

ab126420 – EGFR (Tyr1068) Human In-Cell ELISA Kit

Instructions for Use

For the qualitative measurement of Human EGFR (Tyr1068) phosphorylation in adherent cell lines.

This product is for research use only and is not intended for diagnostic use.

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1. BACKGROUND

Abcam's EGFR (Tyr1068) Human In-Cell ELISA Kit is designed for the qualitative measurement of EGFR (Tyr1068) phosphorylation in adherent cell lines.

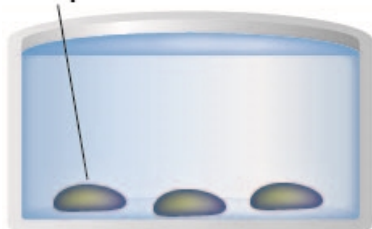
In Abcam's In-Cell EGFR (Tyr1068) ELISA kit, cells are seeded into a 96 well tissue culture plates. The cells are fixed after various treatments, inhibitors or activators. After blocking, Anti Phospho-EGFR (Tyr1068) or Anti-EGFR primary antibody is pipetted into the wells and incubated. The wells are washed, and HRP-conjugated anti-rabbit IgG (secondary antibody) is added. The wells are washed again, a TMB substrate solution is added, and color develops in proportion to the amount of protein. The Stop Solution changes the color from blue to yellow. The intensity of the color is measured at 450 nm.

Protein phosphorylation is instrumental in the regulation of protein activity within a cell. It plays important roles in the living cells including proliferation, differentiation and metabolism. A large number of protein kinases and phosphatases have been extensively investigated, and have been shown to be involved in signal transduction pathways.

Abcam's EGFR (Tyr1068) Human In-Cell ELISA Kit is a very rapid, convenient and sensitive assay kit that can monitor the activation or function of important biological pathways in cells. It can be used for measuring the relative amount of EGFR (Tyr1068) phosphorylation, as a result of various treatments, inhibitors (such as siRNA or chemicals), or activators in cultured Human cell lines. By determining EGFR protein phosphorylation in your experimental model system, you can verify pathway activation in your cell lines without spending excess time and effort preparing cell lysates and performing a Western Blot analysis.

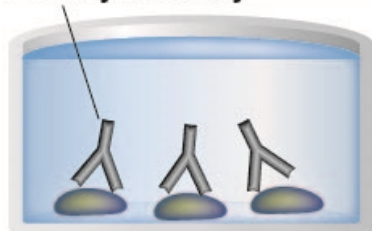
2. ASSAY SUMMARY

Sample



Seed cells and incubate overnight. Apply treatment activators or inhibitors. Fix cells with Fixing Solution. Incubate at room temperature. Add Quenching Buffer. Incubate at room temperature. Add Blocking Buffer. Incubate at 37°C.

Primary Antibody



Add prepared Anti-Phospho-EGFR (Tyr1068) or Anti-EGFR to each well used. Incubate at room temperature.

Labeled HRP-Conjugate



Empty and wash each well. Add prepared HRP-Anti-rabbit IgG. Incubate at room temperature.

Substrate

Colored Product



Empty and wash each well. Add the TMB One-Step Substrate Reagent to each. Incubate at room temperature. Add Stop Solution to

each well. Immediately begin recording the color development.

3. PRECAUTIONS

Please read these instructions carefully prior to beginning the assay.

All kit components have been formulated and quality control tested to function successfully as a kit. Modifications to the kit components or procedures may result in loss of performance.

4. STORAGE AND STABILITY

Store kit at -20°C immediately upon receipt.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in section 9. Reagent Preparation.

