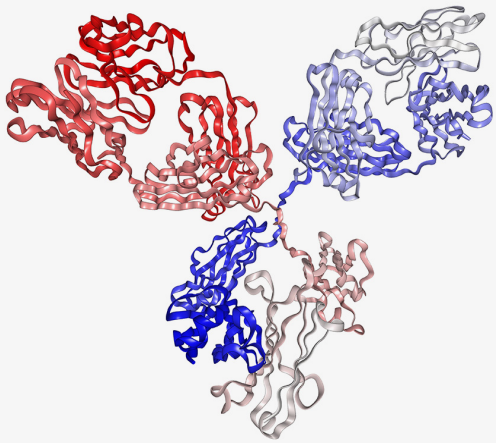

Tag-lite® two-cell assay: a valuable tool for protein drug discovery

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Introduction

This application note describes the development of a cell-based TR-FRET assay to determine the simultaneous binding of an antibody to its antigen and huFcγRIIIa present on different cells.

Antibodies are central mediators of the immune system, with immunoglobulin G (IgG) being the most dominant. They comprise of an antigen binding part (consisting of two Fab arms) and an Fc portion (1), which can interact with Fcγ receptors (FcγRs) present on various immune cells such as NK cells, macrophages, monocytes and neutrophils. Binding of IgG/antigen complexes to membrane-bound FcγRs triggers a cellular immune response (2). Alterations in the Fc portion of the antibody by introducing mutations can lead to complete abolition of FcγR binding.

However, in order to evaluate the influence of such changes to an antibody's performance in a cell-based system, a sensitive and quantifiable means of analysis is required.

The Tag-lite technology, developed by Cisbio Bioassays, is a combination of a classical TR-FRET (time-resolved fluorescence resonance energy transfer) and SNAP-tag® technology (New England Biolabs), which allows antigens present on the cell surface to be labeled with a fluorescent donor or acceptor dye. The assay involves the generation of two labeled cell populations; one cell type with terbium (Tb; donor fluorophor) labeled human FcγRIIIa (huFcγRIIIa), and a second with surface displayed antigen Y labeled with SNAP-Red (acceptor fluorophor). In the presence of an antibody which can bind both labeled proteins, the cells will come into close enough proximity to allow energy transfer between fluorophores (Figure 1).

The combination of this assay system with the excellent sensitivity and flexible wavelength and bandwidth selection offered by the Infinite® M1000 PRO multimode reader provides an invaluable tool for the assay development process.

