CAR T-Cell Analysis with Modular Antibodies Using SpyTag Protein Ligation

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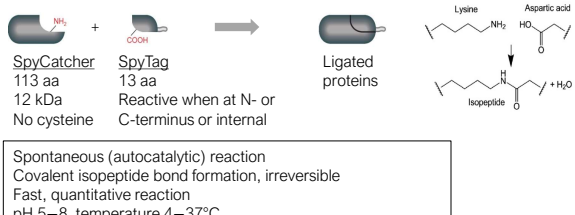
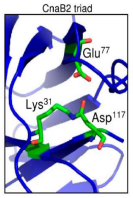
Introduction

Antibodies play a crucial role in the analysis of chimeric antigen receptor (CAR) T-cells. Particularly of interest is the class of anti-idiotypic antibodies that are directed against the CAR since these enable the specific detection of the modified T-cells using flow cytometry. Here we present the generation of specific anti-CAR T-cell antibodies and demonstrate how CAR T-cell analysis benefits from our modular antibody assembly technology using SpyTag-SpyCatcher protein ligation.

SpyTag Technology – Fast, Spontaneous Reaction

SpyTag technology is based on the SpyTag peptide and SpyCatcher protein, which are derived from the fibronectin-binding protein (FbaB) of *Streptococcus pyogenes* (Spy). The FbaB protein contains an intrachain isopeptide bond between the sidechains of a lysine and an aspartic acid within the Ig-like collagen adhesin domain. The SpyTag peptide incorporates the aspartic acid residue, and the SpyCatcher has the lysine residue; when the SpyTag and SpyCatcher are mixed, the isopeptide bond is formed between these amino acids.





Zakeri B et al. (2012). Peptide tag forming a rapid covalent bond to a protein, through engineering a bacterial adhesin. PNAS 109, E:690–697.

- Spontaneous (autocatalytic) reaction
- Covalent isopeptide bond formation, irreversible
- Fast, quantitative reaction
- pH 5–8, temperature 4–37°C
- Robust to buffer conditions, Ca^{2+}/Mg^{2+} not needed
- Robust to detergents
- Reaction occurs also inside cells (in vivo)

Fig. 1. SpyTag-SpyCatcher protein ligation.

Modular Antibodies – One Antibody, Multiple Formats in an Instant

The 14 amino acid SpyTag2 peptide is genetically fused to the C-terminus of the recombinant Fab heavy chain (Figure 2A). A set of SpyCatchers has been produced, both unconjugated and site-specifically labeled with HRP, biotin, or another label of choice (Figure 2B). Variants include the BiSpyCatcher, where two SpyCatchers are genetically linked to allow formation of a bivalent Fab_2, and the FcSpyCatcher, where a SpyCatcher is fused to an immunoglobulin Fc domain, making a full-length Ig-like molecule after the reaction with two SpyTagged Fabs. A Fab antibody with a SpyTag forms a covalent isopeptide bond to the chosen Catcher, enabling site-directed conjugation or fast conversion to bivalent Fab or a full-length Ig-like molecule within an hour (Figure 2C).

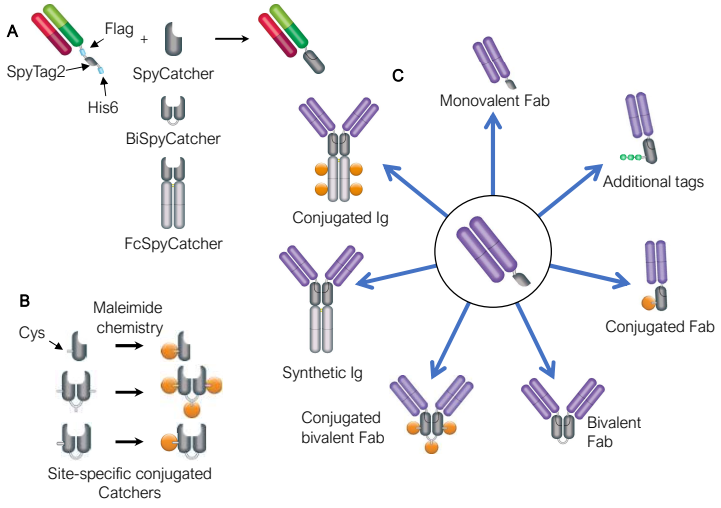


Fig. 2. Conversion of a Fab antibody with a SpyTag to multiple different formats using SpyCatchers. A, a recombinant Fab antibody with a SpyTag2 can be coupled to a SpyCatcher, BiSpyCatcher, or FcSpyCatcher reagent; **B**, SpyCatcher and BiSpyCatcher modified to add cysteine residues can be conjugated to a label of choice using maleimide chemistry; **C**, the monovalent Fab with SpyTag (center) can be converted to multiple different formats via formation of a covalent peptide bond with the chosen Catcher.

Hentrich et al. (2021). Periplasmic expression of SpyTagged antibody fragments enables rapid modular antibody assembly. Cell Chemical Biology 28, 1–12.

Generation of Anti-CAR T-Cell Antibodies

The HuCAL PLATINUM® phage display library was used to select specific antibodies. In three rounds of panning on the scFv or biotinylated scFv and blocking with an unrelated scFv, anti-idiotypic antibodies were enriched. Screening of 368 clones was done by ELISA on scFv and negative controls and by flow cytometry on scFv expressing cells using *Escherichia coli* lysates containing Fab. Hits were then ranked according to their k_{off}-rate and the 20 best candidates were sequenced. Unique antibodies were expressed and purified via His-tag IMAC followed by quality control (QC) and further antibody characterization.

