Human Fc gamma RIIIA / CD16a (F176) binding Kit (TR-FRET)

Pack Size: 100 tests & 500 tests

Catalog Number：FRT-06

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 functional performance evaluation of antibody drug candidates, and also high-throughput screening of anti-human CD16a (F176) antibodies. It can also be used as a universal detection tool to identify the ability of antibody drugs to bind to human CD16a (F176).

It is intended for research use only (RUO).

BACKGROUND

Fc gamma receptors (FcγRs) are membrane anchored proteins expressed in many immune effector cells and mediate antibody functions. The human FcγRs consists of several activating receptors, namely FcγRI (CD64), FcγRIIa (CD32a), FcγRIIc (CD32c), FcγRIIIa (CD16a), one inhibitory receptor FcγRIIb (CD32b), and one receptor with unclear functions FcγRIIIb (CD16b).

FcγRIIIa (CD16a) is a transmembrane receptor with a short C-ter cytoplasmic tail and possesses two extracellular Ig-like domains, which bind to IgG with low affinity, it can interact with all of 4 subclasses of human IgGs including IgG1, IgG2, IgG3, and IgG4, although IgG1 and IgG3 show the highest affinity.

FcγRIIIa (CD16a) is expressed on macrophages, mast cells, and NK cells. Cross-linking of the receptor by immune complexes can trigger various effector functions, such as phagocytosis, degranulation, and antibody-dependent cell-mediated cytotoxicity (ADCC).

Human Fc gamma RIIIA / CD16a (F176) binding kit (TR-FRET) takes advantage of binding of Europium-chelate labeled human Fc gamma RIIIA / CD16a (F176) (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 gamma RIIIA / CD16a (F176) and antibody drug candidates. It is designed to facilitate the ADCC functional performance evaluation of antibody drug candidates, and also high-throughput screening of anti-human CD16a antibodies within 0.5-1 hours. It is highly sensitive, has a short detection time and easy to use.

PRINCIPLE OF THE ASSAY

Human Fc gamma RIIIA / CD16a (F176) binding kit (TR-FRET) is based on TR-FRET technology (Time-Resolved Fluorescence Resonance Energy Transfer). Use the mixture of biotinylated human Fc gamma RIIIA / CD16a (F176)

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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 RIIIA / CD16a (F176) Protein Europium-chelate (Donor) and incubate at room temperature for 0.5 hours.

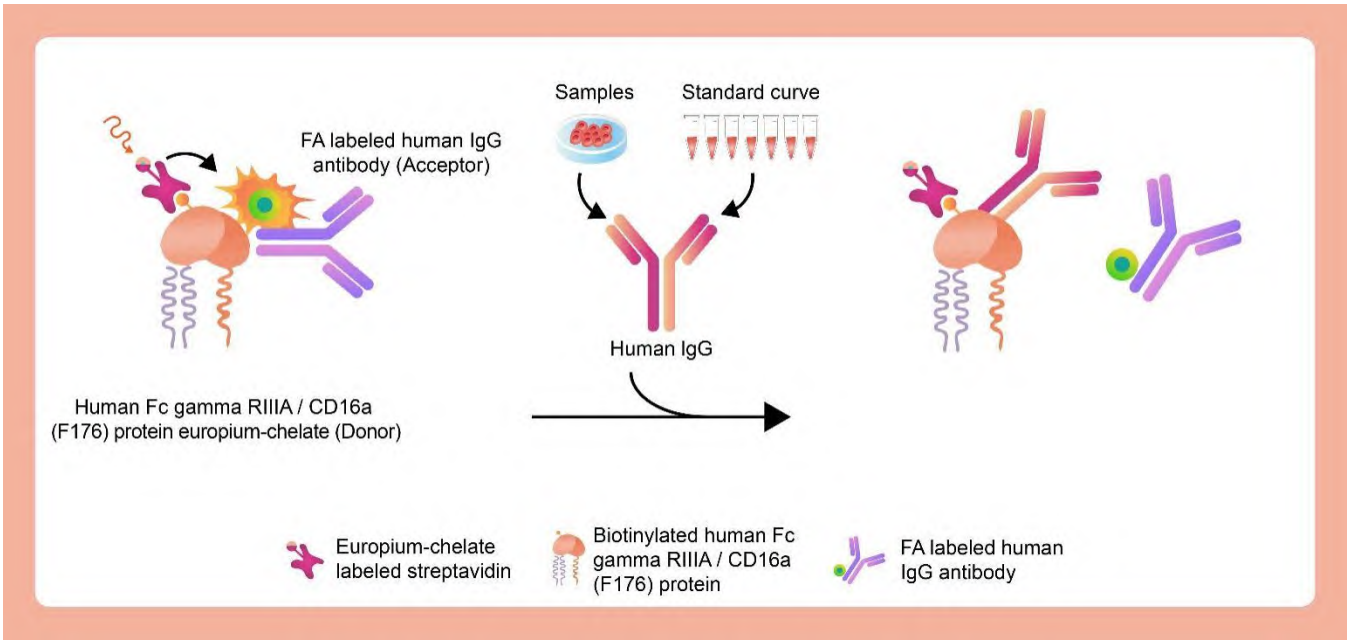
2) Add FA labeled human IgG antibody (Acceptor) and incubate at room temperature for at least 0.5 hours.

