**TECHNICAL DATA SHEET**

# THUNDER™ Human PD-L1 TR-FRET Biomarker Assay Kit

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**CATALOG NUMBERS**

**KIT-PDL1-100** (100 tests)
**KIT-PDL1-500** (500 tests)
**KIT-PDL1-2500** (2500 tests)
**KIT-PDL1-5000** (5000 tests)
**KIT-PDL1-10000** (10000 tests)

Store at -80°C.
For research use only.
Not for use in diagnostic procedures.

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## PRODUCT DESCRIPTION

This assay kit is designed for the quantitative determination of **human Programmed death-ligand 1 (PD-L1)** in cell culture supernatants using a simple, rapid and sensitive immunoassay based on the homogeneous (no-wash) THUNDER™ TR-FRET technology. The kit does not include a recombinant **human PD-L1** standard, which must be purchased separately from R&D Systems® (catalogue number 156-B7).

## SPECIFICITY

This assay kit contains two specific and selective antibodies that recognize both natural and recombinant **human PD-L1** in cell culture supernatant samples.

## SPECIES REACTIVITY

Human (Swiss-Prot Acc. Q9NZQ7; Entrez Gene Id 29126).

## TR-FRET ASSAY PRINCIPLE

The THUNDER™ **human PD-L1** assay kit is a homogeneous time-resolved Förster resonance energy transfer (TR-FRET) sandwich immunoassay (Figure 1). The assay workflow consists of a two-step reagent addition (Figure 2). **Human PD-L1** in the cell supernatant sample is detected with a pair of fluorophore-labeled antibodies in a simple "add-incubate-measure" format (no wash steps). One antibody is labeled with a donor fluorophore (Europium chelate; Eu-Ab1) and the second with a far-red acceptor fluorophore (FR-Ab2). The binding of the two labeled antibodies to distinct epitopes on the target cytokine takes place in solution and brings the two dyes into close proximity. Excitation of the donor Europium chelate molecules with a flash lamp (320 or 340 nm) or a laser (337 nm) triggers a FRET from the donor to the acceptor molecules, which in turn emit a TR-FRET signal at 665 nm. Residual energy from the Eu chelate generates light at 615 nm. The signal at 665 nm is proportional to the concentration of **human PD-L1** in the cell supernatant. Data can be expressed as either the signal at 665 nm or the 665 nm/615 nm ratio. A distinct advantage of the THUNDER™ TR FRET technology is that it does not require addition of potassium fluoride to ensure signal stability. The non-toxic nature of THUNDER™ makes it an ideal candidate for multiplexing with other assay technologies using a sequential assay protocol.

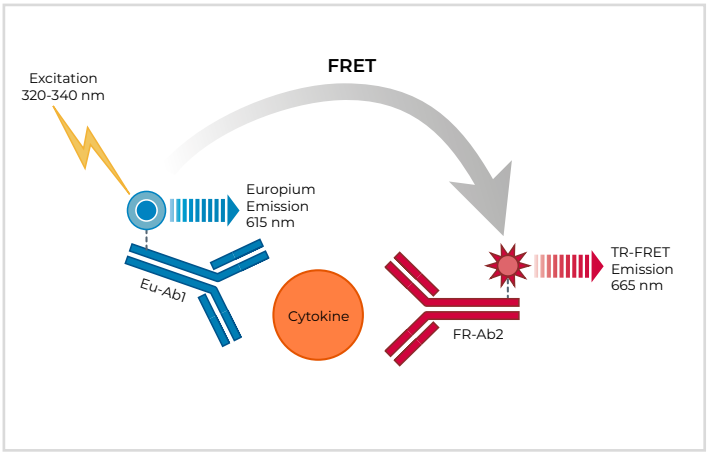


Figure 1 Schematic representation of the TR-FRET assay principle.


Figure 2 THUNDER™ human PD-L1 assay workflow.

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Upon receipt, store kit in the dark at -80°C.

