

## Purification and Assay of Kinase-Active EGF Receptor From Mammalian Cells by Immunoaffinity Chromatography

Gregory J. Wiepz, Arturo G. Guadaramma, David L. Fulgham,  
and Paul J. Bertics

### Summary

The epidermal growth factor (EGF) receptor possesses intrinsic protein-tyrosine kinase activity, and both overexpressed wild-type and mutated forms have been associated with many types of cancers. Therefore, understanding the mechanisms that modulate receptor activity and function is essential to the development of treatments for many of these cancers. However, to address this issue by either conventional or high-throughput screening methods requires the availability of large amounts of highly purified and active EGF receptor.

The technique described in this chapter utilizes immunoaffinity chromatography, which allows for the isolation of highly purified and active preparations of EGF receptor. By immobilizing an antibody that recognizes the ligand-binding domain of the receptor to Sepharose beads, the receptor can be eluted specifically from the antibody by the addition of EGF. This association establishes a unique interaction that ensures the isolation of a highly enriched preparation of EGF receptor. This protocol allows for the purification of large or small batches of receptor that retain their kinase activity. Additionally, this chapter reports on the subsequent steps necessary to characterize the receptor: kinase activity, mass, purity, and the ability of the receptor to undergo autophosphorylation.

**Key Words:** Epidermal growth factor (EGF) receptor; tyrosine kinase; autophosphorylation; immunoaffinity chromatography; receptor purification.

### 1. Introduction

The ability to study enzymes that have been isolated from their natural cellular environment has permitted the extensive evaluation and characterization of the intra- and intermolecular mechanisms regulating enzymatic activity. For example, understanding the internal regulatory mechanisms of the human EGF

From: *Methods in Molecular Biology*, vol. 327: *Epidermal Growth Factor: Methods and Protocols*  
Edited by: T. B. Patel and P. J. Bertics © Humana Press Inc., Totowa, NJ

receptor expressed by many types of cells has led to the generation of numerous potent and highly specific therapeutics for treating various human cancers (**1**). (In this regard, the reader is referred to Chapter 14 regarding the clinical significance of developing agents that target the EGF receptor.)

The EGF receptor is a large transmembrane glycoprotein that resides in the plasma membrane of a wide range of mammalian cells and possesses intrinsic tyrosine kinase activity (**2**). Upon activation by several different ligands, e.g., EGF, transforming growth factor- $\alpha$ , or amphiregulin, the receptor undergoes autophosphorylation on numerous tyrosine residues and tyrosine phosphorylates multiple substrates (**3**). The phosphorylated tyrosine residues in the receptor serve to regulate receptor kinase activity and trafficking and can act as potential docking sites for many cytoplasmic signaling proteins such as Grb2 and phospholipase C (PLC) $\gamma$  (**4–6**).

The ability to investigate substrate, inhibitor, and effector protein interactions with purified receptor *in vitro* has allowed for a more detailed analysis of receptor function. In this regard, multiple in-frame deletions of the EGF receptor have been recently identified in non-small-cell lung cancer patients who were nonsmokers (**7**). These mutations lead to an increase in receptor phosphorylation on tyrosines 992 and 1068, which promotes the increased activation of downstream mediators, such as AKT and STAT5 (**8**). This series of mutations also renders the receptor and the tumor susceptible to a class of tyrosine kinase inhibitors that target the ATP-binding pocket of the EGF receptor (i.e., gefitinib, iressa) (**8,9**). To understand how these mutations alter the ATP-binding site, thereby rendering them susceptible to specific drugs, requires an evaluation of the interaction of the drug with purified receptor.

The method that we employ to isolate functional EGF receptor involves the use of immunoaffinity chromatography (**10**). Once the receptor is solubilized from the cell membrane, it is bound by a Sepharose-linked mouse monoclonal antibody (anti-EGF receptor—clone 528) that recognizes an epitope in the ligand-binding domain. This specific interaction allows for the elution of the receptor from the antibody using recombinant human EGF (rhEGF), i.e., the rhEGF competes with the immobilized antibody for binding to the receptor. The receptor, which remains functional and bound to EGF, is displaced from the resin, and upon addition of the appropriate components (metal ions, ATP, exogenous peptides), its tyrosine kinase activity can be readily measured (**11**).

