# 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 (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).

Human FcγRI (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 FcγRI mainly binds to human monomer IgG1/ IgG3 and mouse IgG2a/IgG3, the binding affinity with human IgG4 is clearly reduced. In contrast, Human FcγRI has no binding to human IgG2.

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

Interaction sites for FcγRs 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 FcγRs 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 FcγRI is to activate myeloid cells to phagocytose IgG1 and IgG-bound target cells via ADCP. The interactions of therapeutic antibodies with FcγRs 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 FcγRI (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 FcγRI and



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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 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 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.



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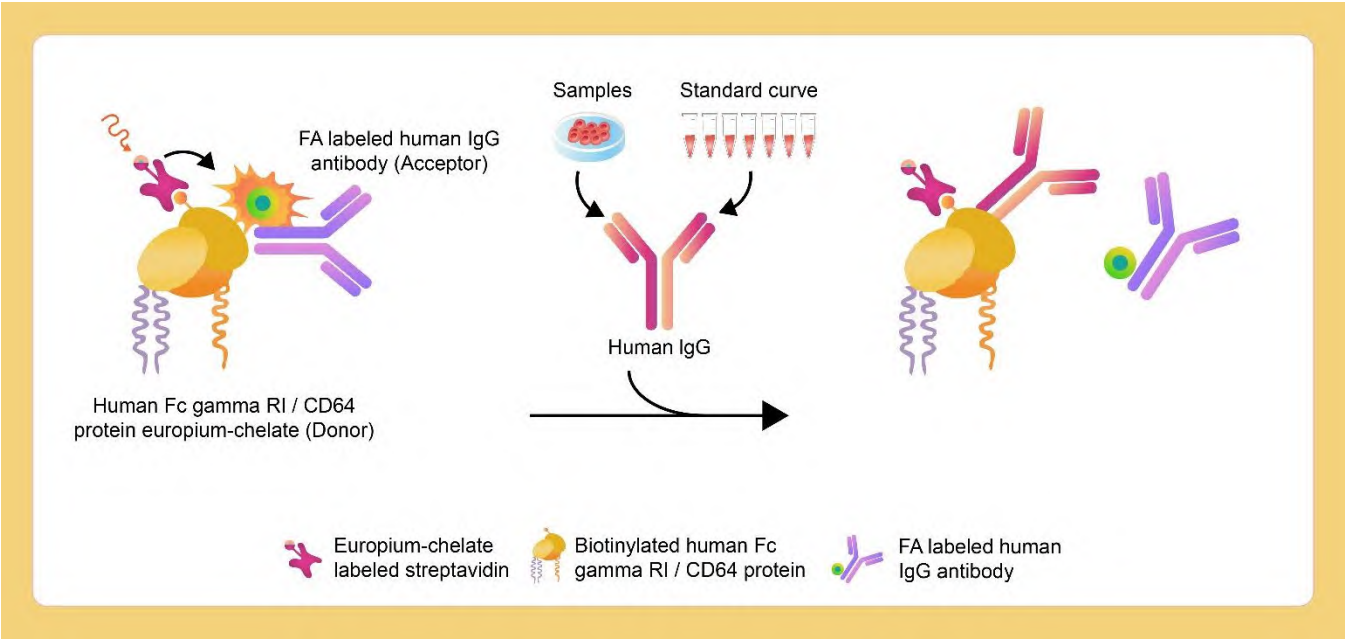
**FIG.1 PRINCIPLE OF THE ASSAY**




## 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℃, avoid light | -70℃, avoid light |
| FRT03-C02 | FA labeled human IgG antibody | 2.4 μg | 12 μg | Powder | 2-8℃, avoid light | -70℃, avoid light |
| FRT03-C03 | Human IgG standard | 100 μg | 50 0μg | Powder | 2-8℃ | -70℃ |
| FRT03-C04 | Sample Dilution Buffer | 10 mL | 10 mL | Liquid | 2-8℃ | 2-8℃ |
| FRT03-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;



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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)

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.