5. MATERIALS SUPPLIED

Item	Quantity	Storage Condition (Before Preparation)
96 well tissue culture plate	1 x 96 wells	-20 °C
Wash Buffer Concentrate A (20X)	1 x 30 mL	-20 °C
Wash Buffer Concentrate B (20X)	1 x 30 mL	-20 °C
Fixing Solution (1X)	1 x 30 mL	-20 °C
Quenching Buffer Concentrate (30X)	1 x 2 mL	-20 °C
Blocking Buffer Concentrate (5X)	1 x 20 mL	-20 °C
Rabbit Anti-Phospho-EGFR (Tyr1068) Concentrate (1,000X)	1 x 6 µL	-20 °C
Rabbit Anti-EGFR Concentrate (1,000X)	1 x 6 µL	-20 °C
HRP-conjugated Anti-rabbit IgG Concentrate (2,000X)	1 x 10 µL	-20 °C
TMB One-Step Substrate Reagent (1X)	1 x 12 mL	-20 °C
Stop Solution (1X)	1 x 14 mL	-20 °C

6. MATERIALS REQUIRED, NOT SUPPLIED

These materials are not included in the kit, but will be required to successfully utilize this assay:

- Model cell line.
- Cell treatments (e.g. Protein tyrosine kinase inhibitors, growth factor or cytokine.)
- 37°C incubator.
- 10, 50, 100, 200 and 1,000 µL adjustable single channel micropipettes with disposable tips.
- Miscellaneous laboratory plastic and/or glass, if possible sterile.
- Absorbent paper.
- Distilled or deionized water.
- Orbital shaker or oscillating rocker.

7. LIMITATIONS

- Bacterial or fungal contamination of either samples or reagents or cross-contamination between reagents may cause erroneous results.
- Disposable pipette tips, flasks or glassware are preferred, reusable glassware must be washed and thoroughly rinsed of all detergents before use.
- Improper or insufficient washing at any stage of the procedure will result in either false positive or false negative results. Completely empty wells before dispensing fresh 1X Wash Buffer. Do not allow wells to sit uncovered or dry for extended periods.

8. TECHNICAL HINTS

- Kit components should be stored as indicated. All the reagents should be equilibrated to room temperature before use.
- Use a clean disposable plastic pipette tip for each reagent, standard, or specimen addition in order to avoid cross-contamination; for the dispensing of the Stop Solution and substrate solution, avoid pipettes with metal parts.
- Thoroughly mix the reagents and samples before use by agitation or swirling.
- All residual washing liquid must be drained from the wells by decantation followed by tapping the plate on absorbent paper. Never insert absorbent paper directly into the wells.
- The TMB solution is light sensitive. Avoid prolonged exposure to light. Also, avoid contact of the TMB solution with metal to prevent color development. Warning TMB is toxic avoid direct contact with hands. Dispose of properly.
- If a dark blue color develops within a few minutes after preparation, this indicates that the TMB solution has been contaminated and must be discarded.
- When pipetting reagents, maintain a consistent order of addition from well-to-well. This will ensure equal incubation times for all wells.
- **This kit is sold based on number of tests. A ‘test’ simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.**

9. REAGENT PREPARATION

Equilibrate all reagents and samples to room temperature (18-25°C) prior to use. Avoid repeated freeze-thaw cycles.

9.1 Wash Buffer A and B

Wash Buffer Concentrate A (20X) or B (20X) should be diluted 20-fold with deionized or distilled water. For example: 25 mL of concentrate + 475 mL of water = 500 mL of 1X working solution.

Note: If the Wash Buffer A (20X) or B (20X) contains visible crystals, warm to room temperature and mix gently until dissolved.

After initial use this item should be stored at 4°C for up to 3 months.

9.2 Quenching Buffer Concentrate

Quenching Buffer Concentrate should be diluted 30-fold with 1X Wash Buffer A before use. For example: 1 mL of concentrate + 29 mL of wash buffer = 30 mL of 1X working solution.

After initial use this item should be stored at 4°C for up to 3 months.

9.3 Blocking Buffer

Blocking Buffer Concentrate (5X) should be diluted 5-fold with deionized or distilled water. For example: 20 mL of concentrate + 80 mL of water = 100 mL of 1X working solution.

After initial use this item should be stored at 4°C for up to 1 month.

9.4 Rabbit Anti-Phospho-EGFR (Tyr1068) Concentrate

Rabbit Anti-Phospho-EGFR (Tyr1068) Concentrate should be diluted 1,000-fold with 1X Blocking Buffer. For example: 2 µL of concentrate + 1,998 µL of 1X Blocking Buffer = 2 mL of 1X working solution.