Antigen: scFv and biotinylated scFv
Screening: ELISA on scFv, biotinylated scFv and unrelated scFv, 196 hits
Secondary screening: flow cytometry on scFv expressing cells, 75 hits
Tertiary screening: k_{off}-rate ranking on Octet RED384; sequencing of 20 best clones; unique antibodies identified, 11
QC: ELISA and flow cytometry

Off-Rate Ranking Data for Unique Antibodies

Sensor Location	Antibody Clone	Antigen	Response [nm]	k_{off} [1/s]
C8	AbD99991ad-1	scFv-bio	0.59	5.35 x 10^{-4}
C9	AbD99992ad-1	scFv-bio	0.79	5.11 x 10^{-4}
G1	AbD99993ad-1	scFv-bio	0.14	3.19 x 10^{-3}
H1	AbD99994ad-1	scFv-bio	0.16	4.07 x 10^{-3}
E1	AbD99995ad-1	scFv-bio	0.25	5.86 x 10^{-3}
B2	AbD99996ad-1	scFv-bio	0.17	3.58 x 10^{-3}
A2	AbD99997ad-1	scFv-bio	0.14	3.72 x 10^{-3}
C1	AbD99998ad-1	scFv-bio	0.11	3.63 x 10^{-3}
F1	AbD99999ad-1	scFv-bio	0.13	4.68 x 10^{-3}
D1	AbD99990ad-1	scFv-bio	0.26	2.47 x 10^{-3}

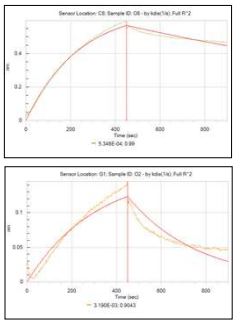


Fig. 3. Primary screening hits were ranked according to their k_{off} values using Octet RED384. k_{off} values for unique antibodies and examples for two sensograms are shown.

TrailBlazer Antibodies – One Production Leads to Three Formats

Each unique antibody clone was expressed in *E. coli* in monovalent Fab format with a SpyTag2. In a coupling reaction taking just one hour, the monovalent Fab was converted to a RPE conjugated bivalent Fab, using BiSpyCatcher2-PE, or to an Ig-like format using human IgG1-FcSpyCatcher3. ELISA with the monovalent Fab format can be used to rank the antibodies according to their binding strength, whereas the bivalent formats (Fab or Ig-like) can be used in applications that benefit from the avidity effect to offer increased sensitivity.

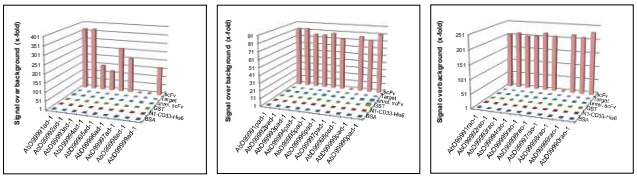
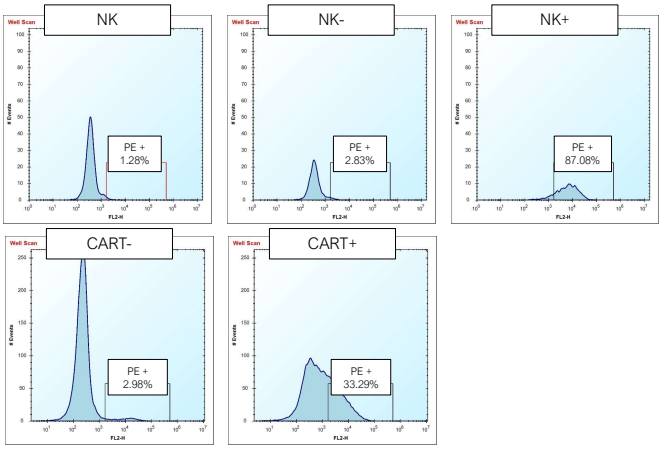


Fig. 4. QC ELISA showing specific binding to the scFv but not to control antigens, using monovalent Fabs (left), bivalent PE-labeled Fabs (center), or Ig-like antibodies (right).

Flow Cytometry

Candidates were further characterized by flow cytometry using an scFv expressing natural killer (NK) cell line, and negative control cells, as well as CAR T-cells (CART+) and unrelated CAR T-cells (CART-). Antibodies in both formats, the Ig-like format and the site-specifically PE-labeled bivalent Fab format, gave similar results.



NK NK cell line
NK- NK cell line transfected with unrelated scFv
NK+ NK cell line transfected with target scFv
CART- CAR T-cells transfected with unrelated scFv
CART+ CAR T-cells transfected with target

Fig. 5. Example of a flow cytometry assay using an antibody in Ig-like format on a NK cell line transfected with the scFv (NK+) and negative controls (top), as well as specific CAR T-cells (CART+) and negative controls (bottom).

Summary

- Fast, well-established platform for the generation of anti-idiotypic antibodies against the scFv of CAR T-cells
- Modular antibody platform results in rapid access to IgGs and conjugated antibodies
- Fast and robust site-specific labeling of antibodies possible using pre-conjugated SpyCatchers
- Bivalent Fabs or synthetic IgGs can be generated within one hour
- Similar or better performance than corresponding controls seen in various assays
- Defined conjugated product:
  - Controlled degree of labeling
  - High batch-to-batch consistency
  - No modification of antibody binding site

Future developments: establish a universal antibody engineering toolbox that simplifies antibody conjugation and switching between multiple stable formats.