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

- When the sample does not contain human Fc gamma RIIIA / CD16a (F176) binding components, the donor and acceptor are in close proximity because of the binding of human Fc gamma RIIIA / CD16a (F176) and FA labeled Human IgG1 antibody. The 620 nm signal emitted by the donor under specific light source excitation is received by the acceptor, emitting a 665 nm signal.

- When the sample contains human Fc gamma RIIIA / CD16a (F176) binding components, the components inhibit the binding between the donor and acceptor and thereby prevents FRET from occurring.

FIG.1 PRINCIPLE OF THE ASSAY



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MATERIALS PROVIDED

TABLE 1. MATERIALS PROVIDED

Catalog	Components	Size (100 tests)	Size (500 tests)	Format	Storage	
					Unopened	Opened
FRT06-C01	Human Fc gamma RIIIA / CD16a (F176) Protein Europium-chelate	100 tests	500 tests	Powder	2-8℃, avoid light	-70℃, avoid light
FRT06-C02	FA Labeled Human IgG Antibody	100 tests	500 tests	Powder	2-8℃, avoid light	-70℃, avoid light
FRT06-C03	Human IgG Standard	400 μg	2 mg	Powder	2-8℃	-70℃
FRT06-C04	Sample Dilution Buffer	10 mL	10 mL	Liquid	2-8℃	2-8℃
FRT06-C05	Detection Buffer	10 mL	10 mL	Liquid	2-8℃	2-8℃

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 665nm/620nm;
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℃-8℃ 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.

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REAGENT PREPARATION

1. Bring all reagents and samples to room temperature (20℃-25℃) 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 vertexing. The reconstituted stock solutions should be stored at -70℃. It is recommended not to freeze-thaw more than 2 times.

***Note**: Human RIIIA / CD16a (F176) 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.	
FRT06-C01	Human Fc gamma RIIIA / CD16a (F176) Protein Europium-chelate	100 tests	60 µL water	500 tests	300 µL water	/
FRT06-C02	FA Labeled Human IgG Antibody	100 tests	60 µL water	500 tests	300 µL water	/
FRT06-C03	Human IgG Standard	400 µg	200 µL water	2 mg	1000 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 (FRT06-C03) 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.

