TECHNICAL DATA SHEET

LANCE® Ultra Research Reagents

Research Use Only. Not for use in diagnostic procedures.

Phosphorylated TBK1 (S172) LANCE Ultra Cellular Detection Kit

Product number: TRF4031

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Product Information

Application: This kit is designed for the detection of phosphorylated TBK1 (S172) in cell lysates using a homogeneous LANCE Ultra assay (no wash steps).

Typical Performance
(Undiluted positive control lysate versus Buffer):

Signal/Background: 6.9
Z': 0.91

Figure 1. Typical positive control lysate (MCF-7 cells stimulated with Calyculin A) diluted in lysis buffer. The data was generated using a white Optiplate™-384 microplate and read on an EnVision™ Multilabel Plate Reader equipped with TR-FRET laser option. Total signal, signal/background window, and sensitivity may vary with other instruments. Positive control lysate is not supplied with the kit and must be purchased separately.

Storage: Store kit in the dark at +4°C.

Stability: This kit is stable for at least 6 months from the manufacturing date when stored in its original packaging and the recommended storage conditions.
Quality Control

Lot to lot consistency is confirmed in a LANCE Ultra assay. S/B and Z' were measured on the EnVision Multilabel Plate Reader equipped with TR-FRET laser option. We certify that these results meet our quality release criteria. Maximum counts, Signal/Background, and Z' values may vary between lots and instrument used. For quality control purposes, Z' is calculated by comparing 12 replicates of undiluted positive lysates versus 12 replicates of lysis buffer. Data is calculated and presented ratiometrically by dividing the signal at 665 nm by the signal at 615 nm and multiplied by 10,000.

Analyte of Interest

TANK-binding kinase 1 (TBK1) is auto-phosphorylated at Ser172. It contributes to multiple pathways. Activation of TBK1 leads to the activation of the TLR3/4 and cGAS-STNG signaling pathways. Ultimately activation of TBK1 leads to IRF3 activation. Phosphorylation of optineurin and p62 by TBK1 marks proteins to be directed to the autophagosome.

Description of the LANCE Ultra Assay

LANCE® and LANCE® (Lanthanide chelate excite) Ultra are homogeneous (no wash) TR-FRET (time-resolved fluorescence resonance energy transfer) technologies. One antibody of interest is labeled with a donor fluorophore (a LANCE Europium chelate) and the second antibody is labeled with an acceptor fluorophore [ULight™ dye]. Upon excitation at 320 or 340 nm, energy can be transferred from the donor Europium chelate to the acceptor fluorophore if sufficiently close for FRET (~10 nm). This results in the emission of light at 665 nm. Data is represented as ratiometric (665/615 nm X 10,000).

![LANCE assay principle](image)
### Kit Content: Reagents and Materials

<table>
<thead>
<tr>
<th>Kit components</th>
<th>TRF4031C (500 assay points**)</th>
<th>TRF4031M (10 000 assay points**)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LANCE Ultra Eu-labeled Anti-TBK1 (S172) Antibody stored in TSA, 0.1% BSA</td>
<td>10 µL @ 500 nM (1 clear tube, yellow cap)</td>
<td>200 µL @ 500 nM (1 clear tube, yellow cap)</td>
</tr>
<tr>
<td>LANCE Ultra ULight-labeled Anti-TBK1 Antibody stored in TSA, 0.1% BSA</td>
<td>100 µL @ 500 nM (1 brown tube, blue cap)</td>
<td>2 X 1000 µL @ 500 nM (2 brown tubes, green caps)</td>
</tr>
<tr>
<td>LANCE Detection Buffer (10X) *</td>
<td>1.8 mL, 1 small bottle</td>
<td>250 mL, 1 large bottle</td>
</tr>
<tr>
<td>LANCE Ultra Lysis Buffer 1 (5X)</td>
<td>10 mL, 1 small bottle</td>
<td>100 mL, 1 large bottle</td>
</tr>
</tbody>
</table>

* Extra detection buffer can be ordered separately (cat # CR97-100C: 1.8 mL or cat # CR97-100: 250 mL). Extra Lysis Buffer can be ordered separately (cat # TRF001C: 10 mL or cat # TRF001F: 100 mL).

