Rapid Generation of Bispecific Antibodies for High-Throughput Screening with SpyLock Technology

Christian Hentrich, Mateusz Putyrski, Hanh Hanuschka, Melissa Wich, Achim Knappik, Francisco Ylera

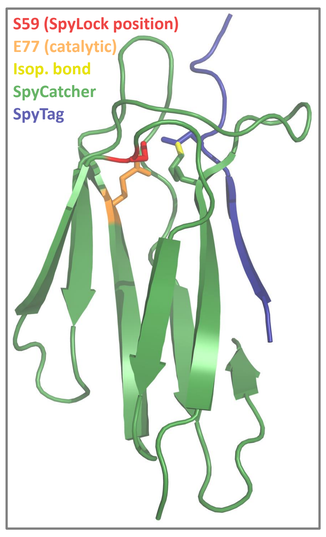
Bio-Rad Laboratories, Inc., Neuried, Germany

Introduction

The SpyCatcher-SpyTag reaction is a fast and specific way to covalently link two proteins and has proven useful for the site-specific labeling and oligomerization of antibodies. Here, we have developed a method to reversibly inhibit the reactivity of SpyCatcher by creating a mutant version that reacts with a disulfide-forming small molecule, thereby abolishing reactivity. Upon reaction with a reducing agent, the small molecule is removed, and reactivity is restored. We call such a lockable SpyCatcher "SpyLock".

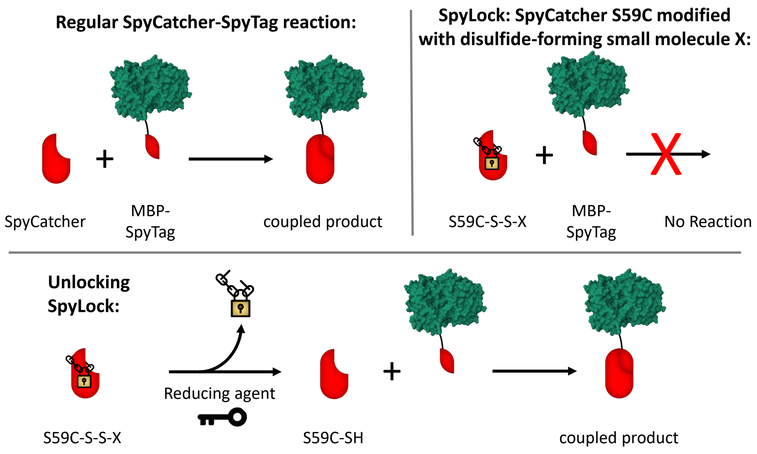
A dimeric fusion of SpyLock and regular SpyCatcher offers an attractive route to bispecific antibodies. SpyTagged antibody fragments specifically ligate to the regular SpyCatcher domain but not to the unreactive SpyLock. After the first ligation, the SpyLock domain is unlocked and an added second antibody will specifically ligate to this domain, thereby creating a bispecific antibody. The "building" of bispecific antibodies is fast (90 minutes) and only requires the presence of SpyTag on each antibody.

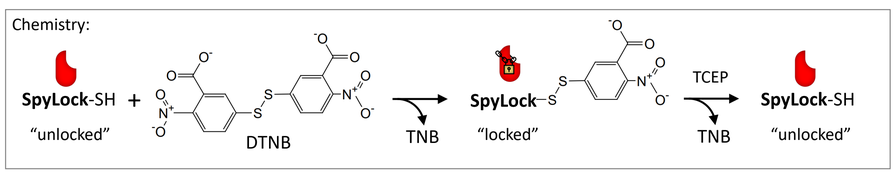
This approach is particularly advantageous for screening antibody pairs recognizing most suitable epitopes or with optimally matched affinities, which can then be further assessed in their final bispecific format. The SpyLock technology seamlessly integrates with Bio-Rad's Pioneer™ Antibody Discovery Platform but can be applied to all recombinant antibodies.

