Active EGF receptor EIA Kit Manual

Active EGF receptor

EIA KIT

An enzyme immunoassay kit
for the quantitative determination of
Phosphorylated EGF receptor
For research use only. Not for use in diagnostic or therapeutic procedures.

Catalog Number. 40-831-160008
For 96 assays

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Introduction

The epidermal growth factor (EGF) receptor is a glycoprotein that is consist of 130 kDa protein (1186 amino acid residues) and 40 kDa sugar chain. EGF receptor molecule is made up of four domains; glycosylated extracellular domain that is the binding site of EGF and TGFα, a transmembrane domain, tyrosin kinase domain and auto-phosphorylation domain. Tyrosin kinase domain is an intracellular domain which is homologous with the oncogene v-erbB product. (1)

The binding of EGF to the receptor results in DNA reproduction and cell proliferation. During this step, ruffuring of cell membrane, phosphorylation of EGF receptor, internalization, pH change, enzyme activation, re-organization of actin filament, and oncogene protein induction are occured. Although the mechanism of the EGF signal transduction is not clear, it is assumed that EGF binding stimulation should initiate a series of signal transduction through tyrosine auto-phosphorylation and phosphorylation of interacting proteins.(2)

The half-life of EGF receptor on the cell surface is approximately 20 hours. When the receptor accepts the ligand, the degradation of the receptor is accelerated

(degraded approximately in 5 hours).(3)

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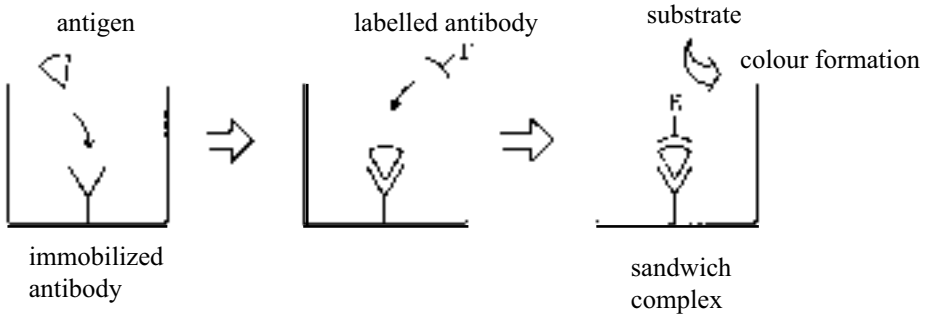
The human squamous epithelium carcinoma cell line, A431, possess many EGF receptor, 5-10 times of standard cell line's. (2-3 x 10^6 / cell)
This kit enables to measure the quantity of phosphorylated EGF receptor by ELISA. It is useful for analysis of receptor phosphorylation and for *in vitro* screening of specific inhibitors to EGF receptor kinase.

Intended use

The Active EGF receptor EIA Kit is an *in vitro* enzyme immunoassay (EIA) kit for quantitative determination of human Phosphorylated EGF receptor in serum, cell lysate and other biological fluids.
This kit is for research use only. It is not for use in diagnostic or therapeutic procedures.

Principle

The active EGF receptor EIA Kit is a solid phase EIA based on a sandwich method that utilizes mouse monoclonal anti human EGF receptor antibody and anti phosphotyrosine antibody to detect phosphrylated and activated EGF receptor by two-step procedure. The mouse monoclonal anti-human EGF receptor (B4G7) is immobilized onto the microtiter plate and blocked against non-specific binding. Samples and standards are added into each wells and incubated. The second step is to wash the plate and to add the second anti-phosphotyrosine (PY20) antibody labelled with peroxidase (POD). During this incubation, phosphorylated EGF receptor is bound to anti-EGF receptor (solid phase) on one side and tagged on the other by POD-anti-phosphotyrosin. The reaction between POD and substrate (H_2O_2, and 3,3',5,5'-tetramethylbenzidine) results in colour development with intensities proportional to the amount of phosphorylated (named active) EGF receptor present in samples and standards. The amount of active EGF receptor can be quantitated by measuring the absorbance using an EIA plate reader. Accurate sample concentrations of active EGF receptor can be determined by comparing their specific absorbances with those obtained for the standards plotted on a standard curve.



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Reagents and materials
Each active EGF receptor EIA Kit includes reagents sufficient for 96 wells. The expiration date for the complete kit is stated on the outer box label and the recommended storage temperature is 2 - 8°C.