# TECHNICAL DATA SHEET

HUMAN PD-L1 TR-FRET ASSAY KIT

## THUNDER™ ASSAY PROTOCOL

**This protocol must be read in its entirety prior to beginning the assay.**

- We cannot guarantee the performance of the product outside the conditions detailed in this Assay Protocol.
- Bring all reagents to room temperature prior to running the assay.
- Centrifuge all tubes before use to improve recovery of content (2000x g, 10-15 sec).
- Use ultrapure water (Milli-Q® grade water; 18 MΩ cm) to dilute Assay Buffer.
- It is recommended to test all standards in triplicate and samples at least in duplicate.

| KIT COMPONENTS | 100 points* | 500 points* |
|---|---|---|
| Eu-labeled anti-hPD-L1 antibody (Eu-Ab1) | 5 µL (1 clear tube, red cap) | 25 µL (1 clear tube, red cap) |
| Acceptor-labeled anti-anti-hPD-L1 antibody (FR-Ab2) | 20 µL (1 brown tube, blue cap) | 100 µL (1 brown tube, blue cap) |
| Assay Buffer 3 (5X)** | 1 mL (1 tube, yellow cap) | 3 mL (3 tubes, yellow cap) |

* The number of assay points is based on an assay volume of 20 µL in low-volume 384-well assay plates using the kit components at the recommended concentrations.

** Extra Assay Buffer can be ordered separately (cat # TRFRET-AB3).

| ADDITIONAL MATERIALS REQUIRED | Recommended source | Catalog No. |
|---|---|---|
| Recombinant human PD-L1 | R&D Systems | 156-B7 |
| Ultrapure laboratory grade water | Many options available | NA |
| Low-volume 384-well microplate, white | PerkinElmer<br>Greiner<br>Corning | 6007290<br>784075<br>4513 |
| Multi- and single-channel pipettes | Many options available | NA |
| Adhesive sealing film for plates | Many options available | NA |
| A plate reader equipped with the TR-FRET option | Many options available | NA |

## REAGENT PREPARATION

- The instructions described below are for testing the entire number of assay points in each kit. Adjust volumes accordingly for testing of fewer assay points.
- Prepare only as much reagent as is needed on the day of the experiment.

### STEP 1 PREPARATION OF 1X ASSAY BUFFER 3

- Mix end-over-end the 5X Assay Buffer 3 before use.
- The thawed 5X Assay Buffer 3 can be stored at 4°C for 1-2 weeks. For longer periods of time, buffer should be stored at -80°C.
- Unused 1X Assay Buffer 3 can be stored at 4°C for 2 days.

**For the 100-point kit**
Add 1 mL of 5X Assay Buffer 3 to 4 mL of water.

**For the 500-point kit**
Add 3 mL of 5X Assay Buffer 3 to 12 mL of water.

### STEP 2 PREPARATION OF PD-L1 STANDARD SERIAL DILUTIONS

**IMPORTANT NOTE**

*The analyte human PD-L1 is not included in the kit. The kit has been validated using human PD-L1 recombinant protein from R&D Systems® (Catalog number 156-B7). Other commercial recombinant human PD-L1 may also work but have not been tested with this kit.*

- Prepare working standards just before use and use within one hour. Do not store working standards.
- Each well requires 15 µL of working standard.
- Change tip between each standard dilution.
- Mix each tube thoroughly before the next transfer.
- The following section describes the preparation of a standard curve for triplicate measurements (recommended).

**NOTE**

*The Working Stock Solution should be used within one hour or aliquoted into screw-capped polypropylene vials and stored at -80°C for long term storage.*

## REAGENT PREPARATION (CONTINUED)

**A.** Follow the manufacturer's instructions to reconstitute the analyte (typically at 100-200 µg/mL) to create a Stock Solution.

**B.** Create a 3 µg/mL Working Stock Solution by diluting the Stock Solution in either 1X Assay Buffer 3 if the standard curve is done in Assay buffer or in culture medium with 10% fetal bovine serum (FBS) if the standard curve is done in culture medium. Use this Working Standard Solution to prepare a standard dilution series.

**C.** Label 11 tubes, one for each working standard. Alternatively, serial dilutions can be conducted using a polypropylene 96-well plate.

