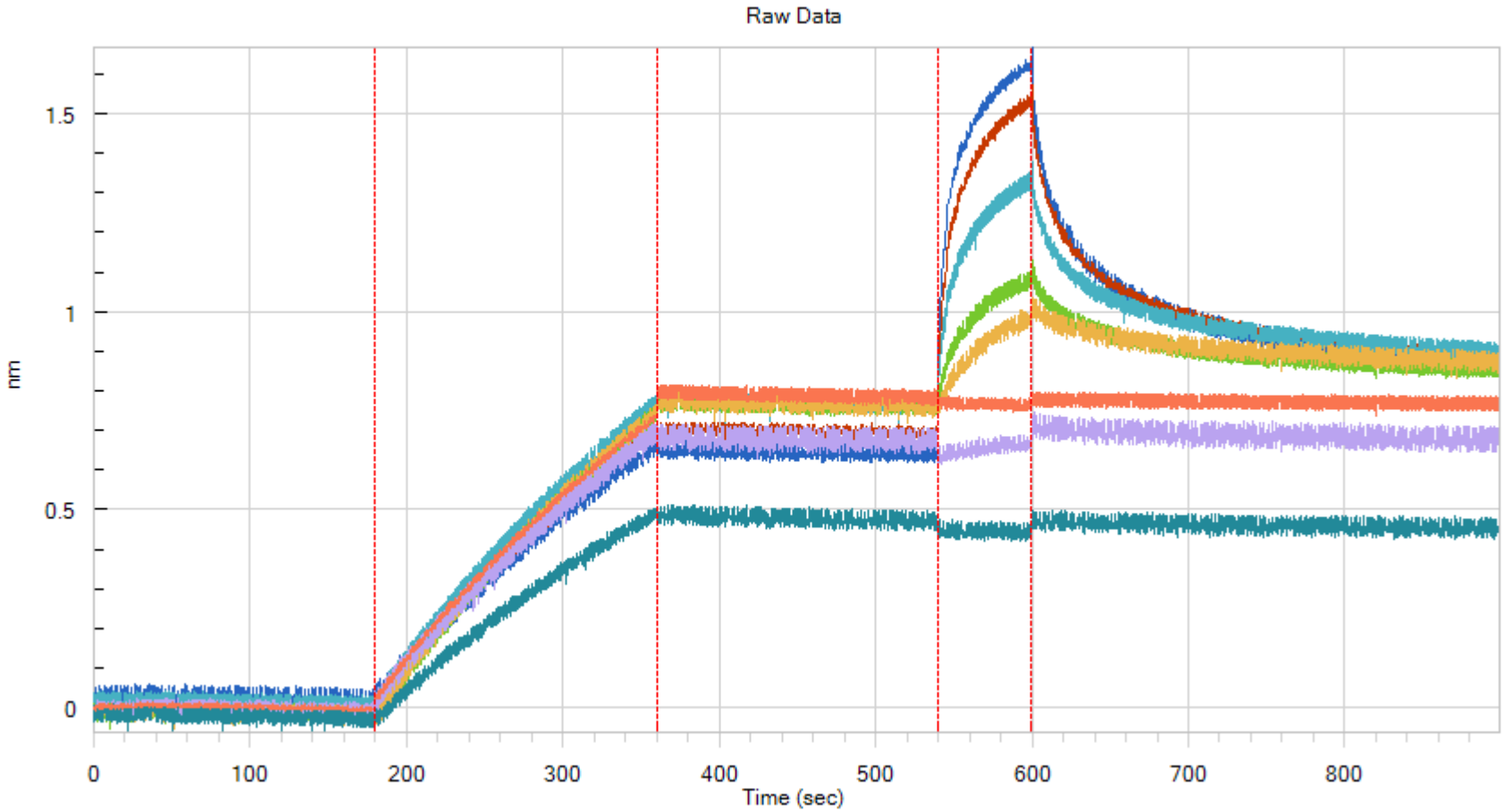
K_D determination of originator mAb Fc/FcRγIIIa interaction

Biosimilar Candidate Number	K_D (M)
1	**$1.9x10^{-5}$**
2	$2.3x10^{-6}$
3	$4.6x10^{-6}$