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

Sodium azide should not be added to the stock reagents. High concentrations of sodium azide (> 0.001 % final in the assay) might decrease the signal.
Specific additional required reagents and materials:

The following materials are recommended:

<table>
<thead>
<tr>
<th>Item</th>
<th>Suggested source</th>
<th>Catalog #</th>
</tr>
</thead>
<tbody>
<tr>
<td>VICTOR Nivo™, EnVision, EnSight™, or any Multilabel Plate Reader equipped with TR-FRET option</td>
<td>PerkinElmer Inc.</td>
<td>Please consult our website</td>
</tr>
<tr>
<td>TopSeal-A PLUS Adhesive Sealing Film</td>
<td>PerkinElmer Inc.</td>
<td>6050185</td>
</tr>
<tr>
<td>Tissue culture treated clear SpectraPlates™, for culturing cells when using the 2-plate protocol</td>
<td>PerkinElmer Inc.</td>
<td>6005650</td>
</tr>
<tr>
<td>White OptiPlate-384, for LANCE Ultra detection assays when using the 2-plate protocol</td>
<td>PerkinElmer Inc.</td>
<td>6007290</td>
</tr>
<tr>
<td>White CulturPlate-384 when using the 1-plate protocol</td>
<td>PerkinElmer Inc.</td>
<td>6007680</td>
</tr>
<tr>
<td>Positive Control Cell Lysate (MCF-7 cells treated with Calyculin A)</td>
<td>PerkinElmer Inc.</td>
<td>TRF4031S</td>
</tr>
</tbody>
</table>

**Recommendations**

**General recommendations:**

- The volume indicated on each tube is guaranteed for single pipetting. Multiple pipetting of the reagents may reduce the theoretical amount left in the tube.
- Re-suspend all reagents by vortexing before use.
- Centrifuge all tubes before use to improve recovery of content (2000x g. 10-15 sec).
- Use Milli-Q® grade H₂O (18 MΩ·cm) to dilute Detection and Lysis Buffers.
- When diluting the samples, change tips between each standard or sample dilution. When loading reagents in the assay microplate, change tips between each standard or sample addition and after each set of reagents.
- When reagents are added to the microplate, make sure the liquids are at the bottom of the well.
- Small volumes may be prone to evaporation. It is recommended to cover microplates with TopSeal-A Adhesive Sealing Films to reduce evaporation during incubation. LANCE Ultra TR-FRET assays cannot be read with the TopSeal-A Film attached. Please remove before reading.
- LANCE signal can be detected using any multimode plate reader equipped with TR-FRET capabilities. Recommended readers are the EnVision, VICTOR Nivo, or EnSight Multilabel Reader equipped with TR-FRET. Use an excitation wavelength of 320 or 340 nm to excite the LANCE Europium chelate. We recommend you read this assay in dual emission mode, detecting both the emission from the Europium donor fluorophore at 615 nm, and the acceptor fluorophore (at 665 nm for ULight dye). The 665/615 nm x 10,000 calculation is used to process your data.
- Signal will vary with temperature and incubation time. For consistent results, identical incubation times and temperatures should be used for each plate.
- The representative data shown in this technical data sheet are provided for information only.
Cell Handling and Lysis recommendations:

- Evaporation can be problematic with cells cultured in microtiter plates. For overnight incubation, it is recommended to add warm PBS or sterile water to unused wells. For longer incubation periods, a sterile breathable sealing membrane (Corning, cat. #3345) can be used to cover the plate. Alternatively, cells can be cultured in larger wells, and/or in a larger volume of culture medium.

- Phosphatase Inhibitors such as NaF and activated Na$_3$VO$_4$ can be added to lysis buffers to protect kinases without affecting LANCE detection.

- An incubation of 30 minutes is usually sufficient for cell lysis. However, the optimal lysis incubation time should be determined by each investigator using a time course study.

- A starving step with serum-free medium may be required depending on your target/cell line and should be evaluated in a separate experiment.

- For 2-plate protocols with adherent cells: cell seeding densities of 40K cells/well are usually sufficient for most cell lines. However, optimization of cell seeding density is recommended.

- For 1-plate protocols with suspension cells: Cell seeding densities of 100K cells/well are usually sufficient for most cell lines. However, optimization of cell seeding density is recommended.