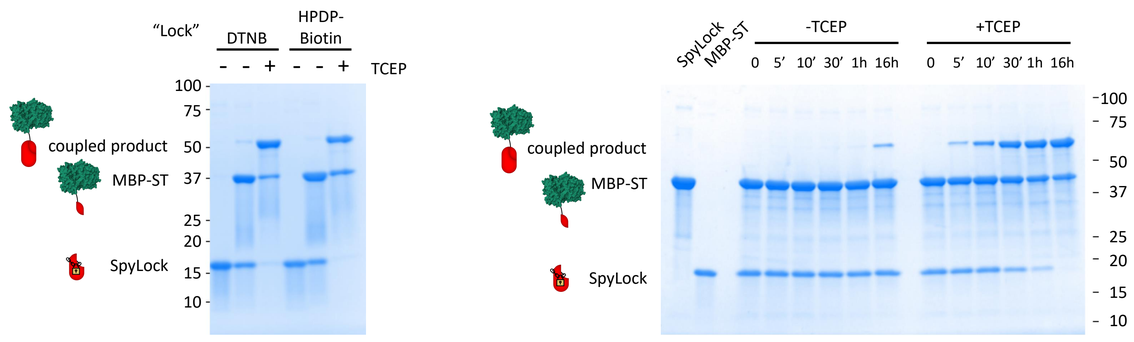


SpyLock Technology

The SpyCatcher with an S59C mutation can be chemically locked and unlocked. If the sulfhydryl group is modified with a small molecule, the isopeptide-bond-forming SpyCatcher-SpyTag reaction is strongly inhibited. By attaching the small molecule to the cysteine via a disulfide bond, this inhibition is reversible. Examples of such disulfide-bond-forming molecules are DTNB (Ellman's reagent) or HPDP-biotin. Treating a thus modified SpyCatcher S59C ("SpyLock") with a reducing agent such as TCEP removes the small molecule from the protein and fully restores reactivity of the SpyLock.





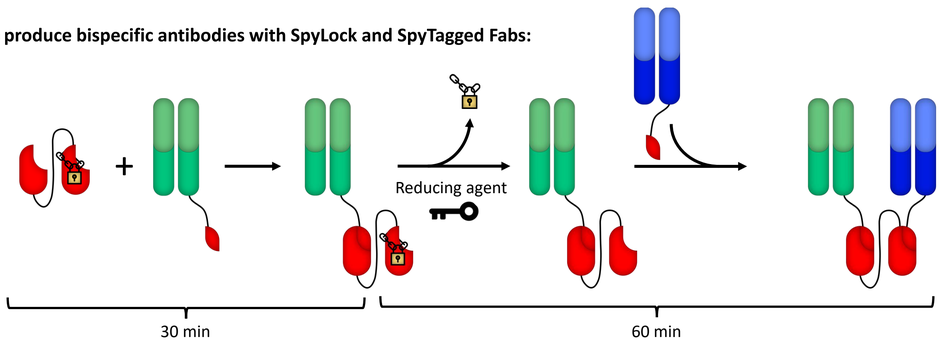


SDS-PAGE showing SpyLock modified with DTNB or HPDP-biotin incubated with an excess of MBP-SpyTag for 1 hr, in the presence or absence of the reducing agent TCEP.

SDS-PAGE showing a time course of SpyLock modified with HPDP-biotin incubated with MBP-SpyTag, in the absence or presence of the reducing agent TCEP. MBP-SpyTag was used in twofold molar excess.

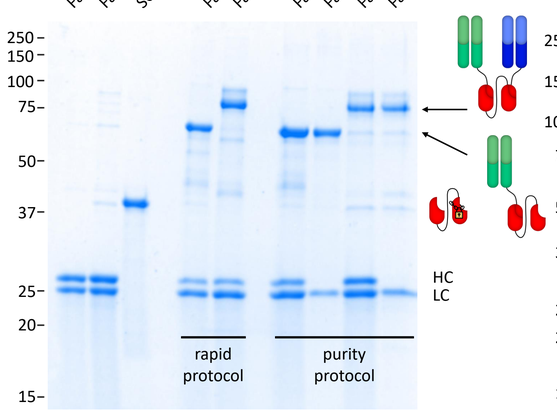
Bispecifics with SpyLock

Workflow to produce bispecific antibodies with SpyLock and SpyTagged Fabs:

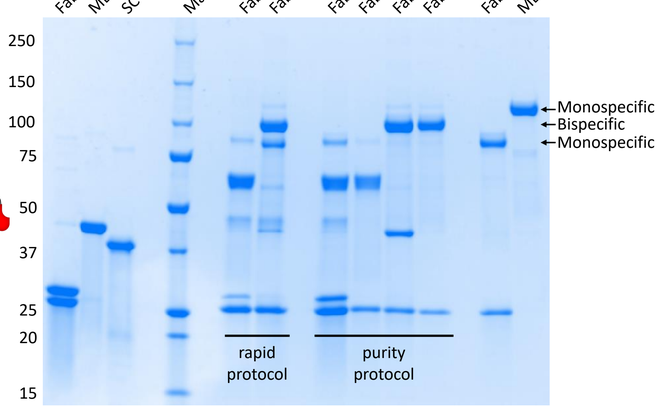


The SpyLock technology can be used to generate bispecific antibodies very rapidly. A SpyCatcher-SpyLock (SC-SL) dimer can be reacted with a first antibody, followed by unlocking with reducing agent and simultaneous reaction with a second antibody. We have developed a rapid protocol, which can be performed in 90 min as well as a high purity protocol which includes purification steps and usually yields more than 95% bispecific antibody. Remaining reducing agent can easily be quenched. Both protocols are compatible with automation.

