

**PerkinElmer Life and Analytical Sciences, Inc.**



**IMINOBIOTIN PROTEIN LABELING KIT  
TOOL FOR REVERSIBLE IN GEL BIOTIN  
DETECTION AND PLASMA MEMBRANE  
PROTEIN ISOLATION**

**CATALOG NUMBER: PRD100A001KT**

**For Laboratory Use  
Caution: Research Chemicals for Research Purposes Only**

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## **ABOUT THE USER MANUAL**

### **The manual describes the following methods:**

- Iminobiotin labeling of proteins (Section 1).
- Selective labeling of cell surface molecules. The basis of the labeling technique is the covalent attachment of an amine-reactive compound, containing iminobiotin, onto the cell surface proteins (Section 2).
- Options for enrichment of the labeled plasma membrane proteins are briefly delineated (Section 3). These include conventional centrifugation methods, differential detergent extraction and affinity purification using streptavidin- or avidin-based capture matrices.
- The electrophoretic analysis of iminobiotin-labeled proteins, such as cell-surface molecules, is described. The labeled proteins are visualization after PAGE by staining with Ember™ Biotin Binder dye (Section 4). This dye is conveniently detected based upon its fluorescence properties.
- The subsequent de-staining of the separated protein bands for total protein visualization or further microchemical analysis (MS) is described (Section 5).

This manual assumes that the user has a working knowledge of commonly practiced protein sciences procedures such as performing polyacrylamide gel electrophoresis (PAGE), and peptide mass profiling by mass spectrometry.

## ABBREVIATIONS

BSA	bovine serum albumin
CCD	charge-coupled device
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
LC	liquid chromatography
LED	light-emitting diode
MALDI-TOF	matrix-assisted laser desorption time-of-flight
MS	mass spectrometry
MSDS	Materials Data Safety Sheet
MS/MS	tandem mass spectrometry
NHS	N-hydroxysuccinimidyl
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PMT	photomultiplier tube
RT	room temperature
RFU	relative fluorescent units
SDS	sodium dodecyl sulfate
TRIS	tris(hydroxymethyl)aminomethane
UV	Ultraviolet
YAG	Yttrium-aluminum garnet

## SAFETY INFORMATION

**Waste Disposal:** Dispose of any materials in accordance with local, state and federal guidelines

## INTRODUCTION

## Overview

### Iminobiotinylation of Proteins (Section 1)

Biotin is a low molecular weight compound which can be conjugated to many proteins, peptides, glycoproteins, and amino acids by a variety of reaction chemistries. The high-affinity binding of biotin to avidin, streptavidin, and related proteins and the inherent signal amplification of the biotin-avidin system has made it useful for a wide variety of immunological and other protein-based methodologies. However, one problem with the biotin/biotin-binding protein interaction is that it is essentially irreversible under physiological conditions. Avidin has a strong affinity with biotin ( $K_a = 10^{15} \text{ M}^{-1}$ ). Avidin also forms a complex with 2-iminobiotin, the cyclic guanidino analog of biotin ( $K_a = 10^8 \text{ M}^{-1}$  at pH 9.5). The avidin-iminobiotin complex is converted to avidin-biotin complex in the presence of biotin because of the differences in binding constants of these molecules for avidin. Binding of avidin and streptavidin to the 2-iminobiotin is pH-dependent, affinity decreases as the pH is lowered. The pH-dependent interaction of 2-iminobiotin with avidin also makes de-staining possible. At high pH values, the free base form of 2-iminobiotin retains the high affinity selective avidin binding property of biotin, whereas at acidic pH values, the salt form of the biotin analog interacts poorly with avidin.

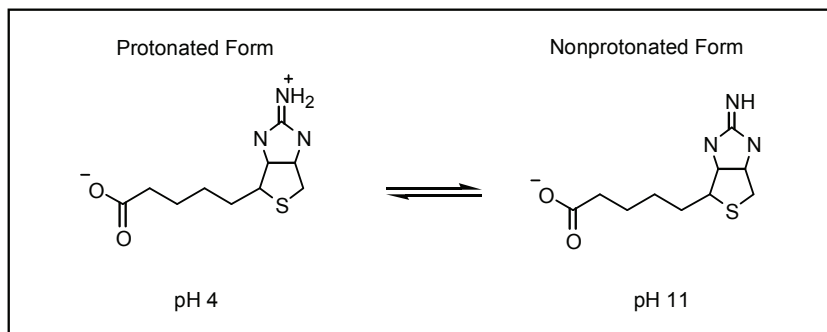


Figure 1: **Diagram of the 2-iminobiotin molecule at low and high pH values.**

In proteins, the NHS-ester activated iminobiotin reagent (N-hydroxysuccinimidyl-iminobiotin trifluoroacetamide) reacts almost exclusively with  $\alpha$ -amino groups at the N-termini of proteins/peptides and with  $\epsilon$ -amino groups of lysine residues to form stable amide linkages that are fairly resistant to hydrolysis. Primary amines are abundant, widely distributed and easily modified because of their

reactivity and their location on the surface of proteins. In aqueous environments the trifluoroacetamide moiety hydrolyzes to the original iminobiotin, producing a structure suitable for avidin binding. There is a spacer arm of 13.5 Å between the reacted primary amine and the iminobiotin moiety. NHS-Iminobiotin can be used for applications that require mild dissociation conditions from avidin or streptavidin or in applications that cannot tolerate the reducing conditions required to break the disulfide bond of a cleavable biotinylation reagent. Disulfide-containing cleavable biotinylation reagents are not stable under the standard running conditions of reducing SDS-PAGE, making NHS-iminobiotin a better labeling reagent when selective detection of the labeled proteins is the objective.