**D.** Prepare serial dilutions for the standard curve as indicated in the table below using either 1X Assay Buffer 3 or culture medium with 10% FBS (for testing of culture supernatant samples):

| Tube | Volume of hPD-L1 | Vol. of 1X Assay Buffer or culture medium +10% FBS (µL) | [hPD-L1] in standard curve (g/mL in 15 µL) |
|---|---|---|---|
| 1 | 10 µL diluted hPD-L1 (3E-6 g/mL) | 90 | 3.0E-7 |
| 2 | 30 µL of tube 1 | 60 | 1.0E-7 |
| 3 | 30 µL of tube 2 | 70 | 3.0E-8 |
| 4 | 30 µL of tube 3 | 60 | 1.0E-8 |
| 5 | 30 µL of tube 4 | 70 | 3.0E-9 |
| 6 | 30 µL of tube 5 | 60 | 1.0E-9 |
| 7 | 30 µL of tube 6 | 70 | 3.0E-10 |
| 8 | 30 µL of tube 7 | 60 | 1.0E-10 |
| 9 | 30 µL of tube 8 | 70 | 3.0E-11 |
| 10 | 30 µL of tube 9 | 60 | 1.0E-11 |
| 11* | 0 | 70 | 0 |

* In order to calculate the Limit of Detection (LOD), three additional zero standard tubes (12-14) are assayed in triplicate (12 wells in the 384-well plate).

## STEP 3 PREPARATION OF 8X ANTIBODY DETECTION MIX

- Prepare and mix just before use.
- Each well requires 2.5 µL of 8X Eu-Ab1 Antibody Solution and 2.5 µL of 8X FR-Ab2 Antibody Solution.
- The unused 8X Working Antibody Solutions may be stored at 4°C for 2 days or aliquoted and stored at -80°C. Avoid repeated freeze/thaw cycles.

### For the 100-point kit

**NOTE**
*Due to the low reagent volumes in the 100-point kit, the antibodies are diluted with 1X Assay Buffer 3 directly in the vial.*

**A.** Add 255 µL of 1X Assay Buffer 3 into the vial containing 5 µL of Eu Ab1 stock solution

**B.** Add 240 µL of 1X Assay Buffer 3 into the vial containing 20 µL of FR Ab2 stock solution.

### For the 500-point kit

**A.** Mix gently 1,275 µL of 1X Assay Buffer 3 with the 25 µL of Eu Ab1 stock solution.

**B.** Mix gently 1,200 µL of 1X Assay Buffer 3 with the 100 µL of FR-Ab2.

## SAMPLE PREPARATION

- Each well requires 15 µL of sample (cell supernatant).
- Just after their collection, put the samples at +4°C and assay immediately or aliquot and store -80°C. Avoid repeated freeze/thaw cycles.
- Do not use a water bath to thaw samples. Thaw samples at room temperature.
- If the analyte concentration in the sample possibly exceeds the highest point of the standard curve, prepare one or more 10-fold dilutions of the sample. Prepare serial dilutions using the same medium used to culture the cells.

## ASSAY PROCEDURE

- Samples and standards must be assayed at least in duplicate each time the assay is performed.
- When loading reagents in the low-volume 384-well microplate, change tips between each standard or sample addition and after each set of reagents.
- If using a multichannel pipettor, always use a new disposable reagent reservoir.
- When reagents are added to the microplate, make sure the liquids are at the bottom of the well.

**A.** Add 15 µL of each working standard or 15 µL of cell supernatant sample.

**B.** Add 2.5 µL of 8X Eu-Ab1 Antibody Solution to each of the assay wells.

**C.** Cover the plate with a plate sealer and incubate 30 minutes at room temperature.

**D.** Gently remove the adhesive plate sealer. Add 2.5 µL of 8X FR-Ab2 Antibody Solution to each of the assay wells.

**E.** Cover the plate with a plate sealer and incubate 2 hours at room temperature.

**F.** Gently remove the adhesive plate sealer. Read plate on a TR-FRET compatible microplate reader.

**NOTE** *The same plate can be read several times without any negative effect on the assay performance.*

## TR-FRET PLATE READER SETTINGS

For optimal assay performance, we recommend that you read the TR FRET assays at two wavelengths, detecting both the emission from the Europium chelate donor fluorophore at 615 nm, and the acceptor fluorophore at 665 nm. The following instrument settings are provided as guidelines.

| | TR-FRET Compatible Plate Reader* | |
|---|---|---|
| **Parameter** | **Flash lamp excitation** | **Laser excitation** |
| Excitation filter | 320 nm (or 340 nm) | Not applicable |
| Emission filter | 615 nm (or 620 nm) | 615 nm (or 620 nm) |
| Delay time | 90 µs | 50 µs |
| Flash energy level | 100% or High | 100% |
| Number of flashes | 100-200 | 20 |
| Window (integration time) | 300 µs | 100 µs |

* These settings are provided as guidelines only. Settings should be optimized for each reader. For optimal settings, visit **bioauxilium.com/resources**.

