Chapter 14

Site-Selective Enzymatic Labeling of Designed Ankyrin Repeat Proteins Using Protein Farnesyltransferase

Yi Zhang, Shelby Auger, Jonas V. Schaefer, Andreas Plückthun, and Mark D. Distefano

Abstract

Affinity agents coupled to a functional moiety play an ever-increasing role in modern medicine, ranging from radiolabeled selective binders in diagnosis to antibody–drug conjugates in targeted therapies. In biomedical research, protein coupling to fluorophores, surfaces and nanoparticles has become an integral part of many procedures. In addition to antibodies, small scaffold proteins with similar target binding properties are being widely explored as alternative targeting moieties. To label these binders of interest with different functional moieties, conventional chemical coupling methods can be employed, but often result in heterogeneously modified protein products. In contrast, enzymatic labeling methods are highly site-specific and efficient. Protein farnesyltransferase (PFTase) catalyzes the transfer of an isoprenoid moiety from farnesyl diphosphate (FPP) to a cysteine residue in a C-terminal CaaX motif at the C-terminus of a protein substrate. The addition of only four amino acid residues minimizes the influence on the native protein structure. In addition, a variety of isoprenoid analogs containing different bioorthogonal functional groups, including azides, alkynes, and aldehydes, have been developed to enable conjugation to various cargos after being incorporated onto the target protein by PFTase. In this protocol, we present a detailed procedure for labeling Designed Ankyrin Repeat Proteins (DARPins) engineered with a C-terminal CVIA sequence using an azide-containing FPP analog by yeast PFTase (yPFTase). In addition, procedures to subsequently conjugate the labeled DARPins to a TAMRA fluorophore using strained-promoted alkyne–azide cycloaddition (SPAAC) reactions as well as the sample preparation to evaluate the target binding ability of the conjugates by flow cytometry are described.

Key words Enzymatic protein labeling, Protein farnesyltransferase, Isoprenoid analogs, Site-specific conjugation, DARPins, SPACC, Flow cytometry

1 Introduction

With the rapid development of targeted therapeutics and diagnostic agents in modern medicine, and the large number of procedures in biomedical research requiring specific binding reagents, there is an increasing demand for protein-based conjugates. To label a protein of interest, conventional chemical modification methods exploit the side-chain functional groups of certain amino acids. Two of

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the commonly targeted residues include cysteines that can undergo Michael-type addition with maleimides [1] and lysines reacting with *N*-hydroxylsuccinimide esters [2]. However, due to the lack of specificity, when multiple copies of the same amino acid are present on the target protein, which often is the case for lysine residues, a heterogeneously labeled product is obtained. As to reactions with maleimides, although they are highly specific toward cysteine residues under acidic and neutral conditions, alkylation of primary amines, such as the side chain amines in the lysine residues, can also occur at a higher pH (pH > 8) [3] or excess reaction time, resulting in a mixture of differently modified proteins. While such complex compositions obviously complicate the characterization, an uncontrolled conjugation can also result in reduced efficacy of therapeutic proteins compared to counterparts modified using site-specific labeling methods [4].

To reliably achieve site-specific conjugation, enzymatic labeling methods have been developed and are widely utilized [5]. Since most of the enzymes employed for protein labeling have a preferred recognition sequence [5], the modification site as well as the labeling stoichiometry can be precisely controlled. In addition, reactions catalyzed by enzymes occur under mild conditions with fast rates. To date, several enzymes have been employed for protein labeling purpose, including sortase A [6, 7], transglutaminase [8, 9], protein farnesyltransferase (PFTase) [10, 11], and tubulin tyrosine ligase [12] as well as related methods such as expressed protein ligation [13].