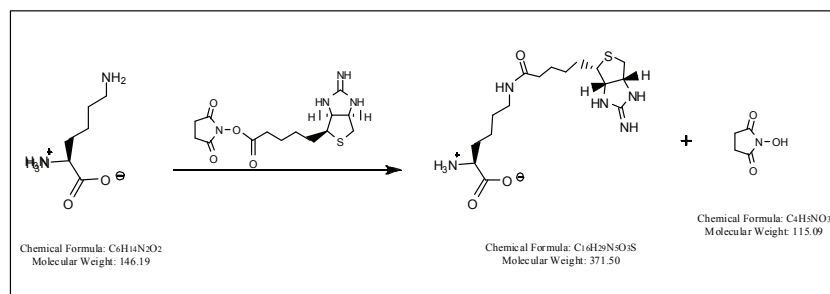


Figure 2: **Diagram of the reaction of 2-iminobiotin with lysine.**

The positive charge of the lysine residue is regenerated upon reaction of NHS-iminobiotin with proteins (Patton et al, 1989). Though the primary amino group from lysine is replaced with an imino group from the biotin analog, this does not produce a significant alteration in protein isoelectric point within the pH range of 3.0-9.5 that is normally used for isoelectric focusing separations. Thus, the labeling reaction is fully compatible with two-dimensional gel electrophoresis. It should be noted that the mass of the modified amino group increases by 225 Daltons upon covalent modification with iminobiotin. While this increase in mass is too small to detect by PAGE, the modification must be considered in mass spectrometry experiments.

The iminobiotinylated molecules can be selectively visualized using a fluorophore-labeled reporter conjugate after PAGE (See "Staining with Ember™ Biotin Binder dye" below). No transfer of proteins by Western blotting is necessary, as detection is achieved directly within the gels. In addition, the iminobiotin allows for subsequent de-staining of the labeled protein bands of interest (see "De-Staining Gels" below). The de-staining removes more than 90% of the fluorophore-labeled conjugate, serving to begin preparation of the sample for further

downstream processing, such as counter-staining with a total protein dye, matrix assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI-TOF-MS)-based peptide mass fingerprinting or tandem mass spectrometry based peptide sequencing.

### **Iminobiotinylation of Plasma Membrane (Section 2)**

Plasma membrane proteins, such as G protein-coupled and tyrosine kinase receptors represent important members of the "druggable" proteome. Defining the complement of plasma membrane proteins displayed on living cells is crucial to discovering diagnostic disease biomarkers, therapeutic agents and drug receptor candidates. With the Iminobiotin Protein Labeling Kit, a modified affinity tagging procedure is employed to selectively label cell surface proteins. In conjunction with a differential detergent extraction kit, protein subcellular compartmentalization may be monitored as well.

Plasma membranes are closed structures that surround the cell itself, having a cytosolic face (the side oriented toward the interior of the cell) and an extracellular face (the side presented to the environment). Since the iminobiotinylation reaction is targeted towards extracellularly-exposed lysine residues, it should be possible to map the topology of plasma membrane proteins by locating trypsin-resistant modified lysine residues using mass spectrometry. Iminobiotinylated lysine residues will not be cleaved by this protease, whereas arginine residues will remain unaffected.

In addition to the labeling of plasma membrane proteins, this kit is suitable for labeling any biomolecule wherein reversible binding to avidin, streptavidin or other biotin-binding protein is desirable, such as for affinity precipitation or affinity chromatography applications. Labeling of protein lysates permits sensitive and reversible detection of total protein profiles by PAGE.

### **Enrichment Options for Labeled Plasma Membrane Proteins (Section 3)**

Proteins redistribute in response to a variety of physiological stimuli. Activation of numerous cellular regulatory pathways is accompanied by the translocation of key proteins from one region of the cell to another. Additionally, clustering of plasma membrane receptors by an extracellular ligand, often leads to the association of specific integral transmembrane proteins with the underlying cytoskeleton. Despite their obvious regulatory significance, dynamic changes in protein compartmentalization have not routinely been monitored quantitatively by combining selective plasma membrane labeling, subcellular fractionation and PAGE.

A number of experimental protocols may be pursued to enrich plasma membrane proteins after iminobiotinylation, including differential

centrifugation and avidin- or streptavidin-based affinity chromatography using the High Capacity Streptavidin Coated Plates (PerkinElmer Cat. # 6006625). Fractionation by sequential extraction of cells or tissues with detergent-containing buffers allows partitioning of proteins into functionally distinct compartments that can readily be evaluated by 1-D or 2-D gel electrophoresis. Unlike most other subcellular fractionation methods, detergent fractionation preserves the integrity of the cytoskeletal compartment. The regulatory significance of the cytoskeletal compartment is increasingly being recognized in processes such as endocytosis, exocytosis, mitosis, cytokinesis, chemotaxis, signal transduction, and protein biosynthesis. Differential detergent extraction is simple, independent of time-consuming ultracentrifugation or cumbersome washing steps, amenable to low quantities of cells and fully compatible with commonly employed electrophoretic procedures. Labeling plasma membrane proteins with iminobiotin selectively highlights plasma membrane proteins in the various detergent fractions, and provides the opportunity to enrich these proteins further using subsequent affinity isolation procedures employing immobilized streptavidin or avidin.

#### **Staining with Ember™ Biotin Binder Dye (Section 4)**

Extracellularly-exposed plasma membrane proteins (or any iminobiotinylated molecule) from the subcellular fractions may subsequently be selectively visualized using a fluorophore-labeled reporter conjugate, Ember™ Biotin Binder dye, after PAGE. Overall, combining the labeling procedure with the cited subcellular fractionation procedure improves detection of low abundance plasma membrane proteins and allows monitoring of changes in their association with the underlying cytoskeleton. The outlined labeling and detection workflow should be suitable for the characterization of membrane proteins by a variety of standard gel-based proteomics methods. Alternatively, the labeled material may be enriched further by avidin- or streptavidin-based affinity chromatography, such as the High Capacity Streptavidin Coated Plates (PerkinElmer Cat. # 6006625) and analyzed by PAGE or LC/MS.

