

## Product Contents

### EGF Receptor:

Part No.	Size (units)
V555A	10

**Description:** Epidermal Growth Factor Receptor (EGF Receptor) is a cell-surface glycoprotein composed of a single polypeptide chain (170kDa) that binds the EGF peptide (6.1kDa). The EGF Receptor is found in numerous tissues of the body where the number of molecules can range from 20,000 to 200,000 per cell (1). The EGF Receptor consists of an extracellular ligand binding domain, a single transmembrane region and a cytoplasmic intrinsic tyrosine kinase domain.

Upon ligand binding, the EGF Receptor autophosphorylates, causing the tyrosine kinase domain of the EGF Receptor to become active. Ligands that bind the Receptor are EGF, Transforming Growth Factor  $\alpha$  (TGF $\alpha$ ), vaccinia virus growth factor and amphiregulin (1,2). The activated tyrosine kinase domain can phosphorylate a number of substrates, such as phospholipase C- $\gamma$ 1, *ras* GTPase-activating protein, the proto-oncogene *c-erb* B-2, lipocortin I, the serine/threonine kinases MAPK and *raf* (2).

EGF Receptor is immunopurified from the A431 cell line following a procedure detailed by Weber *et al.* (3). The purified EGF Receptor possesses tyrosine kinase activity due to the bound EGF; however, the EGF Receptor has not been autophosphorylated.

**Molecular Weight:** Unmodified protein, 131.4kDa; glycoprotein, 170kDa.

**Source:** Immunopurified from A431 cells.

**Stability:** This preparation is stable to at least three freeze-thaw cycles.

**Storage Buffer:** 20mM HEPES (pH 7.4), 1mM EDTA, 1mM DTT, 130mM NaCl, 10% (v/v) glycerol, 0.05% Triton® X-100 and 5 $\mu$ M EGF.

**Storage Conditions:** See the Product Information Label for storage recommendations. Avoid multiple freeze-thaw cycles and exposure to frequent temperature changes.

**Substrate for Functional Tyrosine Kinase Assay:** Angiotensin II (DRVYIHPF) 1046.2Da.

**Unit Definition:** One unit is the amount of kinase required to transfer 1pmol of phosphate to Angiotensin II in 1 minute at 30°C.

**Unit Definition Assay Buffer:** 5mM HEPES (pH 7.4), 50 $\mu$ M Na<sub>3</sub>VO<sub>4</sub>, 5mM MgCl<sub>2</sub>, 2mM MnCl<sub>2</sub>, 1.7mg/ml BSA, 20 $\mu$ M ATP, 250mM ammonium sulfate and 3mM Angiotensin II.

Part# 9PIV555

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**Promega**

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## Quality Control Assay

**Concentration:** See Product Information Label for lot-specific information.

**Protein Concentration:** See Product Information Label for lot-specific information.

**Specificity:** This receptor preparation is free of detectable serine kinase activity as tested by incubation of 5 $\mu$ l of EGF Receptor in a 20 $\mu$ l reaction with test peptides for Protein Kinase C (Cat.# V5261) and cAMP-Dependent Protein Kinase Catalytic Subunit (Cat.# V5161) for 2 hours at 30°C. This test is performed according to the non-radioactivity protocols of Promega's PepTag® Kinase Assay Systems (Cat.# V5330, V5340).

## References

1. Todderud, G. and Carpenter, G. (1989) Epidermal growth factor: The receptor and its function. *Biofactors* **2**, 11–5.
2. Carpenter, G. and Cohen, S. (1990) Epidermal growth factor. *J. Biol. Chem.* **265**, 7709–12.
3. Weber, W., Bertics, P.J. and Gill, G.N. (1984) Immunoaffinity purification of the epidermal growth factor receptor. Stoichiometry of binding and kinetics of self-phosphorylation. *J. Biol. Chem.* **259**, 14631–6.

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## I. Assay Protocol to Determine Tyrosine Kinase Activity of EGF Receptor

### Materials to Be Supplied by the User

(Solution compositions are provided in Section III.)