A. Materials provided

Plate 1. Antibody Coated Microtiterplate - 1 plate (8 well x 12 strips)
The plate coated with murine monoclonal antibody to EGF receptor (B4G7). Store at 2 - 8°C.

Vial 2. Antibody-POD Conjugate - 1 vial (11 ml x 1)
The vial contains lyophilized horseradish peroxidase (POD) conjugated murine monoclonal antibody to Phosphotyrosine(PY20).
Store at 2 - 8°C. Avoid prolonged exposure to light.

Vial 3. Standard - 1 vial (1 ml x 1)(the concentration(fmol/ml) is shown on the label by each lot.)
The vial contains lyophilized native EGF receptor from A431.

Vial 4. Sample Diluent - 2 vials (11 ml x 2)
Each vial contains protein in a buffered solution. Use for Zero standard, and for dilution of the standard (vial 3) and samples which are above the calibration curve. Store at 2 - 8°C.
White precipitate may be generated during storage, but it does not affect the reaction. In this case, mix well before use.

Vial 5. Substrate Solution - 1 vial(12 ml x 1)
Each vial contains hydrogen peroxide and tetramethylbenzidine in a buffered solution. Store at 2 - 8°C.

Vial 8. Receptor Extraction Buffer-1 vial(11 ml x 1)
The vial contains detergent and tyrosine phosphatase inhibitor to prepare cell lysate. Store at 2 - 8°C.

B. Materials required but not provided

1. Reagents
   - Washing Buffer: Phosphate-buffered Saline (PBS)containing 0.1%Tween20 (Dissolve 8.0 grams of NaCl, 0.2 grams of KCl, 2.9 grams of $Na_2HPO_4 \cdot 12H_2O$ and 0.2 grams of KH_2PO_4,1ml of Tween20 in 1000 ml of distilled water.)
   - Stop Solution : 1 N H_2SO_4

2. Materials
   - Precision pipettes with disposable tips: 20 and 100 µl micropipettes, 10 - 200 µl adjustable multiwell pipetter or 20 and 100 µl multiwell pipetters
   - Beakers, flasks, cylinders necessary for preparation of reagents

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- Disposable pipettes and test tubes
- Microtiter plate reader for measurement of absorbance at 450 nm
- Graph paper

Precautions

- Do not mix reagents from different kit lots.
- Do not use reagents beyond expiration date on label.
- In order to avoid reagent contamination, use disposable pipette tips and/or pipettes.
- Sodium azide inactivates POD. Solutions containing sodium azide should not be used in this assay.
- Do not expose Substrate Solution to strong light during storage or incubation.
- Avoid contact of Substrate and Stop Solution with skin or mucous membranes. If these reagents come into contact with skin, wash thoroughly with water. Do not pipette by mouth. Do not smoke, eat, or drink in area where specimens or kit reagents are handled. Take special care in handling blood samples, since all blood fluids should be considered as potentially infectious.
- Avoid contact of Substrate and Stop Solution with any metal surfaces.
- Do not use the Substrate solution if its colour is changed to thick blue.

Specimen collection and handling

Venous blood samples are collected aseptically. Remove the serum from the clot or red cells, respectively, soon after clotting and separation. Samples containing a visible precipitate must be clarified prior to use in the assay. Do not use grossly hemolyzed or lipemic specimens. Samples may be stored up to 24 hours at 4°C. If the length of time between sample collection and assay is to exceed 24 hours, samples should be stored frozen under -20°C for optimal results. Excessive freeze-thaw cycles should be avoided. Prior to assay, frozen samples should be brought to room temperature slowly, and gently mixed by hand. Do not thaw samples in a hot bath. Do not vortex or sharply agitate.

[Method of preparing adhesion cell lysate(ex. A431)]

. Culture A431 cells on a ϕ90mm dish up to confluent.(average cell number 10^7 cells)
. Remove the supernatant (no washing). Add 1 ml of Receptor extraction buffer (vial 8) and recover the cell solution from the dish with Cell Scraper. Transfer the solution to a 1.5 ml microcentrifuge tube.
. Spin at 4°C for 5 min. at 10000 g and collect the supernatant as a sample.
. A431 cell lysate sample shoud be diluted for 10-50 times because of high productivity of EGF receptor. Many other cell lysate can be used for assay without dilution.

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[Method of preparing suspension cell lysate]
- Transfer cells to a microcentrifuge tubes, and spin at 300g.
- Remove the supernatant (no washing). Add 1 ml of Receptor extraction buffer (vial 8) and suspend the cell by pipeting.
- Spin at 4°C for 5min. at 10000g and use the supernatant as a sample.