#### **De-Staining Gels (Section 5)**

De-staining of the labeled protein bands of interest removes more than 90% of the fluorophore-labeled conjugate, serving to begin preparation of the sample for further downstream processing, such as total protein profile detection using a protein stain, matrix assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI-TOF-MS)-based peptide mass fingerprinting or tandem mass spectrometry-based peptide sequencing.

### Kit Contents, Storage and Reagent Preparation

Upon receiving the shipping container, store it at 2-8°C.

Reagents supplied are intended FOR LABORATORY USE. Kits are shipped under ambient conditions.

2.5 ml	Ember™ Biotin Binder dye	1 vial. To be diluted 1:100 in Gel Staining Buffer for iminobiotinylated protein staining before use
10 vials	NHS-Iminobiotin	1.8 mg per vial, vacuum dried. Each to be re-constituted in 12 µl DMSO before use
0.2 ml	Positive Control*	Iminobiotinylated BSA (100 µg/ml). Load 400-500 ng per lane after dilution with PAGE loading buffer
0.2 ml	Negative Control**	Unlabeled ovalbumin (100 µg/ml). Load 400-500 ng per lane after dilution with PAGE loading buffer
50 ml	Gel Staining Buffer (20x)	To make 1x Gel Staining Buffer, dilute contents of the bottle to approximately 900 ml with purified H <sub>2</sub> O. <b>Adjust the pH to 10.5± 0.2 with NaOH.</b> Bring the pH adjusted 1x buffer to 1 liter with purified H <sub>2</sub> O
100 ml	Gel De-Staining Solution A Concentrate (5x)	Dilute 1 volume of Gel De-Staining Solution A Concentrate with 4 volumes of purified H <sub>2</sub> O. <b>Adjust the pH to 4.0± 0.2.</b> The 1x De-Staining Solution is to be used to dilute the De-Staining Solution B Concentrate (100x). See below
5 ml	Gel De-Staining Solution B Concentrate (100x)	Add 1 volume of Gel De-Staining Solution B Concentrate (100x) to 99 volumes of the 1x Gel De-Staining Solution A (above).

\* Stains with Ember™ Biotin Binder dye after PAGE and will de-stain according to the provided protocol.

\*\* Will not stain with Ember™ Biotin Binder dye after PAGE.

**ADDITIONAL MATERIALS & EQUIPMENT REQUIRED, BUT NOT SUPPLIED**

- DMSO
- 1X PBS (without calcium or magnesium)
- 0.1M solution of glycine in PBS
- Centrifuge capable of holding 1.5-2.0 ml centrifuge tubes and generating forces up to 12,000 x g.
- Rocking / Rotating Platform Shaker
- Cellular Protein Fractionation kit. (PerkinElmer, Cat. # PRD101A001KT)
- Avidin- or streptavidin-based chromatographic support, such as the High Capacity Streptavidin Coated Plates. (A pack of five, PerkinElmer Cat. # 6006625)
- Biotinylated Protein Molecular Weight Markers (PerkinElmer, Cat. # NEL310)
- Total protein assay kit, such as the EZQ™ Protein Quantitation Kit (Invitrogen Corp. Cat. # R33200)
- 4-12% polyacrylamide mini-gels and gel electrophoresis buffers
- Total protein gel stain, such as SYPRO® Ruby protein gel stain, silver stain or colloidal Coomassie™ Blue stain
- Fluorescent gel imaging system and associated analysis software

## **SECTION 1: IMINOBOTINYLATION OF PROTEINS, ANTIBODIES, AND OTHER MACROMOLECULES CONTAINING PRIMARY AMINE RESIDUES**

The kit is designed for the labeling of 1-2 mg of protein with 2-iminobiotin in a 0.5 to 1 ml reaction volume. When the NHS-Iminobiotin is reconstituted and used as directed, there will be at least a 40-fold molar ratio of labeling reagent to protein.

Buffers containing primary amine residues, such as TRIS, should be avoided as they will compete with the protein's primary amine groups for labeling. If necessary, the protein may require dialysis into a buffer devoid of primary amines, such as phosphate buffered saline, prior to labeling.

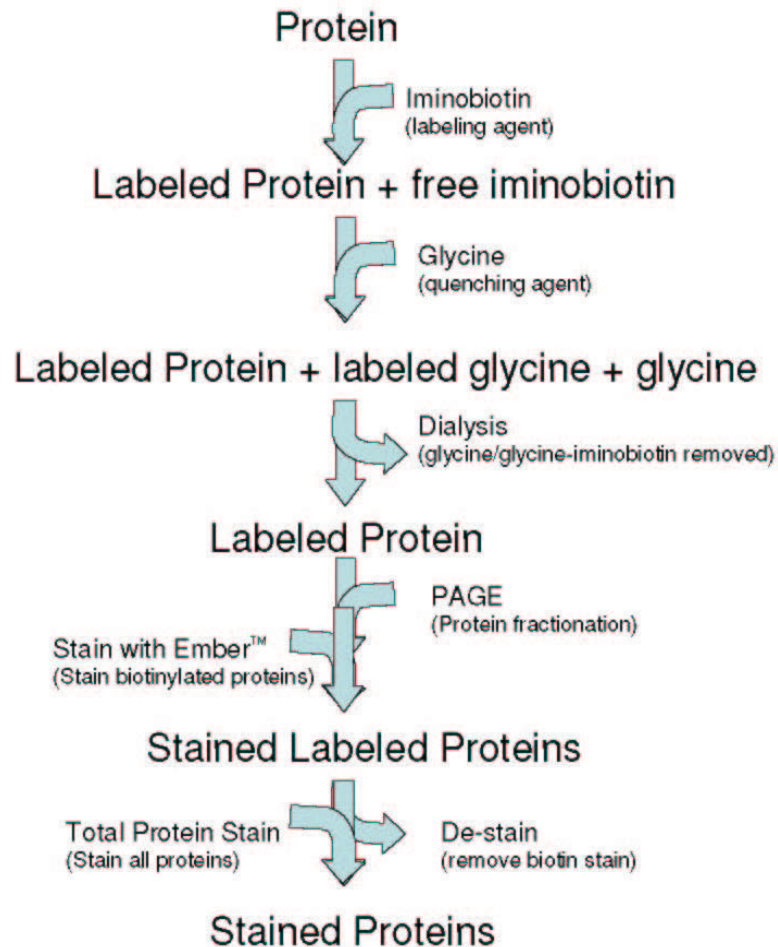
Optimization for your particular protein or peptide may be required, since the number of primary amine groups available for conjugation will vary.

