

Human Fc gamma RI / CD64 binding kit (TR-FRET)

Pack Size: 100 Tests & 500 Tests

Catalog Number: FRT-03

IMPORTANT: Please carefully read this manual before performing your experiment.

For Research Use Only. Not For Use In Diagnostic Or Therapeutic Procedure

INTENDED USE

This kit is designed to facilitate the ADCC and ADCP functional performance evaluation of antibody drug candidates, and also high-throughput screening of anti-human CD64 antibodies. It can also be used as a universal detection tool to identify the ability of antibody drugs to bind to human CD64. It is for research use only (RUO).

BACKGROUND

Fc gamma receptors (Fc γ Rs) are membrane anchored proteins expressed in many immune effector cells and mediate antibody functions. The human Fc γ Rs consists of several activating receptors, namely Fc γ RI (CD64), Fc γ RIIa (CD32a), Fc γ RIIc (CD32c), Fc γ RIIIa (CD16a), one inhibitory receptor Fc γ RIIb (CD32b), and one receptor with unclear functions Fc γ RIIIb (CD16b).

Human Fc γ RI (CD64) is a 70kDa transmembrane glycoprotein, a member of the Ig superfamily, with three C2 structures in the extracellular region. It is the only high-affinity Fc receptor for both the monomeric IgG and immune complex (IC). Human Fc γ RI mainly binds to human monomer IgG1/ IgG3 and mouse IgG2a/IgG3, the binding affinity with human IgG4 is clearly reduced. In contrast, Human Fc γ RI has no binding to human IgG2.

Human Fc γ RI is mainly expressed on monocytes/macrophages, dendritic cells (DCs), and activated neutrophils, but the expression levels are different.

Interaction sites for Fc γ Rs are indicated to be located to the hinge proximal region of IgG-Fc via their extracellular domains (ECDs). Within IgG, the fragment antigen binding (Fab) region contains the paratope, and can exert direct effects through binding interactions with antigen. Meanwhile, the fragment crystallizable (Fc) region interacts with Fc γ Rs to mediate indirect effector functions such as antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP) and these interactions are important for the efficacy and safety of therapeutic antibodies. One of the major functions of Human Fc γ RI is to activate myeloid cells to phagocytose IgG1 and IgG-bound target cells via ADCP. The interactions of therapeutic antibodies with Fc γ Rs are measured in vitro as indicators of antibody functional performance.

The Human Fc gamma RI / CD64 binding kit (TR-FRET) takes advantage of binding of Europium-chelate labeled Fc γ RI (donor) and FA labeled Human IgG1 antibody (acceptor) in a homogeneous (no wash) TR-FRET (Time-Resolved Fluorescence Resonance Energy Transfer) competition assay to measure the interaction between

human Fc γ RI and antibody drug candidates. It is designed to facilitate the ADCC and ADCP functional performance evaluation of antibody drug candidates, and also high-throughput screening of anti-human CD64 antibodies within 0.5-1 hours. It is highly sensitive, has a short detection time and easy to use.

PRINCIPLE OF THE ASSAY

This Human Fc gamma RI / CD64 binding kit (TR-FRET) is based on TR-FRET technology (Time-Resolved Fluorescence Resonance Energy Transfer). Use the mixture of biotinylated human Fc gamma RI / CD64 and Europium-chelate labeled streptavidin as the donor, FA labeled Human IgG1 antibody as the acceptor.

Your experiment will include 3 simple steps:

1) Mix the sample or Human IgG standard in the kit with Human Fc gamma RI / CD64 Protein Europium-chelate (Donor) and incubate at room temperature for 0.5 hours.