Following elution of the receptor, the preparation is stored at  $-80^{\circ}\text{C}$  in the presence of several stabilizing components. The receptor is very stable at this temperature and remains active for at least 1 yr. Following purification, the receptor is quantified regarding mass, purity, kinase activity, and the ability to autophosphorylate. Although this method is described for large-scale purification of EGF receptor, it works equally well on a smaller scale to isolate multiple mutants/variants of the receptor for characterization.

The ability to purify the EGF receptor is critical to the development of drugs that specifically target the EGF receptor kinase and/or protein interaction motifs. Additionally, to better identify and characterize inhibitors directed toward the kinase activity of the EGF receptor, methods that employ high-throughput screening can be facilitated by the ready availability of purified receptor (**12**). (The reader is directed to Chapter 3 by Lafky et al. for a discussion of other receptor-quantification methods, such as those required for the detection of soluble EGF receptor forms.)

## 2. Materials

### 2.1. Receptor Purification

1. Mammalian cells expressing EGF receptors (e.g., A431 cells).
2. Monoclonal antibody 528 which specifically recognizes the ligand binding site of the human EGF receptor: LabVision, CA (MS-268).
3. Recombinant human EGF: Upstate Biologicals Inc. (01-407).
4. CnBr-activated sepharose 4B beads—Sigma (C-9142).
5. Receptor buffer (20 mM hydroxyethyl piperazine ethane sulfonate [HEPES], pH 7.4, 130 mM NaCl, 1 mM ethylenediaminetetraacetic acid [EDTA], 1 mM dithiothreitol [DTT], 10% glycerol, 0.05% Triton X-100).
6. Homogenization buffer: 40 mM HEPES, pH 7.4, 10 mM ethylene glycol-bis(2 aminoethylether)-*N,N,N',N'*-tetraacetic acid [EGTA], 2% Triton X-100, 20% glycerol.
7. Cell harvesting buffer (10 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.4, 145 mM NaCl, 2.5 mM EGTA, 2.5 mM EDTA).
8. Receptor buffer/1 M NaCl: receptor buffer, 1 M NaCl.
9. Receptor buffer/1 M urea: receptor buffer, 1 M urea.
10. Receptor buffer/8 M urea: receptor buffer, 8 M urea.
11. Elution buffer: receptor buffer, 25 µg/mL rhEGF.
12. Bead storage buffer: receptor buffer, 0.05% sodium azide.
13. Coupling buffer: 100 mM NaHCO<sub>3</sub>, 50 mM NaCl.
14. Blocking buffer: 0.5 M ethanolamine in ddH<sub>2</sub>O.
15. Protease/phosphatase buffer: 2 mM Na<sub>3</sub>VO<sub>4</sub>, 100 mM NaF, 10 mM *p*-nitrophenyl phosphate, 1% aprotinin, 2 mM DTT, 0.2 mM leupeptin, 8 mM benzamidine (*see* Note 1).

### 2.2. Assay Buffers

1. Standard assay mix: 20 mM HEPES, pH 7.4, 0.2 mM Na<sub>3</sub>VO<sub>4</sub>, 20 mM MgCl<sub>2</sub>, 8 mM MnCl<sub>2</sub>, 2% (v/v) aprotinin, 0.1 mM leupeptin.
2. Angiotensin II mix: 20 mM HEPES, pH. 7.4, 3 mM angiotensin II, 50% standard assay mix.
3. ATP mix: 20 mM HEPES, pH 7.4, 30 µM ATP, 25% standard assay mix, 0.75 µCi [ $\gamma$ <sup>32</sup>P]ATP/µL.
4. 20% trichloroacetic acid (TCA) in ddH<sub>2</sub>O.