### ***Protein Labeling with Iminobiotin***

The following procedure is applicable to the iminobiotinylation of most proteins, including antibodies and lectins.

1. Dissolve 1-2 mg of protein in 0.5-1 mL of primary amine-free buffer (pH 7.2-8.0)
2. Re-constitute one of the vials of NHS-Iminobiotin labeling reagent with 12  $\mu$ l of 99%+ analytical grade DMSO.
3. Allow the vacuum-dried reagent to reconstitute for 2-5 minutes, and then mix gently by pipetting the reagent up and down inside the vial.
4. Add 4-6  $\mu$ l of the NHS-Iminobiotin labeling reagent to the tube containing the protein.
5. Vortex the sample tube until it is totally dissolved.
6. Incubate for 2 hours at room temperature. Limited data has shown that the incubation may alternatively be performed at 2-8° C overnight.
7. After incubation, quench the labeling reaction by adding 0.1 mL of a 100 mM solution of glycine in 1X PBS.
8. If desired, any remaining unreacted biotin may be removed by using a commercially available low molecular weight cutoff dialysis cassette, such as a concentrator with 10 kDa molecular weight cut-off.

Iminobiotin labeling can be evaluated using PAGE by staining with Ember™ Biotin Binder (Section 4) followed by de-staining and total protein staining (Section 5).



## SECTION 2: IMINOBIOTINYLATION OF PLASMA MEMBRANES

Representative procedures for iminobiotinylation of plasma membrane proteins from adherent or suspension cell lines are presented. If desired, the restriction of labeling to the plasma membrane may be verified by fluorescence microscopy using fluorescein- or Alexa Fluor-488-conjugated streptavidin (See Figure 3 in Gauthier et al, 2004).

### ***Preparation of Adherent Cells for Labeling***

1. Remove the culture media from the tissue culture flask or dish.
2. Wash the cells in the flask with 1 mL of sterile Dulbecco's PBS for every 15 cm<sup>2</sup> of cell surface area (10 mL for T-150 flask, 5 ml for T-75 flask, etc.). Note: A T-150 flask of an adherent cell line at 60-70% confluence contains a cell count of approximately 5.0 x10<sup>6</sup> to 1.0 x10<sup>7</sup> cells per flask.
3. Repeat the wash step two more times. Monitor the flasks using an inverted light microscope after each wash step to assure that cells are still adherent.

### ***Preparation of Suspension Cells for Labeling***

Count the cells and measure their viability using Trypan Blue dye (Cell viability should be > 90%). Trypan Blue is a dye that is used to determine the viability of cells in tissue culture. Living cells exclude the dye, whereas dead cells will take up the blue dye. The blue stain is easily visible, and cells can readily be counted using an inverted light microscope.

1. Centrifuge the cells at 350 x g for 5 min at room temperature.
2. Wash the cells 3x with 4-5 mL of 37° C Dulbecco's PBS per 5.0x10<sup>6</sup> to 1.0x10<sup>7</sup> cells. Centrifuge at 350 x g.
3. Re-suspend the cells with 1 mL of Dulbecco's PBS per 5.0x10<sup>6</sup> to 1.0x10<sup>7</sup> cells. Count the total cells per mL and measure the cell viability after the last wash step.

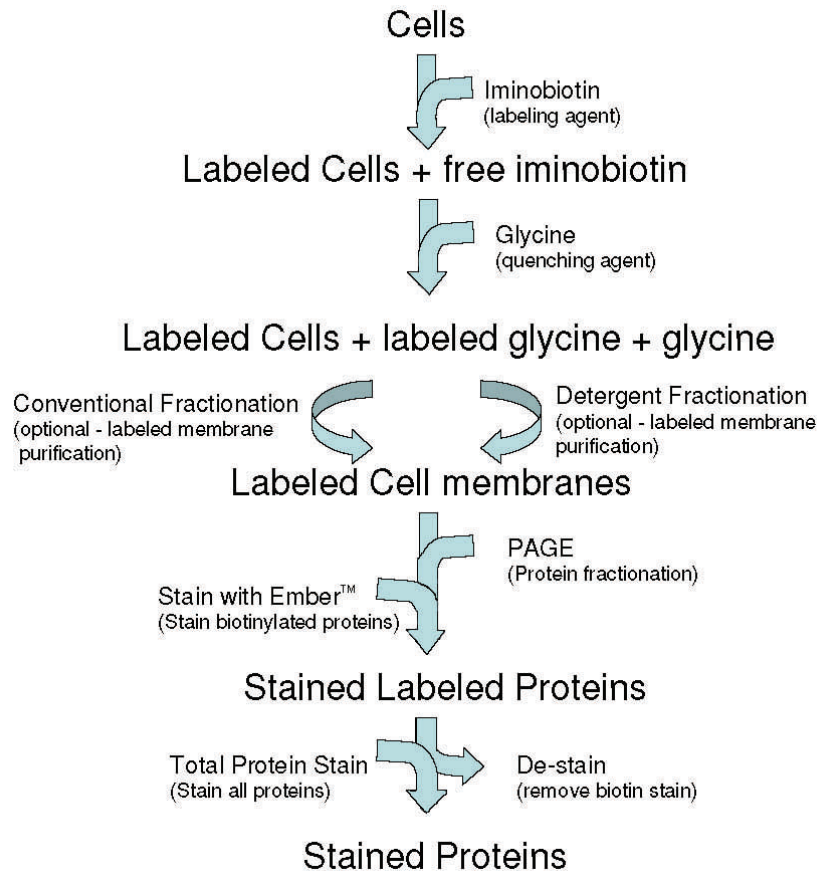
Note: An additional 1 mL of cells may be set aside to serve as an unlabeled "control sample".