NOTE:
- Samples should be prepaerd each time of assay. In case of storing a sample, it should be frozen at -80°C
- Human serum and plasma are used for assay with no dilution or 2-fold dilution.

Preparation of solutions
Note: The following solutions should be prepared directly before use.

Solution 1. Antibody-POD Conjugate Solution
Dissolve the contents of Vial 2 in 11 ml distilled water and mix gently followed by 10 min slowly rolling or occasional mixing, avoiding foam formation.

Solution 2. Standard Solution
Rehydrate Standard (Vial 3) with 1 ml distilled water. Slowly roll for approximately 10 min or let vials to stand and sporadically mix gently. (The concentration (fmol/ml) is shown on the label by each lot.)

Stability of solutions
Solution 1. The reconstituted lyophilisate is stable for 1 week at 4°C and for 1 month when stored at -20°C.
Solution 2. The reconstituted lyophilisate is stable for 2 weeks when stored at -80°C.

Procedure
Double determinations of all samples and standards should be performed.
All of the Kit's contents should be brought to room temperature before use !
For thorough mixing, the microtiter plate can be gently agitated on a plate mixer or by mixing the plate sporadically by hand.

[Enzyme immunoassay]
1. *Sample incubation*: Pipette 100 µl sample and standard(Solution 2)into one well within 5 minutes. Mix, seal the microtiter plate (e.g. with a foil) and incubate for 1 hour at 37°C.
2. Remove sample solution and wash the wells 3 times with ca. 400 µl of washing buffer; between the separate washing steps empty out the microtiter plate and vigorously tap onto paper towl, especially after the last washing.
3. *Antibody-POD conjugate incubation*: Pipette 100 µl of Antibody-POD Conjugate Solution (Solution 1) into one well, mix, seal the microtiter plate (e.g. with a foil) and incubate for 1 hour at 37°C).

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4. Remove sample solution and wash the wells 4 times as described above (It is especially important after this step to thoroughly empty out the remaining fluid before adding the substrate).
5. *Substrate incubation*: Add 100 µl Substrate Solution(vial 5) into each well and incubate at room temperature (20 - 30°C) for 15 min.
6. Add 100 µl Stop Solution(1N H_2SO_4) into each well in the same order as in adding substrate. Tap plate gently to mix.
7. Measure the absorbance at 450 nm with a plate reader. Set zero with distilled water. The absorbance should be read as soon as possible after the completion of the assay. It may be read up to 1 hour after addition of Stop Solution if wells are protected from light at room temperature.
   Note: It is important that Stop Solution is added to wells prior to reading at 450 nm. Addition of Stop Solution causes an increase in absorbance of the substrate Solution and shift in absorbance spectrum.

Results

1. Standard curve
   Record the absorbance at 450 nm for each standard well.
- Average the duplicate values and record the averages.
- Plot the absorbance (vertical axis) versus the Phosphotyrosine concentration
- fmol/ml (horizontal axis) for the standards.

2. Samples
   Record the absorbance at 450 nm for each sample well.
- Average the duplicate values and record the averages.
- Locate the average absorbance value on the vertical axis and follow a horizontal
- line intersecting the standard curve. At the point of intersection read phosphotyrosin concentration of EGF receptor (fmol/ml) from the horizontal axis.

Performance characteristics

1. **Range of standard curve:** 0.1- 5 fmol/ml.
2. **Specificity:** This kit specifically measures phosphorylated EGF receptor. This kit can be used to measure only human phophorylated EGF receptor. Inactive EGF receptor that is dephosphorylated is not detected in this kit.
3. **Assay duration:** Two and half hours after sample incubation.
4. **Total assay capacity:** 96 assays.
5. **Assay capacity for test samples:** If all assay wells (including standards and test samples) are run in duplicate, 40 test samples can be run in duplicate per kit.
6. **Test specimen type:** cell lysate, plasma, serum, of human
7. **Specimen volume required:** If each test sample is run in duplicate, approximately 220 µl (i.e., 100 µl per assay well plus ~10 µl for each sample transfer) is required.
8. **Limitation:** Since conditions may vary from assay to assay, a standard curve must be established for every run. Since cross contamination between reagents will invalidate the test, disposable pipette tips should be used.

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Thorough washing of the wells between incubations is required:
1) Completely empty out the remaining fluid from the well before dispensing fresh wash solution.
2) Use sufficient wash solution for each wash cycle (approximately 400 µl).
3) Do not allow wells to sit uncovered for extended periods between incubation steps.Only samples with absorbance values falling within the range of the standard curve should be assigned a concentration from the curve.