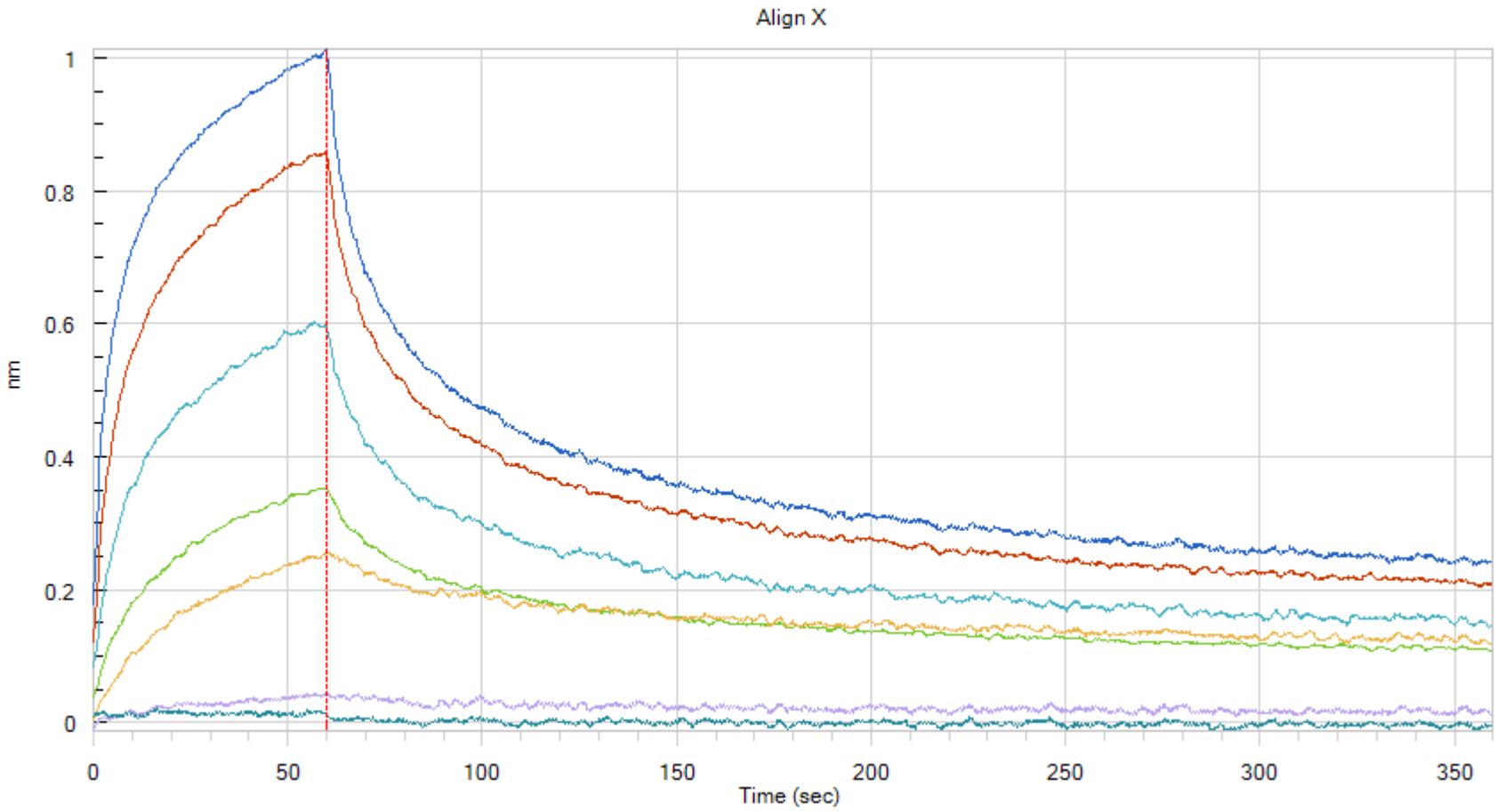
K_D determination of mAb Fc/FcRγIIIa interaction for potential biosimilar candidates

A Funny Thing Happened on the Way to the Binding….