### ***Iminobiotylation of Cell Surface Proteins***

**Note:** All cell work is done at room temperature.

1. Re-constitute one of the vials of NHS-Iminobiotin labeling reagent with 12 µl of 99%+ analytical grade DMSO.
2. Add 12 µl of DMSO reconstituted NHS-Iminobiotin labeling solution to 10 mL of Dulbecco's PBS in an appropriate size polypropylene test tube, adding 6 µl at a time, with mixing by inversion and vortexing until the iminobiotin is completely dissolved. A negative control labeling solution may also be prepared at this time by dissolving 12 µl of DMSO without NHS-iminobiotin in 10 mL of 1X PBS.
3. For an adherent cell line, add 1 mL of the PBS-diluted labeling solution directly to the flask for every 15 cm<sup>2</sup> of cell surface

- area (10 mL for T-150 flask, 5 mL for T-75 flask, etc.). Mix the flask by gently swirling the labeling solution in them. For a suspension cell line, add 10 mL of the diluted labeling solution per mL of cell suspension. Mix the tubes gently by inversion.
- Proceed with the labeling reaction in either the flasks (adherent cell line) or the centrifuge tubes (suspension cell line) for 1 hour at RT. The flasks or reaction tubes must be placed on a rocking platform shaker during labeling. The negative control for labeling should be processed in an identical manner.
  - After 1 hour, quench the iminobiotinylation reaction by adding 2 mL 0.1M solution of glycine in PBS per 10 mL of reaction mixture and gently mix.



### **SECTION 3: ENRICHMENT OPTIONS FOR LABELED PLASMA MEMBRANE PROTEINS**

Plasma membranes only represent roughly 2-5% of a total cell mass. Thus, while it is possible to analyze labeled cell lysates by PAGE directly after solubilization in electrophoresis buffer, it is advisable to enrich the plasma membrane proteins first, in order to enhance detection of the labeled proteins. Strategies for plasma membrane protein enrichment should be tailored to the experimental objectives.

#### ***Plasma Membrane Enrichment Strategies***

Conventional methods of subcellular fractionation such as differential centrifugation may be employed subsequent to labeling plasma membrane proteins with iminobiotin. Differential centrifugation combined with purification in sucrose, Percoll, metrizamide or Nycodenz gradients (rate zonal and isopycnic) are commonly used to isolate specific subcellular organelles, including plasma membrane proteins. Relatively large amounts of cells are needed for these procedures and separation of the organelle of interest typically requires 30 minutes to 2 hours. This separation time is generally too slow for monitoring dynamic signal transduction events in cells. The cytoskeletal compartment is lost using these procedures as microfilament, microtubule and intermediate filament networks are disassembled during the initial cell lysis step, prior to centrifugation. Centrifugation procedures are also notoriously difficult to automate, an important consideration in proteomics workflows.

Alternatively, iminobiotinylated plasma membrane proteins may be isolated directly by avidin- or streptavidin-based affinity capture. For example, streptavidin-agarose resin is suitable for isolating iminobiotinylated proteins. Typically, matrices consist of streptavidin covalently attached to 4% agarose beads through a 15-atom spacer arm. These matrices can bind 2- 8 mg of iminobiotinylated protein per mL of resin. The High Capacity Streptavidin Coated Plate (PerkinElmer Cat. # 6006625) is an especially convenient device for enriching the iminobiotinylated plasma membrane proteins. Since only the iminobiotinylated proteins are enriched by affinity capture approaches, it may be difficult to evaluate regulatory proteins associated with the plasma membrane on the cytoplasmic face of the plasma membrane using the affinity chromatography strategy.

Subsequent to labeling with 2-iminobiotin, eukaryotic cells may be fractionated into four distinct protein fractions: cytosolic, membrane/organelle, nuclear and cytoskeletal using a method that employs

detergents to sequentially extract proteins from small numbers of starting. With adherent cells, the sequential extraction is performed directly in the tissue culture dish, without removing the cells. At each step of the extraction procedure the insoluble cellular fractions remain attached to the plate, until the appropriate extraction reagent is applied. For suspension-grown cells, extraction starts with gentle sedimentation and washing of the cells. The stepwise extraction delivers all four protein fractions from a single specimen.

Fractionation by sequential extraction of cells or tissues with detergent-containing buffers allows partitioning of proteins into functionally distinct compartments that can readily be evaluated by PAGE. Unlike most other subcellular fractionation methods, detergent fractionation preserves the integrity of the cytoskeletal compartment. PerkinElmer offers Cellular Protein Fractionation kit (PerkinElmer Cat. # PRD101A001KT) that is suitable for this approach. Procedures for fractionating adherent and suspension cells are provided with the kit.

