Protein - Protein Interactions: Development of FRET Assay Measuring CD40L and 5C8 Interactions

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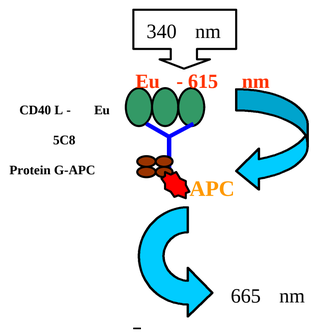
Abstract:

A FRET (Fluorescence resonance energy transfer) assay has been developed which measures the interaction of CD40L and 5C8, a monoclonal antibody to CD40L. CD40L is a member of the TNF family and has properties characteristic of TNF family members including (i) trimeric structure and (ii) low affinity ligand-receptor interactions resulting in complex binding models. The FRET assay capitalizes on the high affinity interaction(s) of 5C8 and CD40L. The high affinity 5C8/CD40L interaction made possible the development of a homogenous HTS compatible assay. The FRET assay can be used to identify molecules that interact with CD40L. The CD40/5C8 FRET assay could serve as a template for the development of HTS compatible assays for additional members of the TNF family

Figure 1: CD40 Ligand FRET assay



Materials and Methods

- Chimeric murine/human 5C8 (Biogen, Inc.)
- Phycolink Protein G xl-APC (Prozyme)
- CD40L –Eu conjugate (EG&G Wallac, special order)
- 100 mM Hepes, pH 7.2
- black Optiplate (96, 384) (Packard)
- LJL Analyst 96-384 and Criterion Host software (LJL Biosystems)

Prebind 5C8 and Protein G-APC in Hepes pH 7.2 buffer at desired concentrations. Incubate at room temperature for 30 minutes. Add CD40L-Eu to 5C8/ProteinG-APC complex. Incubate at room temperature for 30 minutes. Read plate in LJL Analyst in time resolved fluorescence mode using 340 nm and 615/665 nm for excitation and emission, respectively. Calculate FRET ratios (F665/F615): ratio = acceptor/(donor/1000).