5. Autophosphorylation assay mix: 20 mM HEPES, pH 7.4, 2  $\mu$ M ATP, 10 mM  $\text{MgCl}_2$ , 4 mM  $\text{MnCl}_2$ , 100  $\mu$ M  $\text{Na}_3\text{VO}_4$ , 1% (v/v) aprotinin, 0.1 mM leupeptin, 0.3  $\mu$ Ci [ $\gamma^{32}\text{P}$ ]ATP/ $\mu$ L.
6. Sample buffer: 20 mM Tris pH 8.0, 1.5 mM EDTA, 20 mM DTT, 2% sodium dodecyl sulfate [SDS], 20% glycerol, 0.2% bromophenol blue.
7. Sample buffer without DTT: same recipe as above without the DTT and without EDTA.

### 2.3. Additional Key Reagents

1. ATP: Adenosine 5'-triphosphate, magnesium salt (Sigma, A9187).
2. Angiotensin II: human synthetic, acetate salt (Sigma, A9525).
3. [ $\gamma^{32}\text{P}$ ]ATP: Amersham Pharmacia (PB10168).
4. Phosphocellulose paper (P81): Whatman (3698-915).
5. Polyacrylamide gel electrophoresis (PAGE) staining reagents: Sypro Orange, Molecular Probes (S6650).
6. All chemicals unless otherwise stated are purchased from Sigma.

### 2.4. Equipment

1. Refrigerated centrifuge: table top (3200g; e.g., Beckman Allegra 6R).
2. Centrifuge: super speed (capable of 20,000g; e.g., Sorval RC5B).
3. Centrifuge: microfuge (Eppendorf 5415 or 5420).
4. Centrifuge: radioactive microfuge (Eppendorf 5415).
5. Sonicator: Branson Sonifier-250 with microtip.
6. X-ray film developer or a phosphoimager.
7. Polyacrylamide gel electrophoresis (PAGE) equipment.
8. Gel imaging and quantifying equipment.
9. Scintillation counter and scintillation fluid.

## 3. Methods

### 3.1. Cell Culture

Depending on the amount of receptor required, the cell type and cell culture method will vary. To isolate a large amount of wild-type receptor, we prepare approx 5 g of an A431 cell pellet. To achieve this yield, we culture about 250, 15-cm tissue culture plates in groups of 50 plates over a period of several months.

#### 3.1.1. Cell Splitting and Culturing

As the cells approach confluence, they are harvested by rinsing the plate with the cell harvesting buffer and then incubating the plates in the same buffer for 10–15 min at 4°C. The cells are then harvested by scraping with a hard edge scraper. The cells are collected, combined into 50-mL polypropylene centrifuge tubes, and centrifuged at 500g (~1500 rpm in a tabletop refrigerated cen-

trifuge) for 10 min at 4°C. The supernatant is removed, and the pellets are frozen at -80°C (see **Note 2**).

### 3.2. Bead Preparation

This procedure requires 24 h of preparation time. All washes of the beads will occur in a 15-mL conical polypropylene tube centrifuged at 500g (1500 rpm in a tabletop refrigerated centrifuge) for 5 min at 4°C. This protocol is specifically designed to prepare 1 g of CNBr-activated Sepharose beads that will swell to a slurry volume of 3.5 mL, which in turn will then be coupled with 10 mg of anti-EGF receptor antibody. Smaller volumes can be scaled down to accommodate fewer beads and less antibody (see **Note 3**).

1. Lyophilized CNBr-activated beads are suspended in 40 mL of 0.1 M HCl for 1 h to swell and remove the stabilizer. This procedure is performed in a 50-mL graduated cylinder. Invert the beads five times; after settling, the buffer is poured off, which contains the fines, fragments of beads, and debris. Resuspend the beads in 40 mL of buffer again, and after they settle pour off the buffer. Repeat this step three times. Resuspend the beads in coupling buffer (10 mL) and transfer to a 15-mL conical centrifuge tube.
2. Wash the beads two times by centrifugation (500g, 5 min) with 10 mL of coupling buffer.
3. After the final wash add an equal volume of coupling buffer to the bead volume (~3.5 mL). Add the antibody solution (approx 10 mg) directly to the beads. Incubate at room temperature for 2 h with constant inversion.
4. Centrifuge the beads (500g, 5 min) and retain the supernatant (to check for antibody coupling efficiency).
5. Wash the beads three times with 10 mL of the coupling buffer and discard the supernatants.
6. Block the unbound active sites on the beads with three bead volumes of blocking buffer (0.5 M ethanolamine) overnight with constant inversion at 4°C.
7. The next day and before each use, wash the beads 10 times with 10 mL of receptor buffer.