### ***Gel Electrophoresis***

Regardless of the enrichment approach utilized to recover the iminobiotinylated proteins, this kit is designed to permit detection of the iminobiotinylated proteins by PAGE. LC-based analysis of the iminobiotinylated proteins is also possible, but this approach will not take advantage of the fluorescence-based Ember™ Biotin Binder dye included in the kit. Prepare and run SDS-PAGE or 2-D PAGE according to standard protocols, per manufacturer's instructions. Since many plasma membrane proteins are relatively high molecular weight glycoproteins, it is advisable to employ 4-12% gradient PAGE gels to facilitate their resolution. Perform the selective staining of iminobiotinylated plasma membrane proteins with Ember™ Biotin Binder dye, using continuous, gentle agitation (e.g., on an orbital shaker at 50 rpm), as described below.

### **SECTION 4: STAINING WITH EMBER™ BIOTIN BINDER DYE**

PerkinElmer® Ember™ Biotin Binder dye selectively, but reversibly binds to iminobiotinylated proteins with high affinity. Staining is performed directly in PAGE gels, without the need for electroblotting proteins to nitrocellulose or polyvinylidene difluoride (PVDF) membranes. The optimized staining protocol for detection of iminobiotinylated proteins is as follows:

1. Clean and thoroughly rinse the staining dishes before use. Residual detergent in staining dishes will compromise the

detection of proteins. Polypropylene dishes, such as Rubbermaid® Servin' Saver™ containers, are optimal containers for staining because the high-density plastic adsorbs only a minimal amount of the dye. For small gels, circular staining dishes provide the best fluid dynamics on orbital shakers, resulting in less dye aggregation and better staining. Alternatively, the plastic lids of a pipette tip boxes make suitable staining dishes for mini-gels, if properly covered with aluminum foil. Clean and rinse the staining containers well before use, as detergent and other stains can interfere with staining. One may rinse the containers with ethanol just prior to use.

2. Fix the proteins in the gel with 50% isopropanol in water for 15 minutes. Pour the fixative solution into a small, clean plastic dish. Make sure the gel is totally submerged in the fixative.
3. Carefully remove the 50% isopropanol fixative.
4. Place the gel into a 1:100 dilution of the Ember™ Biotin Binder dye conjugate in diluted Gel Staining Buffer, pH 10.5 and stain the gel overnight. For one standard-size mini-gel, use ~80 mL of stain. Protect the gel and staining solution from light at all times by covering the container with a lid or with aluminum foil. Make sure the gel is totally submerged and gently agitate in staining solution at room temperature (50 RPMs on an orbital shaker) overnight.
5. Carefully remove the Ember™ Biotin Binder dye solution after overnight staining.
6. Wash the gels twice for 15 min each with 60-80 ml of diluted Gel Staining Buffer, pH 10.5 using gentle agitation. Note that no dye is added at this point. This step simply removes excess dye from the gel using the Gel Staining Buffer. Make sure the gel is totally submerged in the buffer.
7. Images should be acquired as soon as possible after staining. Always store gels in the dark to prevent photo bleaching. When gels are stored in the staining solution, the signal may decrease somewhat after several days; however, depending upon the amount of protein in the bands of interest, gels may retain a usable signal for many weeks. Gels may be dried between sheets of cellophane, although there is sometimes a slight decrease in sensitivity.
8. Image the stained gel on a laser-based gel scanner equipped with a 532 nm YAG laser and PMT. Alternatively, use a xenon arc lamp-based or green LED-based CCD-camera gel imager equipped with an appropriate green fluorescent excitation filter and red fluorescent emission filter (optimal wavelengths below). A mid-range UV transillumination-based gel imaging system

may also be used to detect Ember™ Biotin Binder dye, but with poorer detection sensitivity compared with the cited visible light imaging configurations. Because of the lower fluorescence signal obtained by UV illumination, it is recommended to take advantage of the integrating capability of a film or CCD camera and use long exposure times to improve the sensitivity of detection. This often makes bands detectable that are not visible to the naked eye when viewing gels on a UV light box. Images are best obtained by digitizing at about 1024 × 1024 pixels resolution with 12- or 16-bit gray scale levels per pixel. Ember™ Biotin Binder dye absorbs maximally at 540 nm and emits maximally at 590 nm. Contact the gel imager manufacturer for recommendations on appropriate filter sets. Generally, filter sets appropriate for Pro-Q® Diamond phosphoprotein gel stain or cyanine-3 dye are appropriate for imaging Ember™ Biotin Binder dye (Figure 3). PMT or CCD camera-based image analysis systems can gather quantitative information that will allow comparison of fluorescence intensities among different bands or spots. It is recommended that images be saved as .jpg and 16 bit .tiff files.

