INSTRUCTIONS

Pierce® Near Infrared In-Cell ELISA Kits

2144.1

Number	Description
62201	**In-Cell ELISA Near Infrared Detection Kit,** sufficient for 4 × 96 wells
62210	**Pierce EGFR Near Infrared In-Cell ELISA Kit,** sufficient materials for 1 × 96 wells
62211	**Pierce ERK1/2 Near Infrared In-Cell ELISA Kit,** sufficient materials for 1 × 96 wells
62212	**Pierce S6 Near Infrared In-Cell ELISA Kit,** sufficient materials for 1 × 96 wells
62213	**Pierce STAT6 Near Infrared In-Cell ELISA Kit,** sufficient materials for 1 × 96 wells
62214	**Pierce STAT3 Near Infrared In-Cell ELISA Kit,** sufficient materials for 1 × 96 wells
62220	**Pierce AKT Near Infrared In-Cell ELISA Kit,** sufficient materials for 1 × 96 wells
62221	**Pierce p53 Near Infrared In-Cell ELISA Kit,** sufficient materials for 1 × 96 wells
62222	**Pierce GSK3α/β Near Infrared In-Cell ELISA Kit,** sufficient materials for 1 × 96 wells
62223	**Pierce Cleaved Caspase 3 Near Infrared In-Cell ELISA Kit,** sufficient materials for 1 × 96 wells
62224	**Pierce Cleaved PARP Near Infrared In-Cell ELISA Kit,** sufficient materials for 1 × 96 wells

Kit Contents

Blocking Buffer, 50mL
20X Tris Buffered Saline, 50mL
Surfact-Amps® 20 (10% Tween®-20 Detergent), 10mL
Surfact-Amps X-100 (10% Triton® X-100 Detergent), 10mL
DyLight® 680 Goat Anti-Mouse IgG (H+L), 55µL
DyLight 800 Goat Anti-Rabbit IgG (H+L), 55µL
Thin Plate Seal Assembly, 8 each
Components included only in target specific kits:
Antibody #1, see vial label
Antibody #2, see vial label

Storage: Upon receipt, store all components except antibodies at 4°C. Store the antibodies at temperatures indicated on the antibody vial. Allow buffers to warm to room temperature before use. See the Solution Preparation Section for storage and stability of prepared solutions. Kit is shipped with an ice pack.

Warning: Completely read these instructions and the accompanying material safety data sheets before using this product. Reagents provided are not for diagnostic use in humans or animals.

Introduction

The Thermo Scientific Pierce In-Cell ELISA Near Infrared Detection Kits provide a simple and convenient method for quantifying intracellular proteins in whole cells. This kit enables simultaneous detection of post-translational-modified protein (PTM) and the corresponding unmodified protein, or two different targets within the same well. Simultaneously detecting targets within an ELISA well eliminates variability caused by differences in cell plating. The expression levels of the protein(s) are monitored using primary antibodies specific to the targets (see the Important Product Information section for antibodies included in each kit) and corresponding species-specific near-infrared Thermo Scientific DyLight-Conjugated Secondary Antibodies. Relative protein activation or inactivation is determined as a ratio of modified protein levels to the corresponding unmodified protein measured using a modification-specific antibody and an antibody to unmodified protein, respectively.

Traditionally, relative protein levels in various samples or PTMs were assessed by performing time-consuming Western blots, which are semi-quantitative and have low throughput. In contrast, the in-cell ELISA method enables accurate quantitation using an infrared imaging system. The assay is performed in a 96- or 384-well microplate, is scalable, and conserves cell culture and treatment reagents. Furthermore, the assay is amenable to automation, which is ideal for siRNA studies and drug screens.

Important Product Information

- The Pierce In-Cell ELISA Kits are offered in two formats: without primary antibodies (Product No. 62201) or with target-specific antibodies (Product No. 62210-14; 62220-24)
- The excitation/emission maxima are 692/712nm for DyLight 680 Dye and 777/794nm for DyLight 800 Dye.
- Each Pierce Target-Specific Kit contains two antibodies (Table 1). Please see our web site for detailed background information for each target and kit-specific data.

Table 1. Target-specific antibodies included in each kit.