9.**Notes :** When an antigen level in an unknown organic specimen is observed to be elevated as compared to the calibration range of the standard curve, it is recommended to dilute the samples properly with the dilution solution included in the kit and assay them again in another run.

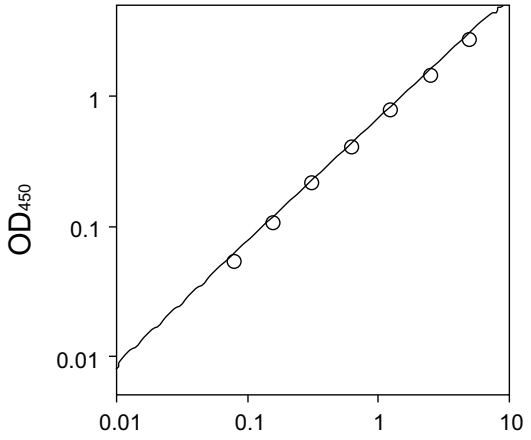
Basal data

Unknown stimulation than EGF binding may phosphorylate other tyrosine sites on EGF receptor. The Standard supplied in this kit is shown with the amount of phosphorylated tyrosine in EGF receptor. (See 6 "Structure of EGFr in page 10.)

1. Typical standard curve

Each laboratory should establish its own normal range for EGFr.

Typical Standard Curve
(Do Not Use To Calculate Unknowns)



Log (y) = - 0.209 + 0.941Log (x)
r = 1.000

Phosphorylated Tyrosine in EGFr (fmol/ml)	5.00	2.50	1.25	0.625	0.312	0.156	1.178
A_{450}	2.727	1.443	0.779	0.404	0.215	0.106	0.054

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2. Intra-assay precision (n=16)

Assay was carried out with 16 replicates of 3 samples containing different concentration of active EGF receptor. (Sample: A431 cell extract)

	Ave. (fmol/ml)	S.D. (fmol/ml)	CV (%)
Sample A	1.940	0.115	5.9
Sample B	0.541	0.026	4.8
Sample C	0.167	0.009	5.4

Inter-assay precision (performance 3 times)

Assay to assay precision with one laboratory was evaluated in three independent experiments over 3 days.

	Ave. (fmol/ml)	S.D. (fmol/ml)	CV (%)
Sample A	1.940	0.095	4.9
Sample B	0.522	0.026	5.0
Sample C	0.162	0.006	3.4

3. Recovery test

The recovery of active EGF receptor was tested by adding two samples out of ten different level in various matrices.

(unit:fmol/ml)

Sample A	Sample B	A+B Measured	A +B Calculated	Recovery (%)
2.640	0.000	1.440	1.320	109.1
2.640	2.640	2.520	2.640	95.5
2.640	1.360	1.890	2.000	94.5
2.640	0.636	1.590	1.640	97.1
2.640	0.288	1.420	1.460	97.0
2.640	0.142	1.340	1.390	96.3
1.360	0.000	0.675	0.680	99.3
1.360	1.360	1.330	1.360	97.8
1.360	0.636	0.946	0.998	94.8
1.360	0.288	0.790	0.824	95.9
1.360	0.142	0.720	0.751	95.9
0.636	0.000	0.317	0.318	99.7
0.636	0.636	0.634	0.636	99.7
0.636	0.288	0.471	0.462	101.9
0.636	0.142	0.390	0.389	100.3
0.288	0.000	0.151	0.144	104.9
0.288	0.288	0.299	0.288	103.8
0.288	0.142	0.218	0.215	101.4
0.142	0.000	0.072	0.071	101.4
0.142	0.142	0.141	0.142	99.3

* Recovery rate = (A + B Measureed) / (A + B Calculated) x 100

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4. Influence of coexistence

Co-existing substance is shown in its final concentration.

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5. Epitope of the antibodies in this kit

The first antibody : Anti-human EGF receptor antibody(B4G7)

Anti human EGF receptor antibody (B4G7) recognizes EGF binding domain peptide on the human EGF receptor.(4) B4G7 antibody reacts with low affinity type receptor in living cells. But in cell lysate such as prepared with methods in page 4, this antibody reacts both with high and low affinity receptors.

B4G7 antibody does not inhibit the DNA reproduction.