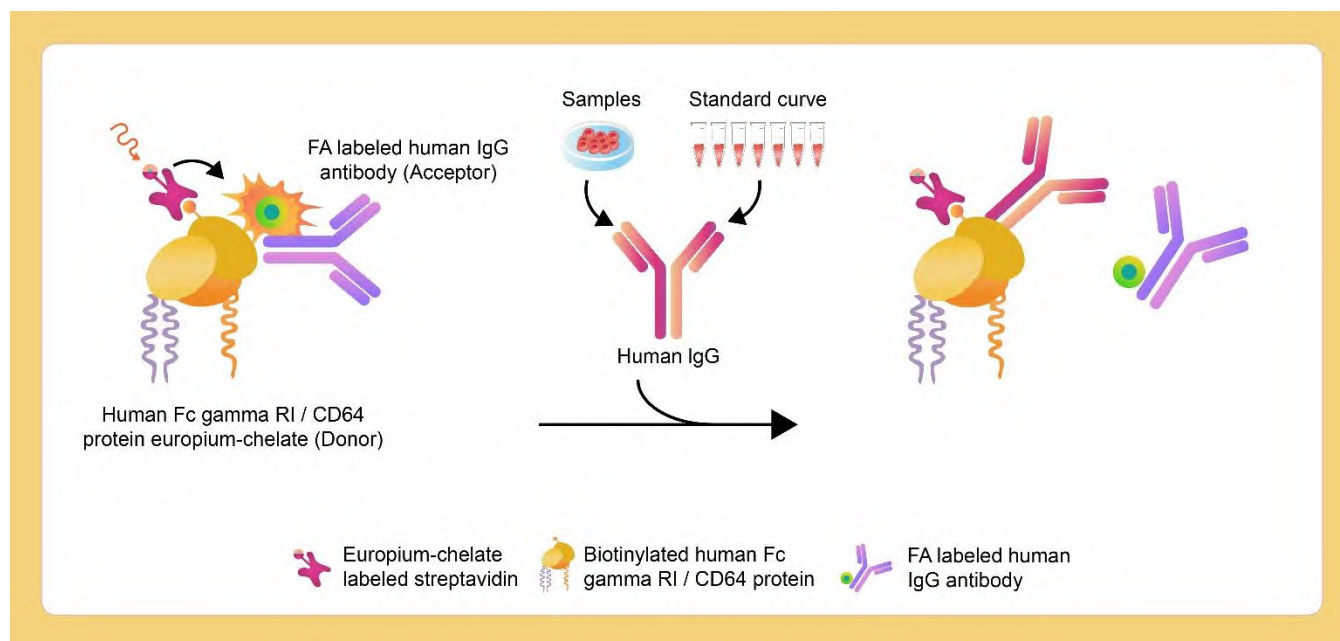
2) Add FA labeled human IgG antibody (Acceptor) and incubate at room temperature for at least 0.5 hours.

3) Use the TR-FRET module of a microplate reader to read the fluorescence signal at 665 nm and 620 nm. Calculate the Ratio based on the formula $\text{Ratio} = \frac{\text{Signal } 665 \text{ nm}}{\text{Signal } 620 \text{ nm}} \times 10^4$. The Ratio value is negatively correlated with the antibody content in the sample.

— When the sample does not contain human Fc gamma RI / CD64 binding components, the donor and acceptor are in close proximity because of the binding of human Fc gamma RI / CD64 and FA labeled Human IgG1 antibody. The 620nm signal emitted by the donor under specific light source excitation is received by the acceptor, emitting a 665nm signal.

— When the sample contains human Fc gamma RI / CD64 binding components, the components inhibit the binding between the donor and acceptor and thereby prevents FRET from occurring.