Note: Briefly centrifuge at ~1,000g before opening to ensure maximum recovery.

After initial use this item should be stored at -20°C for up to 3 months.

9.5 **Rabbit Anti-EGFR Concentrate**

Rabbit Anti-EGFR Concentrate should be diluted 1,000-fold with 1X Blocking Buffer. For example: 2 µL of concentrate + 1,998 µL of 1X Blocking Buffer = 2 mL of 1X working solution.

Note: Briefly centrifuge at ~1,000g before opening to ensure maximum recovery.

After initial use this item should be stored at -20°C for up to 3 months.

9.6 **HRP-conjugated Anti-rabbit IgG Concentrate**

Anti-rabbit IgG Concentrate should be diluted 2,000-fold with 1X Blocking Buffer. For example: 5 µL of concentrate + 9,995 µL of 1X Blocking Buffer = 10 mL of 1X working solution.

Note: Briefly centrifuge at ~1,000g before opening to ensure maximum recovery.

After initial use this item should be stored at -20°C for up to 3 months.

9.7 **Fixing Solution**

Fixing Solution (1X) is supplied ready to use.

After initial use this item should be stored at 4°C for up to 3 months.

9.8 **TMB One-Step Substrate Reagent**

TMB One-Step Substrate Reagent (1X) is supplied ready to use.

After initial use this item should be stored at 4°C for up to 3 months.

10. ASSAY PROCEDURE

- **Equilibrate all materials and prepared reagents to room temperature prior to use.**
- **It is recommended to assay all controls and samples in duplicate.**
- **ALL incubations and wash steps must be performed under gentle rocking or rotation (~1-2 cycles/second).**

10.1 Seed 100 μ L of 20,000 cells into each well in a 96 well plate and incubate for overnight at 37°C, 5% CO₂.

Notes: The optimal cell number used will depend upon the cell line and the relative amount of protein phosphorylation. More or less cells may be used, determined by the end user.

The cells can be starved 4 to 24 hours dependent on cell lines prior to treatment (inhibitor or activator).

Optional: If seeding HUVECs, HMEC-1 or other loosely attached cells, coat the Uncoated 96-Well Microplate by adding 100 μ L poly-L-Lysine into each well and then follow manufacturer's instructions. A pre-coated microplate or other poly-lysine treated tissue culture plate may be used in place of the provided Uncoated 96-Well Microplate.

10.2 Apply various treatments, inhibitors (such as siRNA or chemicals) or activators according to manufacturer's instructions.

Dissolve your inhibitors or activators into serum free cell culture medium before treating the cells (unless otherwise stated in the manufacturer's instructions.)

10.3 Discard the cell culture medium by flipping the microplate upside down and gently tapping the bottom of the microplate over a sink.

10.4 Wash by pipetting 200 μ L of the prepared 1X Wash Buffer A into each well. Discard the wash buffer (same as step 10.3) and wash 2 more times for a total of 3 washes using fresh wash buffer each time. After the final wash, gently blot the

microplate onto a paper towel to remove any excess/remaining buffer.

To avoid cell loss, do not pipette directly onto the cells. Instead, gently dispense the liquid down the wall of cell culture wells. Also avoid the use of vacuum suction or too forcefully tapping the microplate when discarding any solution.

- 10.5 Add 100 μ L of Fixing Solution into each well and incubate for 20 minutes at room temperature with shaking.
- 10.6 Repeat wash step 10.4.
- 10.7 Add 200 μ L of prepared 1X Quenching Buffer and incubate for 20 minutes at room temperature.
- 10.8 Wash the plate 4x with 1X Wash Buffer A, then tap the plate upside down to remove all wash buffer.
- 10.9 Add 200 μ L of prepared 1X Blocking Buffer and incubate for 1 hour at 37°C.
- 10.10 Wash 3x with 200 μ L 1X Wash Buffer B, then tap the plate upside down to remove all of excess wash buffer.
Note: If needed, the microplate may be stored at -80°C for several days after this wash.
- 10.11 Add 50 μ L of 1X Anti-Phospho-EGFR (Tyr1068) or 1X Anti-EGFR to corresponding wells and incubate for 2 hours at room temperature with shaking.
- 10.12 Wash 4x with 200 μ L 1X Wash Buffer B, then tap the plate upside down to remove all wash buffer.
- 10.13 Add 50 μ L of 1X Anti-rabbit IgG (HRP-conjugated secondary antibody) and incubate for 1 hour at room temperature.
- 10.14 Wash 4x with 200 μ L 1X Wash Buffer B then tap the plate upside down to remove all of excess wash buffer.
- 10.15 Add 100 μ L of TMB to each well and incubate for 30 minutes with shaking at room temperature in the dark.
- 10.16 Add 50 μ L of stop solution to each well and read at 450 nm, measure OD immediately.

