INSTRUCTIONS

Thermo SCIENTIFIC

Pierce Colorimetric In-Cell ELISA Kits

2143.2

Number	Description
62200	**In-Cell ELISA Colorimetric Detection Kit,** sufficient materials for 4 × 96 wells
62205	**Pierce EGFR Colorimetric In-Cell ELISA Kit,** sufficient materials for 1 × 96 wells
62206	**Pierce ERK1/2 Colorimetric In-Cell ELISA Kit,** sufficient materials for 1 × 96 wells
62207	**Pierce S6 Colorimetric In-Cell ELISA Kit,** sufficient materials for 1 × 96 wells
62208	**Pierce STAT6 Colorimetric In-Cell ELISA Kit,** sufficient materials for 1 × 96 wells
62209	**Pierce STAT3 Colorimetric In-Cell ELISA Kit,** sufficient materials for 1 × 96 wells
62215	**Pierce AKT Colorimetric In-Cell ELISA Kit,** sufficient materials for 1 × 96 wells
62216	**Pierce p53 Colorimetric In-Cell ELISA Kit,** sufficient materials for 1 × 96 wells
62217	**Pierce GSK3 α/β Colorimetric In-Cell ELISA Kit,** sufficient materials for 1 × 96 wells
62218	**Pierce Cleaved Caspase 3 Colorimetric In-Cell ELISA Kit,** sufficient materials for 1 × 96 wells
62219	**Pierce Cleaved PARP Colorimetric 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

HRP Conjugate, 0.14mL

TMB Substrate, 58mL

TMB Stop Solution, 55mL

Janus Green Whole-Cell Stain, 50mL

Elution Buffer, 2 × 30mL

Thin Plate Seal Assembly, 8 each

Components included only in the 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 Colorimetric Detection Kit is a simple and convenient method for quantifying intracellular proteins in whole cells. To perform the assay, cells are first plated, treated and fixed. Expression of the protein(s) of interest is monitored in wells of a microplate using target-specific primary antibodies (see the Important Product Information section for antibodies included in each kit) and a horseradish peroxidase (HRP)-conjugated detection reagent. The kit is supplied with a whole-cell stain to control for differences in cell plating, which is important when measuring relative levels of a protein with different treatments or assessing its post-translational modification (PTM) form. After staining, the results are analyzed by normalizing the absorbance (HRP activity) values to cell number, which adjusts for the cell plating differences among the wells.

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 a standard ELISA plate reader. 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. 62200) or with target-specific antibodies (Product No. 62205-62209; 62215-62219).
- The HRP Conjugate that is included in each kit will recognize and bind primary antibodies (IgG) from several species, including mouse and rabbit IgG.
- Each 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
62205	EGFR	#1: Anti-Phospho EGFR (Y1173) Antibody #2: Anti-EGFR Antibody
62206	ERK1/2	#1: Anti-ERK 1 & 2 (Thr 202/Tyr 204) Antibody #2: Anti-ERK1/2 Antibody
62207	S6	#1: Anti-Phospho S6 (Ser 235/236) Antibody #2: Anti-S6 Antibody
62208	STAT6	#1: Anti-Phospho STAT6 (Y641) Antibody #2: Anti-STAT6 Antibody
62209	STAT3	#1: Anti-Phospho STAT3 (Y705) Antibody #2: Anti-STAT3 Antibody
62215	AKT	#1: Anti-Phospho AKT (S473) Antibody #2: Anti-AKT Antibody
62216	p53	#1: Anti-p53 Antibody #2: Anti-Alpha Tubulin Antibody
62217	GSK3 α/β	#1: Anti-Phospho GSK3 a/b (S21/9) Antibody #2: Anti-GSK3 Antibody
62218	Cleaved Caspase 3	#1: Anti-Cleaved Caspase 3 Antibody #2: Anti-Alpha Tubulin Antibody
62219	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, quench endogenous peroxidase and block nonspecific sites with blocking buffer.
3. Detect targets with primary antibodies and the HRP-conjugate.
4. Measure absorbance at 450nm.
5. Stain cells with whole-cell stain (steps 5-7 are optional).
6. Elute whole-cell stain.
7. Measure absorbance at 615nm.
8. Analyze results.

Additional Materials Required

- Disposable reagent reservoirs (Thermo Scientific ImmunoWare Reagent Reservoirs, Product No. 15075)
- Standard ELISA reader for measuring absorbance at 450nm and 615nm
- Methanol-free formaldehyde (Thermo Scientific 16% Formaldehyde, Product No. 28906), diluted to 4%
- 30% hydrogen peroxide (H_2O_2, Sigma Product No. H1009), diluted to 1%
- 96-well cell culture clear-bottom microplates, such as black collagen-coated plates (Nunc, Product No. 152036), clear collagen-coated plates, (BD, Product No. 354407), black clear-bottom plates, (Perkin-Elmer, Product No. 6005182) or clear plates (Corning, Product No. 3596)