FIG.1 PRINCIPLE OF THE ASSAY



MATERIALS PROVIDED

TABLE 1. MATERIALS PROVIDED

Catalog	Components	Size (100 Tests)	Size (500 Tests)	Format	Storage	
					Unopened	Opened
FRT03-C01	Human Fc gamma RI / CD64 Protein Europium-chelate	0.6 µg	3 µg	Powder	2-8°C, avoid light	-70°C, avoid light
FRT03-C02	FA labeled human IgG antibody	2.4 µg	12 µg	Powder	2-8°C, avoid light	-70°C, avoid light
FRT03-C03	Human IgG standard	100 µg	50 0µg	Powder	2-8°C	-70°C
FRT03-C04	Sample Dilution Buffer	10 mL	10 mL	Liquid	2-8°C	2-8°C
FRT03-C05	Detection Buffer	10 mL	10 mL	Liquid	2-8°C	2-8°C

MATERIALS REQUIRED BUT NOT PROVIDED

1. Single channel or multichannel pipettes with 10 µL, 200 µL and 1000 µL precision;
2. 10 µL, 200 µL and 1000 µL pipette tips;

3. Microporous plate shaker;
4. Microplate reader with TR-FRET module which can detect signals at 665 nm/620 nm;
5. Test Tubes;
6. Timer;
7. White plate (96 or 384-well low volume white plate): For example, HTRF 96-well, white plate, low volume (Revvity, Cat. No. 66PL96100); White Opaque 384-well Microplate (Perkinelmer, Cat. No. 6007299);
8. Deionized or distilled water for reconstitute.

STORAGE AND VALIDITY INSTRUCTIONS

1. Unopened kit should be stored at 2°C-8°C upon receiving.
2. Find the expiration date on the outside packaging and do not use reagents past their expiration date.
3. The opened kit should be stored per components table. The shelf life is 30 days from the date of opening.

REAGENT PREPARATION

1. Bring all reagents and samples to room temperature (20°C-25°C) before use.
2. Reconstitute the provided lyophilized materials to stock solutions with water as recommended in Table 2 and solubilize for 15 to 30 minutes at room temperature with occasional gentle mixing. Avoid vigorous shaking or vortexing. The reconstituted stock solutions should be stored at -70°C. It is recommended not to freeze-thaw more than 2 times.

Note: Human Fc gamma RI / CD64 Protein Europium-chelate and FA labeled human IgG antibody stock solution should be protected from light.

TABLE 2. RECONSTITUTION METHODS FOR 100 TESTS AND 500TESTS

Catalog	Components	Size (100 Tests)		Size (500 Tests)		Stock Solution Conc.
		Amount	Reconstitution Buffer and Vol.	Amount	Reconstitution Buffer and Vol.	
FRT03-C01	Human Fc gamma RI / CD64 Protein Europium-chelate	0.6µg	60 µL water	3µg	300 µL water	10 µg/mL
FRT03-C02	FA labeled human IgG antibody	2.4µg	60 µL water	12µg	300 µL water	40 µg/mL
FRT03-C03	Human IgG standard	100µg	50 µL water	500µg	250 µL water	2000 µg/mL

RECOMMENDED PROTOCOL

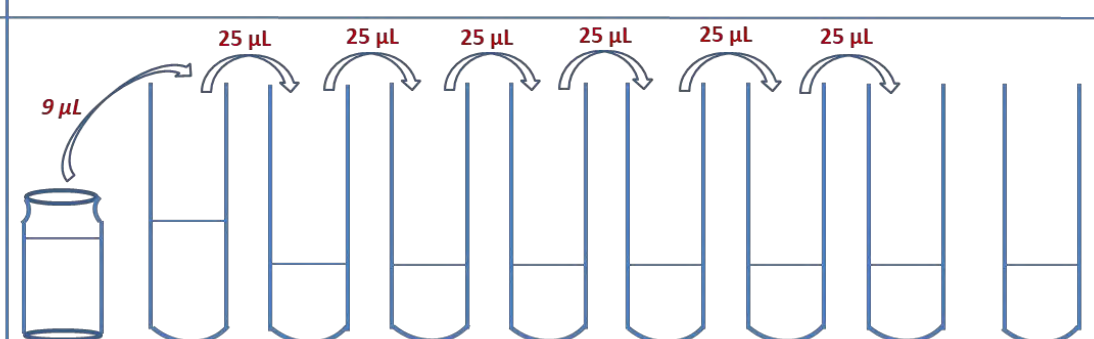
1. Add Samples

1.1 Make series dilution of the samples as appropriate.

1.2 If you intend to use the provided Human IgG standard as a reference (Std.), you may dilute the antibody as recommend in FIG. 2. Dilute the sample to be tested appropriately using the Sample Dilution Buffer.

