**ABSTRACT** Programmed cell death protein 1 (PD1) is an immune checkpoint receptor that regulates T cell response. Its ligand, programmed death-ligand 1 (PD-L1), is commonly over-expressed on the tumor cell surface. When PD1 is bound to PD-L1, T cell response is suppressed, contributing to tumor immune resistance. Checkpoint inhibitors blocking PD1/PD-L1 complex formation are generating considerable interest in cancer immunotherapy.

The HTRF PD1/PD-L1 Binding Assay is designed to measure the interaction between PD1 and PD-L1 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**

2 µL compound/antibody
4 µL of PD-L1-Tag1
4 µL of PD1-Tag2

Incubate 15 min @ RT

5 µL of anti-Tag1-Eu³⁺
5 µL of anti-Tag2-XL665
or
10 µL of pre-mixed Anti-tag detection reagents

Incubate 2 hours @ RT

Reagents should be dispensed in the following order:

- 2 µL compound/antibody or diluent buffer.
- 4 µL PD-L1-Tag1.
- 4 µL PD1-Tag2.

Incubate at RT for 15min.

- 5 µL of anti-Tag1-Eu³⁺ and 5 µL of anti-Tag2-XL665 or 10 µL of pre-mixture of two conjugates.

Seal the plate and incubate at RT for 2 hours.

Remove the plate sealer and read the fluorescence emission.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Inhibitor</th>
<th>Tag1-PD-L1</th>
<th>Tag2-PD1</th>
<th>Anti-Tag1-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>
<td></td>
</tr>
<tr>
<td>Positive control</td>
<td></td>
<td>4 µL</td>
<td>4 µL</td>
<td>5 µL</td>
<td>5 µL</td>
<td>2 µL</td>
<td></td>
</tr>
<tr>
<td>Negative control</td>
<td></td>
<td>4 µL</td>
<td>5 µL</td>
<td>5 µL</td>
<td>6 µL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cryptate control</td>
<td></td>
<td>4 µL</td>
<td>5 µL</td>
<td>10 µL</td>
<td>5 µL</td>
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<tr>
<td>Buffer control</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).
PD1/PD-L1 BINDING ASSAY

In order to optimize protein concentrations, PD1 was titrated from 0 to 400 nM at a saturating concentration of PD-L1 (20 nM) and PD-L1 was titrated from 0 to 50 nM with a saturating concentration of PD1 (200 nM). The two proteins were incubated for 2 hours at room temperature.

Data were calculated by fitting specific signal to One-site specific binding curve.

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

Optimal concentrations of PD1 and PD-L1 were defined at 50 nM and 5 nM respectively for inhibition tests.

INHIBITION OF PD1/PD-L1

The inhibitory effects of small molecules, human and mouse blocking antibodies of PD1 and PD-L1 were tested at 5 nM PD-L1 and 50 nM PD1. An assay window around 10 was obtained.