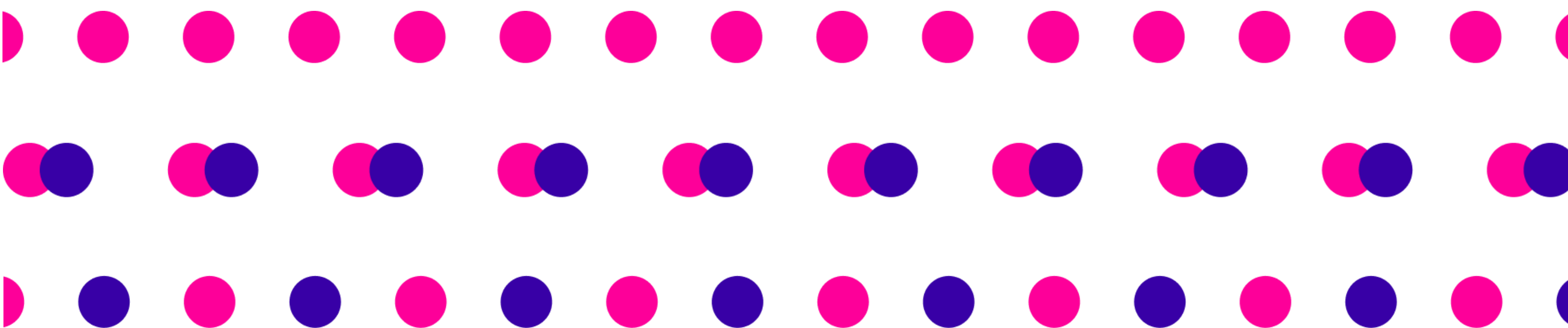
Fluidic
Sciences

APPLICATION NOTE

Binding of Fc-engineered IgG antibodies to FcRn

Fc enginerring of IgGs to extend antibody serum half-life or to modulate immune-receptor binding is an essential part of antibody drug development. However, quantifying human IgG-Fc receptor binding using surface-based, kinetic biomolecular interaction analysis has proven difficult due to reagent heterogeneity resulting in complex binding kinetics with multiple K_D values. Because of these challenges, scientists in many cases resort to reporting derived qualitative binding parameters which are difficult to compare across different labs or studies. In this application note, we present a straightforward equilibrium binding assay that yields easy to interpret and universally comparable K_D values for IgG–Fc receptor binding affinities. The assay utilizes microfluidic diffusional sizing (MDS) as a robust and physically meaningful way to investigate biomolecular interactions. An experiment requires only 20 minutes of hands-on time per IgG–Fc receptor pair and uses nanogram and low microgram amounts of Fc receptor and IgG, respectively.



Introduction

Therapeutic monoclonal antibodies are widely used in many disease areas including cancer, auto-immune disease, and infectious disease. All FDA-approved therapeutic antibodies are members of the IgG class. IgG molecules are multifunctional and multivalent as they are composed of two identical fragment antigen binding (Fab) domains and a single fragment crystallizable (Fc) domain. The two Fab domains are responsible for antigen recognition while the Fc domain is involved in humoral and effector cell functions. Fc engineering[1] is often required to modulate the function of therapeutic antibodies to either promote or abolish antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent cellular phagocytosis (ADCP) or to modify serum half-life, for example.

The neonatal Fc receptor (FcRn) is responsible for controlling serum IgG concentrations. The key is that IgG molecules only bind to FcRn at pH <6.5, whereas they have very low affinities at physiological pH of 7.4. IgGs circulating in the blood stream get internalized in acidic FcRn-containing endosomes by circulating endothelial cells and monocytes. In the acidic endosome, monomeric IgGs bind to FcRn and are recycled by being released back into circulation at physiological pH. Increasing the binding affinity to FcRn increases the recycled fraction of antibody and so extends the IgG half-life. Polymeric IgGs bound to antigens in immune complexes do not bind FcRn and so are degraded in lysosomes during antigen clearance.