PFTase catalyzes the transfer of the isoprenoid unit from farnesyl diphosphate (FPP) to a cysteine residue near the C-terminus of the protein substrate, resulting in thioether bond formation. The enzyme recognition sequence is composed of a C-terminal CaaX motif, where "C" is a cysteine residue, "a" represents aliphatic amino acids, and "X" determines whether the protein is a substrate for PFTase (X = Ala, Ser, or Met) or geranylgeranyltransferase type I (X = Leu, Ile, or Phe) [14]. Since the enzyme is highly selective toward its recognition sequence, only the cysteine residue within the CaaX motif will be modified even in the presence of other cysteines in the protein of interest. It has been shown that the incorporation of the CaaX motif, such as the CVIA sequence which is found in a naturally prenylated peptide [15], onto the C-terminus of a given protein of interest makes it a substrate for PFTase [16, 17]. The addition of only four amino acids minimizes potential perturbation to the structure and function of the genetically engineered target proteins. While the PFTase active site cannot accommodate large moieties such as biotin, drugs or fluorophores (although it can be modified to do so via protein engineering [18, 19]), the enzyme does accept smaller modifications of the isoprenoid substrate. To apply the PFTase reaction for protein modification, our laboratory and others have developed a

variety of isoprenoid analogs containing different bioorthogonal functional groups, including azides [20, 21], alkynes [22, 23], and aldehydes [10, 24]. These analogs can be efficiently incorporated to targeted proteins bearing a C-terminal CaaX motif, enabling subsequent conjugation for different applications.

Using the PFTase labeling strategy, protein conjugation to various functionalities has been achieved, including fluorophores [10], DNA oligonucleotides [25], PEG polymers [26], and solid surfaces [27]. More recently, protein–drug conjugates created via the PFTase labeling method have also been reported as potential targeted therapeutics [28]: a repebody was engineered with a C-terminal CVIM sequence, enzymatically labeled with a ketone functional group by PFTase and subsequently reacted with aminooxy-functionalized monomethyl auristatin F (MMAF) to yield a stable and homogeneous protein–drug conjugate with anti-tumor activities in vivo.

As a robust and general labeling method, we applied the PFTase enzyme to modify Designed Ankyrin Repeat Proteins (DARPins) (Fig. 1) which can be selected in vitro for different target binding with high affinity and specificity and that have been used for a variety of applications [29]. As a class of small binding protein scaffolds, DARPins are extremely stable, can easily be engineered as well as expressed in *E. coli* in high yield [30].

In this protocol, we describe in detail the procedure to label DARPins bearing an engineered C-terminal CVIA sequence with an azide-containing isoprenoid analog using PFTase. The subsequent conjugation of the azide-modified DARPins to a dibenzocyclooctyne (DBCO)-functionalized fluorophore is also explained. Finally, we present the sample preparation procedure to examine the target binding ability of the DARPin-fluorophore conjugates by flow cytometry using an epithelial cell adhesion molecule (EpCAM)-binding DARPin [31] as an example.

2 Materials

2.1 Reagents and Buffers for Protein Labeling

1. DARPin engineered with a C-terminal CVIA sequence, stored in PBS_{400} supplemented with 10% glycerol.
2. C10-Azi analog **1** (Fig. 1). The synthesis procedure has been previously published [32]. Analog **1** is stored in 25 mM NH_4HCO_3 solution with concentrations typically ranging from 1 mM to 10 mM.
3. Yeast farnesyltransferase (yPFTase). The expression and purification methods have been previously published [18]. The yPFTase is stored in a storage buffer (25 mM Tris–HCl, pH 7.5, 100 mM·NaCl, 2.5 μM·$ZnCl_2$, 2.5 mM·$MgCl_2$, 1 mM β-mercaptoethanol, 50% glycerol (v/v)) with concentrations typically ranging from 200 μM to 500 μM.