Construction of bispecific antibodies with SpyLock:



Purity of bispecific SpyLock constructs:



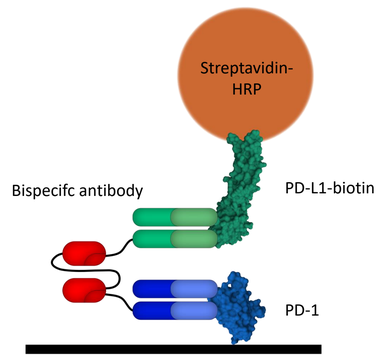
Anti-PD-1 – PD-L1 Bispecificity Confirmed by Bridging ELISA

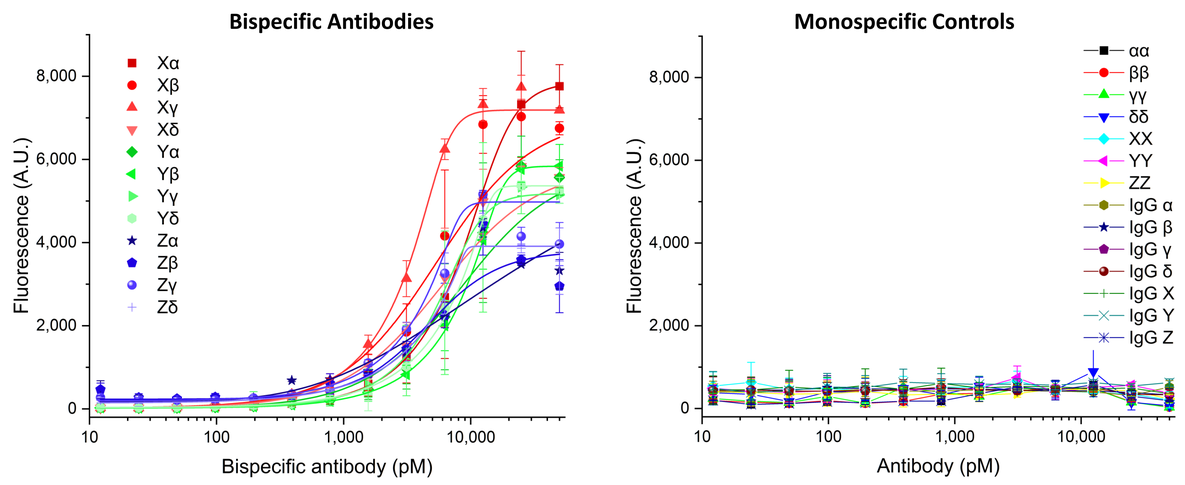
Programmed cell death protein 1 (PD-1) and its ligand, PD-L1, are widely recognized targets for cancer immunotherapy. Although they naturally interact, their intrinsic affinity is relatively low, approximately 4 µM. Perhaps counterintuitively, bispecific targeting of PD-1 and PD-L1 has shown enhanced anti-tumor activity in mouse models when compared to combined antibody treatment or individual monotherapies, consequently sparking interest in the development of such bispecifics (Kotanides H et al., Cancer Immunol Res, 2020, 8, 1,300-1,310).

To showcase the SpyLock technology, we generated Fab fragments derived from three anti-PD-L1 and four anti-PD-1 therapeutic antibodies and used them to construct 12 unique bispecific antibodies with the SpyLock technology.

We employed a bridging ELISA to test for bispecificity. In this assay, the various bispecific antibodies were added to a plate with immobilized PD-1. Bispecificity was then confirmed by their ability to bind added PD-L1-biotin, which was subsequently detected using streptavidin-HRP. As expected, monospecific controls — either as BiCatcher-dimerized Fabs or as full-length IgGs — did not generate any detectable signals in this assay.