Product #	Target	Antibodies Included
62210	EGFR	#1: Anti-Phospho EGFR (Y1173) Antibody #2: Anti-EGFR Antibody
62211	ERK1/2	#1: Anti-ERK 1 & 2 (T 202/Tyr 204) Antibody #2: Anti-ERK1/2 Antibody
62212	S6	#1: Anti-Phospho S6 (S 235/236) Antibody #2: Anti-S6 Antibody
62213	STAT6	#1: Anti-Phospho STAT6 (Y641) Antibody #2: Anti-STAT3 Antibody
62214	STAT3	#1: Anti-Phospho STAT3 (Y705) Antibody #2: Anti-STAT3 Antibody
62220	AKT	#1: Anti-Phospho AKT (S473) Antibody #2: Anti-AKT Antibody
62221	p53	#1: Anti-p53 Antibody #2: Anti-Alpha Tubulin Antibody
62222	GSK3 α/β	#1: Anti-Phospho GSK3 α/β (S 21/9) Antibody #2: Anti-GSK3 α/β Antibody
62223	Cleaved Caspase 3	#1: Anti-Cleaved Caspase 3 Antibody #2: Anti-Alpha Tubulin Antibody
62224	Cleaved PARP	#1: Anti-Cleaved PARP Antibody #2: Anti-Alpha Tubulin Antibody

Procedure Summary

1. Prepare plates (i.e., plate and treat cells as desired, and then fix them using 4% PFA).
2. Permeabilize and block nonspecific sites with blocking buffer.
3. Detect targets with primary antibodies and the corresponding Dylight Dye-conjugated secondary antibodies.
4. Scan the plate on an infrared imaging system.
5. Analyze results.

Additional Materials Required

- Disposable reagent reservoirs (Thermo Scientific ImmunoWare Reagent Reservoirs, Product No. 15075)
- Infrared imaging system (e.g., LI-COR Biosciences Odyssey® or Aerius® Infrared Imaging System)
- Methanol-free formaldehyde (Thermo Scientific 16% Formaldehyde, Product No. 28906), diluted to 4%
- 96-well cell culture clear-bottom microplates, such as black collagen-coated plates (Nunc, Product No. 152036) or black clear-bottom plates, (Perkin-Elmer, Product No. 6005182)

Precautions

- All samples and reagents must be at room temperature (20-25°C) before use in the assay.
- If using a multichannel pipette, always use a new disposable reagent reservoir.
- To avoid cross-contamination use new disposable pipette tips for each transfer and a new adhesive plate cover for each antibody incubation step.
- Take care not to let plate dry at any time during the assay.
- Avoid exposing reagents to excessive heat or light during storage and incubation.
- Avoid microbial contamination of reagents. Do not mix reagents from different kit lots. Do not combine leftover reagents with those reserved for additional plates.
- Individual components may contain antibiotics and preservatives. Wear gloves while performing the assay to avoid contact with samples and reagents. Please follow proper disposal procedures.
- Dispense and equilibrate to room temperature only the reagent volumes required for the number of plates being used.
- Briefly centrifuge the tubes of primary antibody before use.

In-Cell ELISA Protocol

- Perform all incubations with gentle shaking on a plate shaker.
- To remove the plate contents, rapidly invert the plate over a waste receptacle. Tap the inverted plate gently three times on a paper towel or other absorbent material to remove any remaining solution.
- Perform each wash step for 5 minutes with gentle shaking on a plate shaker.

A. Solution Preparation (per 96-well plate)

1X Tris Buffered Saline (TBS)	Add 2.5mL of 20X TBS to 47.5mL of ultrapure water. Store buffer at 4°C for up to 7 days.
4% Formaldehyde	Add 2.75mL of 16% methanol-free formaldehyde to 8.25mL of 1X TBS. Prepare solution just before each assay.
1X Permeabilization Buffer	Add 0.11mL of Surfact-Amps X-100 Detergent to 11mL of the 1X TBS. Store this buffer at 4°C for up to 7 days.
1X Wash Buffer	Add 7.5mL of 20X TBS to 141mL of ultrapure water. Add 1.5mL of Surfact-Amps 20 Detergent. Store buffer at 4°C for up to 7 days.
Diluted Primary Antibody	Add 3mL of Blocking Buffer to 3mL of 1X Wash Buffer. Dilute the primary antibody with this solution to the dilution stated on the antibody vial.* This volume is sufficient for one-96 well microplate. Adjust the volume of the antibody solution based on the number of wells being tested.
Secondary Antibody Mix	Add 12 µl of DyLight 800 Goat Anti-Rabbit IgG and DyLight 680 Goat Anti-Mouse IgG to 12mL of 1X Wash Buffer and mix gently. Prepare solution just before each assay. Protect the solution from light. If using only one primary antibody, add only the corresponding species-specific secondary antibody.

