TECHNICAL DATA SHEET

LANCE® *Ultra* Research Reagents

Research Use Only. Not for use in diagnostic procedures.

Phosphorylated SMAD2 (S465/S467) LANCE *Ultra* Cellular Detection Kit

Product number: TRF4012 C/M

Lot number: 2538321

Manufacturing date: January 28, 2019

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○ Product Information

Application: This kit is designed for the detection of phosphorylated SMAD2 (S465/S467) in cell lysates using a homogeneous LANCE *Ultra* assay (no wash steps).

Typical Performance (Undiluted positive control lysate versus Buffer): Signal/Background: 17.5

Z': 0.83

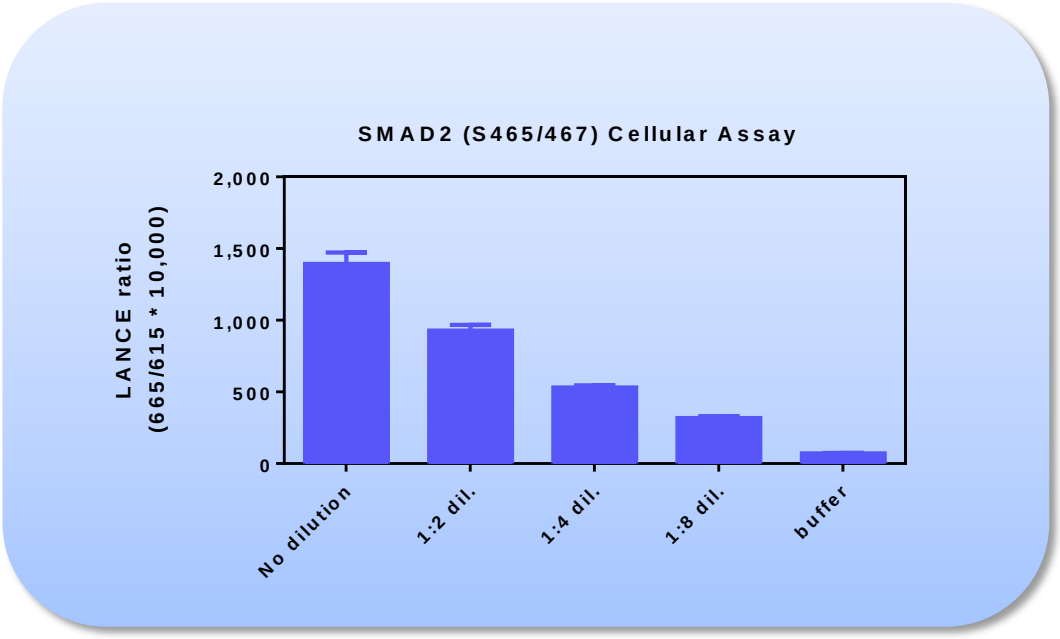


Figure 1. Typical positive control lysate (HeLa stimulated with TGFβ) diluted in lysis buffer. The data was generated using a white Optiplate™-384 microplate and read on an EnVision™ Multilabel Plate Reader equipped with TR-FRET laser option. Total signal, signal/background window, and sensitivity may 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.

Signal/Background: 17.6
Z': 0.85

Analyte of Interest

SMAD proteins are transcriptional activators activated by bone morphogenic proteins (BMPs). These ligands induce dimerization of SMAD proteins, whereby they translocate to the nucleus and bind target genes. SMAD1,2, and 3 constitute receptor-regulated SMADs.

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 [U*Light*™ 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).

