Human FcRn binding Kit (TR-FRET)

Pack Size: 100 Tests & 500 Tests

Catalog Number：FRT-01

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 half-life evaluation of antibody drug candidates, and also high-throughput screening of FcRn inhibitors. It can also be used as a universal detection tool to identify the ability of antibody drugs to bind to FcRn. It is for research use only (RUO).

BACKGROUND

FcRn is a heterodimer comprising a β2-microglobulin (β2m) light chain and a major histocompatibility complex class I-like heavy chain. It is widely accepted that the interaction of IgGs with the Fc FcRn plays a critical role in regulating IgG homeostasis in vivo. FcRn interacts with the CH2-CH3 portion of the Fc domain of IgGs in a tightly regulated pH-dependent manner with high affinity binding occurring at an acidic (pH 6.0) and weak to no binding interactions as the pH is raised to neutral (pH 7.4). FcRn is responsible for the extended serum half-life of IgG and also serum albumin, and for the transport across endothelial and epithelial barriers, increasing the overall bioavailability of IgG and serum albumin. The binding affinity between FcRn and IgG are commonly used to characterize the metabolic levels of antibody drugs.

The Human FcRn Binding Kit (TR-FRET) takes advantage of binding of Europium-chelate labeled FcRn (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 FcRn and antibody drug candidates or FcRn inhibitors. It is designed to facilitate the half-life evaluation of antibody drug candidates, and also high-throughput screening of FcRn inhibitors within 0.5-1 hours. It is highly sensitive, has a short detection time and easy to use.

PRINCIPLE OF THE ASSAY

This Human FcRn binding Kit (TR-FRET) is based on TR-FRET technology (Time-Resolved Fluorescence Resonance Energy Transfer). Use the mixture of biotinylated FcRn 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 FCRN&B2M Heterodimer 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 $= \dfrac{\text{Signal 665 nm}}{\text{Signal 620 nm}} \times 10^{4}$. The Ratio value is negatively correlated with the antibody content in the sample.

— When the sample does not contain FcRn binding components, the donor and acceptor are in close proximity because of the binding of FcRn 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 FcRn binding components, the components inhibit the binding between the donor and acceptor and thereby prevents FRET from occurring.

FIG.1 PRINCIPLE OF THE ASSAY
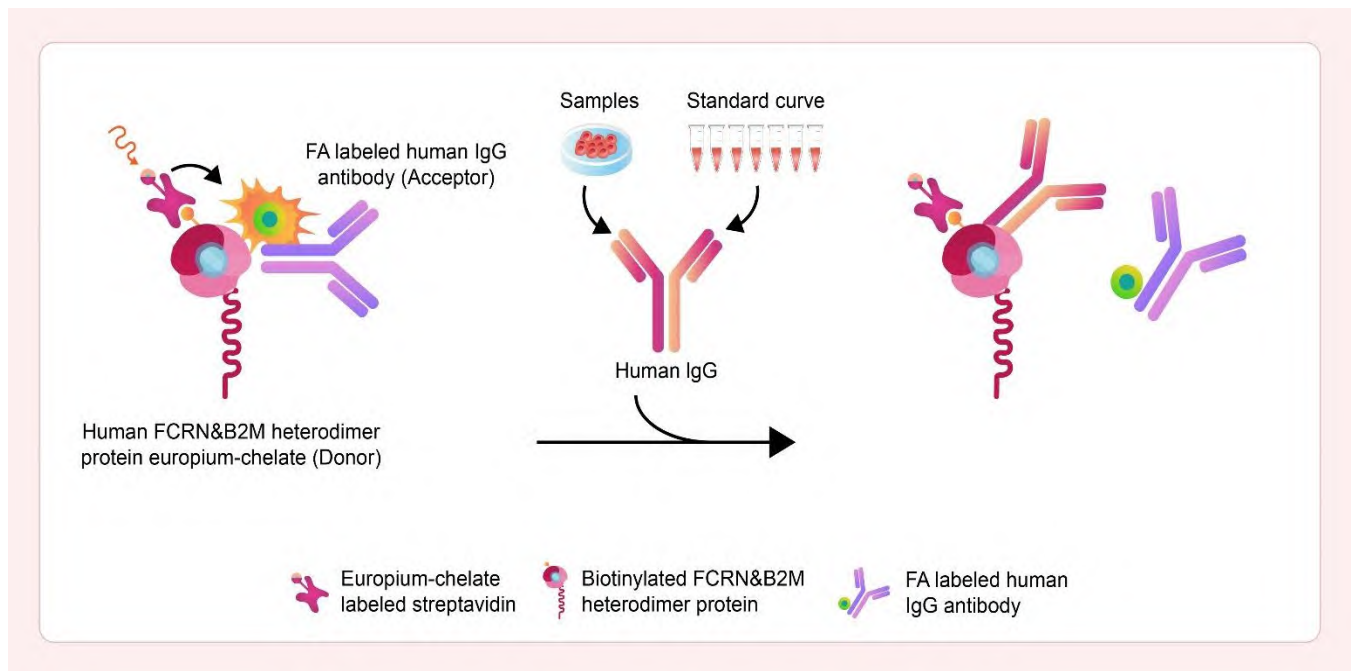
MATERIALS PROVIDED