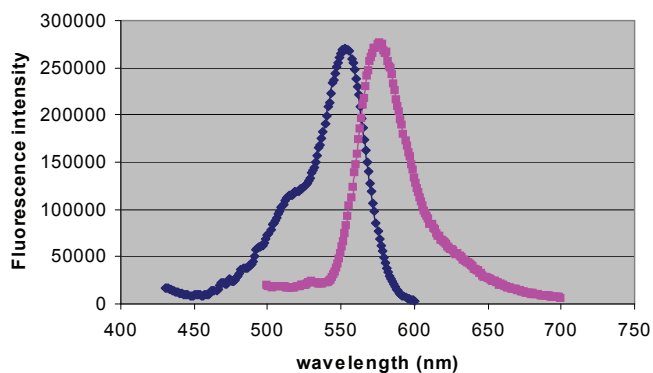
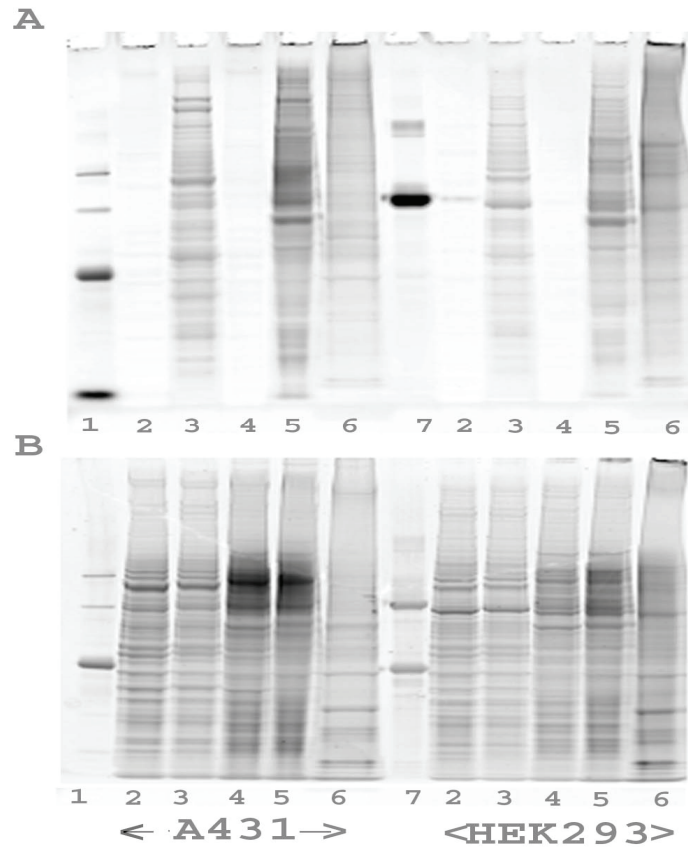


Figure 3. **Excitation (540 nm) and Emission (590 nm) spectra of Ember™ Biotin Binder dye.**



**Figure 4. SDS PAGE Patterns of Subcellular Fractions of Cells Labeled with Iminobiotin and Stained with Ember™ Biotin Binder Dye (Top Figure A) and for Total Protein with SYPRO® Ruby protein gel stain. (Bottom Figure B).** A431 cells (adherent) and HEK293 (loosely-adherent) cells were labeled for 1 hour at RT and fractionated using the Cellular Protein Fractionation kit (PerkinElmer Cat. # PRD101A001KT). The cytoskeletal fraction was treated with DNase and RNase prior to dissolving in hot SDS. Lane 1. Biotinylated Molecular Weight Markers (PerkinElmer Cat. # NEL310) 200 ng loaded; Lane 2. Cytosolic fraction: 1 hr DMSO only; Lane 3. Cytosolic fraction: 1 hr iminobiotinylation; Lane 4. Membrane fraction: 1 hr DMSO only; Lane 5. Membrane fraction: 1 hr iminobiotinylation; Lane 6. Cytoskeletal fraction: from labeled cell preparation; Lane 7. Positive Controls and Negative Controls.

### Quantitation of Signal from Staining

Integrated fluorescent signal intensity, which is proportional to the amount of labeled protein in the band, can be quantified using standard image analysis software using the saved 16 bit tiff image files. Ember™ Biotin Binder dye exhibits a high linear correlation between the base-10 logarithm of signal (RFU) and the base-10 logarithm of the protein (BSA) concentration over a 2-3 log unit range (Figure 5). Limits of detection are typically in the 1-2 ng range.

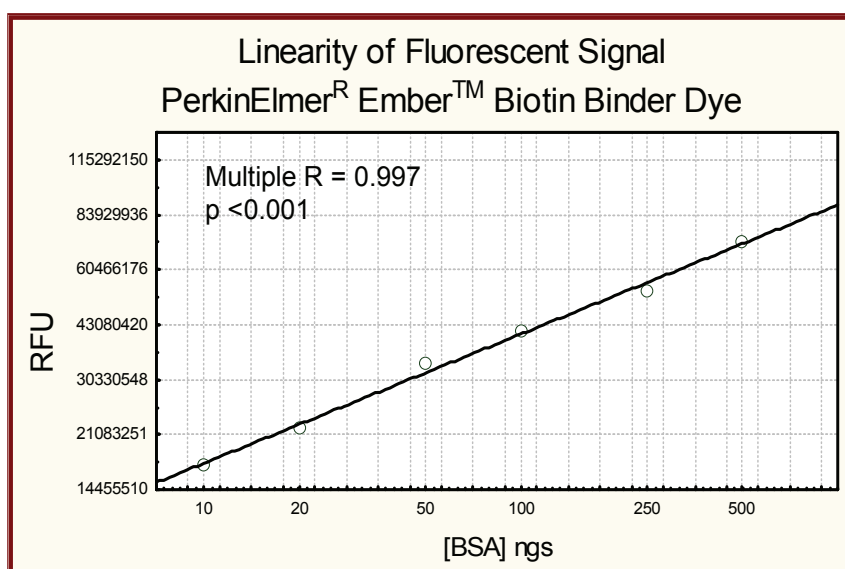


Figure 5. Linearity of Fluorescent Signal of the Ember™ Biotin Binder dye.

### SECTION 5: DE-STAINING

De-staining Ember™ Biotin Binder dye allows the PAGE gels to be subsequently re-stained for total protein, eliminating the need to run two gels, one for iminobiotin-labeled proteins and one for the total protein control. In this instance, the entire mini-gel is typically de-stained. In addition, de-staining is needed in order to facilitate analysis of proteins by mass spectrometry. Selected bands of interest may be excised from the gel using a suitable cutting device, such as a scalpel or robotic spot-cutting instrument.