Figure 2: FRET measurement of 5C8 binding to CD40L-Eu

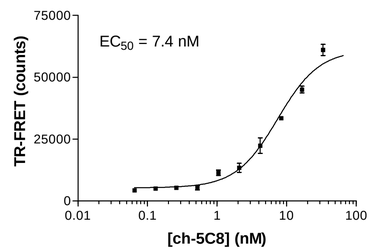


Figure 3: Titration of 5C8 and Protein G-APC

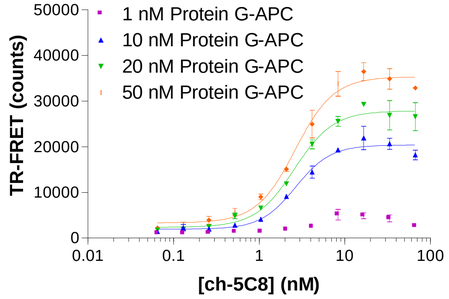


Figure 4: Titration of 5C8 and Protein G-APC

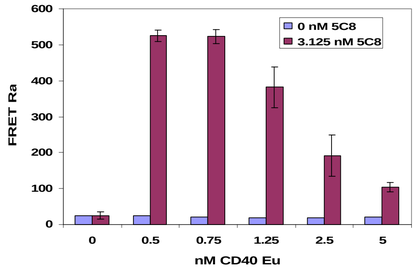


Figure 5: Time course of CD40L-Eu/5C8 interaction

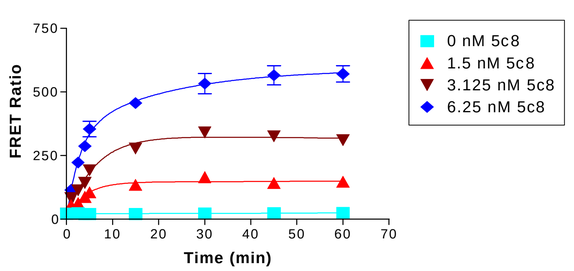
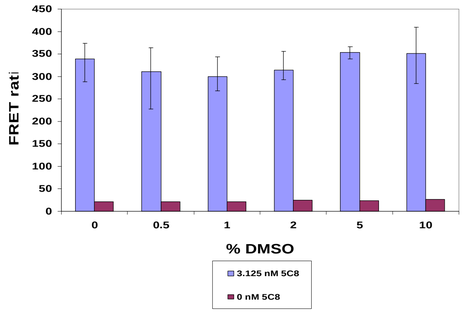


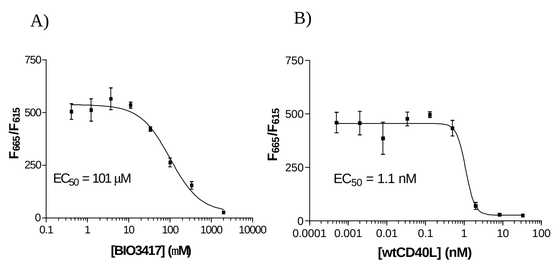
Figure 6: Assay tolerance to DMSO



Results and discussion:

The FRET pair Europium (Eu)(1) and Allophycocyanin (APC) was used for the development of the CD40L and 5C8 assay (Figure 1). The binding activity of Eu-CD40L to a CD40 fusion protein was shown to be comparable to the binding of wild type CD40L by a competition ELISA based assay (data not shown)(2). Titrating 5C8 and measuring the FRET signal (Figure 2) identified the optimal concentration range of 5C8/CD40L binding to be 1 – 10 nM. In order to identify the optimal concentrations for CD40L-Eu, Protein G-APC and 5C8 pairing, these reagents were titrated and FRET signal measured (Figure 3, 4). The FRET signal measured is proportional to the concentration of Protien G-APC. A signal to background ratio of 10:1 is obtained using 3 nM 5C8 and 50 nM Protein G-APC. CD40L-Eu was titered based upon the optimized concentrations of 5C8 and Protein G-APC, 3 nM and 50 nM, respectively. A robust signal to background ratio of 20:1 is obtained using 0.25 nM CD40L-Eu, 3 nM 5C8 and 50 nM Protein G-APC.

Figure 7: IC_{50} measurements of active molecules



Having optimized for the concentrations of CD40L-Eu, 5C8 and Protein G-APC, the time course of the solution phase binding of CD40-Eu and 5C8 was measured (Figure 5). The reaction is rapid and equilibrium binding is observed within 15 minutes. A 30-minute reaction time was chosen due to ease of scheduling and the stability of the reaction once equilibrium is reached.

The tolerance of the assay to DMSO, assay miniaturization and assay reproducibility were evaluated to ensure the assay could be used for HTS purposes. The assay tolerated high concentrations of DMSO (minimal loss of FRET signal at 10% DMSO)(Figure 6).

To confirm that the optimized CD40L/5C8 FRET assay measured the binding interactions between CD40L and 5C8, the assay was run in the presence of a small molecule, BIO3417 (Figure 7a) and wild type CD40L (Figure 7b). Inhibition is observed with both CD40L and BIO3417.

Summary:

- **A robust FRET assay has been developed that can be used to identify molecules that interact with CD40L**
- **The assay has a signal to background of 20:1**
- **The assay has been successfully miniaturized to a 384-well format**

References:

1. Mathis, G. (1995) Probing molecular interactions with homogeneous techniques based on rare earth cryptates and fluorescence energy transfer. *Clin. Chem.* 41:1391.

2. Lederman, S. et. al. (1994) T-BAM/CD40-L on Helper T Lymphocytes Augments Lymphokine-Induced B Cell Ig Isotype Switch Recombination and Rescues B Cells from Programmed Cell Death. *J. Immunol.* Mar 1;152(5):2163-71.