* If using Product No. 62201, dilute the antibody as indicated by the supplier.

B. Assay Procedure

1. Plate 10,000 cells/well in a 96-well plate. Incubate plate overnight at 37°C in 5% CO_2. Use only cells growing in log phase at a passage number ≤ 15.

   Note: Plate enough wells to perform the experiment in triplicate and include appropriate controls such as nonspecific signal (treat with all reagents except the primary antibody).

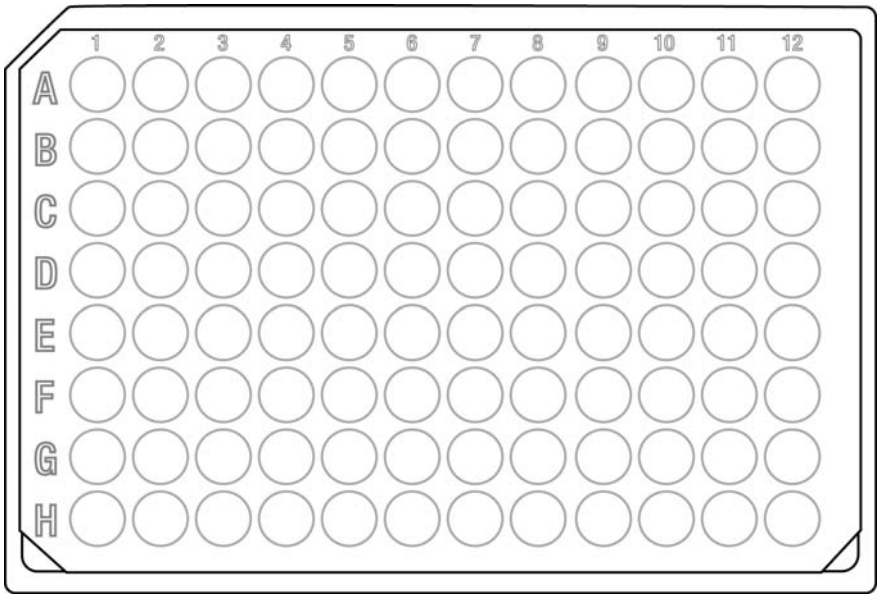
2. Apply cell treatment.
3. Remove the media and add 100µL of 4% formaldehyde to each well. Incubate the plate in a fume hood at room temperature for 15 minutes.

   Note: Formaldehyde and its vapors are highly toxic. Perform steps involving formaldehyde in a fume hood. Discard the formaldehyde waste according to your local regulations.

4. Remove formaldehyde and wash plate twice with 100µL/well of 1X TBS.
5. Remove 1X TBS, add 100µL/well of 1X Permeabilization Buffer and incubate for 15 minutes at room temperature.
6. Remove Permeabilization Buffer and wash plate once with 100µL/well of 1X TBS.
7. Remove 1X TBS, add 200µL/well of Blocking Buffer and incubate at room temperature for 60 minutes.
8. Remove Blocking Buffer and add 50µL/well of Primary Antibody Solution. Seal the plate with a Plate Sealer and incubate overnight at 4°C.
9. Remove Primary Antibody Solution and wash plate three times with 100µL/well of 1X Wash Buffer.
10. Remove Wash Buffer and add 100µL/well of the Secondary Antibody Mix. Incubate for 60 minutes at room temperature.
11. Remove the Secondary Antibody Mix and wash plate three times with 200µL/well of 1X Wash Buffer.
12. Scan the plate on the infrared imaging system according to the manufacturer's instructions. The excitation/emission maxima are 692/712nm for DyLight 680 Dye and 777/794nm for DyLight 800 Dye.
13. Use the software provided by the manufacturer to analyze the results. For example, when using a LI-COR Biosciences instrument, use the ICW software provided.