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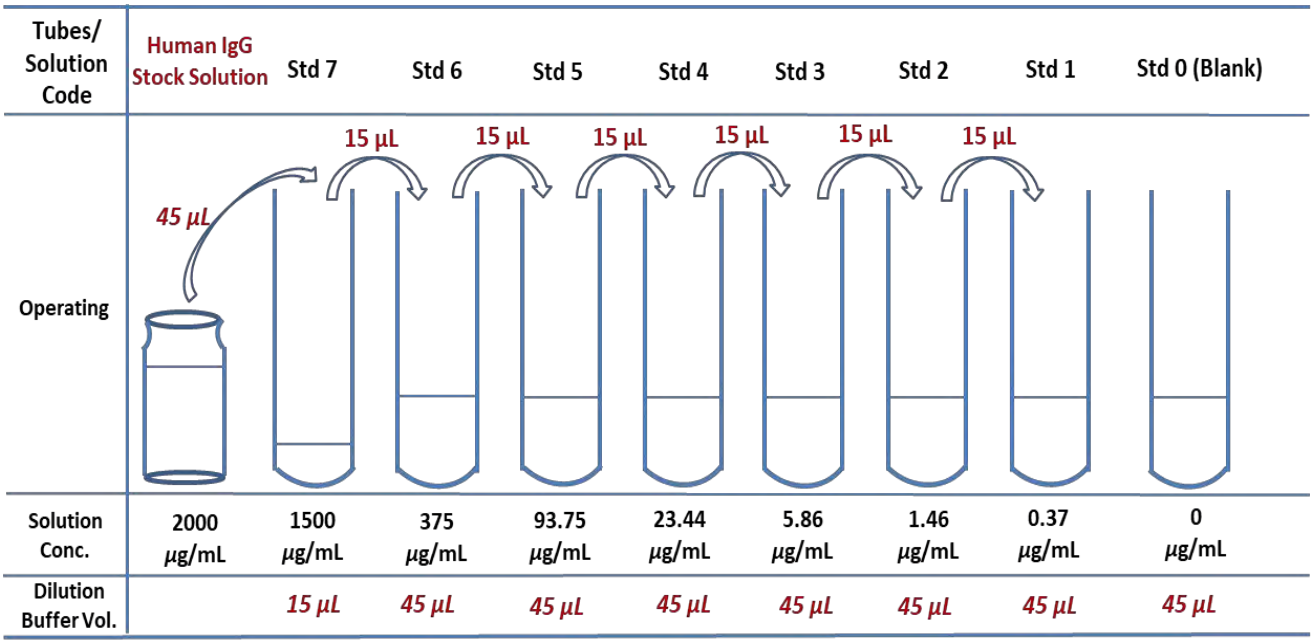
FIG.2 PREPARATION OF 1:4 SERIAL DILUTIONS OF THE HUMAN IGG STANDARD



2. Add Donor

Dilute **Human Fc gamma RIIIA / CD16a (F176) Protein Europium-chelate** stock solution 10 times 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℃-25℃) 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 10 times 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℃-25℃) 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.

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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	…	…

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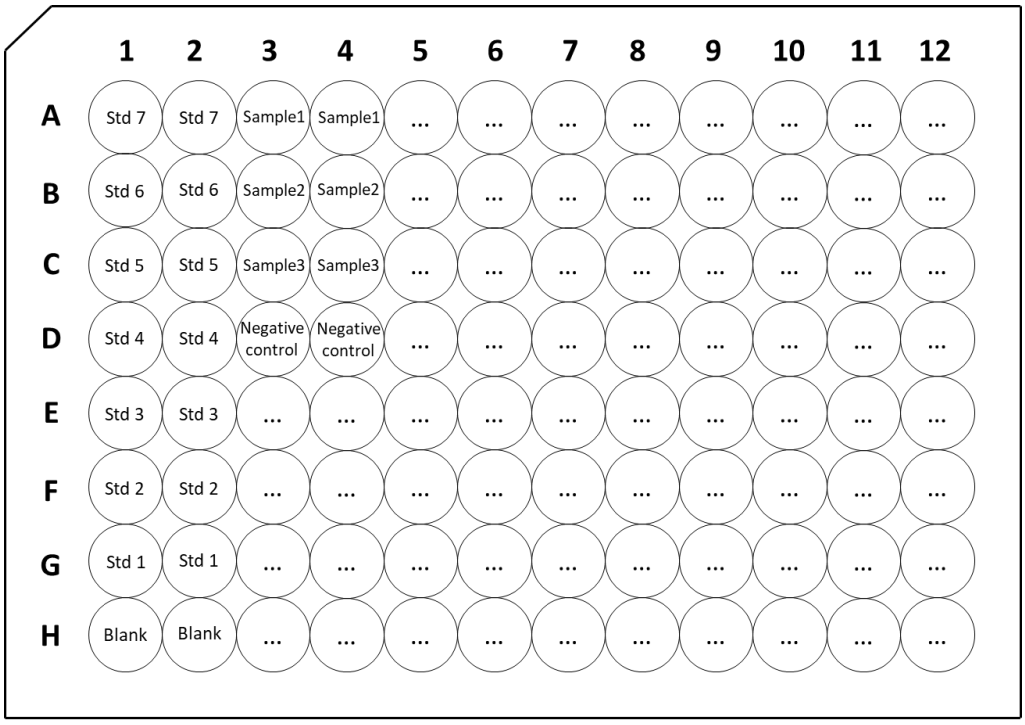
FIG.3 PLATE LAYOUT



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 $\text{Ratio} = \frac{\text{Signal 665 nm}}{\text{Signal 620 nm}} \times 10^4$.