## 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 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



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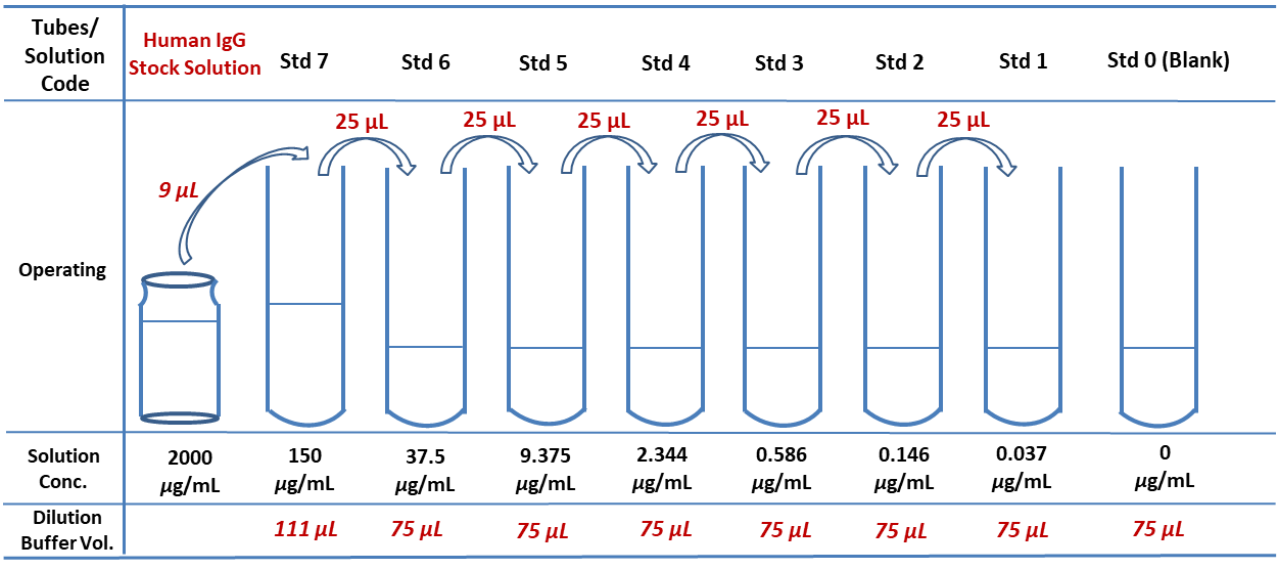
## 1. Add Samples

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**




## 2. Add Donor

Dilute **Human Fc gamma RI / CD64 Protein Europium-chelate** stock solution (10 µg/mL) to 1 µg/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℃-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 (40 µg/mL) to 4 µg/mL with **Detection Buffer** to make Acceptor working solution. The working solution should be prepared immediately before use and should not be stored.



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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.

**TABLE 3. SAMPLES ADDING TO MICROPLATE**

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

**FIG.3 PLATE LAYOUT**



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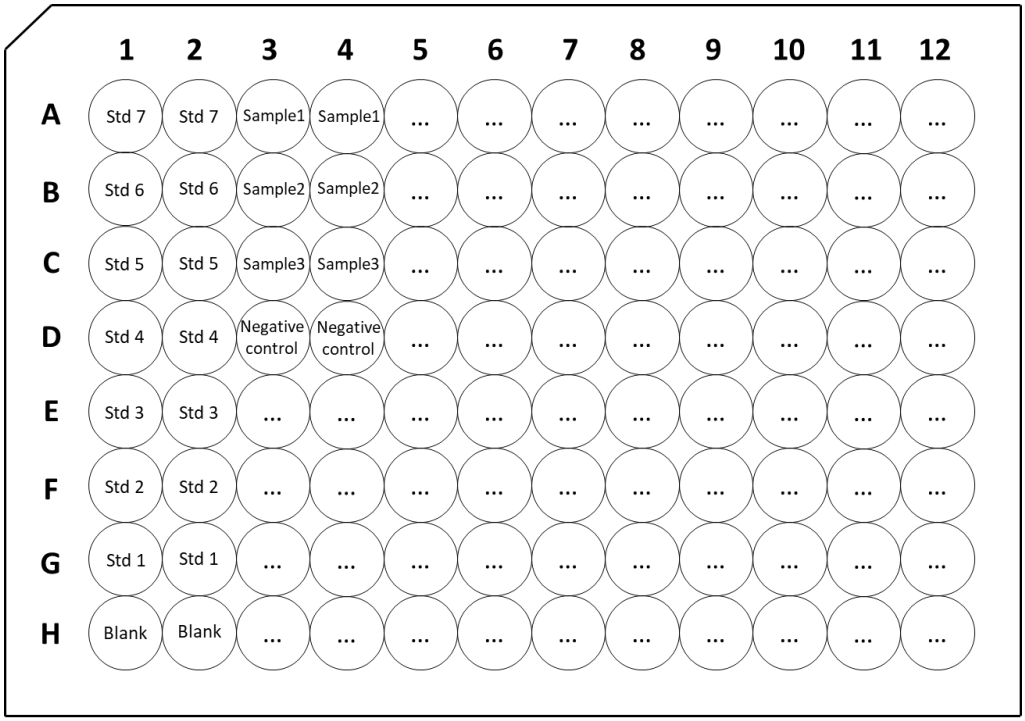