- Angiotensin II (Sigma Cat.# A9525)
- 20% trichloroacetic acid (TCA)
- [ $\gamma$ -<sup>32</sup>P]ATP (at 3,000Ci/mmol, 10mCi/ml)
- 2% phosphoric acid
- phosphorylation buffer
- 0.5% phosphoric acid
- 1.5M ammonium sulfate
- liquid scintillation fluid
- BSA (10mg/ml)
- Whatman® P-81 phosphocellulose paper
- deionized water

### A. Standard EGF Receptor Phosphorylation Assay of Angiotensin II

The following is an enzymatic assay for EGF Receptor tyrosine kinase activity. To produce quantitatively accurate results, all of the phosphorylation reactions must be incubated uniformly. Begin timing the phosphorylation reaction upon adding the phosphorylation buffer to the reaction tube. **Note:** To determine whether a protein is a potential EGF Receptor phosphorylation substrate, substitute this protein for Angiotensin II. The amount of sample protein to be assayed in the phosphorylation reaction must be determined empirically.

- Set up the following 50 $\mu$ l reactions, on ice, in 1.5ml microcentrifuge tubes in the order shown below:

	Volume	Final Concentration*
18mM Angiotensin II	10 $\mu$ l	3mM
BSA (10mg/ml)	10 $\mu$ l	1.7mg/ml
1.5M ammonium sulfate	10 $\mu$ l	250mM
phosphorylation buffer	20 $\mu$ l	

\*Final concentration is based on the 60 $\mu$ l phosphorylation reaction volume.

- Remove a reaction tube from the ice, place at 30°C and incubate for 3 minutes. Add 10 $\mu$ l EGF Receptor (see Note 1) to the reaction tube to initiate the reaction. Begin timing the enzymatic reaction.
- Incubate the 60 $\mu$ l phosphorylation reaction at 30°C for 5 minutes.
- Terminate the phosphorylation reaction by adding 20 $\mu$ l of 20% TCA to the tube and place on ice (see Note 2). Process the remaining samples, one at a time, starting at Step 2. This will ensure that all samples are processed uniformly. Incubate all terminated reactions on ice for 5 minutes before proceeding to Step 5.
- Spin the terminated phosphorylated reaction tubes at top speed in a microcentrifuge for 5 minutes.
- Remove 60 $\mu$ l of the phosphorylation mixture from each tube and place into a new microcentrifuge tube that contains 60 $\mu$ l of deionized water and mix (see Note 3).
- Remove 50 $\mu$ l from each phosphorylation reaction mixture and spot onto a piece of correspondingly labeled Whatman® paper.
- To remove unincorporated phosphate, wash the sample papers in 200ml of 0.5% phosphoric acid for 5 minutes at room temperature. Repeat washing with fresh 0.5% phosphoric acid for a total of four washes.
- Place the sample papers into correspondingly labeled scintillation vials containing 5ml of scintillation fluid and count. The cpm generated are produced by bound phosphorylated Angiotensin II.
- To determine total cpm/pmol of ATP (Standard) add 5 $\mu$ l phosphorylation buffer to two labeled scintillation vials that contain 5ml of scintillation fluid and count. Use the average cpm of these samples as the Standard.

#### Notes:

- To include a phosphorylation negative control (Blank), substitute 10 $\mu$ l of deionized water for the 10 $\mu$ l of EGF Receptor.
- The TCA precipitation eliminates the EGF Receptor that has been radiolabeled by autophosphorylation. The phosphorylated substrate remains in the supernatant. To determine the amount of phosphorylated EGF Receptor, as well as phosphorylated substrate, do not perform the TCA precipitation (Step 4), but add 20 $\mu$ l of 2% phosphoric acid directly to the reaction tube and proceed to Step 6.
- The 60 $\mu$ l of deionized water is necessary to dilute the reaction mixture to ensure that the phosphorylated substrate binds to the Whatman® paper.