TABLE 1. MATERIALS PROVIDED

Catalog	Components	Size (100 Tests)	Size (500 Tests)	Format	Storage	
					Unopened	Opened
FRT01-C01	Human FCRN&B2M Heterodimer Protein Europium-chelate	2.7 μg	13.5 μg	Powder	2-8℃, avoid light	-70℃, avoid light
FRT01-C02	FA labeled human IgG antibody	2.4 μg	12 μg	Powder	2-8℃, avoid light	-70℃, avoid light
FRT01-C03	Human IgG standard	200 μg	1 mg	Powder	2-8℃	-70℃
FRT01-C04	Sample Dilution Buffer	10mL	10 mL	Liquid	2-8℃	2-8℃
FRT01-C05	Detection Buffer	10mL	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 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℃-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 FCRN&B2M Heterodimer Protein Europium-chelate and FA labeled human IgG antibody stock solution should be protected from light.

TABLE 2. RECONSTITUTION METHODS FOR 100 TESTS AND 500 TESTS

Catalog	Components	Size (100 Tests)		Size (500 Tests)		Stock Solution Conc.
		Amount	Reconstitution Buffer and Vol.	Amount	Reconstitution Buffer and Vol.	
FRT01-C01	Human FCRN&B2M Heterodimer Protein Europium-chelate	2.7 μg	60 μL water	13.5 μg	300 μL water	45 μg/mL
FRT01-C02	FA labeled human IgG antibody	2.4 μg	60 μL water	12 μg	300 μL water	40 μg/mL
FRT01-C03	Human IgG standard	200 μg	100 μL water	1 mg	500 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 a 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	30 µL	15 µL	15 µL	15 µL	15 µL	15 µL	15 µL		
Solution Conc.	2000 µg/mL	1000 µg/mL	250 µg/mL	62.5 µg/mL	15.625 µg/mL	3.9 µg/mL	0.98 µg/mL	0.24 µg/mL	0 µg/mL
Dilution Buffer Vol.		30 µL	45 µL	45 µL	45 µL	45 µL	45 µL	45 µL	45 µL

2. Add Donor

Dilute **Human FCRN&B2M Heterodimer Protein Europium-chelate** stock solution (45 µg/mL) to 4.5 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°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 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. 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
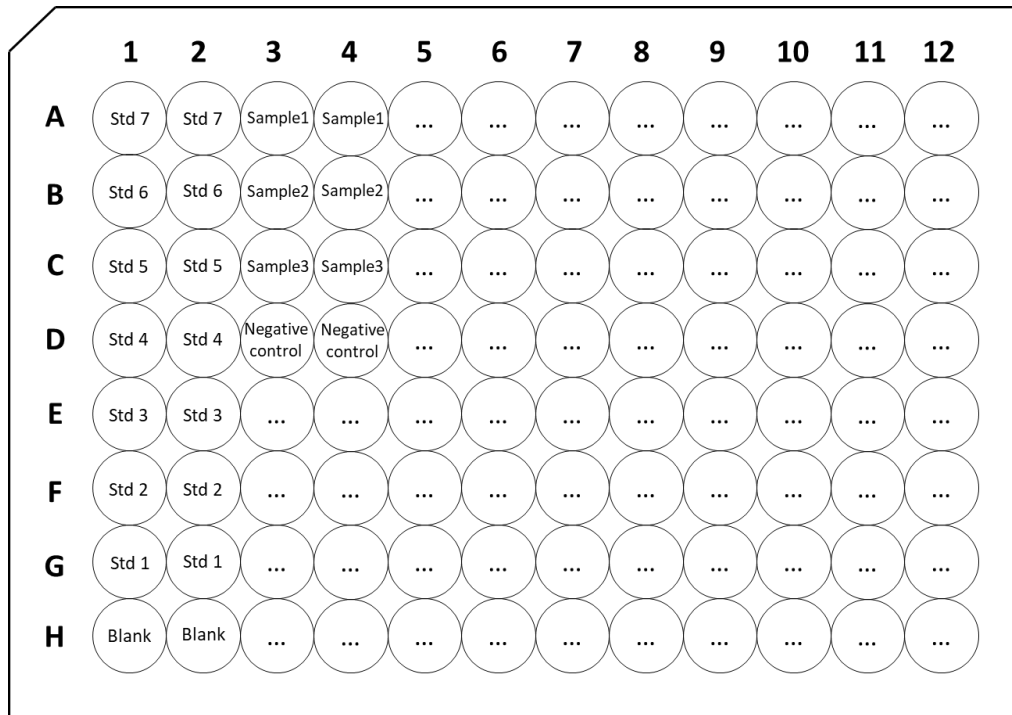
4. Data Recording