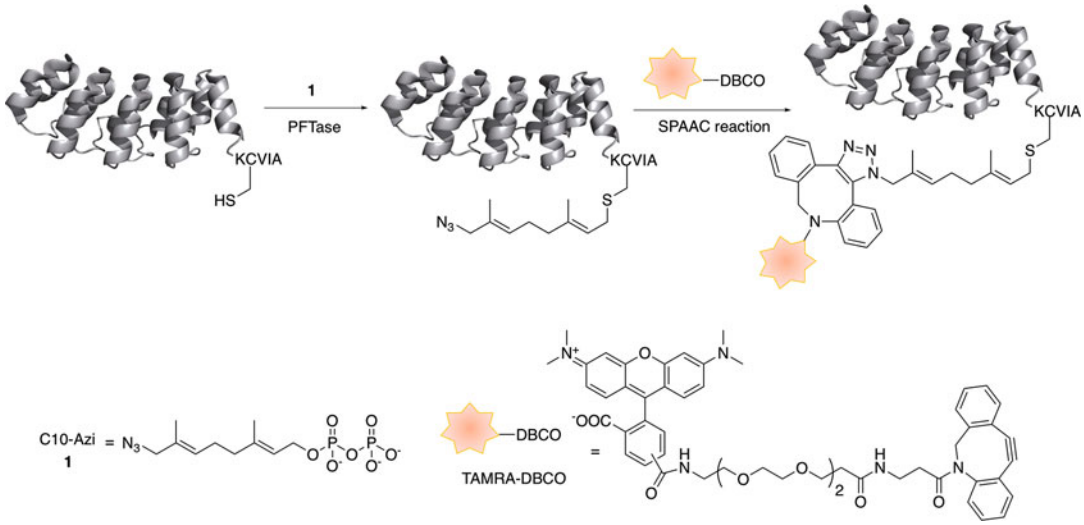

Fig. 1 Schematic summary of the enzymatic labeling of DARPins engineered with a C-terminal CVIA sequence to C10-Azi analog **1** by using PFTase and subsequent conjugation with DBCO-TAMRA using the SPAAC reaction

4. Bradford assay dye (Bio-Rad).
5. TAMRA-DBCO (Click Chemistry Tools LLC.).
6. Prenylation buffer (6×): 300 mM Tris–HCl, pH 7.5, 120 mM·KCl, 60 mM $MgCl_2$, 30 mM dithiothreitol (DTT), 60 μM·$ZnCl_2$ (*see* **Note 1**).
7. Phosphate-buffered saline (1× PBS): 8.1 mM·Na_2HPO_4, 1.5 mM·KH_2PO_4, pH 7.4, 137 mM·NaCl, 2.7 mM·KCl.
8. PBS_{400}: 1× PBS, 400 mM NaCl (total NaCl concentration).
9. Buffers for LC-MS analysis. Buffer A: H_2O with 0.1% formic acid (FA); Buffer B: acetonitrile with 0.1% FA.
10. 4× Laemmli loading buffer: 8% (w/v) sodium dodecyl sulfate, 40% (v/v) glycerol, 0.04% (w/v) bromophenol blue, 200 mM Tris–HCl, pH 6.8 (no reducing agent present).
11. 0.5 M DTT (*see* **Note 1**).
12. Molecular weight ladder (e.g. precision plus protein dual color standards (Bio-Rad)).
13. Coomassie Blue stain solution: 0.2% (w/v) Coomassie Brilliant Blue R250, 45% (v/v) H_2O, 45% (v/v) methanol, 10% (v/v) acetic acid.
14. Destaining buffer: 60% H_2O, 30% methanol and 10% acetic acid (v/v).
15. PBSA buffer: 1× PBS containing 1 mg/mL bovine serum albumin (BSA).

2.2 Reagents and Buffers for Mammalian Cell Cultures

1. DMEM: Dulbecco's Modified Eagle's Medium.
2. FBS: fetal bovine serum.
3. Pen/Strep: 10,000 U penicillin, 10 mg/mL streptomycin.
4. 10× trypsin (2.5%), without EDTA.
5. Versene buffer: 1× PBS, 0.6 mM EDTA.

2.3 Instruments

1. Amicon Ultra centrifugal filters (0.5 mL, 3 kDa molecular weight cut off, Millipore Sigma).
2. NAP-5 columns (GE Healthcare).
3. Isotemp water bath.
4. Microcentrifuge.
5. Microplate reader.
6. UV-Vis spectrometer.
7. Fluorescence scanner: Typhoon FLA 9500 with 532 laser and LPG (575 LP) filter.
8. LC-MS instrument: Agilent 1100 Series LC/MSD trap SL (equipped with a UV-Vis detector); column: Zorbax 300 SB-C8 capillary 0.3 × 100 mm, 3.5 μm (Agilent); and sample vials: interlocked vials with 300 μL fused glass insert (Chrome Tech).
9. CO_2 incubator, microscope and biosafety hood.
10. Flow cytometer (equipped with 561 nm laser): BD LSRFortessa H0081.

3 Methods

3.1 Enzymatic Labeling of DARPin with C10-Azi Analog 1

The following protocol describes a 2 mL-scale prenylation reaction.