## Assay Procedure

<table>
<thead>
<tr>
<th>Format</th>
<th># of data points</th>
<th>Final</th>
<th>Sample</th>
<th>Eu-Antibody/ULight-Antibody MIX</th>
<th>Plate recommendation</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRF4031C</td>
<td>250</td>
<td>40 µL</td>
<td>30 µL</td>
<td>10 µL</td>
<td>White OptiPlate-96 (cat # 6005290) White ½ AreaPlate-96 (cat # 6005560)</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>20 µL</td>
<td>15 µL</td>
<td>5 µL</td>
<td>White OptiPlate-384 (cat # 6007290) White CulturPlate-384 (cat # 6007680)</td>
</tr>
<tr>
<td></td>
<td>1 250</td>
<td>8 µL</td>
<td>6 µL</td>
<td>2 µL</td>
<td>ProxiPlate™-384 Plus (cat # 6008280) White OptiPlate-384 (cat # 6007290) White CulturPlate-384 (cat # 6007680)</td>
</tr>
<tr>
<td></td>
<td>2 500</td>
<td>4 µL</td>
<td>3 µL</td>
<td>1 µL</td>
<td>White OptiPlate-1536 (cat # 6004290)</td>
</tr>
<tr>
<td>TRF4031M</td>
<td>10 000</td>
<td>20 µL</td>
<td>15 µL</td>
<td>5 µL</td>
<td>White OptiPlate-384 (cat # 6007290) White CulturPlate-384 (cat # 6007680)</td>
</tr>
<tr>
<td></td>
<td>25 000</td>
<td>8 µL</td>
<td>6 µL</td>
<td>2 µL</td>
<td>ProxiPlate-384 Plus (cat # 6008280) White OptiPlate-384 (cat # 6007290) White CulturPlate-384 (cat # 6007680)</td>
</tr>
<tr>
<td></td>
<td>50 000</td>
<td>4 µL</td>
<td>3 µL</td>
<td>1 µL</td>
<td>White OptiPlate-1536 (cat # 6004290)</td>
</tr>
</tbody>
</table>
**General Lysis Protocol:** Cells are lysed in 1X lysis buffer.

*Each protocol described below is designed for 500 assay points.*

**Lysate Preparation (2-plate protocol for adherent cells):**

**Stimulation:**

1. **Plate** 50 µL of cells in 96-well tissue culture treated plate (allow adherence overnight in CO₂ atmosphere)
2. **Add** 50 µL of 2X stimulator in cell culture media
3. **Incubate** while shaking at 350 rpm for 10 minutes at 23 °C
4. **Carefully remove** cell supernatant and discard
5. **Add** 50 µL of 1X lysis buffer (Recommended: LANCE Ultra Lysis Buffer 1)
6. **Incubate** while shaking plate at 350 rpm for 30 minutes at 23 °C
7. **Transfer** 15 µL of lysate to 384-well plate

**Inhibition:**

1. **Plate** 50 µL of cells in 96-well tissue culture treated plate (allow adherence overnight in CO₂ atmosphere)
2. **Add** 25 µL of 3X inhibitor in cell culture media
3. **Incubate** while shaking at 350 rpm for 30 minutes at 23 °C
4. **Add** 25 µL of 4X stimulator in cell culture media
5. **Incubate** while shaking at 350 rpm for 10 minutes at 23 °C
6. **Carefully remove** cell supernatant and discard
7. **Add** 50 µL of 1X lysis buffer (Recommended: LANCE Ultra Lysis Buffer 1)
8. **Incubate** while shaking plate at 350 rpm for 30 minutes at 23 °C
9. **Transfer** 15 µL of lysate to 384-well plate
Lysate Preparation (1-plate protocol for suspension cells):

**Stimulation:**

1. **Plate** 8 μL of cells in 384-well OptiPlate

2. **Add** 4 μL of 3X stimulator in cell culture media

3. **Incubate** while shaking at 350 rpm for 10 minutes at 23 °C

4. **Add** 3 μL of 5X lysis buffer (Recommended: LANCE Ultra Lysis Buffer 1)