Symbol	Antigen	International nonproprietary name
X	PD-L1	atezolizumab
Y	PD-L1	avelumab
Z	PD-L1	durvalumab
α	PD-1	cemiplimab
β	PD-1	dostarlimab
γ	PD-1	nivolumab
δ	PD-1	pembrolizumab

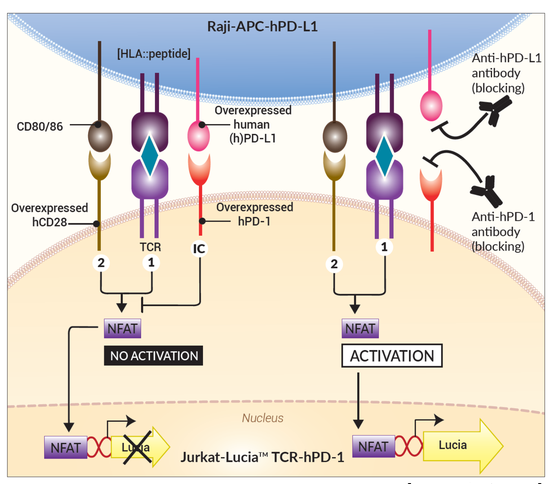




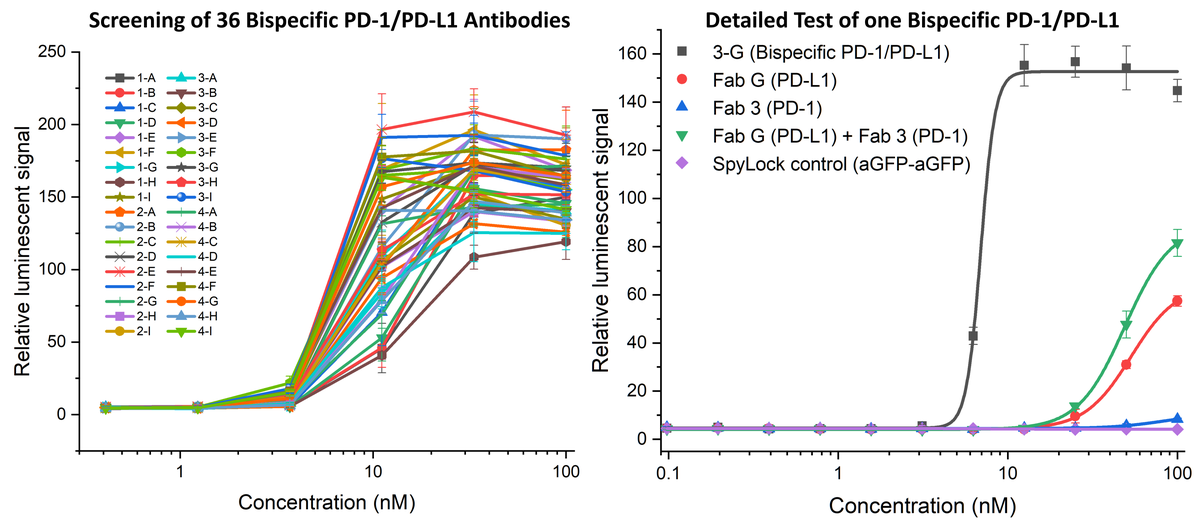
PD-1 – PD-L1 Bispecifics: Performance in Cellular Bioassay

To demonstrate the compatibility of SpyLock technology with cellular assays, we used the PD-1/PD-L1 blockade bioassay from InvivoGen. In this assay, antigen-presenting Raji cells are co-incubated with Jurkat cells expressing antigen-specific TCR and luciferase as a reporter of T-cell activation. Only upon inhibition of the PD-1 immune checkpoint the luciferase is expressed and bioluminescence can be measured.

Using the SpyLock platform, four anti-PD-1 antibodies (Fab 1-4) and nine anti-PD-L1 antibodies (Fab A-I) derived from the Pioneer Library were used to construct 36 bispecific antibodies. All bispecific constructs were tested in the bioluminescent assay and displayed distinct efficacies of the checkpoint inhibition. Additionally, one selected pair of antibodies was assayed in more detail and compared to the activity of the monovalent parental Fabs and their equimolar mixture, as well as to a SpyLock control ('bispecific' construct based on an anti-GFP Fab). The bispecific anti-PD-1/PD-L1 reagent was markedly more potent in the assay than the controls.



[source: Invivogen]



Bioluminescent PD-1/PD-L1 blockade assay: all measurements were performed in triplicate and are depicted as mean ±SD of % signal elicited by treating the cells with 50 nM dostarlimab.

Summary

SpyLock is a reversibly inhibited SpyCatcher. In the locked form, it practically does not react with SpyTag. It can be unlocked with a reducing agent.

SpyLock can be used to generate bispecific antibodies. The process offers the following advantages:

- Fast reaction, bispecific antibodies in 90 minutes
- Only a single antibody format needed: SpyTagged antibody fragments
- Easily scalable, high-throughput compatible

If you are interested in using SpyLock technology in a collaborative project, please contact francisco_ylera@bio-rad.com

Bio-Rad Laboratories, Inc. | Life Science Group | 2000 Alfred Nobel Drive | Hercules, CA 94547 USA