## DATA ANALYSIS

- TR-FRET data are typically calculated and presented ratiometrically using the following formula: [(665 nm/615 nm) x 1,000]
- Alternatively, the signals at 665 nm can be used directly to process your data.
- Calculate the TR-FRET ratio for each well.
- Since TR-FRET signal is read in a time-resolved mode, background substraction is usually not necessary.
- Create a standard curve by analyzing data according to a nonlinear regression using the 4 parameter logistic equation (sigmoidal dose-response curve with variable slope) and a $1/Y^2$ data weighting. For correct analysis, the highest standard point should be removed from the curve if it is present after the hook point (i.e., if it shows lower counts compared to the next standard).
- Do not use a value of zero for the zero standard. Use instead the actual background values obtained with the zero standard.
- If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

## TYPICAL STANDARD CURVE

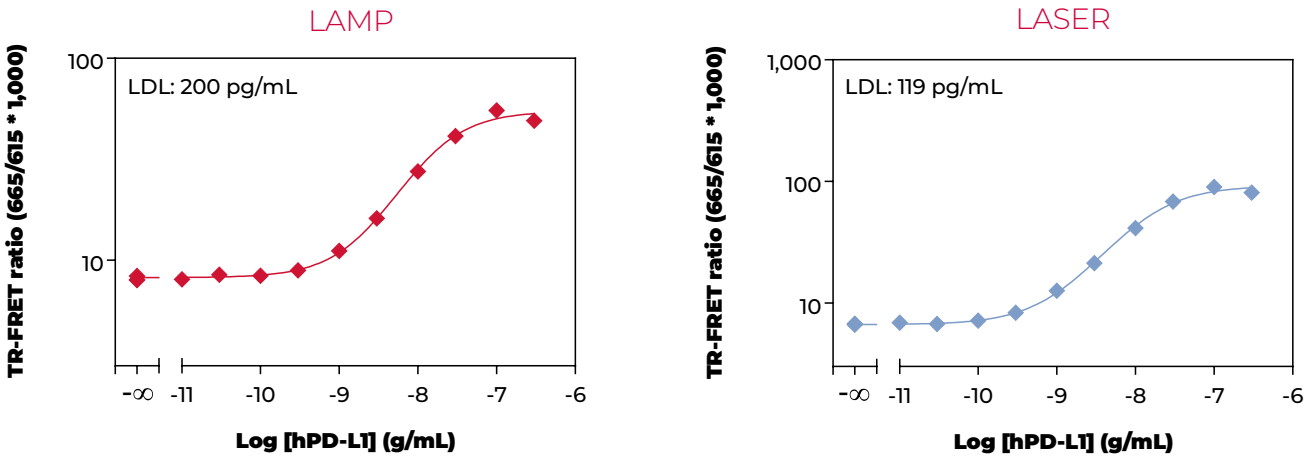


Figure 3 Typical nonlinear human PD-L1 standard curves in Assay Buffer 3. Left: lamp excitation, Right: laser excitation. Data represent the mean ± standard deviation of triplicate measurements (3 wells) for each standard. The data was generated using a low-volume 384-well white plate read on the EnVision® equipped with TR-FRET option.

## VALIDATION DATA

The THUNDER™ human PD-L1 assay kit has been validated using human PD-L1 protein from R&D Systems® (Catalog number 156-B7 NOT included in the kit).

| HUMAN PD-L1 THUNDER™ ASSAY SUMMARY | | |
|---|---|---|
| Assay Type | Homogeneous sandwich immunoassay | |
| Format | 384-well plate | |
| Hands-on time | 15 minutes | |
| Incubation time | 2.5 hours | |
| Sample type (volume) | Cell culture supernatant (15 µL) | |
| Specificity | Natural and recombinant human PD-L1 | |
| | **Laser excitation** | **Lamp excitation** |
| Limit of Detection (LOD) | 119 pg/mL | 200 pg/mL |
| Lower Limit of Quantification (LLOQ) | 609 pg/mL | 1 219 pg/mL |
| $EC_{50}$ | 15.7 ng/mL | 17.5 ng/mL |
| Dynamic Range | 119 – 100,000 pg/mL | 200 – 100,000 pg/mL |

## SENSITIVITY

The Limit of Detection (LOD) was calculated by adding two standard deviations to the mean background counts of 12 zero standard replicates and calculating the corresponding concentration on the standard curve.