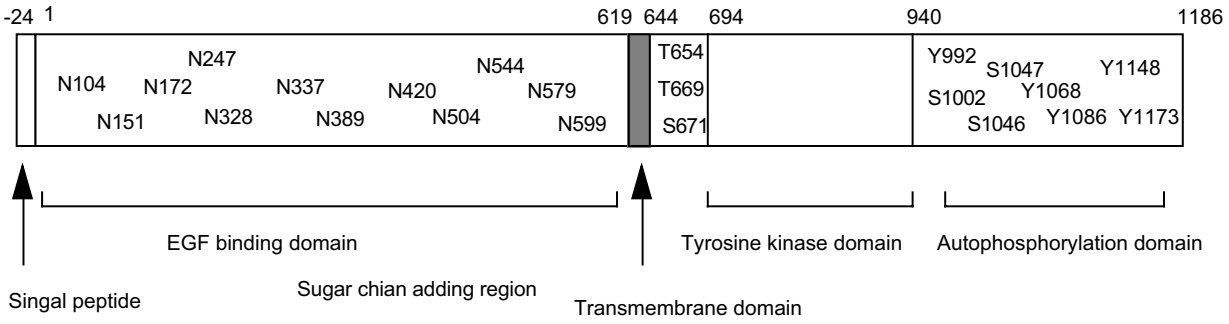
In immunoprecipitation study using A431 cell extract, it is confirmed that B4G7 antibody specifically reacts with MW170 kDa and 160 kDa protein of EGF receptor.

The labelled antibody : Anti-phosphotyrosin antibody (PY20)-HRP

Anti-phosphotyrosin antibody (PY20) is specific to phosphotyrosin without reacting phosphoserine nor phosphothreonin nor dephosphorylated tyrosin.(5)

PY20 antibody reacts with only MW170kDa protein in B4G7 immunocomplex using A431 lysate. MW160 kDa protein is regarded as a fragment of MW170 kDa.

6.Structure of EGF receptor and it's position of phosphorylation



N : Asparagine
T : Threonine
S : Serine
Y : Tyrosine

Main phosphorylated amino acid residue (T:Threonin,S:Serin,Y:Tyrosin) position are located in auto-phosphorylation domain of EGF receptor. By accepting the EGF molecule, phosphorylation of EGF receptor is accelerated, and signals are transfered by phosphorylation.

Using phosphorylated peptide mapping method, Shimizu and his co-workers have analyzed the position of phosphorylated amino acids in the receptor of NA cells which were highly expressing EGF receptor after stimulated with EGF or H_2O_2.(6)

According to their reports, in NA cells, T^{669},S^{1046},S^{1047} are phosphorylated in normal condition, and S^{671},T^{669},S^{1046},S^{1047},Y^{1173} are phosphorylated with H_2O_2 stimulation and T^{654},T^{669},Y^{1173} and the other Y with EGF stimulation.

Thus, it is confirmed that tyrosin phosphorylation is remarkably triggered by EGF or H_2O_2 exposure.

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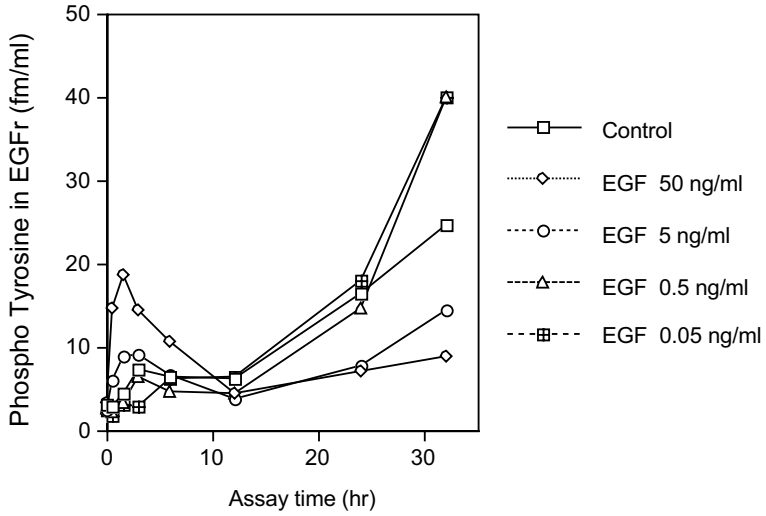
7. Application examples