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℃-25℃) 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.

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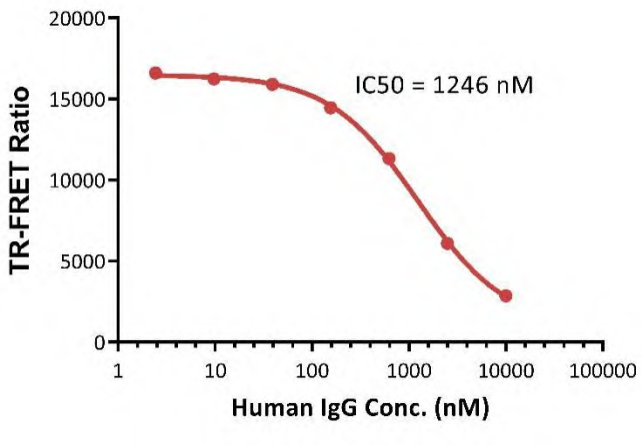
TYPICAL DATA

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 Labtech CLARIOstar Plus. This following data is for reference only.

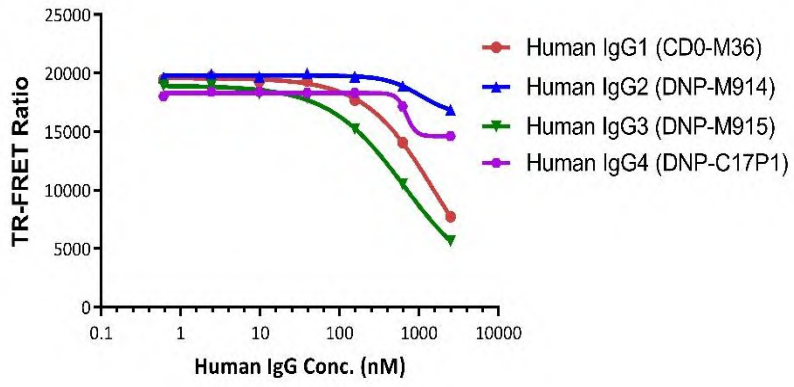


Human IgG standard Conc.	Human IgG standard Conc.	Signal 665 nm	Signal 620 nm	Ratio
1500 μg/mL	10000 nM	22122	77591	2850.2
375 μg/mL	2500 nM	45001	73775	6099.2
93.75 μg/mL	625 nM	76485.5	67568.5	11328.4
23.44 μg/mL	156.25 nM	93230	64486.5	14451.1
5.86 μg/mL	39.06 nM	102997	64751.5	15902.0
1.46 μg/mL	9.77 nM	108662.5	66954.5	16233.9
0.37 μg/mL	2.44 nM	104305	62797	16605.7
0 μg/mL	0 nM	106611	65198.5	16351.9

DIFFERENT ANDIBODY SUBTYPES DATA

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

As shown in the following figure, human CD16a (F176) binds to human IgG1, IgG2, IgG3 and IgG4 with low affinity, and IgG1 and IgG3 show the higher affinity than IgG2 and IgG4.



Antibody	IC50 (nM)
Human IgG1 (CD0-M36)	1524
Human IgG2 (DNP-M914)	1048
Human IgG3 (DNP-M915)	651.6
Human IgG4 (DNP-C17P1)	NA

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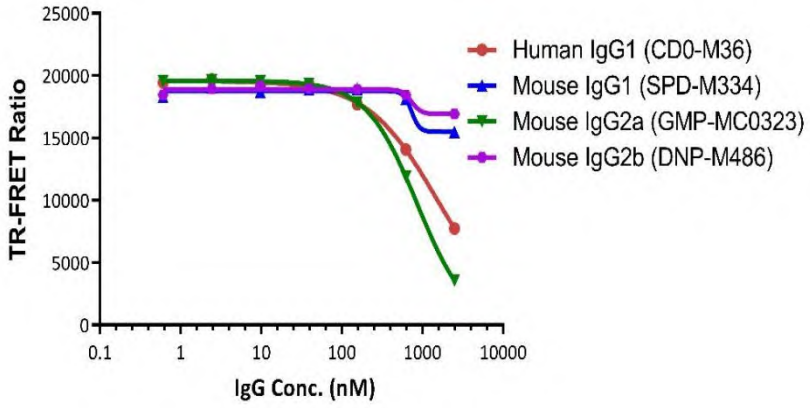
SPECIES SELECTIVITY