## 4. Data Recording

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 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°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.



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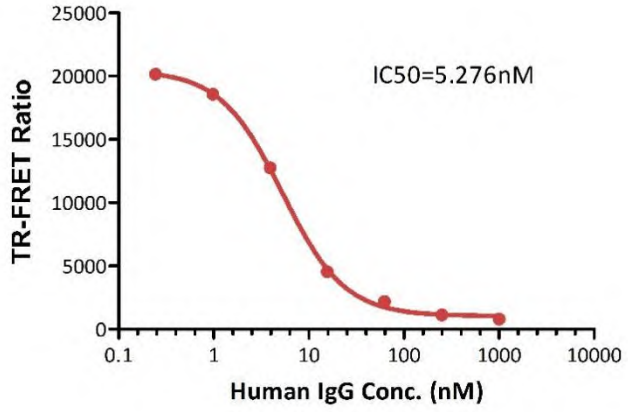
## TYPICAL DATA

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 CLARIOstar Plus. This following data is for reference only.

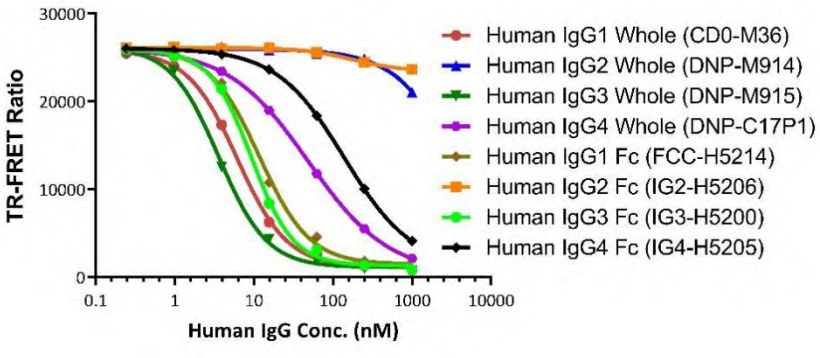



| Human IgG standard Conc. | Human IgG standard Conc. | Signal 665 nm | Signal 620 nm | Ratio |
|---|---|---|---|---|
| 150 μg/mL | 1000 nM | 1935 | 24398 | 793 |
| 37.5 μg/mL | 250 nM | 2819 | 24758 | 1139 |
| 9.375 μg/mL | 62.5 nM | 5371 | 24461 | 2196 |
| 2.344 μg/mL | 15.625 nM | 10816 | 23776 | 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