After each purification, the beads are washed with 10 mL of receptor buffer/8 M urea, followed by 10 washes of receptor buffer (10 mL), and stored in bead storage buffer (10 mL) at 4°C for up to 1 yr.

### 3.3. Receptor Isolation and Elution

To isolate the receptor from the plasma membrane, the cells are sonicated in the presence of Triton X-100, protease, and phosphatase inhibitors. The receptor is then combined with the beads so that it may bind to the agarose bound antibody. Following the incubation for the specific interaction between the receptor and the antibody, the beads are washed under stringent conditions (1 M

NaCl, 1 M urea) to remove all nonspecifically bound cell products. Finally, the receptor is displaced from the antibody with EGF, which interacts with the same binding site as the antibody.

1. Upon thawing, the cells are combined with an equal volume of homogenization buffer that includes the protease inhibitor buffer (*see Note 4*).
2. The cell solution is then disrupted using a probe sonicator for  $3 \times 20$  s bursts at a setting of 3 (30–50% output; *see Note 5*).
3. Centrifuge the cell homogenate at 20,000g for 15 min at 4°C. Retain the supernatant, which is where the receptor will predominantly reside. Discard the pellet.
4. Add the supernatant to the prepared beads and incubate overnight at 4°C with constant inversion (*see Note 6*).
5. After the overnight incubation, the beads are washed according to the following protocol. All centrifugations are 500g for 5 min performed in a 15-mL centrifuge tube. The volume of each of the washes is 15 mL minus the volume of the beads (e.g., fill the tube up for each wash).
6. Centrifuge the beads and collect and retain the supernatant, which will contain any unbound receptor (*see Note 7*).
7. Wash the beads 10 times with receptor buffer.
8. Wash the beads three times with receptor buffer /1 M NaCl.
9. Wash the beads three times with receptor buffer.
10. Wash the beads three times with receptor buffer/1 M NaCl.
11. Wash the beads three times with receptor buffer.
12. Wash the beads three times with receptor buffer /1 M urea.
13. Wash the beads five times with receptor buffer.

### 3.3.2. Receptor Elution

To elute the receptor from the beads, add an equal volume of the elution buffer and mix by constant inversion at room temperature for 25 min. Centrifuge the beads and keep the supernatant, which will contain the receptor. Repeat this step and combine the two elution supernatants (*see Note 8*).

### 3.3.3. Receptor Storage

Freeze the supernatants in small aliquots (20–500  $\mu$ L) and store at  $-80^{\circ}\text{C}$  (*see Note 9*).

### 3.3.4. Bead Storage

The beads are then washed with receptor buffer/8 M urea (*see Note 10*). Next, the beads are washed 10 times with receptor buffer and finally suspended in twice the bead volume of bead storage buffer. The beads can be used for up to 1 yr after preparation or for a total of six separate purifications. However, this will depend on the quality of the bead preparation, storage, and usage. Additionally, the receptor yield should be evaluated after every purification, as described below (*see Note 11*).

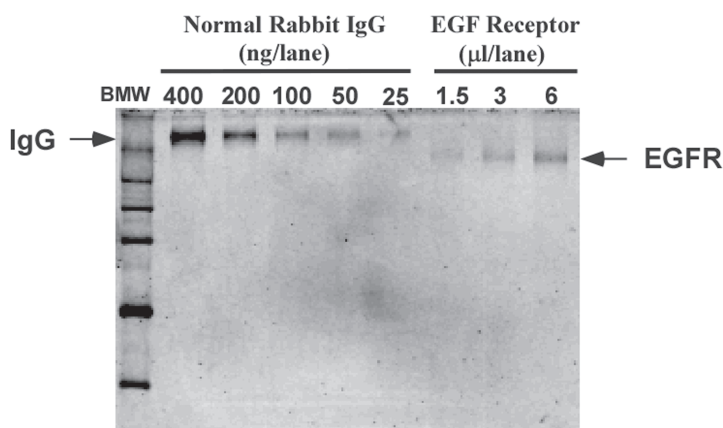


Fig. 1. Determination of epidermal growth factor (EGF) receptor mass and purity. Various amounts of the purified EGF receptor and rabbit IgG were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The gel was processed as described and stained with Sypro orange, and the bands were visualized on a Hitachi FMBIO II laser scanner and quantified using the National Institutes of Health Software Image. The data were then processed in Microsoft Excel to produce a standard curve for the IgG to correlate with the EGF receptor values.