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

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.

TYPICAL DATA

For each experiment, a standard curve needs to be set for each microplate, 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.
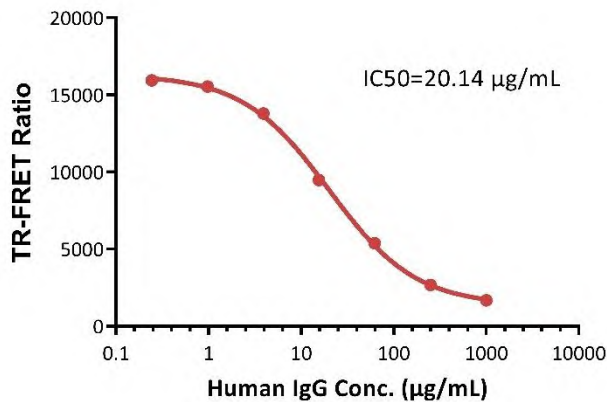
Human IgG standard Conc.	Signal 665 nm	Signal 620 nm	Ratio
1000 μg/mL	3488	20864	1672
250 μg/mL	5713	21352	2676
62.5 μg/mL	10745	19997	5373
15.625 μg/mL	17787	18770	9476
3.9 μg/mL	25657	18594	13799
0.98 μg/mL	28226	18167	15537
0.24 μg/mL	28672	17991	15937
0 μg/mL	29559	18091	16339

## DIFFERENT SUBCLASSES OF HUMAN IGG FC PROTEINS DATA

The kit has been used to detect different subclasses of Human IgG Fc proteins (Human IgG1, Human IgG2, and Human IgG4), which exhibit different IC50 results as expected.
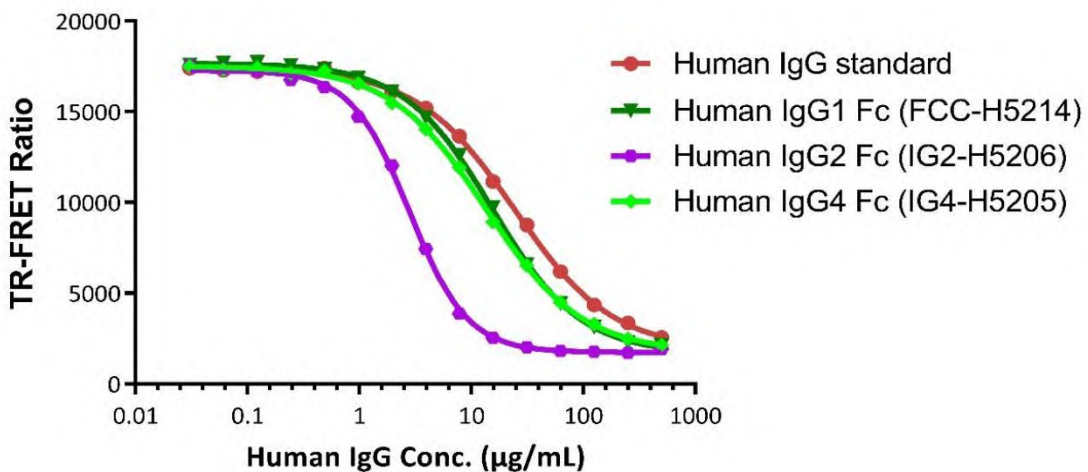
Human IgG Fc proteins	Molecular Weight	IC50 (µg/mL)-TR-FRET	IC50 (nM)-TR-FRET
Human IgG standard	150 KDa	25.01 µg/mL	166.73 nM
Human IgG1 Fc, Tag Free (Cat. No. FCC-H5214)	26.1 KDa	15.31 µg/mL	598.59 nM
Human IgG2 Fc, Tag Free (Cat. No. IG2-H5206)	25.72 KDa	2.794 µg/mL	108.63 nM
Human IgG4 Fc, Tag Free (Cat. No. IG4-H5205)	25.8 KDa	13.57 µg/mL	525.97 nM