An extended serum half-life is often thought to be beneficial for therapeutic antibodies as it should increase efficacy by sustaining serum concentrations, decreasing dosing frequency, and lowering cost to the patient and health system. In consequence, the Fc-engineered triple mutant IgG-YTE (M252Y/S254T/T256E)[2] was developed which showed increased binding affinity to FcRn and a 4-fold extended half-life in serum.

For antibodies that target antigens on the surface of immune cells, for example PD-1 blockers, it is essential to abolish immune effector functions such as ADCC and/or ADCP. To eliminate these pathways, Fc-engineering was employed to create another triple mutant molecule named IgG-TM (L234F/L235E/P331S)[3] which lacks immune receptor binding.

Despite the high therapeutic relevance of IgG–Fc receptor binding, measuring these interactions often proves to be challenging. In this application note, we present a straightforward in-solution assay based on microfluidic diffusional sizing (MDS) to determine binding affinities of Fc-engineered IgGs to FcRn in comparison with wild type IgG.

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Key Advantage

MDS does not suffer from challenges faced by surface-based, kinetic Fc receptor binding assays such as complex binding kinetics due to reagent heterogeneity[4] or varying results depending on the assay setup used. MDS delivers easy to interpret K_D values that provide quantitative information on the binding properties of Fc engineered antibodies.

Results

To analyze FcRn binding to IgGs, binding curves were measured for fluorescently labeled FcRn with different variants of IgG (Figure 1). Specifically, we used IgG wild type, IgG-TM, which lacks immune receptor binding, IgG-YTE, which has an extended serum half-life, and YTE-TM which combines both properties.