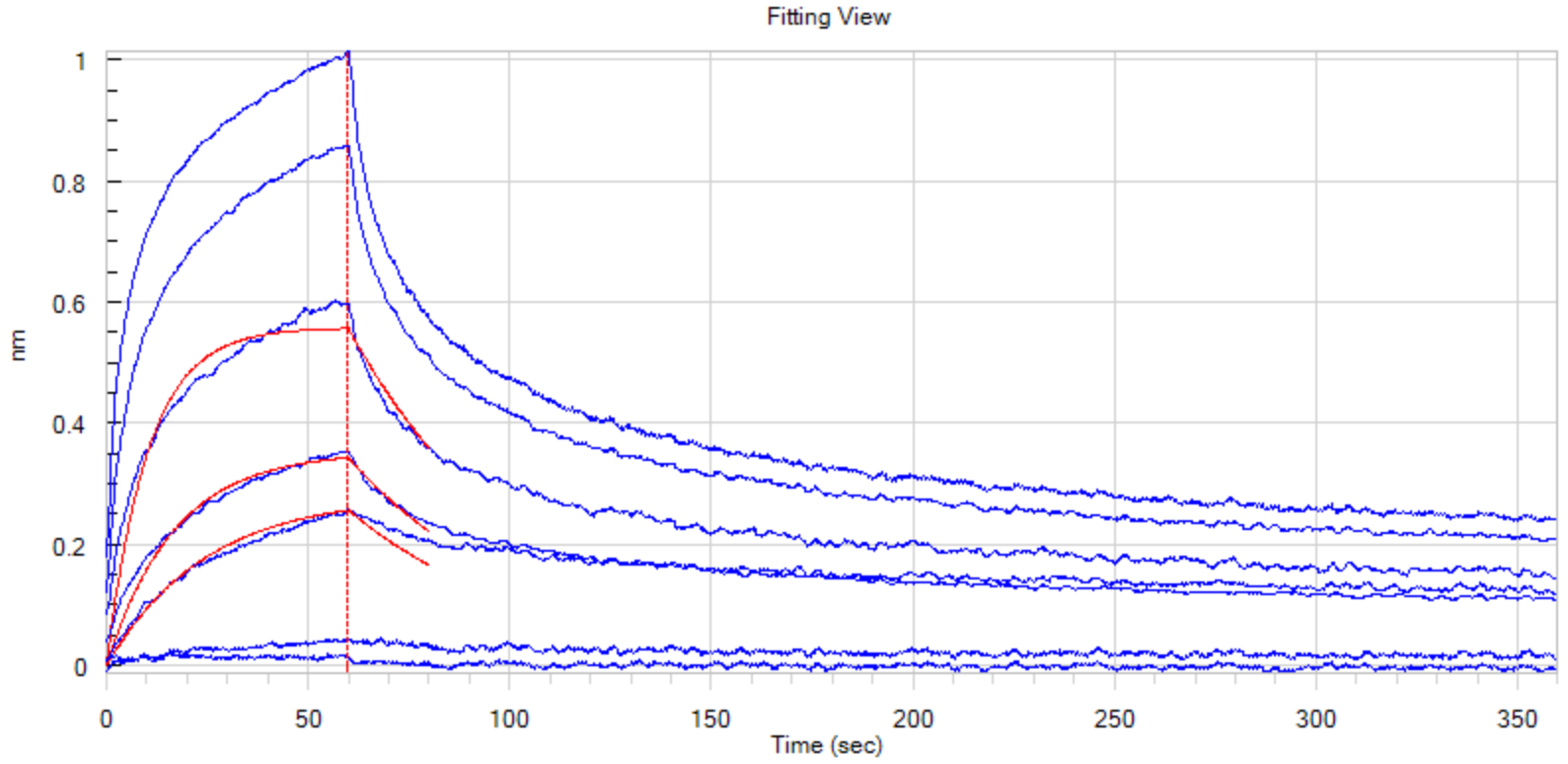
CD32b/c (FcγRIIB/C) Analysis



CD32b/c (FcγRIIB/C) Analysis



CD32b/c (FcγRIIB/C) Analysis

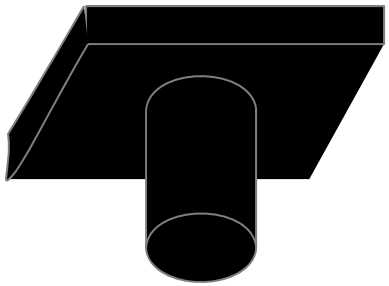


CD32b/c (FcγRIIB/C) Analysis

Loading Sample ID	Conc. (nM)	Response	KD (M)	KD Error	Full X^2	Full R^2	kon(1/Ms)	kon Error	kdis(1/s)	kdis Error	Rmax	Rmax Error
FcgRIIB/C	1000	0.9796										
FcgRIIB/C	500	0.8321										
FcgRIIB/C	250	0.5802	7.210E-08	4.053E-09	0.096064	0.981154	3.04E+05	1.25E+04	2.20E-02	8.43E-04	0.7187	0.015
FcgRIIB/C	125	0.3363	7.210E-08	4.053E-09	0.096064	0.981154	3.04E+05	1.25E+04	2.20E-02	8.43E-04	0.5557	0.0176
FcgRIIB/C	62.5	0.2356	7.210E-08	4.053E-09	0.096064	0.981154	3.04E+05	1.25E+04	2.20E-02	8.43E-04	0.6042	0.0251
FcgRIIB/C	31.3	0.0369										
FcgRIIB/C	15.6	0.0125										

Answer to the Mystery.....

Look carefully at the plate you use!!!



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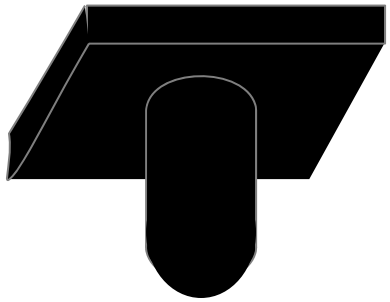

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discover more.

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