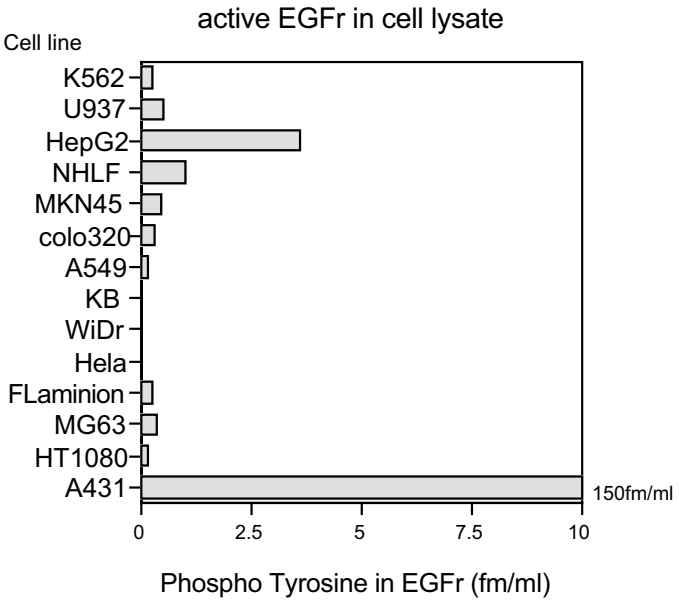
1: Quantitation of phosphorylated EGF receptor in time course after stimulation with EGF

The active EGF receptor production in A431 cells (overexpressing EGF receptor cells) was quantitated after stimulation with EGF at various concentrations.
Some reports claims that the proliferation of cells which are overexpressing EGF receptor can be inhibited by EGF exposure in reverse. (7,8)
The result obtained with this kit shows that activation of EGF receptor is also inhibited by high concentation of EGF in A431 cells.



2: Quantitation of Active EGF receptor in various cell lysate

Phosphorylated EGF receptor in several human cultured cell lysate was quantitated. All samples were prepared by using 10^6 cells/1 ml receptor extraction buffer. The 100 µl of each lysate was applied to the assay. Active EGF receptor level of A431 lysate was the highest incomparably among the other cells. No active EGF receptor was detected in the supernatant of cultured cells of A431. (data not shown)



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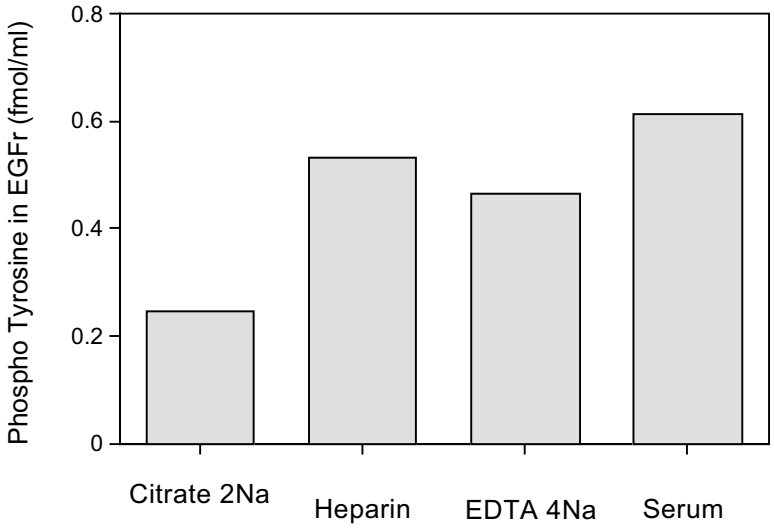
3: Quantitation of phosphorylated EGF receptor in human blood samples
This kit can use human blood as a sample. The effect of anticoagulants was studied using blood sample prepared from one person. (The data is shown as below). It is recommended to use serum directly (no dilution) as a sample.



Storage and Stability
This kit is shipped at 2-8°C and should be stored at 2-8°C if not used. Under this condition, the kit is stable until the expiry date printed in the box label.

Protocol summary
1. Prepare all reagents as directed in the Package Insert.
2. Bring all reagents to room temperature and prepare the solutions.
3. Add 100 µl of Standard or sample to appropriate wells, and incubate for 1 hours at 37°C.
4. Remove sample solution and wash the wells 3 times with 400 µl of washing buffer.
5. Add 100 µl of antibody-POD conjugate solution into wells and incubate at 37°C for 1 hour.
6. Aspirate solution from wells. Wash 4 times with 400 µl of washing buffer per wells, aspirating thoroughly between washes.
7. Add 100 µl of Substrate Solution to each well. Incubate 15 minutes at room temperature.
8. Add 100 µl of Stop Solution to all wells. Mix gently.
9. Read at 450 nm as soon as possible.

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