### 3.4. Receptor Characterization

Quantification of the purified receptor involves determining three separate parameters: receptor mass and purity, kinase activity, and the ability to undergo autophosphorylation. From these data the quality and quantity of the receptor can be established.

#### 3.4.1. Measurement of EGF Receptor Mass

Because the receptor was eluted with buffer containing 25  $\mu\text{g/mL}$  of rhEGF, the mass must be estimated by gel electrophoresis comparing the receptor mass to a protein standard, preferably of similar molecular weight. For this determination, a standard solution of rabbit IgG is used. Because the IgG is made up of heavy and light chains, when prepared in the absence of a reducing agent the molecule will remain intact and migrate at a similar rate to EGF receptor in a 10% polyacrylamide gel (*see Fig. 1*). This procedure also gives you an estimate of purity of the receptor.

1. Prepare a 10% polyacrylamide gel.
2. Prepare the IgG standards so that you load 400, 200, 100, 50, 25 ng/well. The volume will depend on the type of gel system and comb size that you use. These standards are prepared in sample buffer that does not contain any reducing agents (DTT) or EDTA.



3. Prepare dilutions of purified EGF receptor (e.g., 6, 3, 1.5  $\mu\text{L}$  of receptor/well).
4. Run the gel and stain (*see Note 12*).
5. Quantify the bands for the receptor and the IgG standard by densitometry and determine the protein mass for the EGF receptor per microliter (*see Note 13*).

#### 3.4.2. Measurement of Receptor Kinase Activity

Kinase activity is determined by quantifying the ability of the purified receptor to phosphorylate angiotensin II under very specific assay conditions. Although there are a number of new techniques to assess the activity of the receptor (*12*), the method that we employ is the transfer of a radioactive phosphate from  $\gamma^{32}\text{P}$ -ATP to angiotensin II. Although fairly simple to conduct, this assay does require the use of radioactive materials and the associated equipment and regulatory clearance.

This assay is performed by adding various concentrations of the receptor with the appropriate assay components under very specific conditions (i.e., time and temperature). The timing of the assay is extremely critical, as it is important to assess receptor kinase activity using linear initial velocity conditions (*12*). Modifying the concentration of the receptor, substrate, ATP, or the time and the temperature will alter the measured activity of the receptor.

**Steps 1–3** are performed on ice.

1. Make the appropriate dilutions of the receptor in receptor buffer. The receptor must be diluted so that each tube will receive exactly 20  $\mu\text{L}$ . Maintain the tubes on ice at all times (*see Table 1*) (*see Note 14*).
2. Aliquot to all tubes (1.5-mL polypropylene centrifuge tubes) 20  $\mu\text{L}$  of angiotensin II.
3. Add the receptor to the appropriate tubes (20  $\mu\text{L}$ ) or control solution (receptor buffer)
4. Warm the ATP mixture in a 30°C water bath for 5 min.
5. Each tube must undergo a 2 min prewarming step prior to receiving the warm ATP mixture to initiate the reaction, which is then incubated at 30°C for exactly 5 min. To facilitate the completion of the assay, the tubes are staggered by 15–30 s so that multiple tubes can be incubating at the same time but are all stopped at the specified time. (*see Table 1*).
6. The reactions are terminated by the addition of 20  $\mu\text{L}$  of 20% trichloroacetic acid (TCA) and placed at 4°C.
7. Upon completion of the assay, each tube is centrifuged in a microfuge on max (>10,000 rpm) for 5 min at room temperature (*see Note 15*).
8. To allow the angiotensin II to bind to the phosphocellulose paper, the TCA must be diluted. The supernatant (50  $\mu\text{L}$ ) is combined with ddH<sub>2</sub>O (50  $\mu\text{L}$ ) in a separate tube, and 90  $\mu\text{L}$  of this solution is spotted onto a 1  $\times$  1 in. piece of phosphocellulose paper that has been prelabeled with the tube number in pencil/pen/marker (*see Note 16*).