DIFFERENT SUBTYPES OF ANTIBODY DATA

The kit has been used to detect different subclasses of Human IgG (Human IgG1, Human IgG2, and Human IgG4), which exhibit different IC50 results as expected.
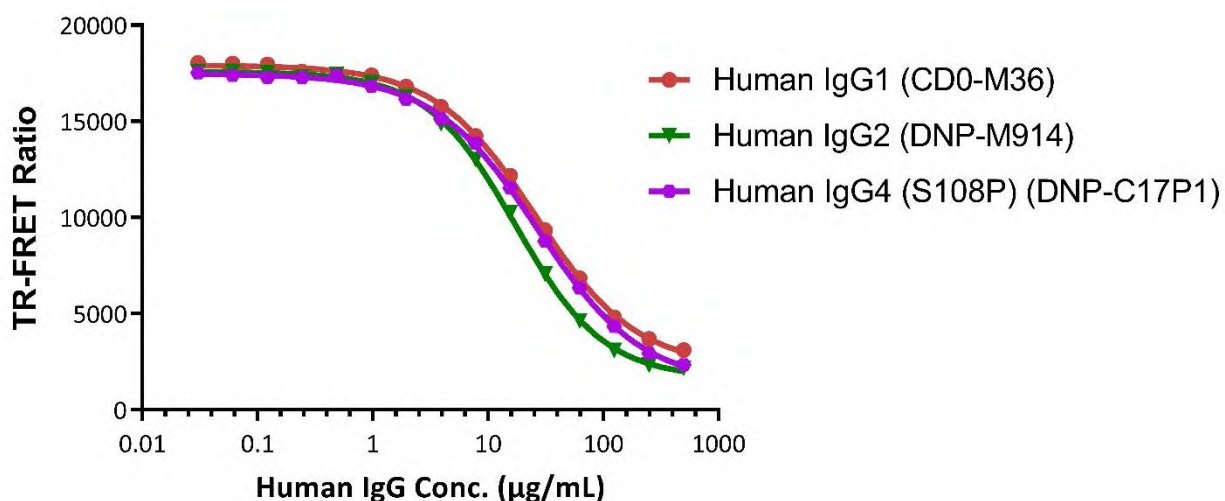
| Antibody	Molecular Weight	IC50 (µg/mL)-TR-FRET	IC50 (nM)-TR-FRET
Human IgG1 (Cat. No. CD0-M36)	150 KDa	26.03 µg/mL	173.53 nM
Human IgG2 (Cat. No. DNP-M914)	150 KDa	17.32 µg/mL	115.47 nM
Human IgG4 (Cat. No. DNP-C17P1)	150 KDa	26.95 µg/mL	179.67 nM