Precautions

- All samples and reagents must be at room temperature (20-25°C) before use in the assay.
- To avoid cross-contamination use new disposable pipette tips for each transfer and a new adhesive plate cover for each antibody incubation step. If using a multichannel pipette, always use a new disposable reagent reservoir.
- 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.
- Do not mix reagents from different kit lots, and discard unused ELISA components after assay completion. Do not combine leftover reagents with those reserved for additional plates.
- Do not use glass pipettes to measure the Substrate Solution. Take care not to contaminate the solution. If solution is blue before use, do not use it.
- 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 Thermo Scientific™ Surfact-Amps™ X-100 Detergent to 11mL of the 1X TBS. Store this buffer at 4°C for up to 7 days.
Quenching Solution	Add 0.38mL of 30% H_2O_2 to 11mL of 1X TBS to make 1% H_2O_2. Prepare solution just before each assay.
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 3 ml 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 target antibody added to 96 wells. Adjust the volume of the antibody solution based on the number of targets and wells being tested.
Diluted HRP Conjugate	Add 30µL of HRP Conjugate to 12mL of 1X Wash Buffer. Prepare solution just before each assay.

*If using Product No. 62200, dilute the antibody as indicated by the supplier.

B. Assay Procedure

1. Plate 10,000 cells/well in a 96-well plate. Incubate plates 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. Include appropriate controls such as nonspecific signal (i.e., wells treated with all reagents except the primary antibody).
2. Apply cell treatment as necessary.
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 100µL/well of Quenching Solution and incubate at room temperature for 20 minutes.
8. Remove Quenching Solution and wash plate once with 100µL/well of 1X TBS.
9. Remove 1X TBS, add 100µL/well of Blocking Buffer and incubate at room temperature for 30 minutes.
10. Remove Blocking Buffer and add 50µL/well of primary antibody. Apply a plate sealer and incubate overnight at 4°C.
11. Remove the primary antibody solution and wash plate three times with 100µL/well of 1X Wash Buffer.

12. Remove Wash Buffer and add 100µL/well of Diluted HRP Conjugate. Incubate for 30 minutes at room temperature.
13. Remove the Diluted HRP Conjugate and wash plate three times with 200µL/well of 1X Wash Buffer.
14. Remove Wash Buffer and add 100µL/well of TMB Substrate. Incubate at room temperature protected from light. Stop the reaction within 15 minutes or when the desired blue color has been achieved.

    Note: Dispense from bottle only the amount of reagent required for the number of wells being used. Do not use a glass pipette to measure the TMB Substrate. Take care not to contaminate remaining substrate solution.
15. Add 100µL/well of TMB Stop Solution. Measure the absorbance at 450nm (A_{450}) within 30 minutes of stopping the reaction.

C. Whole Cell Staining (optional)

1. Empty the plate contents and wash plate twice with 200µL/well of ultrapure water.
2. Remove water and add 100µL/well of Janus Green Whole-Cell Stain. Incubate plate for 5 minutes at room temperature.
3. Remove stain and wash with 3-5 times with 200µL/well of ultrapure water until all excess stain is removed.
4. Remove water and add 100µL/well of the Elution Buffer. Incubate for 10 minutes at room temperature. Measure the absorbance at 615nm (A_{615}).

D. Results Calculation

1. Calculate the average of all replicate background measurements (nonspecific signal control) from each experimental condition.
2. Subtract the background values from all values of the same experimental type.
3. If Janus green staining was performed, normalize the A_{450} values to A_{615} values from corresponding wells to account for differences in cell numbers in various wells by dividing the net A_{450} values by the A_{615} values.
4. Calculate the average A_{450} value for each experimental condition (e.g., with and without treatment) for each target. For assessing target protein modification with treatment, calculate the fold change as a ratio of the treated and nontreated modified protein A_{450} values.

    Note: To compensate for changes in total target protein levels with treatment (irrespective of PTM) measure the levels of non-modified target protein using the same experimental conditions. Normalize the average A_{450} value obtained for the modified protein to that of the average A_{450} value for the total target protein. Use the normalized A_{450} values to calculate the fold change.

Data Template

Date: **Cell Type:**

1 2 3 4 5 6 7 8 9 10 11 12

A

B

C

D

E

F

G

H

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
High background	Excessive primary or secondary antibody concentrations	Perform titration to optimize antibody concentrations
	Wash buffers might be contaminated	Use new wash buffer
	Washing or blocking was inadequate	Increase number of washes or increase the blocking time to 1 hour
	Endogenous HRP in cells was not adequately quenched	Prepare the hydrogen peroxide just before use
	Reused reagent reservoirs and/or plate sealers causing cross-contamination	Use new reservoirs for each step
	Blocker or antibody diluent contained serum	Use only serum-free blocking agents and diluent
No or partial activation	Stimulator is inactive or degraded	Include a positive control to confirm system is working
	Differing stimulator kinetics	Perform a 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**

62201 **In-Cell ELISA Near-Infrared Fluorescence Detection Kit**

62203 **Janus Green Whole Cell Stain,** 50mL

62204 **Anti-α-Tubulin Antibody,** 100µL (200µg/mL)

34028 **1-Step Ultra TMB-ELISA,** 250mL

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

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