1.3 Add 10 μL of sample and standard solution to each well according to our recommendation (FIG. 3) or your own plate setup.

FIG.2 PREPARATION OF 1:4 SERIAL DILUTIONS OF THE HUMAN IGG STANDARD

Tubes/ Solution Code	Human IgG Stock Solution	Std 7	Std 6	Std 5	Std 4	Std 3	Std 2	Std 1	Std 0 (Blank)
Operating									
Solution Conc.	2000 $\mu\text{g}/\text{mL}$	150 $\mu\text{g}/\text{mL}$	37.5 $\mu\text{g}/\text{mL}$	9.375 $\mu\text{g}/\text{mL}$	2.344 $\mu\text{g}/\text{mL}$	0.586 $\mu\text{g}/\text{mL}$	0.146 $\mu\text{g}/\text{mL}$	0.037 $\mu\text{g}/\text{mL}$	0 $\mu\text{g}/\text{mL}$
Dilution Buffer Vol.		111 μL	75 μL	75 μL	75 μL	75 μL	75 μL	75 μL	75 μL

2. Add Donor

Dilute **Human Fc gamma RI / CD64 Protein Europium-chelate** stock solution (10 $\mu\text{g}/\text{mL}$) to 1 $\mu\text{g}/\text{mL}$ with **Detection Buffer** to make Donor working solution. The working solution should be prepared immediately before use and should not be stored. Add 5 μL of Donor working solution to each well. Seal the plate with microplate sealing film and incubate at room temperature (20°C-25°C) for 0.5 hours on orbital shaker at 400-600 rpm to ensure the samples and donor can react adequately.

3. Add Acceptor

Dilute **FA labeled human IgG antibody** stock solution (40 $\mu\text{g}/\text{mL}$) to 4 $\mu\text{g}/\text{mL}$ with **Detection Buffer** to make

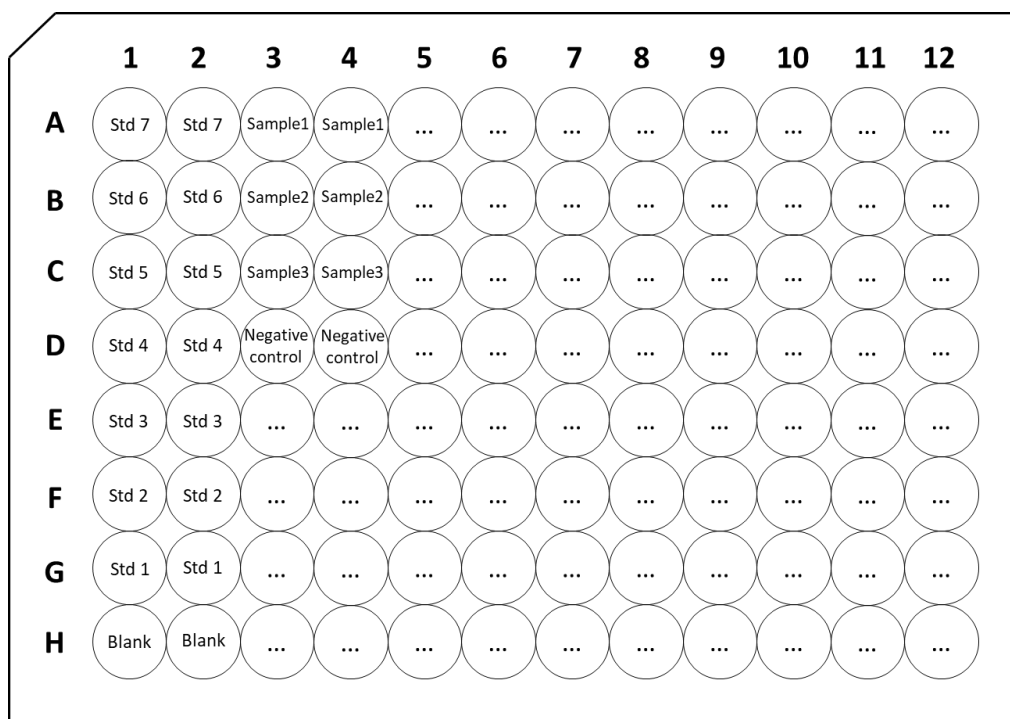
Acceptor working solution. The working solution should be prepared immediately before use and should not be stored. Add 5 µL of Acceptor working solution to each well. Seal the plate with microplate sealing film and incubate at room temperature (20°C-25°C) for 0.5 hours on orbital shaker at 400-600 rpm.