1. Reduction: Prepare the reduction reaction in a 5 mL conical tube. Dilute the DARPin engineered with a C-terminal CVIA sequence to 15 μM in prenylation buffer (6×), which contains DTT. Incubate the tube on ice for 30 min. After the reduction reaction, dilute the reaction mixture 6× by adding 1.65 mL of H_2O. This will result in a 2 mL substrate mixture containing 2.5 μM of reduced DARPin (*see* **Note 2**).
2. Prenylation: Add analog **1** (15 μM final concentration, *see* **Note 3**) and yPFTase (300 nM final concentration, *see* **Notes 4–6**) to initiate the reaction. Incubate the tube in a 32 °C water bath for 6 h (*see* **Note 7**).
3. Analyze the product by injecting the reaction mixture directly into LC-MS without prior purification to confirm the formation of the desired product (*see* an example in Fig. 2b) *see* Subheading 3.4 for detailed LC-MS method.

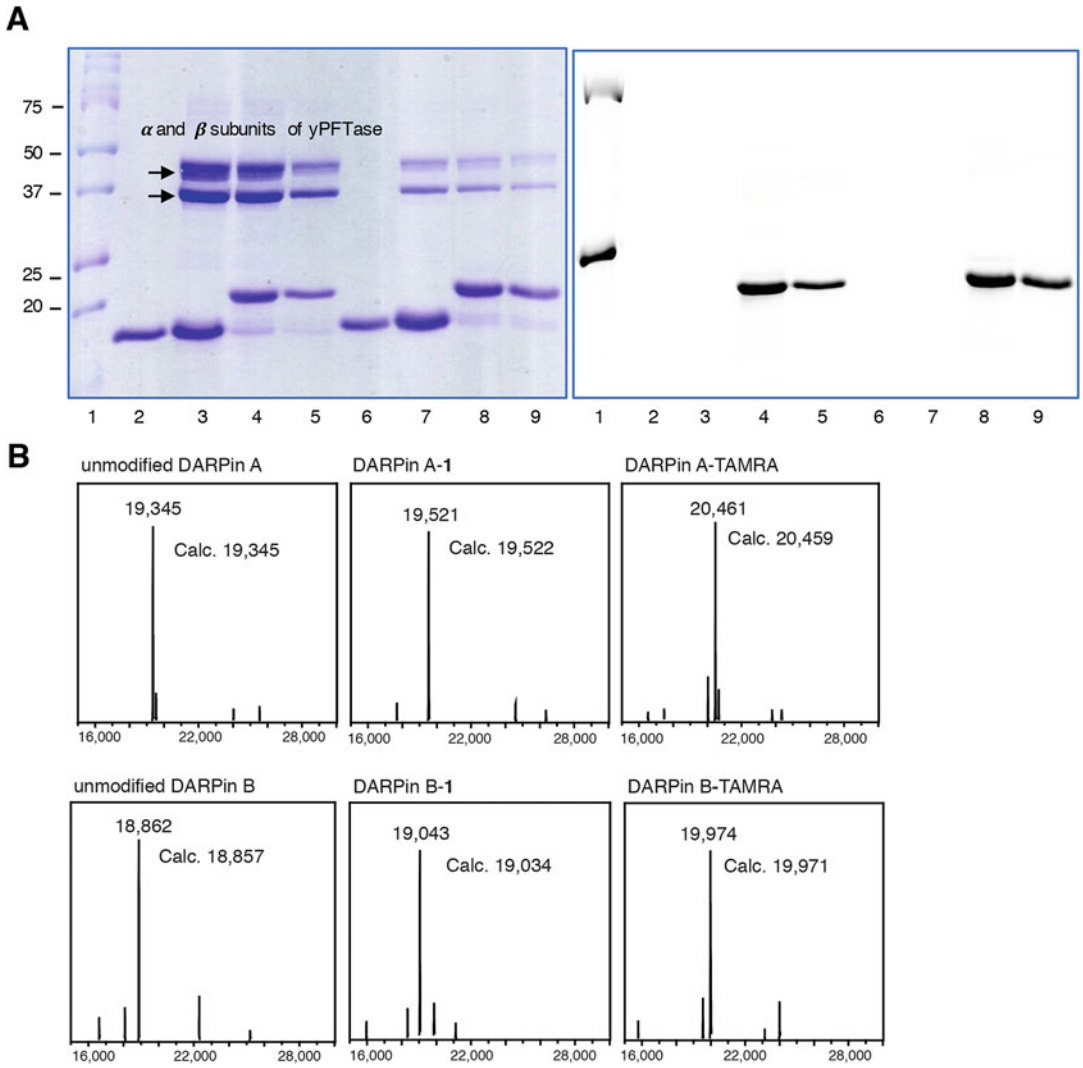

Fig. 2 (**a**) Coomassie-stained gel from SDS-PAGE analysis (left) and in-gel fluorescence imaging (right) of two azide-modified DARPin variants A-**1** and B-**1** and DARPin-TAMRA conjugates A-TAMRA and B-TAMRA. Lane 1: Precision plus protein dual color standards. Lane 2: unmodified DARPin B. Lane 3: DARPin B-**1** prenylation mixture. Lane 4: DARPin B-TAMRA reaction mixture. Lane 5: DARPin B-TAMRA subjected to partial purification (buffer exchange and removal of TAMRA reagent excess) using a NAP-5 column. Lane 6: unmodified DARPin A. Lane 7: DARPin A-**1** prenylation mixture. Lane 8: DARPin A-TAMRA reaction mixture. Lane 9: DARPin A-TAMRA partially purified with NAP-5 column (buffer exchange and removal of TAMRA reagent excess). The fluorescence band from lane 4/5 and lane 8/9 confirmed successful conjugation. It should be noted that the yPFTase concentration in DARPin B labeling was increased from 300 nM to 800 nM to ensure efficient modification. (**b**) ESI-MS of the unmodified DARPins, azide-modified DARPins, and the DARPin-TAMRA conjugates. The observed masses closely match to the calculated values, suggesting successful modifications. The enzymatic prenylation efficiency was quantified based on the absorbance