**Table 1**  
**Typical Protocol for an Epidermal Growth Factor Receptor (EGFR) Assay**

Tube	50% EGFR ( $\mu$ L)	25% EGFR ( $\mu$ L)	12.5% EGFR ( $\mu$ L)	EGFR Receptor buffer ( $\mu$ L)	3 mM AII ( $\mu$ L)	30 $\mu$ M ATP ( $\mu$ L)	20% TCA ( $\mu$ L)	Start (min)	Stop (min)
1	20	—	—	—	20	20	20	2	7
2	20	—	—	—	20	20	20	2.25	7.25
3	—	20	—	—	20	20	20	2.5	7.5
4	—	20	—	—	20	20	20	2.75	7.75
5	—	—	20	—	20	20	20	3	8
6	—	—	20	—	20	20	20	3.25	8.25
7	—	—	—	20	20	20	20	3.5	8.5
8	—	—	—	20	20	20	20	3.75	8.75
Total	40	40	40	40	160	160	160		

9. The paper is allowed to dry and is then washed in a large volume (~300  $\mu\text{L}$ ) of cold 0.5% phosphoric acid three times for 10 min with constant, gentle rocking.
10. The filter papers are air dried, added to 5 mL of scintillation fluid, and the activity on each paper is determined by scintillation counting (*see* **Note 17**).

### 3.4.2.1. SAMPLE CALCULATIONS (**TABLE 2**)

#### 3.4.2.1.1. Determination of ATP-Specific Activity

1. ATP concentration—the total concentration of ATP present in the ATP solution.
2. Total counts—the number of dpm per volume of the ATP solution (i.e., 5  $\mu\text{L}$ ) (*see* **Note 18**).
3. Specific activity—the number of dpm/pmol of ATP. This number is used to convert the number of dpm into the amount of phosphate incorporated into the substrate (angiotensin II).

#### 3.4.2.1.2. Determination of Kinase Activity

1. Disintegrations per minute (DPM)—actual uncorrected counts.
2. DPM corrected—DPM minus the counts present in the blank tubes (average background).
3. pmol  $^{32}\text{P}$ —DPM corrected divided by the specific activity. This value is the amount of phosphate that was transferred to the substrate (angiotensin II).
4. pmol/assay—pmol $^{32}\text{P}$  incorporated multiplied by the % reaction counted.
5. % reaction counted—because only a fraction of the actual assay reaction volume is counted, the cpm needs to be corrected. From the original assay volume, 50  $\mu\text{L}$  is removed and combined with 50  $\mu\text{L}$  of water to dilute the concentration of TCA, which permits the binding of the substrate to the P-81 paper. From this diluted sample, 90  $\mu\text{L}$  is applied to the paper. Therefore, the amount of sample counted is 56% of the original tube ( $0.9 \times 0.625 = 0.56$ ).
6. Velocity (pmol/min)—pmol/assay divided by the time (min), usually 5 min.
7. Average velocity—average of the duplicates for each dilution.
8. Velocity pmol/min/ $\mu\text{L}$ —average velocity divided by the number of  $\mu\text{L}$  of receptor originally used.

### 3.4.3. Receptor Autophosphorylation (**Fig. 2**)

Because the EGF receptor is known to autophosphorylate *in vivo*, evaluation of this parameter reflects the degree to which the receptor has remained intact following purification. This assay is similar to the standard kinase assay, with the exclusion of any additional substrate. Following the reaction, varying amounts of the receptor are separated by SDS-PAGE and exposed to radiographic film for the appropriate amount of time (*see* **Note 19**).