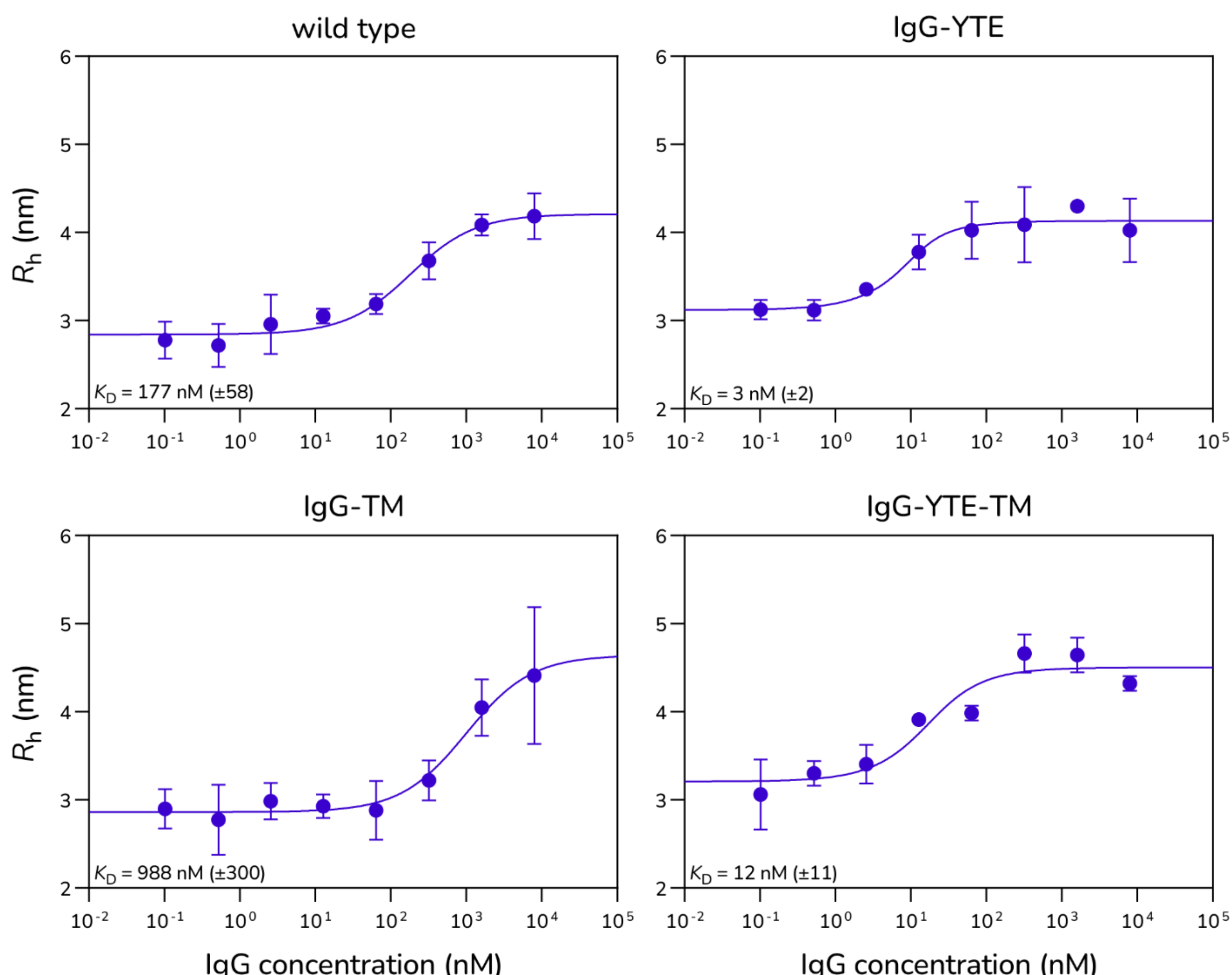

Figure 1. Equilibrium binding curves to analyze FcRn binding of IgG with engineered Fc regions. FcRn labeled with Alexa Fluor™ 488 was used at a constant concentration of 10 nM. Errors on data points are the standard deviation from at least three measurements per point, and uncertainty on K_D values are standard errors from nonlinear least squares fitting.

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To obtain binding curves, the four different IgG molecules were titrated against fluorescently labeled FcRn which was kept at a constant concentration of 10 nM for all curves. Binding was assessed through an increase in average size of the FcRn molecule as determined by MDS on the Fluidity One-M.

As shown in Figure 2, IgG molecules that contain the YTE triple mutation in their Fc domains exhibited the highest binding affinities to FcRn, consistent with the extended half-life reported for this IgG variant. IgG-TM showed a slightly reduced binding affinity to FcRn as compared with IgG wild type. This affinity reduction could however be overcome by combining the TM mutations with the YTE mutations such that IgG YTE-TM benefits from both extended half life and absence of effector functions.

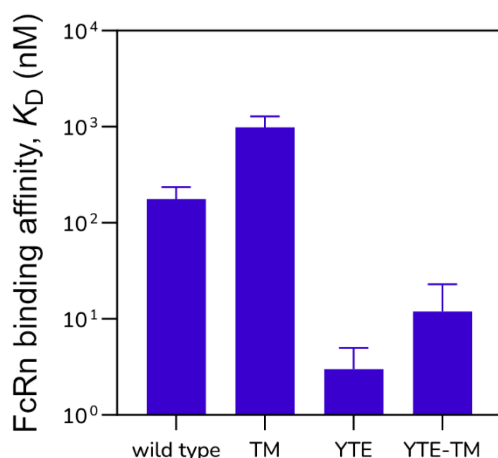

Figure 2. Overview of dissociation constants, K_D, measured for FcRn binding of wild type IgG and three different Fc engineered IgG molecules. IgG-TM exhibits no immune effector function, IgG-YTE has extended serum-half life, and IgG YTE-TM combines the properties of IgG-YTE and IgG-TM.

Conclusion

Here, we present an in-solution based equilibrium FcRn–IgG binding assay which does not suffer from the challenges faced by surface-based, kinetic biomolecular interaction analysis and provides K_D values for quantitative assessment and comparison of binding affinities. The assay requires only 20 min of hands-on time per FcRn–IgG pair and uses only 70 ng of FcRn and 30 µg of IgG.