chromatogram at 280 nm obtained in the LC-MS analysis by comparing the peak areas for the unmodified DARPin and the DARPin-**1**. For DARPin A protein, labeling efficiency ranges from 85% to 90% using 300 nM to 500 nM yPFTase. The prenylation for DARPin B protein is less efficient with conversion around 70% using 500 nM yPFTase. Concerning the SPAAC reactions, when both DARPin A-TAMRA and DARPin B-TAMRA conjugates were subjected to LC-MS analysis, no mass corresponding to the unreacted DARPin A-**1** or DARPin B-**1** conjugates could be detected, suggesting high reaction yield for the SPAAC reactions

4. To concentrate the solution, load 500 μL of the reaction mixture into a 0.5 mL centrifugal filter (3 kDa cutoff) and centrifuge at 13,000 × *g* for 5 min. Refill the centrifugal filter with another 300 μL of the reaction mixture and centrifuge for 5 min. Repeat this procedure until all the reaction mixture solution is concentrated. Concentrate the final solution to approximately 100 μL.
5. To remove excess isoprenoid analogs present in the concentrated solution, wash the centrifugal filter with 400 μL PBS_{400} buffer and centrifuge for 5 min at 13,000 × *g*. Repeat this procedure two more times. Concentrate the final solution to approximately 100 μL.
6. To determine the concentration of the azide-modified DARPin (DARPin-**1**), several steps are employed (*see* **Note 8**).
   (a) Measure the total protein concentration (total protein in mg/mL) in the solution using a Bradford assay with a microplate reader.
   (b) Calculate the theoretical mass ratio of the DARPin protein to the total amount of protein present in the prenylation reaction (**step 2**) based on the amounts of proteins added: R = [mass of DARPin]/([mass of DARPin] + [mass of yPFTase]). Note that the molecular masses of DARPin and yPFTase are approximately 20 and 80 kDa, respectively.
   (c) Determine the prenylation efficiency (E) by comparing the peak area of UV absorption at 280 nm for the azide-modified DARPin and the unreacted DARPin from LC-MS analyses (*see* an example in Fig. 3).
   (d) Calculate the concentration of DARPin-**1** conjugate:

$$[\text{DARPin-}\mathbf{1}] = [\text{total protein}] \times R \times E.$$

3.2 Construction of DARPin-Fluorophore Conjugates Using SPAAC Reaction

1. Prepare a 10 mM stock solution of DBCO-TAMRA in DMSO.
2. To the resulting DARPin-**1** solution (100 μL) in **step 5** of Subheading 3.1 (*see* **Note 9**), add a fivefold (molar ratio) excess of DBCO-TAMRA based on the calculated concentration of DARPin-**1** conjugate.
3. Cover the reaction tube with aluminum foil and incubate it at room temperature for 3 h.
4. To remove excess fluorophore, load the reaction mixture (100 μL) onto a NAP-5 column (*see* **Note 10**), pre-equilibrated with 1× PBS. Add 400 μL of 1× PBS and then elute the proteins with 500 μL of 1× PBS.
5. Analyze the mass of the DARPin-TAMRA conjugates by LC-MS to verify the formation of the desired product.

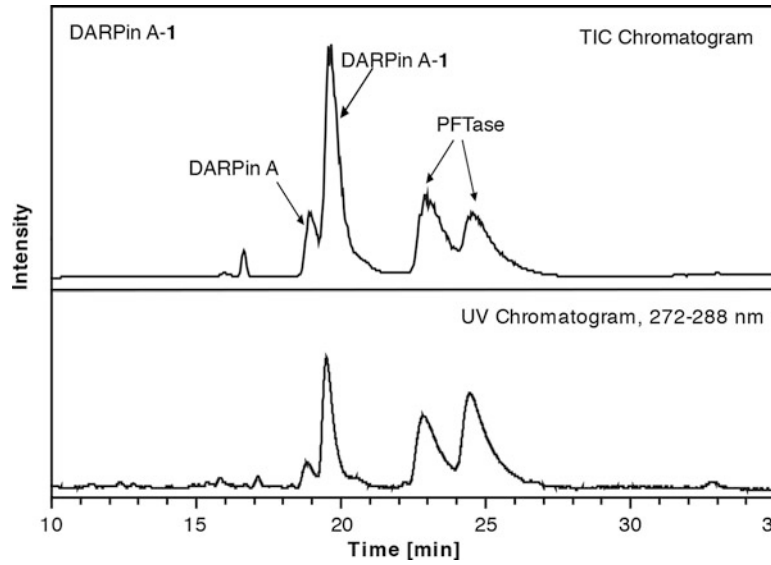

Fig. 3 Total ion chromatogram (TIC) and UV chromatogram of DARPin A-**1** conjugates. The prenylation efficiency estimated from UV chromatogram is approximately 85%

6. Determine the concentration of the DARPin-TAMRA conjugates by measuring the absorbance of the solution in 1× PBS at 555 nm using a UV-Vis spectrophotometer. (ε_{555} = 65,000 $M^{-1} \cdot cm^{-1}$).

3.3 Procedure for Characterization of DARPin-TAMRA Conjugates by SDS-PAGE and In-Gel Fluorescence Imaging

1. Based on the concentration of DARPin-TAMRA conjugates, calculate the volume for 3 μg of conjugates. If the volume is smaller than 10 μL, add H_2O to reach 10 μL.
2. Aliquot the desired amount of DARPin-TAMRA into an Eppendorf tube. Add 5 μL of 4× Laemmli loading buffer and 5 μL of 0.5 M DTT to reach a final volume of 20 μL.
3. Heat the samples at 95 °C for 10 min and load onto a 15% acrylamide (3″ × 4″) SDS-PAGE gel for electrophoresis at a constant voltage of 120 V.
4. Before staining the gel with Coomassie Blue, scan the gel for TAMRA fluorescence (553/575 nm excitation/emission).
5. Stain the gel with Coomassie Blue stain solution for 20 min, followed by destaining in destaining buffer for 2 to 4 h (*see* an example in Fig. 2a).

3.4 Procedure for LC-MS Analysis of Protein Samples

1. Dilute the protein samples to be analyzed to 3 μM (*see* **Note 11**) and centrifuge at 10,000 × *g* for 2 min to remove any precipitate.
2. Aliquot 20 μL of solution into sample vials. Inject 8 μL for LC-MS analysis.