Refer to FIG. 3 and Table 3 for the design of microplate layout according to the experimental requirements, and add the corresponding reaction solution into the corresponding plate wells.

TABLE 3. SAMPLES ADDING TO MICROPLATE

	1	2	3	4
A	10 µL Std7 5 µL Donor working solution 5 µL Acceptor working solution	10 µL Std7 5 µL Donor working solution 5 µL Acceptor working solution	10 µL Sample1 5 µL Donor working solution 5 µL Acceptor working solution	10 µL Sample1 5 µL Donor working solution 5 µL Acceptor working solution
B	10 µL Std6 5 µL Donor working solution 5 µL Acceptor working solution	10 µL Std6 5 µL Donor working solution 5 µL Acceptor working solution	10 µL Sample2 5 µL Donor working solution 5 µL Acceptor working solution	10 µL Sample2 5 µL Donor working solution 5 µL Acceptor working solution
C	10 µL Std5 5 µL Donor working solution 5 µL Acceptor working solution	10 µL Std5 5 µL Donor working solution 5 µL Acceptor working solution	10 µL Sample3 5 µL Donor working solution 5 µL Acceptor working solution	10 µL Sample3 5 µL Donor working solution 5 µL Acceptor working solution
D	10 µL Std4 5 µL Donor working solution 5 µL Acceptor working solution	10 µL Std4 5 µL Donor working solution 5 µL Acceptor working solution	10 µL Sample Dilution Buffer 5 µL Donor working solution 5 µL Detection Buffer	10 µL Sample Dilution Buffer 5 µL Donor working solution 5 µL Detection Buffer
E	10 µL Std3 5 µL Donor working solution 5 µL Acceptor working solution	10 µL Std3 5 µL Donor working solution 5 µL Acceptor working solution
F	10 µL Std2 5 µL Donor working solution 5 µL Acceptor working solution	10 µL Std2 5 µL Donor working solution 5 µL Acceptor working solution
G	10 µL Std1 5 µL Donor working solution 5 µL Acceptor working solution	10 µL Std1 5 µL Donor working solution 5 µL Acceptor working solution
H	10 µL Sample Dilution Buffer 5 µL Donor working solution 5 µL Acceptor working solution	10 µL Sample Dilution Buffer 5 µL Donor working solution 5 µL Acceptor working solution

FIG.3 PLATE LAYOUT



4. Data Recording

Use the TR-FRET module of a microplate reader to read the fluorescence signal at 665nm and 620nm.

5. Calculate Ratio

Calculate the Ratio based on the formula $\text{Ratio} = \frac{\text{Signal } 665 \text{ nm}}{\text{Signal } 620 \text{ nm}} \times 10^4$.

