Abstract
This protocol provides the steps to follow to couple a recombinant Fab antibody incorporating a SpyTag2 (Fab), e.g., format Fab-F-Spy2-H, to a bivalent FcSpyCatcher3 and its derivatives (FcCatcher). These guidelines can also be applied to couple a protein with a reactive SpyTag to an FcCatcher.

Short Protocol
1. Calculate the required volumes of Fab and FcCatcher, starting with the amount of Fab you want to couple (see detailed protocol below for calculations).
2. Mix Fab and FcCatcher.
3. Incubate for 1 hr at RT.

Detailed Method
1. To ensure full bivalency of the final coupled Fab and FcCatcher, it is recommended to start with a 25% molar excess of Fab over SpyCatcher sites. Since each FcCatcher contains two SpyCatcher sites, this equals a 2.5 molar excess of Fab e.g., 1 nmol FcSpyCatcher3 + 2.5 nmol Fab-F-Spy2-H. It is possible to use a 2:1 coupling ratio for Fab:FcCatcher (equals a 1:1 ratio for Fab:SpyCatcher sites), but inaccuracies in protein concentration determination might lead to deviations from this ratio and to unpredictable amounts of uncoupled Fab or FcCatcher.
2. When coupling Fab and FcCatcher in solution, it is recommended to use the FcCatcher at the original concentration supplied, and to adjust the concentration of the Fab to 1 mg/ml if practical. [Note 1, 2]

Note 1: There is no minimum concentration required for coupling, but the coupling reaction is faster when the components are at a higher concentration; the lower the concentration, the slower the reaction.

Note 2: When working with dilute Fab or FcCatcher e.g., immobilized FcCatcher on a resin or ELISA plate, and antibody concentrations in the single- or double-digit μg/ml range, reaction times for complete coupling will be longer and must be determined experimentally.

3. Add the required volume of FcCatcher to the Fab. Mix and incubate for 1 hr at RT. It is not important to stop the reaction after 1 hr, it can be left overnight if desired. [Note 3]
Assuming the FcCatcher is at the original concentration, if the Fab concentration is 1 mg/ml, the volume of FcCatcher required is 1/10th the volume of Fab, i.e. add 10 μl FcCatcher to 100 μl of Fab; if the Fab concentration is 0.5 mg/ml, the volume of FcCatcher required is 1/20th the volume of Fab, i.e. add 5 μl of FcCatcher to 100 μl Fab.

Note 3: This method can be used for coupling FcCatchers to SpyTag1, SpyTag2, and SpyTag3. A longer reaction time is required when coupling to SpyTag1.

To calculate the required volume of FcCatcher when starting with quantities or concentrations different from above:

\[
V(FcCatcher) = \frac{m(Fab) \times 1,000,000}{Mw(Fab) \times c(FcCatcher) \times Valency \times Ratio}
\]

 where:
- \( V(Fab) \) is the volume of Fab (μl)
- \( m(Fab) \) is the amount of Fab (μg)
- \( conc(Fab) \) is the concentration of Fab (mg/ml)
- \( V(FcCatcher) \) is the volume of FcCatcher (μl)
- \( Mw(Fab) \) is the molecular weight of Fab (g/mol)
- \( c(FcCatcher) \) is the molar concentration of FcCatcher (μM)
- \( Valency \) is the number of Catcher sites, 2 per FcCatcher
- \( Ratio \) is the ratio of Fab:Catcher; 1.25 is recommended for bivalent FcCatchers.
**Quality Control**

The success of the reaction can be checked using nonreducing SDS PAGE with Coomassie staining. Run 1-1.5 µg of the coupled product. For comparison, also run the uncoupled Fab and FcCatcher on the same gel.

**Recommended Storage**

For short term use, store aliquots at 2-8°C; for long term storage refer to the conditions recommended on the datasheet for each specific FcCatcher. Avoid repeated freeze-thaw cycles. The addition of 0.1% ProClin 950 as a preservative is recommended for storage for up to one month at 2-8°C.

**Calculating the Molar Concentration of the Coupled Antibody**

\[
c (\text{Product}) = \frac{n (\text{FcCatcher})}{V (\text{FcCatcher}) + V (\text{Fab})} \times \frac{c (\text{FcCatcher}) 	imes V (\text{FcCatcher})}{V (\text{FcCatcher}) + V (\text{Fab})}
\]

- **c(Product)**: Molar concentration of coupled antibody (µM)
- **n(FcCatcher)**: Molar amount of FcCatcher used for the reaction (pmol)

To convert the molar concentration to weight concentration:

\[
\text{conc} = c (\text{Product}) \times Mw (\text{Product})
\]

\[
Mw (\text{Product}) = Mw (\text{FcCatcher}) + 2 \times Mw (\text{Fab})
\]

\[
Mw (\text{FcCatcher}) : \text{see table below}
\]

\[
Mw (\text{Fab}) : \sim 54,000 \text{ g/mol}
\]

**Table 1. FcCatcher products.**

<table>
<thead>
<tr>
<th>Product</th>
<th>Description</th>
<th>Molecular Weight (Da, calculated)</th>
<th>Catalog Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>hlgG1-FcSpyCatcher3</td>
<td>SpyCatcher3 fused to the hinge region, CH2, and CH3 of human IgG1</td>
<td>77,166</td>
<td>TZC009</td>
</tr>
<tr>
<td>hlgG2-FcSpyCatcher3</td>
<td>SpyCatcher3 fused to the hinge region, CH2, and CH3 of human IgG2</td>
<td>76,372</td>
<td>TZC016</td>
</tr>
<tr>
<td>hlgG3-FcSpyCatcher3</td>
<td>SpyCatcher3 fused to the hinge region, CH2, and CH3 of human IgG3</td>
<td>87,394</td>
<td>TZC017</td>
</tr>
<tr>
<td>hlgG4-FcSpyCatcher3</td>
<td>SpyCatcher3 fused to the hinge region, CH2, and CH3 of human IgG4</td>
<td>76,494</td>
<td>TZC018</td>
</tr>
<tr>
<td>hlgG4-Pro-FcSpyCatcher3</td>
<td>SpyCatcher3 fused to the hinge region, CH2, and CH3 of human IgG4-Pro (S228P)</td>
<td>76,514</td>
<td>TZC019</td>
</tr>
<tr>
<td>hlgA-FcSpyCatcher3</td>
<td>SpyCatcher3 fused to the hinge region, CH2, and CH3 of human IgA</td>
<td>79,656</td>
<td>TZC020</td>
</tr>
<tr>
<td>mlgG2a-FcSpyCatcher3</td>
<td>SpyCatcher3 fused to the hinge region, CH2, and CH3 of mouse IgG2a</td>
<td>77,738</td>
<td>TZC012</td>
</tr>
<tr>
<td>rblgG-FcSpyCatcher3</td>
<td>SpyCatcher3 fused to the hinge region, CH2, and CH3 of rabbit IgG</td>
<td>76,132</td>
<td>TZC013</td>
</tr>
</tbody>
</table>


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