3. LC gradient for LC-MS analysis: 0 to 2 min, 10% buffer B; 2 to 23 min, 10 to 70% buffer B; 23 to 25 min, 70 to 90% buffer B; 25 to 35 min, 90% buffer B. Flow rate: 9 μL/min. UV-Vis detection: 280 and 555 nm.

3.5 Procedure for Sample Preparation for Flow Cytometry Analysis to Examine DARPin Binding to Cell Surface EpCAM

1. Prepare TAMRA conjugates of the EpCAM-binding DARPin (DARPin A-TAMRA) as well as of a control DARPin (not binding to any target, DARPin B-TAMRA) using the methods described above.
2. Culture MCF-7 (EpCAM overexpressing cells) and U87-MG cells (EpCAM negative) in DMEM media supplied with 10% FBS and 1% Pen/Strep. Incubate the cells at 37 °C in a moisture-controlled environment with a supply of 4% CO_2.
3. When the cells reach 80% confluence, detach the cells using 1× trypsin solution (diluted in Versene buffer from 10× stock). Count and aliquot 400,000 cells into separate vials (a total of four vials for MCF-7 cells and three vials for U87-MG cells, *see* **Note 12**). These samples should be kept on ice at all times.
4. Centrifuge the cells at 300 × *g* for 3 min. Discard the media. Wash the cells by resuspending in 1 mL of ice-cold PBSA, followed by centrifugation to remove the supernatant.
5. Add 400 μL of the sample solution to each vial so that the concentration of cells is 1×10^6 cells/mL. For the control sample with no conjugate treatment, add PBSA. This sample is used to measure the background fluorescence for each cell line. For the positive binding sample, add 400 μL of 100 nM DARPin A-TAMRA (diluted in PBSA). For the control binding sample, add 400 μL of 100 nM DARPin B-TAMRA (diluted in PBSA).
6. Incubate the cells at 4 °C on a rotary shaker in the dark for 45 min.
7. For blocking experiments with MCF-7 cells (*see* **Note 12**), first incubate the cells in 200 μL of 2 μM unlabeled DARPin A at 4 °C on a rotary shaker in dark. After 15 min, add 200 μL of 200 nM DARPin A-TAMRA for an additional 30 min incubation.
8. Wash the cells with 1 mL ice-cold PBSA and resuspend them in 400 μL PBSA for flow cytometry analysis (*see* an example in Fig. 4).

4 Notes

1. To maintain the reducing ability of DTT, a stock solution of 0.5 M in H_2O is prepared, aliquoted into small volumes (30 μL) and stored at −20 °C. Thaw individual aliquots when needed and discard the tubes after one-time usage.

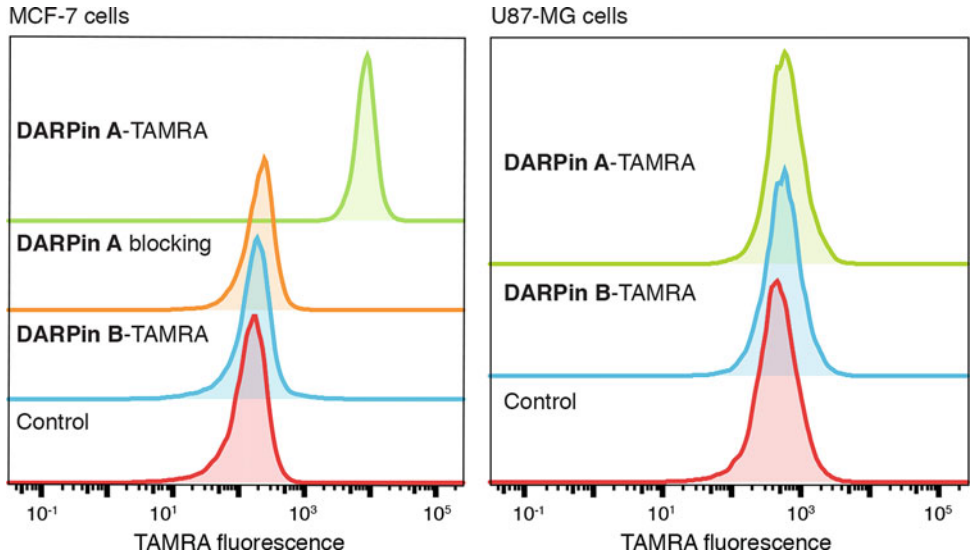

Fig. 4 Evaluation of DARPin A-TAMRA binding to cell surface EpCAM by flow cytometry. Two cell lines were utilized, MCF-7 cells with EpCAM overexpression (left) and U87-MG cells as negative control (right). DARPin A binds to cell surface EpCAM while DARPin B is a control protein that does not bind to any target. The control sample for each cell line contains cells treated with only PBSA buffer. The results confirmed the ability and specificity of DARPin A-TAMRA binding to cell surface EpCAM without non-specific interactions