Data Template

Date: **Cell Type:**



Troubleshooting

Problem	Cause	Solution
No signal or weak signal	Improper reagent preparation or storage conditions	Store reagents as indicated in these instructions
	Reagent contamination/degradation	Aliquot solutions into single-use volumes upon receipt
	Inadequate primary or secondary antibody concentrations	Perform antibody titration
	Cell loss caused by washing	Adjust wash flow rate or use a plate coated with an extracellular matrix, such as collagen 1, or coated with poly-lysine
	Cell loss caused by the treatment	Use less stringent treatment or decrease treatment time
	Cell passage number or other cell handling conditions were not optimal	Make sure the cells are within 15 passages
	Incorrect filters were used	The excitation/emission maxima are 692/712nm for DyLight 680 Dye and 777/794nm for DyLight 800 Dye
High background	Excessive primary or secondary antibody concentrations	Perform titration to optimize antibody concentrations
	Wash buffer might be contaminated	Use new wash buffer
	Washing or blocking was inadequate	Increase number of washes or increase the blocking time to 1 hour
	Reused reagent reservoirs and/or plate sealers causing cross-contamination	Use new reservoirs for each step
No or partial activation	Stimulator is inactive or degraded	Include positive control to confirm system is working
	Differing stimulator kinetics	Perform dose-response experiment to optimize concentration
	Insufficient stimulus	Increase or change stimulus
	Improper cell line used	Make sure the cell line is appropriate for the signaling being tested

Additional Information

Visit our web site for additional information relating to this product including the following:

- Target-specific data
- Application notes and references

Related Thermo Scientific Products

62200 **In-Cell ELISA Colorimetric Detection Kit**

28358 **20X TBS Buffer,** 500mL

28320 **Surfact-Amps 20 (10% Tween-20 Detergent),** 6 × 10mL

28314 **Surfact-Amps X-100 (10% Triton X-100 Detergent),** 6 × 10mL

35568 **Goat Anti-Rabbit IgG (H+L) DyLight 680 Conjugated,** 1mL (1mg/mL)

35571 **Goat Anti-Rabbit IgG (H+L) DyLight 800 Conjugated,** 1mL (1mg/mL)

35518 **Goat Anti-Mouse IgG (H+L) DyLight 680 Conjugated,** 1mL (1mg/mL)

35521 **Goat Anti-Mouse IgG (H+L) DyLight 800 Conjugated,** 1mL (1mg/mL)

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Tween is a registered trademark of ICI Americas.

Triton is a registered trademark of Rohm & Haas.

The anti-phospho EGFR (Y1173) antibody is protected by US patents 5,675,063 and 5.599,681.

The anti-cleaved PARP and anti-alpha tubulin antibodies are protected by US patent 5,675,063.

This product ("Product") is warranted to operate or perform substantially in conformance with published Product specifications in effect at the time of sale, as set forth in the Product documentation, specifications and/or accompanying package inserts ("Documentation") and to be free from defects in material and workmanship. Unless otherwise expressly authorized in writing, Products are supplied for research use only. No claim of suitability for use in applications regulated by FDA is made. The warranty provided herein is valid only when used by properly trained individuals. Unless otherwise stated in the Documentation, this warranty is limited to one year from date of shipment when the Product is subjected to normal, proper and intended usage. This warranty does not extend to anyone other than the original purchaser of the Product ("Buyer").

No other warranties, express or implied, are granted, including without limitation, implied warranties of merchantability, fitness for any particular purpose, or non infringement. Buyer's exclusive remedy for non-conforming Products during the warranty period is limited to replacement of or refund for the non-conforming Product(s).

There is no obligation to replace Products as the result of (i) accident, disaster or event of force majeure, (ii) misuse, fault or negligence of or by Buyer, (iii) use of the Products in a manner for which they were not designed, or (iv) improper storage and handling of the Products.

Current product instructions are available at *www.thermoscientific.com/pierce*. For a faxed copy, call 800-874-3723 or contact your local distributor.