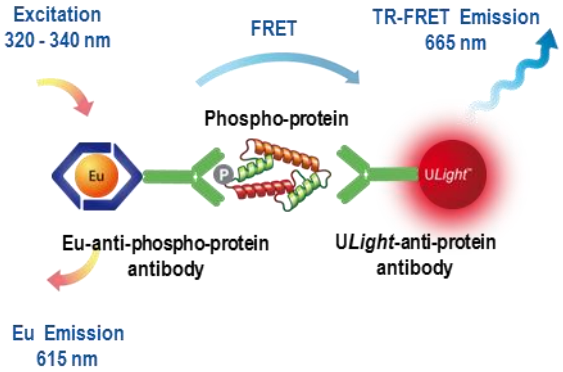


Figure 2. LANCE assay principle

Kit Content: Reagents and Materials

Kit components	TRF4012C (500 assay points**)	TRF4012M (10 000 assay points**)
LANCE *Ultra* Eu-labeled Anti-SMAD2 (S465/S467) Antibody stored in TSA, 0.1% BSA	10 µL @ 500 nM (1 clear tube, yellow cap)	200 µL @ 500 nM (1 clear tube, yellow cap)
LANCE *Ultra* U*Light*-labeled Anti-SMAD2 Antibody stored in TSA, 0.1% BSA	100 µL @ 500 nM (1 brown tube, blue cap)	2 X 1000 µL @ 500 nM (2 brown tubes, green caps)
LANCE Detection Buffer (10X) *	1.8 mL, 1 small bottle	250 mL, 1 large bottle
LANCE *Ultra* Lysis Buffer 1 (5X) *	10 mL, 1 small bottle	100 mL, 1 large bottle

* 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:

Item	Suggested source	Catalog #
VICTOR™ X, VICTOR Nivo™, ViewLux®, EnVision, EnSight™, EnSpire® Multilabel Plate Reader equipped with TR-FRET option	PerkinElmer Inc.	Please consult our website-
TopSeal-A PLUS Adhesive Sealing Film	PerkinElmer Inc.	6050185
Tissue culture treated clear SpectraPlates™, for culturing cells when using the 2-plate protocol	PerkinElmer Inc.	6005650
White OptiPlate-384, for LANCE *Ultra* detection assays when using the 2-plate protocol	PerkinElmer Inc.	6007290
White CulturPlate-384 when using the 1-plate protocol	PerkinElmer Inc.	6007680
Positive Control Cell Lysate (HeLa cells stimulated with TGFβ)	PerkinElmer Inc.	TRF4012S

○ 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 H_2O (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 a VICTOR X, ViewLux, EnVision, EnSpire, 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 U*Light* 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_3VO_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

		Volume			
Format	**# of data points**	**Final**	**Sample**	**Eu-Antibody/U*Light*-Antibody MIX**	**Plate recommendation**
TRF4012C	250	40 µL	30 µL	10 µL	White OptiPlate-96 (cat # 6005290) White ½ AreaPlate-96 (cat # 6005560)
	500	20 µL	15 µL	5 µL	White OptiPlate-384 (cat # 6007290) White CulturPlate-384 (cat # 6007680)
	1 250	8 µL	6 µL	2 µL	ProxiPlate™-384 Plus (cat # 6008280) White OptiPlate-384 (cat # 6007290) White CulturPlate-384 (cat # 6007680)
	2 500	4 µL	3 µL	1 µL	White OptiPlate-1536 (cat # 6004290)
TRF4012M	10 000	20 µL	15 µL	5 µL	White OptiPlate-384 (cat # 6007290) White CulturPlate-384 (cat # 6007680)
	25 000	8 µL	6 µL	2 µL	ProxiPlate-384 Plus (cat # 6008280) White OptiPlate-384 (cat # 6007290) White CulturPlate-384 (cat # 6007680)
	50 000	4 µL	3 µL	1 µL	White OptiPlate-1536 (cat # 6004290)

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:

Plate 50 µL of cells in 96-well tissue culture treated plate (allow adherence overnight in CO_2 atmosphere)

↓

Add 50 µL of 2X stimulator in cell culture media

↓

Incubate while shaking at 350 rpm for 60 minutes at 23 °C

Carefully remove cell supernant and discard

↓

Add 50 µL of 1X lysis buffer (Recommended: LANCE *Ultra* HiBlock Buffer)

↓

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

Transfer 15 µL of lysate to 384-well plate

Inhibition:

Plate 50 µL of cells in 96-well tissue culture treated plate (allow adherence overnight in CO_2 atmosphere)

↓

Add 25 µL of 3X inhibitor in cell culture media

↓

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

Add 25 µL of 4X stimulator in cell culture media

↓

Incubate while shaking at 350 rpm for 60 minutes at 23 °C

Carefully remove cell supernatant and discard

↓

Add 50 µL of 1X lysis buffer (Recommended: LANCE *Ultra* HiBlock Buffer)

↓

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

Transfer 15 µL of lysate to 384-well plate

Lysate Preparation (1-plate protocol for suspension cells):

Stimulation:

Plate 8 µL of cells in 384-well OptiPlate

Add 4 µL of 3X stimulator in cell culture media

Incubate while shaking at 350 rpm for 60 minutes at 23 °C

Add 3 µL of 5X lysis buffer (Recommended: LANCE *Ultra* HiBlock Buffer)

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

Inhibition:

