ABSTRACT CTLA4 (cytotoxic T-lymphocyte-associated protein 4), also known as CD152, is an inhibitor of the immune response expressed by T cells. CTLA4 and CD28 (stimulatory checkpoints) share the same ligands, namely CD80 (B7-1) and CD86 (B7-2), with CTLA4 displaying a greater affinity than CD28 for both thus creating effective ligand binding competition. While CTLA4 can be found on all T cells, it is mainly expressed on Treg where it participates in their inhibition of the immune response.

The HTRF CTLA4/B7-2 Binding Assay is designed to measure the interaction between CTLA4 and B7-2 proteins. Utilizing HTRF (Homogeneous Time-resolved Fluorescence) technology, the assay enables simple and rapid characterization of compound and antibody blockers in a high throughput format.

INHIBITION TEST

Reagents should be dispensed in the following order:

- 2 µL compound/antibody or diluent buffer.
- 4 µL B7-2-Tag1.
- 4 µL CTLA4-Tag2.

Incubate at RT for 15min.

- 5 µL of anti-Tag1-Tb³⁺ or 10 µL of pre-mixture of two conjugates.

Seal the plate and incubate at RT for 1 hour to overnight.

Remove the plate sealer and read the fluorescence emission.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Inhibitor</th>
<th>Tag1-B7-2</th>
<th>Tag2-CTLA4</th>
<th>Anti-Tag1-3 Cryptate</th>
<th>Anti-Tag2-XL665</th>
<th>Diluent</th>
<th>Detection buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>2 µL</td>
<td>4 µL</td>
<td>4 µL</td>
<td>5 µL</td>
<td>5 µL</td>
<td></td>
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</tr>
<tr>
<td>Positive control</td>
<td>4 µL</td>
<td>4 µL</td>
<td>5 µL</td>
<td>5 µL</td>
<td>2 µL</td>
<td></td>
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</tr>
<tr>
<td>Negative control</td>
<td>4 µL</td>
<td>5 µL</td>
<td>5 µL</td>
<td>6 µL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cryptate control</td>
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<td>5 µL</td>
<td>10 µL</td>
<td>5 µL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Buffer control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>10 µL</td>
<td>10 µL</td>
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</tr>
</tbody>
</table>

For 96 & 384-well low volume plates (20 µL).
In order to optimize protein concentrations, the 2 tagged proteins were cross-titrated. B7-2 was titrated from 0 to 60 nM at 3.7, 11.1, 33.3 and 100 nM of CTLA4, and CTLA4 titration, ranging from 0 to 33.3 nM, was achieved at 6.67, 20, and 60 nM of B7-2. The two proteins were incubated for 1 hour at room temperature.

Data were calculated by fitting specific signal to One-site specific binding with hill slope.

Specific signal for each ligand concentration = Total binding - Non specific binding

6 nM CTLA4 and 6 nM B7-2 were chosen for inhibition tests.

**INHIBITION OF CTLA4/B7-2**

The inhibitory effects of Mouse anti-CTLA4 were tested at 6nM CTLA4 and 6 nM B7-7. An assay window around 2 was obtained.