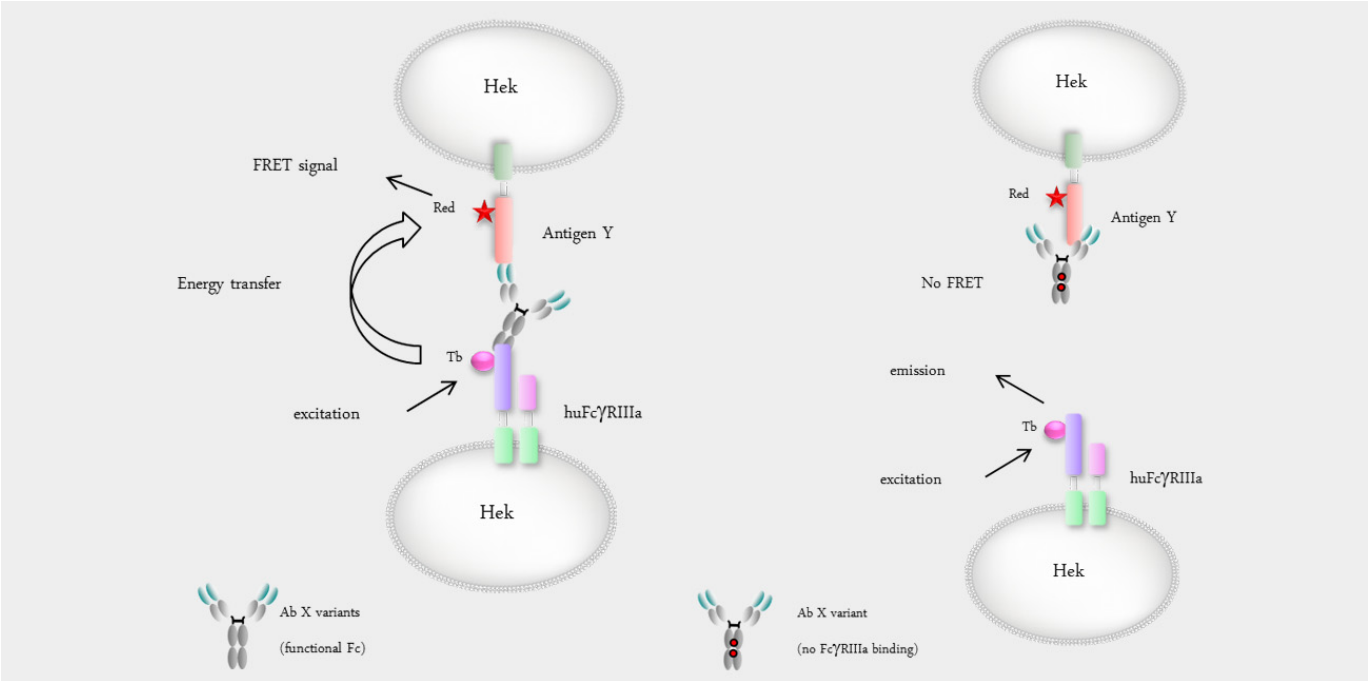


Figure 1 Schematic representations of the Tag-lite two-cell assay. Transfected Hek cells expressing huFcγRIIIa labeled with terbium (donor) are mixed with transfected Hek cells expressing antigen Y labeled with SNAP-Red (acceptor) and antibody X (Ab X) or its variant. After excitation of the Tb, an energy transfer to SNAP-Red can only take place if the antibody binds to both antigen Y and huFcγRIIIa. In case of binding of the antibody to only one of the proteins, the cells do not come into close enough proximity for the energy transfer to occur, and no emission from the acceptor can be detected.

Material and Methods

Equipment

- Infinite M1000 PRO multimode reader (Tecan, Switzerland)
- i-control™ software (Tecan, Switzerland)
- Prism 5™ (GraphPad Software, Inc., California)
- 384-well, Small Volume™ HiBase polystyrene microplates, white (Greiner Bio-One, Germany)

Reagents and culture conditions

- Hek293 EBNA cells (cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10 % FCS in a humidified incubator (5 % CO_2) at 37 °C
- Post-transfection medium: DMEM, 10 % FCS and non-essential amino acid (Sigma-Aldrich, USA)
- Gibco® Opti-MEM® I reduced serum medium, no phenol red (Life Technologies, USA)
- Lipofectamine® 2000 transfection reagent (Life Technologies, USA)
- Gibco® Dulbecco's phosphate-buffered saline (DPBS), no calcium, no magnesium (Life Technologies, USA)
- Tag-lite buffer (Cisbio Bioassays)
- SNAP-Lumi4-Tb (Cisbio Bioassays)
- SNAP-Red (Cisbio Bioassays)
- d2 labeling kit (Cisbio Bioassays)
- huFcγRIIIa-Tb kit (Cisbio Bioassays)

Methods

Preparation of cells

Cells (at 60-80 % confluency) were transfected with 2 ug cDNA encoding for antigen Y fused to SNAP-Tag using Lipofectamine 2000.

After incubation for 24h at 37 °C in a humidified incubator (5 % CO_2), cells were washed with DPBS and labeled with

SNAP-Red (100 nM for one T75 flask) or SNAP-Lumi4-Tb (100 nM for one T75 flask) in Tag-lite buffer.

Following incubation for 1h at 37 °C, cells were washed, counted and frozen at -80 °C in appropriate aliquots. Labeling efficiency was determined by measuring the emission intensity of Tb at 620 nm for 10,000 cells per well.

huFcγRIIIa binding

An antibody X (Ab X) variant was generated by introducing mutations in the Fc portion of the antibody to abolish FcγRIIIa binding. A competitive assay to determine the EC_{50} binding values for Ab X with either wt or variant Fc to huFcγRIIIa was carried out according to the manufacturer's instructions.

Briefly, 10,000 cells per well expressing huFcγRIIIa-Tb were incubated with 50 nM IgG-d2 (provided in the kit) and decreasing concentrations of competing antibody (750-0.18 nM final concentration) at room temperature (RT). Emission intensity was measured at 620 nm and 665 nm at different time points with an Infinite M1000 PRO (reader settings shown in Table 1), and the 665/620 ratio was calculated after background substraction (cells only). Data was presented graphically using Prism 5 (GraphPad).

Mode	Fluorescence Intensity Top	
Excitation Wavelength	340	nm
Emission Wavelength	620 / 665	nm
Excitation Bandwidth	20	nm
Emission Bandwidth	10*)	nm
Gain	232	Optimal (100%)
Number of Flashes	100	
Flash Frequency	100	Hz
Integration Time	500	µs
Lag Time	60	µs
Settle Time	0	ms
Z-Position (Calculated From: L10)	25,018	µm (calculated from well L10)

Table 1 Reader settings for Tecan Infinite M1000 PRO
*) *For low signal intensities 20 nm emission bandwidth can be used.*

Antigen binding

Ab X (wt) was labeled with d2 according to the kit manufacturer's instructions. A binding curve for antigen-expressing cells labeled with Tb was generated to enable determination of the optimal concentration of labeled Ab X for the competition assay.

EC_{50} values were determined by incubation of 2,000 cells/well with 0.77 nM Ab-d2 and decreasing concentrations of the competing Abs (750-0.18 nM final concentration) for 1, 2, 3 and 4 hours at RT. Emission intensities were measured at 620 and 665 nm with the Infinite M1000 PRO, and the 665/620 ratio was calculated after background subtraction (cells only). Data was presented graphically using Prism 5 (GraphPad).