MATRIX EFFECT

Verify potential matrix effects by adding different levels of DEME and FBS to the Sample Diluted buffer.
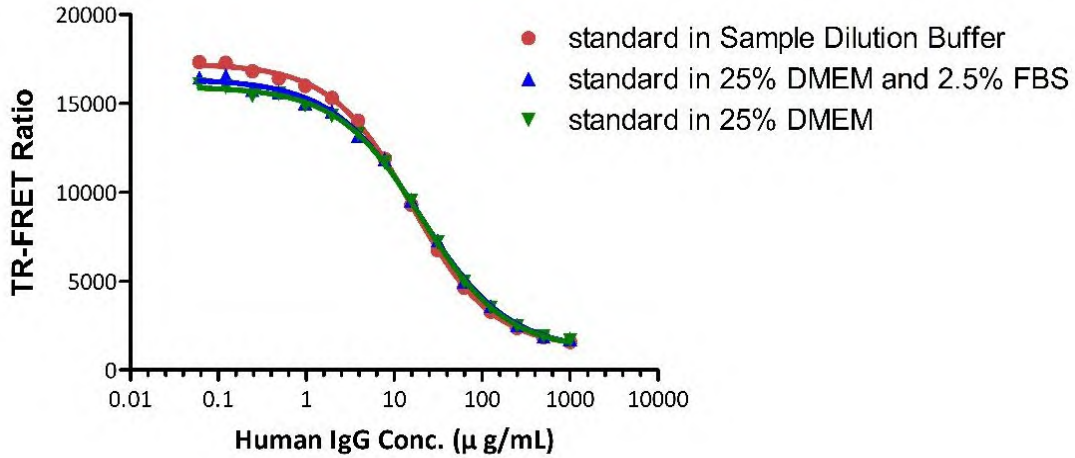
SPECIES SELECTIVITY

The kit is human IgG specific and not compatible with mouse IgG1 and mouse IgG2a.
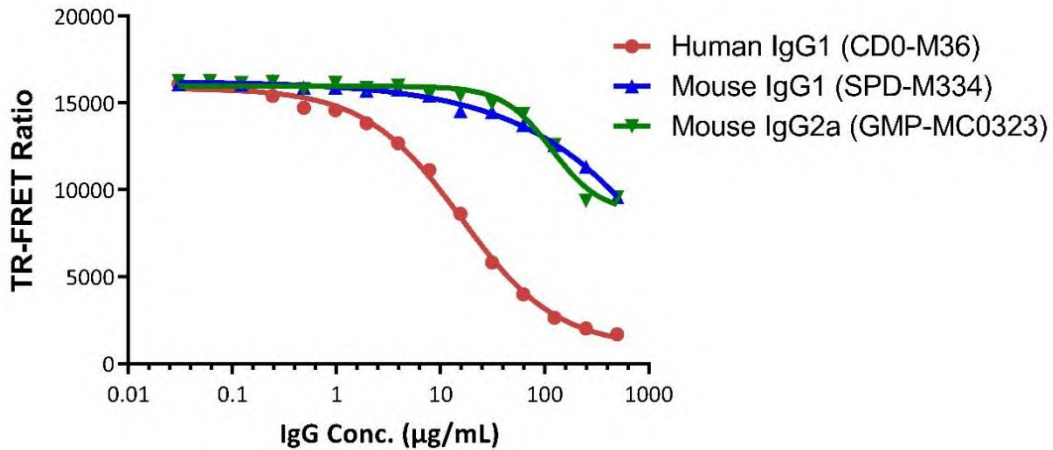

APPLICATION OF ANTIBODY DRUG HALF-LIFE ASSESSMENT

The half-lives of monoclonal antibodies currently in clinical use generally correlate with the binding affinity to FcRn.

The kit has been used to detect three FDA approved antibody drugs of different affinities to FcRn, and the IC50 trends

are consistent with affinity constant from SPR as well as the actual *in vivo* half-life published.