The Lowest Level of Quantitation (LLOQ) was calculated by adding 10 standard deviations to the mean background counts of 12 zero standard replicates and calculating the corresponding concentration on the standard curve.

| | ASSAY BUFFER 3 | | DMEM +10% FBS | | RPMI +10% FBS | |
|---|---|---|---|---|---|---|
| | Laser | Lamp | Laser | Lamp | Laser | Lamp |
| # of standard curves | 10 | 10 | 3 | 3 | 3 | 3 |
| LOD (pg/mL) | 119 | 200 | 347 | 610 | 244 | 520 |
| LLOQ (pg/mL) | 609 | 1219 | 1 912 | 3 594 | 1535 | 2 874 |

## PRECISION

### INTRA-ASSAY PRECISION

Three samples of known concentration (low, medium, high) were tested twenty-one times each on the same plate to assess intra-assay precision. The assay was run in a 384-well plate with standards and samples prepared in Assay Buffer 3. The resulting low %CV (<10%) for each sample indicates good reproducibility within an assay.

| Sample concentration | Low | Med | High |
|---|---|---|---|
| # of samples | 21 | 21 | 21 |
| Mean (pg/mL) | 1 935 | 6 728 | 23 675 |
| SD (pg/mL) | 192 | 340 | 2 165 |
| CV (%) Intra-Assay | 9.9 | 5.0 | 9.1 |

### INTER-ASSAY PRECISION

Three samples of known concentration were tested in separate assays to assess inter-assay precision. A total of eight independent experiments were performed by two operators using two different kit lots. The assay was run in 384-well plates with standards and samples prepared in Assay Buffer 3 and tested in triplicate. The resulting low %CV (<10%) for each sample indicates good reproducibility between assays.

| Sample concentration | Low | Med | High |
|---|---|---|---|
| # of samples | 24 | 24 | 24 |
| Mean (pg/mL) | 2 174 | 7 395 | 23 569 |
| SD (pg/mL) | 216 | 614 | 1 948 |
| CV (%) Inter-Assay | 9.9 | 8.3 | 8.3 |

## RECOVERY

To assess the accuracy of the assay, spike-to-recovery experiments were conducted. Each assay consisted of one standard curve and three spike concentrations (low, medium and high), assayed in triplicate in Assay Buffer 3, DMEM and RPMI (supplemented with 10% FBS). The average recovery was calculated from the average of 3 wells and compared to a standard curve prepared in the corresponding diluent. Data show recovery values within the range of 80–120% demonstrating assay accuracy.

| | Spike level | Expected (pg/mL) | Observed (pg/mL) | Average % Recovery |
|---|---|---|---|---|
| Assay Buffer 3 | High | 30 000 | 32 025 | 107 |
| | Medium | 10 000 | 9 631 | 96 |
| | Low | 3 000 | 3 002 | 100 |
| DMEM + 10% FBS | High | 30 000 | 25 102 | 84 |
| | Medium | 10 000 | 9 691 | 97 |
| | Low | 3000 | 3 339 | 111 |
| RPMI + 10% FBS | High | 30 000 | 28 924 | 96 |
| | Medium | 10 000 | 11 061 | 111 |
| | Low | 3 000 | 3 501 | 117 |

## LINEARITY

To assess the linearity of the assay, samples were spiked with a high concentration of analyte in culture medium supplemented with 10% FBS and diluted with the same culture medium with 10% FBS. The assay was run in a 384-well plate with standards prepared in culture media with 10% FBS. Each sample was measured in triplicate. Data show recovery values within 80-120% confirming assay linearity.

| | Expected (pg/mL) | Observed (pg/mL) | Average % Recovery |
|---|---|---|---|
| DMEM + 10% FBS | 93 800 | 100 179 | 107 |
| | 46 900 | 48 473 | 103 |
| | 23 400 | 25 798 | 110 |
| | 11 700 | 13 366 | 114 |
| | 5 860 | 7 055 | 120 |
| | 2 930 | 2 532 | 86 |
| | 1 460 | 1 587 | 108 |
| RPMI + 10% FBS | 93 800 | 105 476 | 113 |
| | 46 900 | 46 903 | 100 |
| | 23 400 | 24 413 | 104 |
| | 11 700 | 12 946 | 110 |
| | 5 860 | 6 309 | 108 |



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