1. Prepare a 1:5 dilution of Gel De-Staining Solution A Concentrate, pH 4.0, in purified water.
2. Add 1 volume of Gel De-Staining Solution B Concentrate to 99 volumes of this diluted De-Staining Solution.
3. Incubate the stained gel or gel segment in this solution overnight at room temperature using a rocking shaker platform.
4. De-staining can be verified by re-imaging the gel using the same settings as used to obtain the original image of the gel stained with the Ember™ Biotin Binder dye (Figure 6).
5. For gel slices, wash the gel segment briefly with water before trypsinization to prepare peptides for MS analysis.

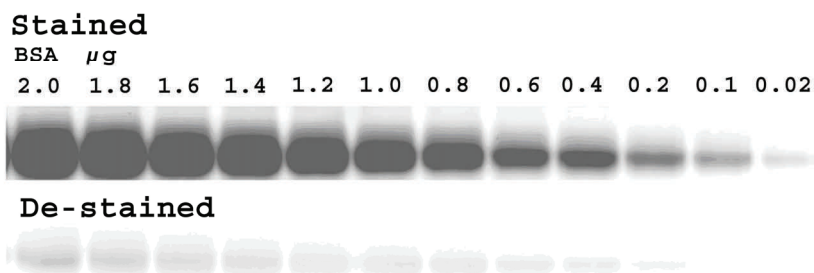


Figure 6. **Extent of Ember™ Biotin Binder dye de-staining.** An image of a PAGE gel with decreasing concentrations of iminobiotinylated BSA separated by PAGE and stained using Ember™ Biotin Binder dye. The same gel was de-stained according to the protocol above and re-imaged (bottom) with the same imager settings.

#### RE-STAINING WITH A TOTAL PROTEIN STAIN

After de-staining the Ember™ Biotin Binder dye, proteins may be re-stained using a total protein stain such as SYPRO® Ruby protein gel stain, silver stain or colloidal Coomassie™ Blue stain. No special precautions are required when post-staining a gel that has previously been stained with Ember™ Biotin Binder dye and then de-stained. Staining for total protein is accomplished by standard methods, according to the stain manufacturer's recommendations. This staining step will allow the visualization of all proteins present, whether iminobiotin-labeled or not. The staining and de-staining procedure does not affect the proteins in the gel (negligible protein loss), allowing

a single gel to be used for detecting iminobiotin labeling and for total protein visualization.

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## **LICENSING**

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**APPENDIX**  
***Troubleshooting***

<b>Problem</b>	<b>Potential Causes and Remedies</b>
<p>No or poor labeling of the proteins.</p>	<ul style="list-style-type: none"> <li>• Inappropriate choice of labeling buffer. Choose an amine-free labeling buffer. Dialyze or desalt the sample into an amine-free buffer at pH 7-9.</li> <li>• Hydrolysis of iminobiotinylation reagent has occurred. Make up fresh labeling reagent and use immediately for labeling.</li> <li>• Insufficient number of primary amines is present on target proteins. Consider converting protein sulfhydryl residues to primary amines using aminoethyl-8 reagent (N-(iodoethyl) trifluoroacetamide) prior to labeling.</li> </ul>
<p>Labeling is not confined to the plasma membrane.</p>	<ul style="list-style-type: none"> <li>• Only label cells demonstrated to display &gt; 90% viability by Trypan Blue Viability assay. (Test for viability prior to and after labeling, then optimize accordingly).</li> <li>• Reduce the time or temperature used for the labeling reaction.</li> </ul>
<p>No or only faint iminobiotinylated bands are detected on the stained gels.</p>	<ul style="list-style-type: none"> <li>• Incorrect pH value for the 1X Gel Staining Buffer was used. Be sure the pH value for the diluted Gel Staining Buffer is adjusted to 10.5.</li> <li>• Insufficient protein was loaded. Verify sufficient protein was applied per lane using a total protein gel stain. (Typically 20-1000 ng of a complex protein sample should be loaded per lane).</li> <li>• Ember™ Biotin Binder dye was diluted excessively. Ensure that you use the stain at the recommended 1:100 dilution. Greater dilution will result in lower fluorescence intensity.</li> <li>• Reconstituted Ember™ Biotin Binder stain was reused to stain additional gels. If used immediately, the staining solution may be re-used once or twice, but detection sensitivity may decline somewhat. Storage of reconstituted stain is not recommended.</li> </ul>

	<ul style="list-style-type: none"> <li>• Failure of detection reagents has occurred. Check expiration dates on staining solutions. Prepare fresh staining solutions.</li> <li>• Reconstituted Ember™ Biotin Binder dye staining solution was heated before or during staining. Never heat the fluorescent stain above 40° C. Do not microwave the stain.</li> <li>• Imaging instrument is not correctly configured. Verify that the imaging instrument is equipped with correct excitation/emission filters, PMT setting and light source for detecting Ember™ Biotin Binder dye.</li> <li>• Imaging system is malfunctioning. Check the instrument manual for troubleshooting.</li> </ul>
<p>High background or non-specific staining is observed on gels.</p>	<ul style="list-style-type: none"> <li>• Residual SDS is interfering with staining. Fix the gel with 50% isopropanol and rehydrate with deionized water, according to the staining protocol.</li> <li>• Ineffective washing after staining due to poor mixing or an improper sized tray. Use sufficient stain and perform staining on a rotary shaker to ensure proper fluid dynamics</li> </ul>
<p>Irregular or non-uniform staining is observed on gels.</p>	<ul style="list-style-type: none"> <li>• Gel is not completely covered with stain or is folded onto itself. Ensure that the gel is completely submerged with enough staining solution, using containers that are large enough so that the gel can move freely in the solution during agitation.</li> <li>• More than one gel is being stained per container. Only stain one gel per container.</li> <li>• Ineffective washing after staining, due to poor mixing or an improper sized tray. Use sufficient stain and perform staining on a rotary shaker to ensure proper fluid dynamics.</li> </ul>

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