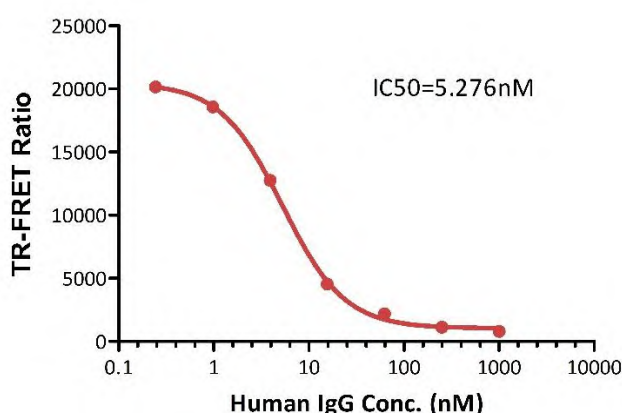
PRECAUTIONS

1. This kit is for research use only and is not for use in diagnostic or therapeutic applications.
2. This kit should be used according to the provided instructions.
3. Do not mix reagents from different lots.
4. Bring all reagents and samples to room temperature (20°C-25°C) before use. If crystals have formed in the buffer solution, incubate until the crystals have completely dissolved. Before use, bring the solution back to room temperature.
5. This kit should be stored at 2°C-8°C.
6. Please prepare the working solution of each component according to the needs of the experiment. All prepared

working solution is for one-time use and cannot be stored.

TYPICAL DATA

For each experiment, a standard curve needs to be set for each micro-plate, and the specific Ratio value may vary depending on different laboratories, testers, or equipment. Different microplate reader and different gain value may give different fluorescence signal. Please adjust parameters according to the equipment manual. Reduce the gain value when the signal is too high. The following data is from the BMG CLARIOstar Plus. This following data is for reference only.

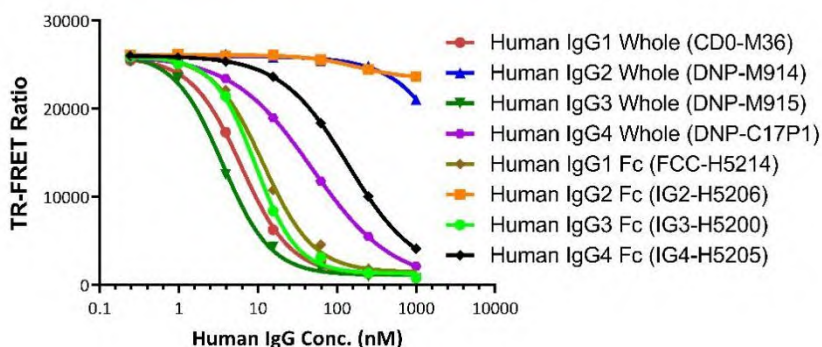


Human IgG standard Conc.	Human IgG standard Conc.	Signal 665 nm	Signal 620 nm	Ratio
150 µg/mL	1000 nM	1935	24398	793
37.5 µg/mL	250 nM	2819	24758	1139
9.375 µg/mL	62.5 nM	5371	24461	2196
2.344 µg/mL	15.625 nM	10816	23776	4549
0.586 µg/mL	3.906 nM	27800	21796	12755
0.146 µg/mL	0.977 nM	36700	19756	18577
0.037 µg/mL	0.244 nM	39438	19572	20150
0 µg/mL	0 nM	39462	19237	20514

DIFFERENT ANDIBODY SUBTYPES DATA

The kit has been used to detect different subclasses of Human IgG Whole and Fc fragment proteins (Human IgG1, Human IgG2, Human IgG3 and Human IgG4), which exhibit different IC50 results as expected.