1. Add 10  $\mu\text{L}$  of the purified receptor at 4°C with 10  $\mu\text{L}$  of the Autophosphorylation buffer.
2. Incubate on ice for 10 min and stop the reaction with 20  $\mu\text{L}$  of sample buffer.
3. Prepare a 10% PAGE and load various volumes of the reaction (e.g., 20, 10, 5, 2.5  $\mu\text{L}$ , which is equivalent to 5, 2.5, 1.25, and 0.63  $\mu\text{L}$  of receptor).

**Table 2**  
**Representative Data from Epidermal Growth Factor Receptor Kinase Assay<sup>a</sup>**

Specific activity						
ATP concentration = 3.00E-05 M %Reaction counted = 0.56						
μL counted = 0.5                      Reaction time (min) = 5						
Total ct	Average ct	dpm/μL	ATP (pmol/μL)	dpm/pmol		
904091	887661	1775322	30	59177.40		
871231						
Tube	Receptor (μL/tube)	DPM	DPM corrected	pmol <sup>32</sup> P	pmol/assay	Velocity pmol/min receptor
1	10	399383	376282	6.3585	11.3545	0.2307
2	10	411449	388348	6.5624	11.7186	
3	5	191287	168186	2.8421	5.0751	0.1918
4	5	172787	149686	2.5294	4.5169	
5	2.5	94680	71579	1.2096	2.1599	0.2357
6	2.5	146797	123696	2.0903	3.7326	
Background 1 = 24589				Average velocity/μL = 0.2		
Background 2 = 21613						
Average background = 23101						

<sup>a</sup>This analysis was performed in an Excel worksheet. DPM, disintegrations per minute; SD, standard deviation.

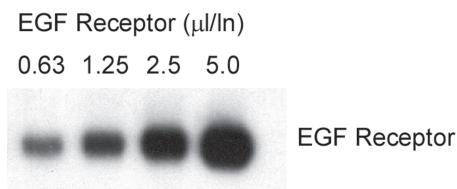


Fig. 2. Epidermal growth factor (EGF) receptor autophosphorylation. The reaction for autophosphorylation was performed as described using purified EGF receptor (43 ng/ $\mu\text{L}$ ). The radiographic film was exposed for 60 min.

4. Run gel for the appropriate time (*see Note 20*).
5. Fix gel with 70% methanol/10% glacial acetic acid for 15 min.
6. Rehydrate gel with ddH<sub>2</sub>O for 20 min.
7. Wrap gel in plastic wrap and expose to film for 1–2 h or overnight. Alternatively, a phosphoimager can be used.

#### 4. Notes

1. As an alternative, protease inhibitor cocktail can be used in place of aprotinin, leupeptin, and benzamidin (i.e., Sigma—Mammalian Protease Inhibitor Cocktail, P8340).
2. These cell pellets are very stable at  $-80^{\circ}\text{C}$  and can be stored for more than a year.
3. The hybridoma clone for this antibody can be purchased from American Type Culture Collection ([www.atcc.org](http://www.atcc.org); HB-8509) and the antibody produced/purified using a hybridoma facility.
4. Make the protease/phosphatase inhibitor buffer just prior to use. Alternatively, a protease inhibitor cocktail can be used (Sigma P8340) with the addition of the phosphatase inhibitors.
5. Always sonicate on ice and allow 1 min between sonications. The probe intensity varies between machines, and the required amount of sonication will need to be determined empirically by checking for intact cells by microscopy.
6. Adjust the volume to 12 mL in a 15-mL tube, or use a smaller tube to decrease the number of beads that stick to the tube.
7. For large preps a considerable amount of receptor will remain in the solution and can be saved and bound to the antibody beads again after the first elution.
8. If there is only a small volume of beads, decrease the tube size to better match the volume.
9. The receptor is commonly stored in multiple small aliquots (25  $\mu\text{L}$ ) to avoid repeated freeze/thaws.
10. After centrifugation, the supernatant containing the denatured protein is retained and can be used as a 170 kDa marker for SDS-PAGE.
11. Due to the nature of the procedure, the volume of beads will go down after each use. However, the remaining beads will retain most of their activity through multiple purifications.