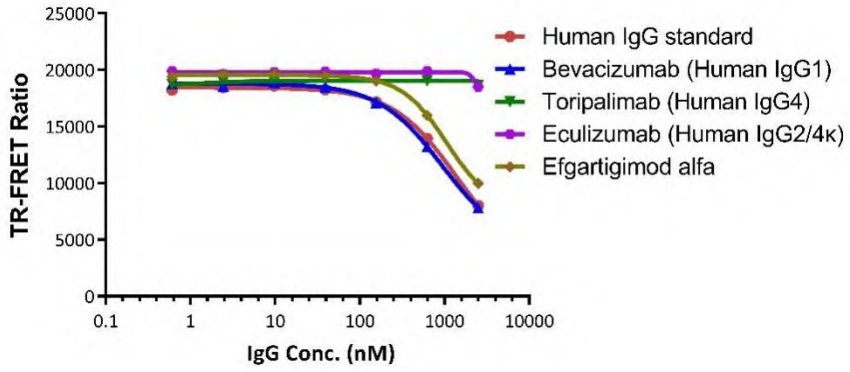
The kit has been used to detect different subclasses of mouse IgG, which exhibit different IC50 results as expected. As shown in the following figure, human CD16a (F176) has very weak or no binding to mouse IgG1, mouse IgG2a, and mouse IgG2b as observed.



Antibody	IC50 (nM)
Human IgG1 (CD0-M36)	1524
Mouse IgG1 (SPD-M334)	NA
Mouse IgG2a (GMP-MC0323)	865.7
Mouse IgG2b (DNP-M486)	NA

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 CD16a (F176). Bevacizumab and Efgartigimod alfa bind to human CD16a (F176) with the nanomolar affinity around 1000 nM. Toripalimab doesn't bind to human CD16a (F176). 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 CD16a (F176).



Antibody	IC50 (nM)
Human IgG Standard	1385
Bevacizumab (Human IgG1)	935.9
Toripalimab (Human IgG4)	NA
Eculizumab (Human IgG2/4κ)	NA
Efgartigimod alfa	1064

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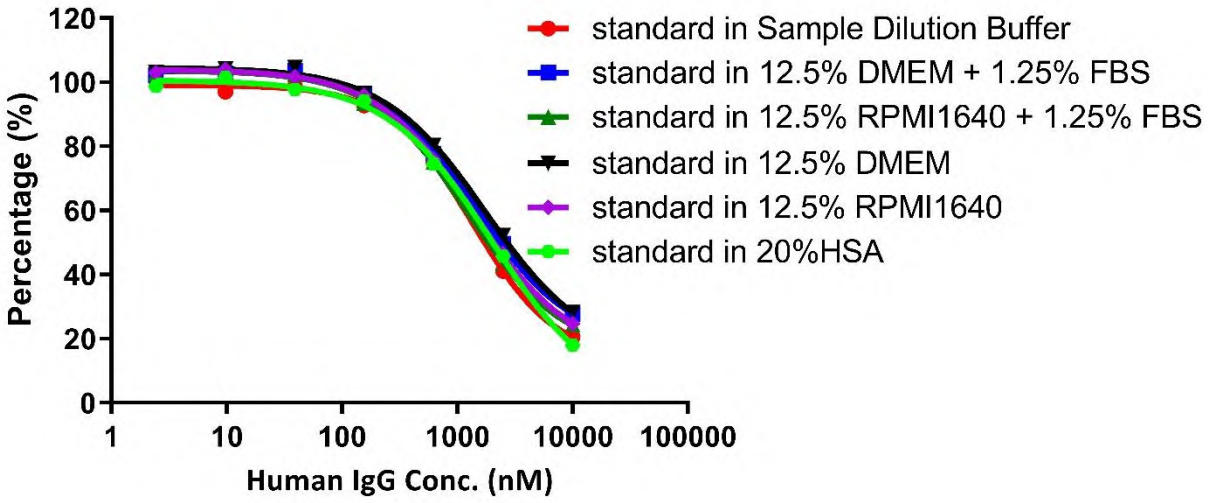
MATRIX EFFECT

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



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