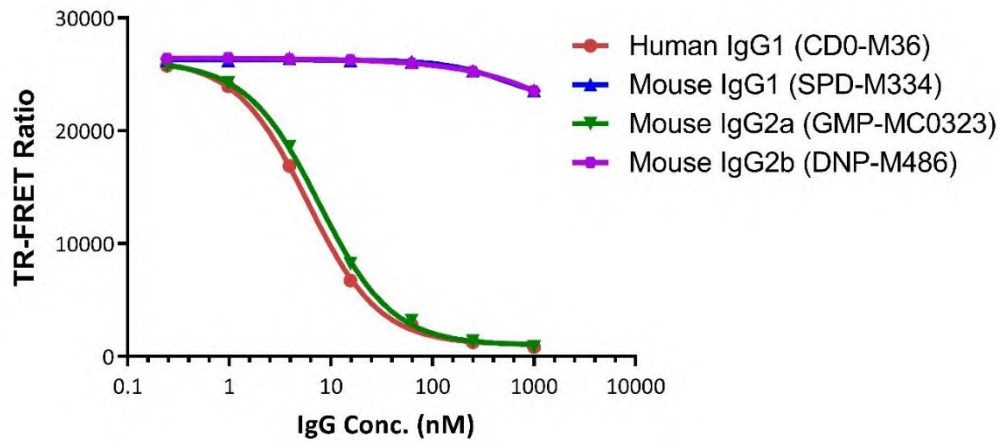
As shown in the following figure, human CD64 is a high affinity receptor that binds to human IgG1, IgG3 and IgG4 with nanomolar affinity, while it has not been shown to bind to human IgG2.



Antibody	IC50 (nM)
Human IgG1 Whole (CD0-M36)	6.183
Human IgG2 Whole (DNP-M914)	No Binding
Human IgG3 Whole (DNP-M915)	3.598
Human IgG4 Whole (DNP-C17P1)	48.83
Human IgG1 Fc fragment (FCC-H5214)	11.74
Human IgG2 Fc fragment (IG2-H5206)	No Binding
Human IgG3 Fc fragment (IG3-H5200)	9.353
Human IgG4 Fc fragment (IG4-H5205)	139.4

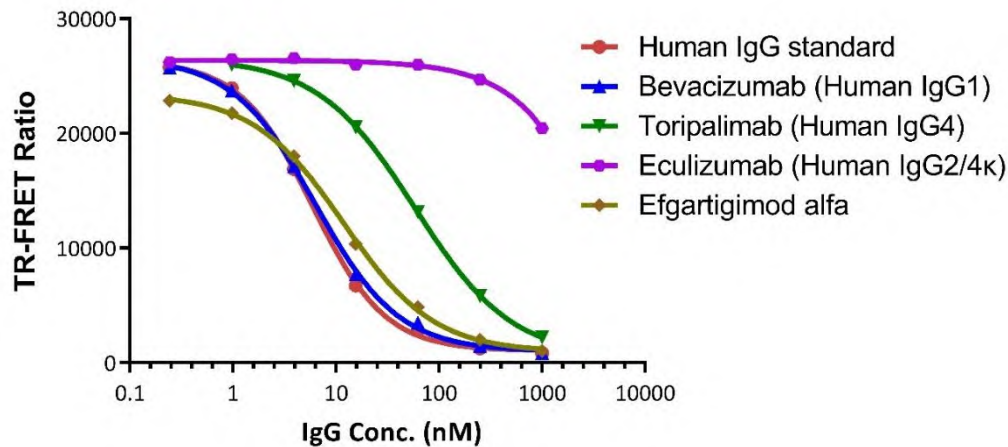
SPECIES SELECTIVITY

The kit has been used to detect different subclasses of mouse IgG (mouse IgG1, mouse IgG2a and mouse IgG2b), which exhibit different IC50 results as expected. As shown in the figure, human CD64 binds to mouse IgG2a with a high affinity, but not mouse IgG1 and mouse IgG2b.



APPLICATION OF FDA APPROVED ANTIBODY DRUGS DETECTION

The kit has been used to detect four FDA approved antibody drugs with different affinities binding to human CD64. Bevacizumab, Toripalimab, and Efgartigimod alfa bind to human CD64 with the nanomolar affinity. The Fc of Eculizumab has been modified into the human IgG2 hinge region and human IgG4 CH2-CH3 region, so it doesn't bind to human CD64.



MATRIX EFFECT

Verify potential matrix effects by adding different levels of DEME, RPMI1640, FBS and HSA to the Sample Diluted buffer.