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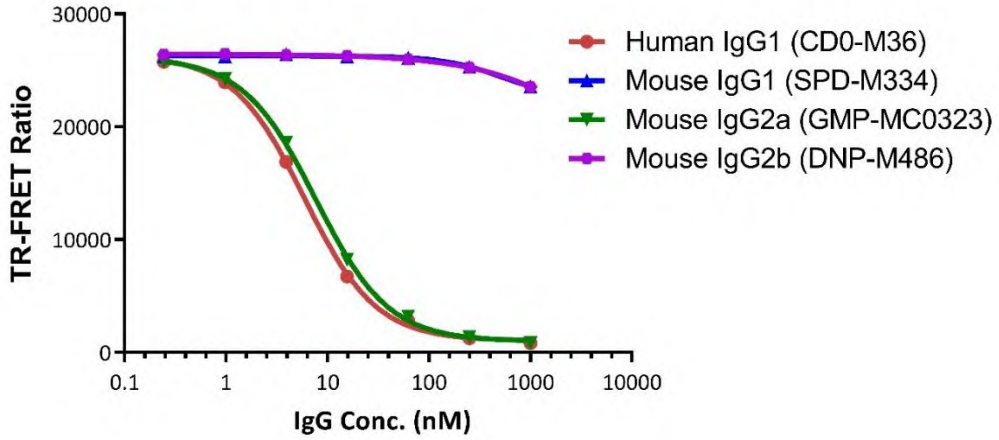
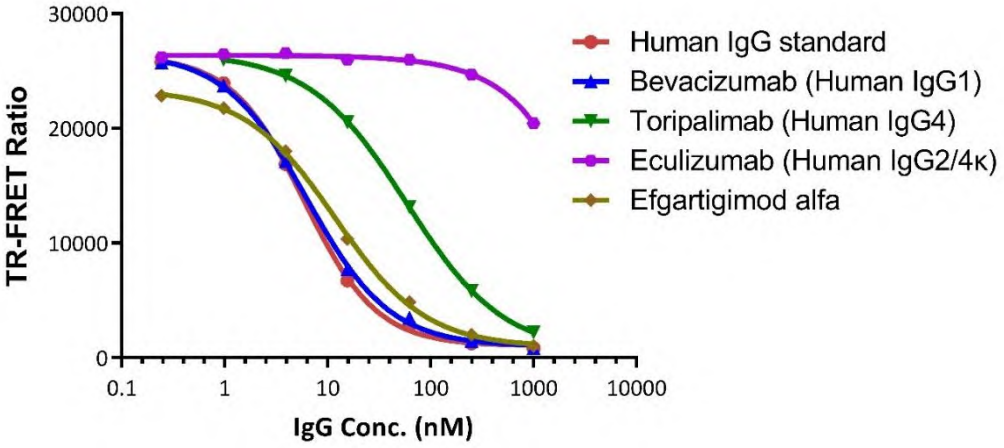
The kit has been used to detect different subclasses of mouse IgG (mouse IgG1, mouse IgG2a and mouse IgG2b), which exhibit different IC50 results as expected. As shown in the figure, human CD64 binds to mouse IgG2a with a high affinity, but not mouse IgG1 and mouse IgG2b.




## 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



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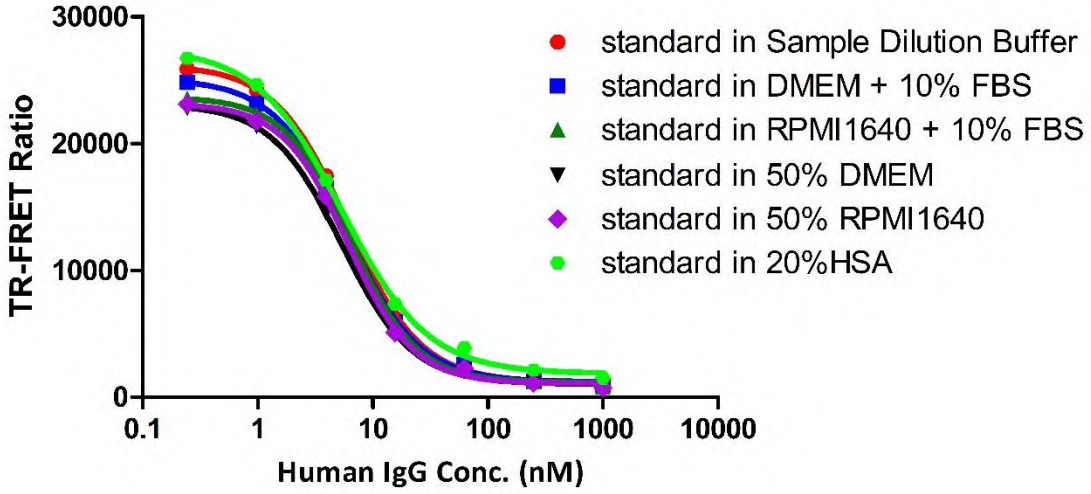
Verify potential matrix effects by adding different levels of DEME, RPMI1640, FBS and HSA to the Sample Diluted buffer.



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