11. TYPICAL DATA

Representative results of an In-Cell EGFR (Tyr1068) ELISA are shown below. Data and notes are provided for demonstration purposes only.

- 20,000 A431 cells were seeded into appropriate wells in a microplate. Cells were incubated at 37°C in 5% CO₂ overnight.
- 50 µL of different concentrations of stimulators (recombinant human EGF 0, 20 or 100 ng/mL in serum free DMEM) were added to appropriate wells. Then incubated for 10 minutes at 37°C.
- Wells were emptied and washed 3 times with 200 µL 1X Wash Buffer A per well. The plate was then tapped upside down to remove all excess wash buffer. The Assay Procedure (see Section 10) was then followed from step 10.5.

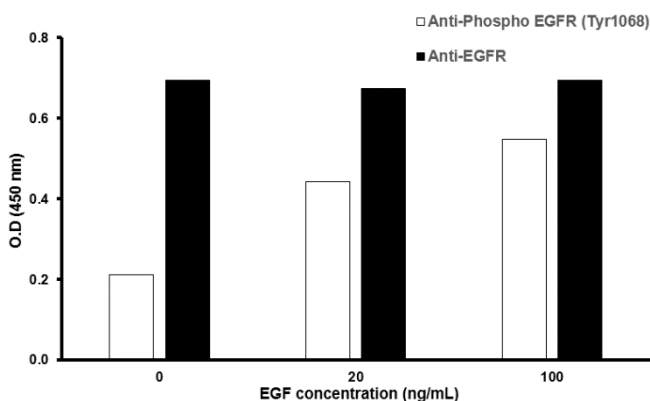


Figure 1. A431 cells were stimulated by different concentrations of EGF for 10 minutes at 37°C.

12. TROUBLESHOOTING

Problem	Cause	Solution
Low signal	Improper storage of the ELISA kit.	Store the kit according to manual instructions. Keep substrate solution in dark.
	Improper dilution.	Ensure correct preparation of antibody and reagents.
	Cells drop off from the wells.	Some of treatments may make cells drop off the wells. Reduce inhibitor or activator concentration.
High background	Inadequate washing	Be sure to remove all of washing solution and follow the recommendations for washing.
	Too many cells	Reduce the cell number.
Large CV	Inaccurate pipetting	Check pipette.
	Remaining wash buffer in the well.	Remove all of wash buffer.
	Cells drop off from the wells.	Please don't directly face the cells with tips when adding reagents or wash buffer.

13. NOTES

UK, EU and ROW

Email: technical@abcam.com | Tel: +44-(0)1223-696000

Austria

Email: wissenschaftlicherdienst@abcam.com | Tel: 019-288-259

France

Email: supportscientifique@abcam.com | Tel: 01-46-94-62-96

Germany

Email: wissenschaftlicherdienst@abcam.com | Tel: 030-896-779-154

Spain

Email: soportecientifico@abcam.com | Tel: 911-146-554

Switzerland

Email: technical@abcam.com

Tel (Deutsch): 0435-016-424 | Tel (Français): 0615-000-530

US and Latin America

Email: us.technical@abcam.com | Tel: 888-77-ABCAM (22226)

Canada

Email: ca.technical@abcam.com | Tel: 877-749-8807

China and Asia Pacific

Email: hk.technical@abcam.com | Tel: 108008523689 (中國聯通)

Japan

Email: technical@abcam.co.jp | Tel: +81-(0)3-6231-0940

www.abcam.com | www.abcam.cn | www.abcam.co.jp