12. Several staining procedures are available to quantify the amount of protein present on the gel. We routinely use Sypro Orange (Molecular Probes, Inc.) and visualize the gel in a Laser scanner (Hitachi FMBIO II). Alternatively, depending on the concentration of protein present, other methods can be employed (i.e., Coomassie, silver stain)
13. Various imaging software programs are available to record and quantify the intensity of each band, such as Image (Mac) and Image J (PC), both of which are available from <http://rsb.info.nih.gov/nih-image/>.
14. Receptor dilution is expressed as a percentage to address a possible range in activity.
15. This procedure will make the centrifuge very radioactive, and appropriate handling procedures must be adhered to.
16. To help spread the 90  $\mu$ L of assay mixture on the filter paper, the papers are suspended on straight pins that are inserted into a Styrofoam block.
17. To facilitate the drying of the phosphocellulose paper, the individual papers are dried under a heat lamp.
18. The number of dpm is equal to the number of cpm in the scintillation counter that we use with the appropriate scintillation cocktail.
19. Alternatively, this detection can be performed on a phosphoimager.
20. Try not to run the dye front off the gel where a major amount of the radioactivity is located. Stop the gel before the dye front comes off the bottom, cut it off, and discard it appropriately.

## Acknowledgments

This work was supported by grants from the National Institutes of Health (CA105730 and GM53271).

## References

1. Lynch, T. J., Bell, D. W., Sordella, R., et al. (2004) Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N. Engl. J. Med.* **350**, 2129–2139.
2. Carpenter, G. and Cohen, S. (1990) Epidermal growth factor. *J. Biol. Chem.* **265**, 7709–7712.
3. Bertics, P. J. and Gill, G. N. (1985) Self-phosphorylation enhances the protein-tyrosine kinase activity of the epidermal growth factor receptor. *J. Biol. Chem.* **260**, 14,642–14,647.
4. Buday, L. and Downward, J. (1993) Epidermal growth factor regulates p21ras through the formation of a complex of receptor, Grb2 adapter protein, and Sos nucleotide exchange factor. *Cell* **73**, 611–620.
5. Wang, X. J., Liao, H. J., Chattopadhyay, A., and Carpenter, G. (2001) EGF-dependent translocation of green fluorescent protein-tagged PLC-gamma1 to the plasma membrane and endosomes. *Exp. Cell. Res.* **267**, 28–36.
6. Zhu, G., Decker, S. J., and Saltiel, A. R. (1992) Direct analysis of the binding of Src-homology 2 domains of phospholipase C to the activated epidermal growth factor receptor. *Proc. Natl. Acad. Sci. USA* **89**, 9559–9563.

7. Pao, W., Miller, V., Zakowski, M., et al. (2004) EGF receptor gene mutations are common in lung cancers from “never smokers” and are associated with sensitivity of tumors to gefitinib and erlotinib. *Proc. Natl. Acad. Sci. USA* **101**, 13,306–13,311.
8. Sordella, R., Bell, D. W., Haber, D. A., and Settleman, J. (2004) Gefitinib-sensitizing EGFR mutations in lung cancer activate anti-apoptotic pathways. *Science* **305**, 1163–1167.
9. Paez, J. G., Janne, P. A., Lee, J. C., et al. (2004) EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science* **304**, 1497–1500.
10. 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**, 14,631–14,636.
11. Gronowski, A. M. and Bertics, P. J. (1993) Evidence for the potentiation of epidermal growth factor receptor tyrosine kinase activity by association with the detergent-insoluble cellular cytoskeleton: analysis of intact and carboxy-terminally truncated receptors. *Endocrinology* **133**, 2838–2846.
12. Beebe, J. A., Wiepz, G. J., Guadarrama, A. G., Bertics, P. J., and Burke, T. J. (2003) A carboxyl-terminal mutation of the epidermal growth factor receptor alters tyrosine kinase activity and substrate specificity as measured by a fluorescence polarization assay. *J. Biol. Chem.* **278**, 26,810–26,816.

Epidermal Growth Factor

Methods and Protocols

Patel, T.B.; Bertics, P.J. (Eds.)

2006, XII, 208 p. 46 illus., 1 illus. in color., Hardcover

ISBN: 978-1-58829-421-0

A product of Humana Press