Plate 8 µL of cells in 384-well Optiplate

Add 2 µL of 5X inhibitor in cell culture media

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

Add 2 µL of 6X stimulator in cell culture media

Incubate while shaking at 350 rpm for 60 minutes at 23 °C

Add 3 µL of 5X lysis buffer (Recommended: LANCE *Ultra* HiBlock Buffer)

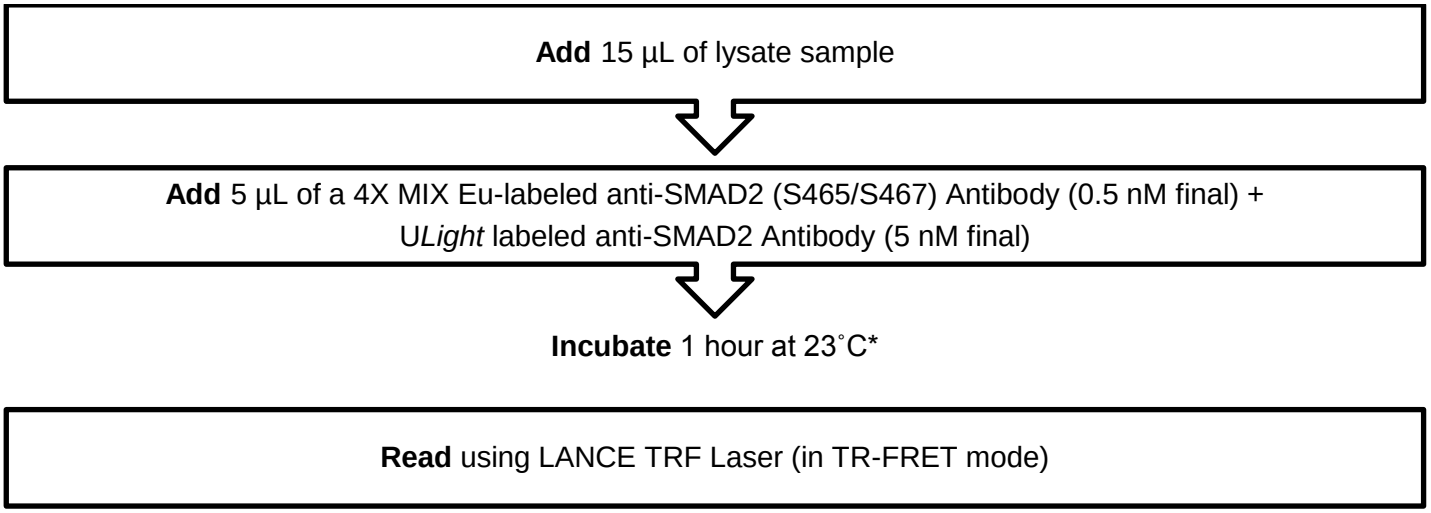
Incubate while shaking plate at 350 rpm for 30 minutes at 23 °C

Reagent Preparation:

1) Preparation of 1X LANCE Detection Buffer:
    a. Add 1 mL of 10X LANCE Detection Buffer to 9 mL H_2O.

2) Preparation of 4X MIX Eu-labeled anti-SMAD2 (S465/S467) Antibody (2 nM) + U*Light* labeled anti-SMAD2 Antibody (20 nM):
    a. Prepare just before use.
    b. Add 10 µL of 500 nM Eu-labeled anti-SMAD2 (S465/S467) Antibody and 100 µL of 500 nM U*Light*-labeled anti-SMAD2 Antibody to 2390 µL of LANCE Detection Buffer

3) In a white Optiplate (384 wells):



***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 U*Light* 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:

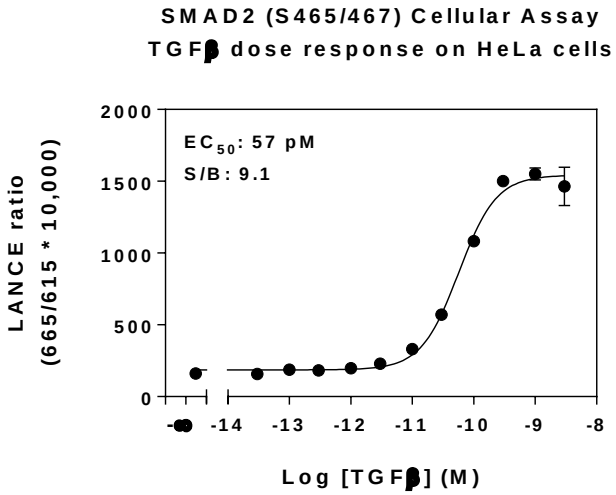


Figure 3: Dose Response Curve HeLa cells stimulated with TGFβ. HeLA cells (80K/well) were treated with increasing concentrations of TGFβ for 60 minutes prior to lysis with LANCE *Ultra* Lysis Buffer 1 for 30 minutes at room temperature.