5. **Incubate** while shaking plate at 350 rpm for 30 minutes at 23 °C

**Inhibition:**

1. **Plate** 8 μL of cells in 384-well OptiPlate

2. **Add** 2 μL of 5X inhibitor in cell culture media

3. **Incubate** while shaking at 350 rpm for 30 minutes at 23 °C

4. **Add** 2 μL of 6X stimulator in cell culture media

5. **Incubate** while shaking at 350 rpm for 10 minutes at 23 °C

6. **Add** 3 μL of 5X lysis buffer (Recommended: LANCE Ultra Lysis Buffer 1)

7. **Incubate** while shaking plate at 350 rpm for 30 minutes at 23 °C
Reagent Preparation:

1) Preparation of 1X LANCE Detection Buffer:
   a. Add 1 mL of 10X LANCE Detection Buffer to 9 mL H₂O.

2) Preparation of 4X MIX Eu-labeled anti-TBK1 (S172) Antibody (2 nM) + ULight labeled anti-TBK1 Antibody (20 nM):
   a. Prepare just before use.
   b. Add 10 µL of 500 nM Eu-labeled anti-TBK1 (S172) Antibody and 100 µL of 500 nM ULight-labeled anti-TBK1 Antibody to 2390 µL of LANCE Detection Buffer

3) In a white Optiplate (384 wells):

   Add 15 µL of lysate sample

   Add 5 µL of a 4X MIX Eu-labeled anti-TBK1 (S172) Antibody (0.5 nM final) + ULight labeled anti-TBK1 Antibody (5 nM final)

   Incubate 2 hours at 23°C*

   Read using LANCE TRF Laser (in TR-FRET mode)

*In order to reduce evaporation, we recommend covering the OptiPlate with TopSeal-A PLUS during the incubation. Longer incubation times can be used and in some cases may improve assay signal/background.

Important: LANCE signal is detected using an EnVision Multilabel Reader equipped with a TR-FRET laser. Use an excitation wavelength of 320 or 340 nm to excite the LANCE Europium chelate. We recommend you read this assay in dual emission mode, detecting both the emission from the Europium donor fluorophore at 615 nm, and the acceptor fluorophore (at 665 nm for ULight dye). Data is calculated and presented ratiometrically by dividing the signal at 665 nm by the signal at 615 nm and multiplying by 10,000.

Data Analysis

- Data is represented ratiometrically. Divide the signal at 665 nm by the signal at 615 nm and multiply by 10,000.
- Analyze data according to a nonlinear regression using the 4-parameter logistic equation (sigmoidal dose-response curve with variable slope) and a 1/Y² data weighting (the values at maximal concentrations of analyte after the hook point should be removed for correct analysis).
Assay Performance Characteristics

LANCE Ultra assay performance described below was determined using the 2-plate protocol.

Dose Response Curve:

![Dose Response Curve](image)

Figure 3: Dose Response Curve MCF-7 cells stimulated with Calyculin A. MCF-7 cells (175K/well) were treated with increasing concentrations of Calyculin A for 45 min at room temperature before lysis with 50 µL of LANCE Ultra Lysis Buffer 1 for 30 min at room temperature.

Inhibition Dose Response:

![Inhibition Dose Response](image)

Figure 4: Inhibition Dose Response Curve MCF-7 cells treated with BX-795 and stimulated with Calyculin A. MCF-7 cells (175K/well) were treated with increasing concentrations of BK-795 for 1 hour at RT. The cells were then stimulated with 1.8 µM of Calyculin A for 45 min at RT before lysis with 50 µL of LANCE Ultra Lysis Buffer 1 for 30 min at room temperature.
Assay Robustness (Z’):

The assay was tested with Stimulated and Unstimulated cell lysates in multiple replicates (n=36) and a Z’ score was determined.

Figure 5: Z’ determination in Stimulated versus Unstimulated cell lysates read using laser or lamp configuration. MCF-7 cells (175K/well) were treated with 2 µM Calyculin A for 45 min at room temperature before lysis with 50 µL of LANCE Ultra Lysis Buffer 1 for 30 min at room temperature. The same plate was read using both the TR-FRET laser or lamp options.

Additional Resources

For more information on optimizing LANCE Ultra Cellular Detection Assays follow the link below:

http://www.perkinelmer.com/LANCECellGuide

You will find additional information regarding LANCE Ultra Assays at:

http://www.perkinelmer.com/askLANCE

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