In the same experiment, the lower the IC50, the stronger binding affinity between IgG antibodies and FcRn, and the longer half-life of the antibody drug. Bevacizumab and Toripalimab have consistent binding affinity with FcRn and have a half-life of about 20 days. The Fc of Eculizumab has been modified to bind relatively weakly to FcRn, resulting in a relatively short half-life of approximately 11 days.
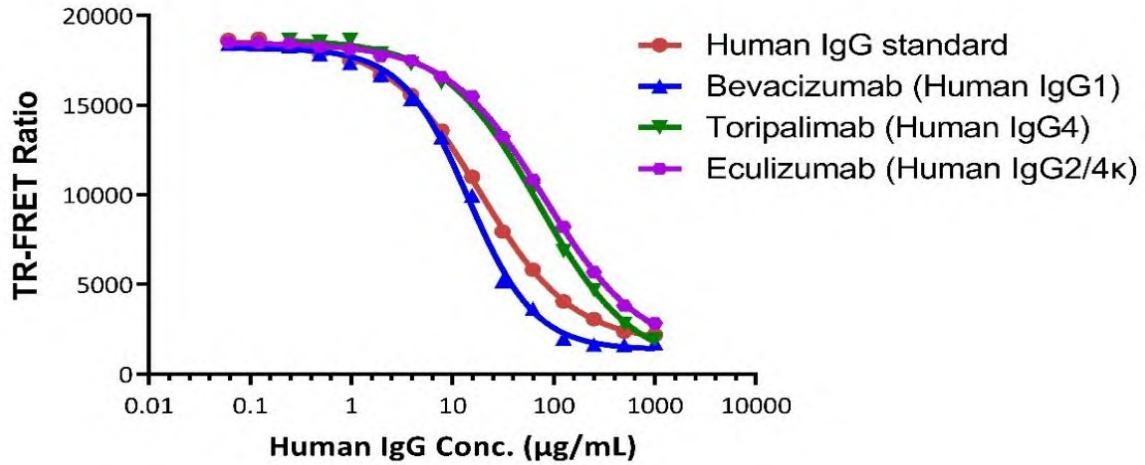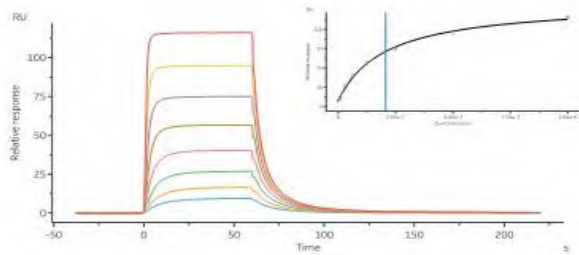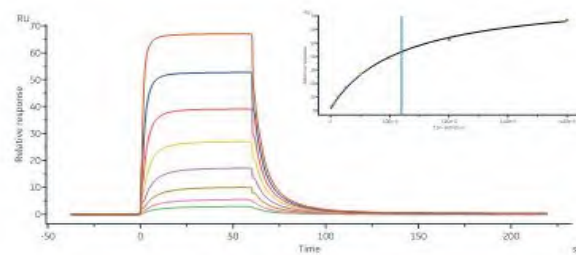

FRT01-C03
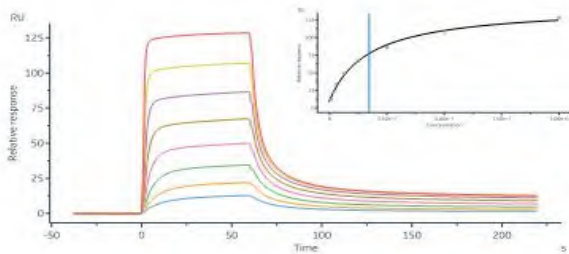
Bevacizumab
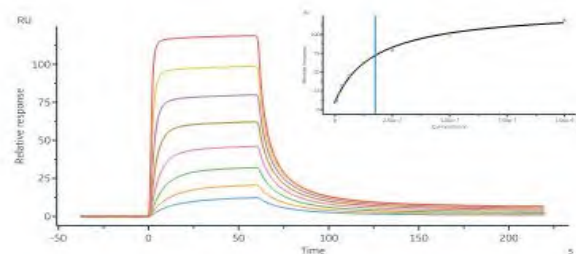
Toripalimab

Eculizumab

Antibody	Molecular Weight	IC50 (µg/mL)-TR-FRET	IC50 (nM)-TR-FRET	KD (M)-SPR	Half-life
Human IgG standard (FRT01-C03)	150 kDa	25.01 µg/mL	124.4 nM	1.73E-07 M	NA
Bevacizumab (Human IgG1)	149 kDa	19.7 µg/mL	97.1 nM	1.76E-07 M	20 days (11 to 50 days)
Toripalimab (Human IgG4)	150 kDa	52.82 µg/mL	430.9 nM	8.29E-07 M	10 ± 1.5 days after the first dose and 18 ± 9.4 days at steady state
Eculizumab (Human IgG2/4κ)	148 kDa	94.95 µg/mL	549.9 nM	1.20E-06 M	11 to 18 days (270 to 414 hours)

APPLICATION OF HIGH-THROUGHPUT SCREENING OF FcRn INHIBITORS

The kit is suitable for the detection of FcRn inhibitors. It shows that both Efgartigimod and its biosimilar, Human IgG1 Fc (C103S, M135Y, S137T, T139E, H316K, N317F) His Tag (Cat. No. IG1-H52H8) exhibit good inhibitory activity in this TR-FRET competition assay.