Inhibition Dose Response:

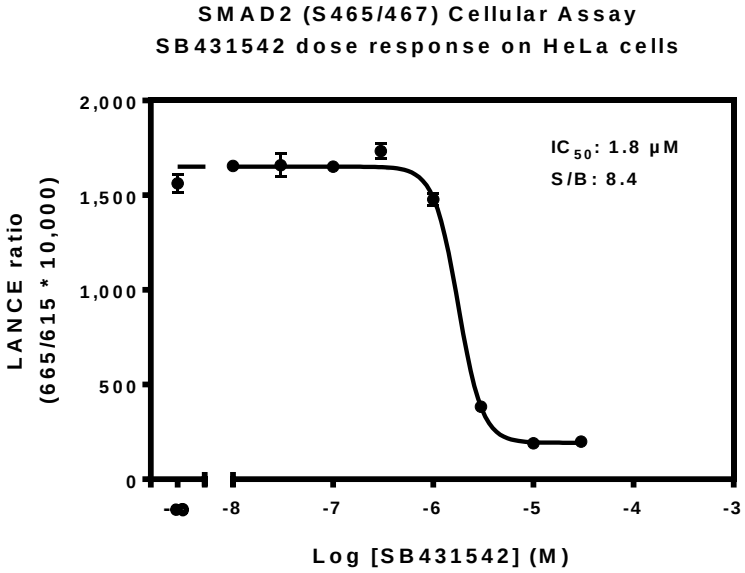


Figure 4: Inhibition Dose Response Curve HeLa cells treated with SB431542 and stimulated with TGFβ. HeLa cells (80K/well) were treated with increasing concentrations of SB431542 and incubated for 30 minutes. The cells were then stimulated with 0.3 nM TGFβ for 60 minutes prior to lysis with LANCE *Ultra* Lysis Buffer 1 for 30 minutes 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.

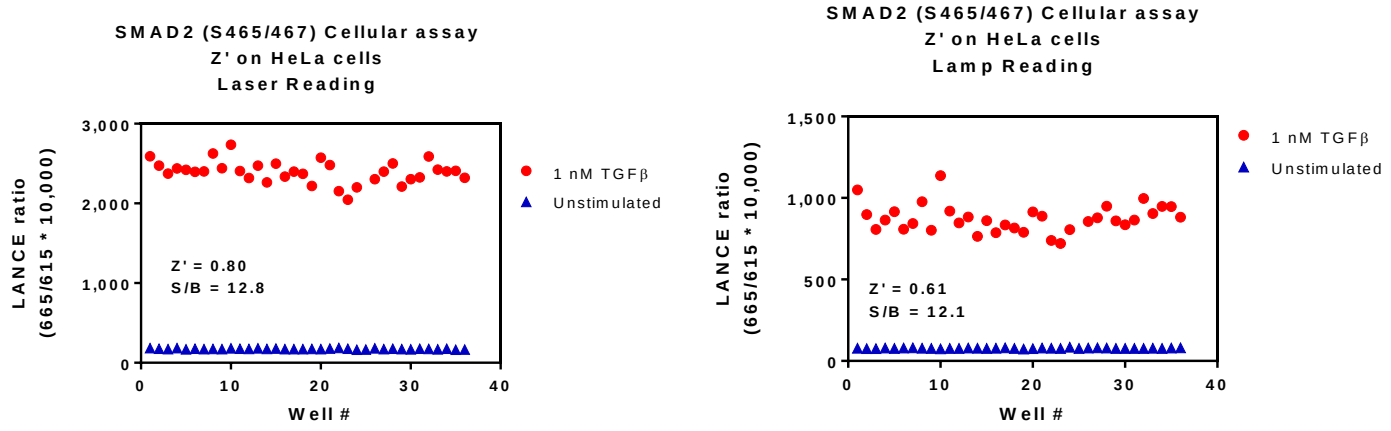


Figure 5: Z' determination in Stimulated versus Unstimulated cell lysates read using laser or lamp configuration. HeLa cells (80K/well) were treated with 1 nM TGFβ for 60 minutes prior to lysis with LANCE *Ultra* Lysis Buffer 1 for 30 minutes at room temperature. The same plate was read using both the 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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