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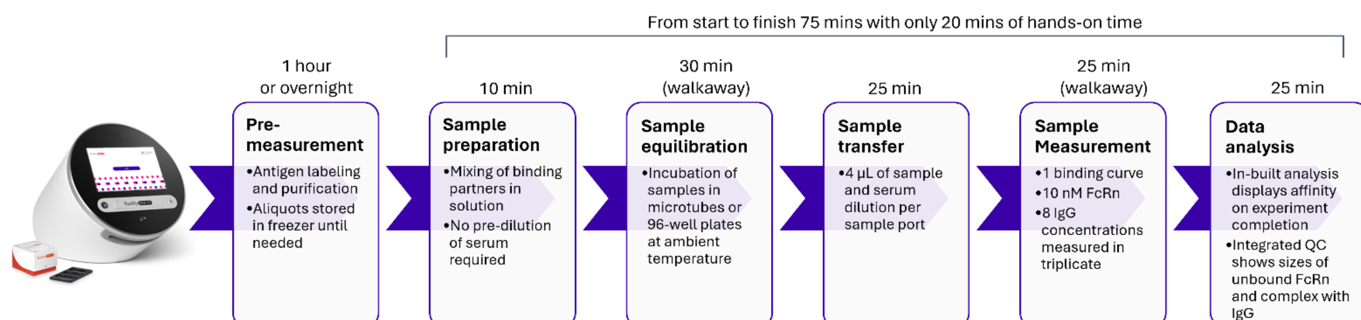
Workflow and assay features



Assay features:

- Quick and easy to perform
- Quantitative binding info (K_D)
- K_D range from pM to µM
- In-built QC due to size measurement
- Required amount of FcRn: 70 ng
- Required amount of IgG: 30 µg

Methods

FcRn labeling with Alexa Fluor™ 488 NHS ester

FcRn (P55899, R&D systems) was fluorescently labeled with Alexa Fluor™ 488 NHS ester (Thermo Fisher). A solution containing 100 µg of FcRn was mixed with dye at a three-fold molar excess in the presence of $NaHCO_3$ (Merck) buffer at pH 8.3 and incubated at 4 °C overnight. Unbound label was removed using a Zeba™ desalting chromatography cartridge (7K MWCO, 1 mL, Thermo Fisher). FcRn was stored at −80 °C in PBS pH 7.4 containing 10% (w/v) glycerol.

FcRn–IgG binding affinity assay

IgGs were diluted into PBS containing 0.05% (v/v) Tween 20 to achieve a five-fold serial dilution series. IgG dilutions were subsequently mixed with a solution of labeled FcRn to obtain a final FcRn concentration of 10 nM.

Samples were incubated for 30 min at ambient temperature prior to measurement. For MDS measurements on a Fluidity One-M instrument, 4 µL of the FcRn–IgG mixtures were loaded on the microfluidic chip having 4 µL of PBS with Tween 20 (0.05% (v/v)) as the auxiliary fluid. Measurements were performed in triplicate using the (2.0–9.3) nm size range and viscosity setting 1.

The binding affinity was quantified in terms of the equilibrium dissociation constant K_D, which was determined by non-linear least-squares fitting. To this end, the concentration of FcRn was set to 10 nM.

Equations

$$f_d = \rho_f + \frac{[B]}{[L]}(\rho_b - \rho_f)$$

F_d	fraction of labeled species that diffused into the auxiliary flow of the microfluidic chip
[L]	total concentration of labeled species
[B]	equilibrium concentration of bound labeled species
ρ_f	fraction of free labeled species
ρ_b	fraction of bound labeled species

$$[B] = \frac{[L] + \alpha[U] + K_D - \sqrt{([L] + \alpha[U] + K_D)^2 - 4\alpha[L][U])}}{2}$$

[U]	total concentration of binding sites in the unlabeled species
α	fractional concentration of unlabeled species in the binding measurement
K_D	dissociation constant (affinity)

Acknowledgements

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