## II. Calculations

- Determine the cpm of phosphate transferred onto the substrate. Subtract the Blank (see Note 1) from the average TCA-precipitated cpm from the phosphorylation reaction (see Step 9) and divide by the percent of phosphorylation reaction mixture that was spotted onto the Whatman® paper. Following the procedure outlined, 31.25% of the phosphorylation mixture was spotted onto the Whatman® paper. **Example:** The phosphorylation of Angiotensin II produced 1,030cpm, the Blank produced 125cpm, and 31.25% of the phosphorylation mixture was spotted onto the Whatman® paper. How many cpm of phosphate were transferred to Angiotensin II by the EGF Receptor?

$$\frac{(\text{phosphorylation reaction cpm}) - (\text{blank cpm})}{\text{percent phosphorylation mixture spotted}} = \text{cpm transferred}$$

$$\frac{(\text{Angiotensin II, 1030cpm}) - (\text{Blank, 125cpm})}{0.3125} = 2,896\text{cpm transferred}$$

- Determine the cpm/pmol of ATP (phosphate) by taking the average cpm of the Standard (see Step 10), multiplying by the dilution factor of four and dividing by the pmol of ATP in the phosphorylation reaction. Following the procedure outlined, 1,200pmol of ATP was present in the phosphorylation reaction. **Example:** The Standard contained 575,000cpm and the Angiotensin II phosphorylation reaction contained 1,200pmol of ATP. What was the cpm/pmol phosphate?

$$\frac{(\text{Average cpm of Standard}) \times \text{dilution factor}}{\text{pmol ATP (phosphate)}} = \text{cpm/pmol phosphate}$$

$$\frac{(\text{Standard, 575,000cpm}) \times 4}{1,200\text{pmol ATP}} = 1,917\text{cpm/pmol phosphate}$$

- Determine the pmol of phosphate transferred onto the phosphorylation substrate by dividing the cpm transferred in the phosphorylation reaction (the result of Calculation 1) by the cpm/pmol ATP (the result of Calculation 2). **Example:** The phosphorylation reaction in Calculation 1 had 2,896cpm transferred and each pmol of phosphate produced 1,917cpm (Calculation 2). How many pmol of phosphate were transferred onto Angiotensin II?

$$\frac{\text{phosphorylation reaction cpm transferred}}{\text{cpm/pmol phosphate}} = \text{pmol phosphate transferred}$$

$$\frac{\text{phosphorylation reaction, 2,896cpm}}{1,917\text{cpm/pmol phosphate}} = 1.51\text{pmol phosphate transferred to Angiotensin II}$$

- Determine units of EGF Receptor used in phosphorylation reaction by dividing the pmol of phosphate transferred to the phosphorylation substrate (result of Calculation 3) by the reaction time. **Example:** 1.51pmol of phosphate was transferred to Angiotensin II within the five-minute incubation time. How many units of EGF Receptor were used in the phosphorylation reaction?

$$\frac{\text{pmol phosphate transferred}}{\text{assay time}} = \text{EGF Receptor units}$$

$$\frac{1.51\text{pmol phosphate transferred}}{5 \text{ minutes}} = 0.302\text{pmol phosphate/minute or } 0.302 \text{ EGF Receptor units}$$

## III. Composition of Buffers and Solutions

### phosphorylation buffer

1–10 × 10 <sup>6</sup> cpm/assay	[ $\gamma$ - <sup>32</sup> P]ATP (3,000Ci/mmol, 10mCi/ml)
60 $\mu$ M	ATP
15mM	MgCl <sub>2</sub>
6mM	MnCl <sub>2</sub>
150 $\mu$ M	sodium orthovanadate
15mM	HEPES (pH 7.4)

### Angiotensin II (DRVYIHPF)

18mM (18.8mg/ml) in deionized water

## IV. Related Products

Product	Size	Cat.#
EGF, Human, Recombinant	100 $\mu$ g	G5021
SignaTEC® Protein Tyrosine Kinase Assay System	96 reactions	V6480
Anti-Phosphotyrosine pAb	200 $\mu$ g	V2171

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