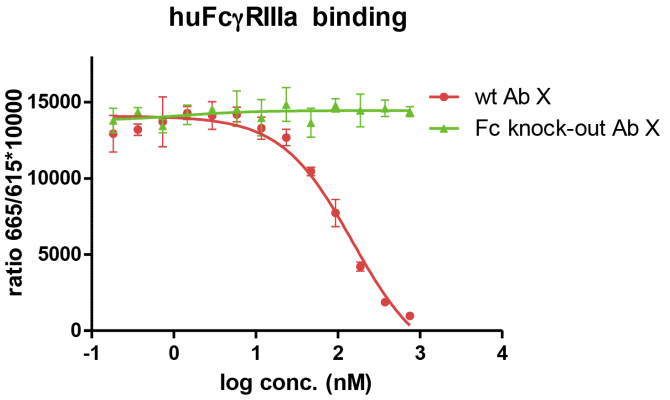
Two-cell assay

20,000 cells expressing antigen Y-Red were mixed with 10,000 huFcγRIIIa-Tb expressing cells, and increasing concentrations (0.015-32 nM) of Ab X (wt or variant) were added. The fluorescence emission intensities were measured at 620 nm and 665 nm with the Infinite M1000 PRO, and the 665/620 ratio was calculated after background subtraction (cells only). The EC_{50} was calculated using Prism 5 sigmoidal dose response (variable slope).

Results and discussion

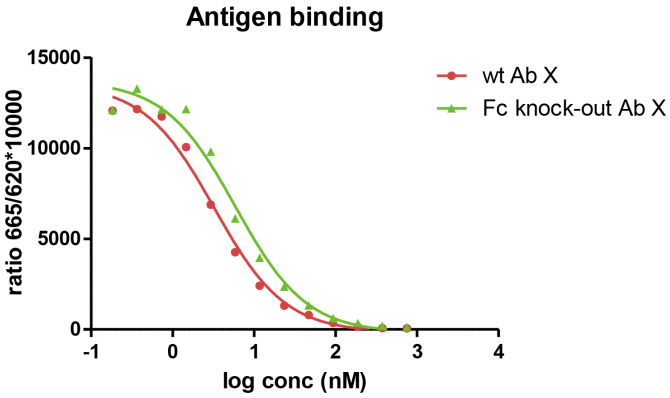
Analysis of Fc receptor binding showed that wt Ab X is able to bind to huFcγRIIIa, whereas the Fc knock-out mutation completely abolished huFcγRIIIa binding (Figure 2), as expected. In contrast, binding to antigen Y was similar for both wt and Ab X variant (Figure 3). As the antigen Y binding moiety for both antibodies is the same, this is also in line with expectations.

The Fc knock-out variant did not lead to the generation of a FRET signal in the two-cell assay (Figure 4). As FRET only occurs when both parts of the antibody interact with their respective binding partners, this goes some way to confirming the specificity of the assay. In contrast, the wt Ab X bound to both huFcγRIIIa and antigen Y, resulting in generation of a FRET signal and a good binding curve.



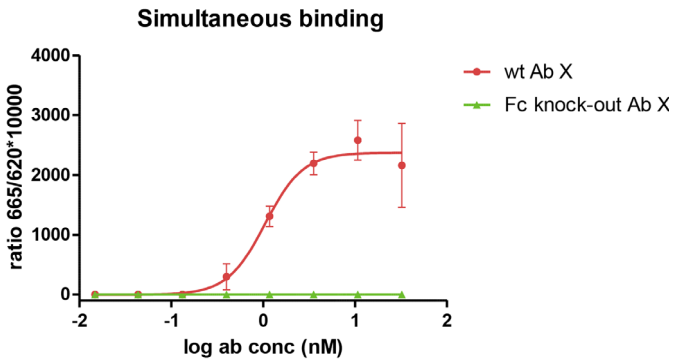
	wt Ab X	Fc knock-out Ab X
EC_{50} (nM)	151.3 (95.17-240.5)	no binding

Figure 2 EC_{50} determination for binding of wt and Ab X variant to huFcγRIIIa by Tag-lite.



	wt Ab X	Fc knock-out Ab X
EC_{50} (nM)	3.25 (2.46-4.28)	5.93 (4.11-8.55)

Figure 3 EC_{50} determination for binding of wt and Ab X variant to antigen Y by Tag-lite.



	wt Ab X	Fc knock-out Ab X
EC_{50} (nM)	1.052 (0.719-1.539)	No FRET

Figure 4 EC_{50} determination of simultaneous binding of wt Ab X and its variant to antigen Y and huFcγRIIIa.

Conclusions

The results presented here demonstrate the suitability of the Infinite M1000 PRO to perform a novel, FRET-based two-cell assay using Tag-lite technology. This will allow more in-depth analysis of protein-protein interactions in a physiologically relevant, cell-based environment.

References

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