2. It is critical to incubate the protein substrate with DTT on ice before the addition of the yPFTase enzyme to fully reduce any disulfide bonds involving the CaaX box and make sure that the cysteine residue is accessible for the prenylation reaction. Unlike in reactions with maleimides, the excess amount of DTT does not need to be removed prior to the prenylation reaction. To maximize the concentrations of DTT and the DARPin protein during reduction (prior to prenylation), the reaction is performed in a concentrated prenylation buffer (6×). Therefore, the tube contains Tris–HCl, pH 7.5 (200 μL of 0.5 M stock), KCl (80 μL of 0.5 M stock), $MgCl_2$ (20 μL of 1 M stock), DTT (20 μL of 0.5 M stock), $ZnCl_2$ (10 μL of 2 mM stock), and DARPin (22 μL of 224 μL stock).

   Alternatively, TCEP can also be used as the reducing agent [33].

3. In addition to the C10-Azi analog **1** employed here, a C15-Azi analog with similar structure but a farnesyl backbone has also been previously reported to be a good substrate for yPFTase and can be used for protein labeling [21]. The precursor to make the C15-Azi analog (one-step esterification reaction of the alcohol precursor to generate the diphosphate compound) is commercially available from Cayman Chemical (farnesyl alcohol azide).

4. In addition to the yPFTase enzyme used in this protocol, rat PFTase (rPFTase) can also be employed to catalyze a prenylation reaction, which is commercially available from Jena Bioscience. However, yPFTase has a higher activity [34] and is more promiscuous toward different isoprenoid analogs than rPFTase.
5. Both the analog **1** and the yPFTase are added directed to the reaction mixture from the stock solution. Since both reagents are diluted approximately 1000-fold, the effect of their corresponding stock solutions to the composition of the prenylation buffer is negligible.
6. If the reaction efficiency results from LC-MS analysis (**step 3**) are not satisfying, increase the amount of yPFTase added to the prenylation reaction. A labeling yield of 90% or higher can be achieved.
7. Since the isoprenoid analog **1** is relatively hydrophobic, protein precipitation might occur after the conjugation depending on the target protein. To determine if there is precipitation, centrifuge the tube after prenylation reaction to check for any visible precipitate.
8. This method can only be used to estimate the concentration of the DARPin-**1** conjugate since it assumes that the DARPin and yPFTase give similar signals in the Bradford assay. However, this is not critical since the purpose of this calculation is to obtain an approximate value to determine the correct amount of DBCO-TAMRA to add for subsequent conjugation. As an alternative, the concentration of the DARPin-**1** conjugate can be estimated from the initial amount used in the prenylation reaction assuming complete prenylation and recovery.
9. The SPAAC reaction is performed in PBS_{400} buffer with high salt concentration that is used to store DARPin proteins (to prevent any potential aggregation of binders at very high concentrations). Alternatively, the reaction can also be carried out in 1× PBS buffer.
10. The NAP-5 column is a simple gel filtration column used to remove excess TAMRA fluorophore from the SPAAC reaction. To remove the yPFTase from DARPin-TAMRA conjugates more quantitatively, preparative size-exclusion columns, such as the Superdex 75 Increase column (GE Healthcare), can be employed on a fast protein liquid chromatography (FPLC) system.
11. Depending on the sensitivity of the LC-MS instrument employed, the concentration and volume of the protein samples being analyzed might have to be adjusted accordingly.

12. In order to check for specific binding, we recommend not just to test the binding of the labeled DARPins on both cell types but also to include a sample where the receptor is blocked in advance with a tenfold excess of unlabeled DARPins in a competition setup.

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