NanoTag Nanobody Tools for *Drosophila In Vitro* and *In Vivo* Studies

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Nanobodies have emerged as powerful protein-binding tools to uncover protein functions. Using functionalized protein binders, proteins of interest can be visualized, degraded, delocalized, or post-translationally modified *in vivo*. We recently reported the use of two short peptide tags, 10-aa 127D01 and 14-aa VHH05, and their corresponding nanobodies, Nb127D01 and NbVHH05, for both *in vitro* and *in vivo* studies in *Drosophila*. Here, we provide detailed protocols for nanobody production and for visualization of proteins of interest in either fixed or live samples. In addition, we include protocols for endogenous protein tagging using CRISPR-mediated genome engineering. © 2022 Wiley Periodicals LLC.

Basic Protocol 1: Nanobody production in S2 cells
Basic Protocol 2: Nanobody expression and purification in bacterial cells
Basic Protocol 3: Immunostaining with nanobodies
Basic Protocol 4: Immunoblotting with nanobodies
Basic Protocol 5: Immunoprecipitation with nanobodies prepared from S2 cells
Basic Protocol 6: Immunoprecipitation with nanobodies prepared from bacteria
Basic Protocol 7: NbVHH05 and Nb127D01 used as chromobodies
Basic Protocol 8: NanoTag trap as a method to alter protein localization
Support Protocol: CRISPR-mediated tagging of endogenous genes with NanoTags

Keywords: CRISPR • *Drosophila* • knock-in • nanobody • NanoTag

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INTRODUCTION

Antibodies allow protein visualization by immunostaining, biochemical studies by immunoprecipitation and immunoblotting, and proteomic studies by immunoprecipitation

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followed by mass spectrometry. Despite their importance in biological studies, antibodies are not available for most *Drosophila* proteins. Currently, most available antibodies are polyclonal and thus are not a renewable resource. Further, renewable monoclonal antibody reagents are available for fewer than 400 fly proteins (Mohr et al., 2021). When we compare this number with the approximately 14,000 protein-coding genes annotated in the fly genome, it becomes clear that the development of additional antibody resources and/or alternative methods is needed to study the function of proteins of interest in detail.

In recent years, nanobodies have emerged as powerful tools to study the function of proteins (Cheloha, Harmand, Wijne, Schwartz, & Ploegh, 2020; Harmansa & Affolter, 2018). Compared to conventional antibodies, which typically have a molecular weight of about 150 to 160 kDa and are composed of four polypeptides (two identical heavy chains and two identical light chains), camelid-derived nanobodies have a smaller size of about 12 to 15 kDa. Given their small size, nanobodies 1) are easy to express in *Escherichia coli*, either alone or fused to a fluorescent marker or enzyme; 2) allow better super-resolution microscopy than antibody-based imaging; and 3) enable binding to epitopes not accessible to full-length conventional antibodies (Fang et al., 2018; Fornasiero & Opazo, 2015; Mikhaylova et al., 2015; Virant et al., 2018). However, because nanobodies mainly bind to structural epitopes, nanobodies reacting to linear epitopes are rarely identified, and only a few linear epitope-binding nanobodies have been discovered (Boersma et al., 2019; De Genst et al., 2010; Götzke et al., 2019; Tanenbaum, Gilbert, Qi, Weissman, & Vale, 2014; Traenkle et al., 2015).

Recently, we reported the application of two linear epitope–recognizing nanobodies, NbVHH05 (Ling et al., 2019) and Nb127D01 (Bradley et al., 2015), and their corresponding NanoTags, VHH05 and 127D01 (Xu et al., 2022), which facilitate almost every application that is possible with conventional antibodies. We showed that both nanobodies can be used for multiplexed immunostaining, immunoblotting, and immunoprecipitation. In addition, NanoTags/nanobodies can be expressed intracellularly, thus facilitating live imaging and protein trapping. They can also be genetically encoded as fluorescent protein fusions (i.e., chromobodies, or CBs), enabling the detection of target proteins that carry NanoTags at their N-terminal, internal, or C-terminal sites. NanoTagged proteins can be further manipulated by nanobodies fused to various subcellular localization signals. Importantly, because NanoTags are small, they can easily be introduced into an endogenous gene locus using CRISPR-mediated gene editing.

Here, we describe protocols for the preparation and use of nanobodies. Nanobodies prepared in *Drosophila* cells (Basic Protocol 1) or bacteria (Basic Protocol 2) can be used for immunostaining, immunoblotting, or immunoprecipitation (Basic Protocols 3 to 6). Nanobodies can be used as CBs to determine the localization of NanoTagged proteins (Basic Protocol 7) or as protein traps to alter protein localization (Basic Protocol 8). To support broad use of NanoTag nanobodies to detect *Drosophila* proteins, we also include a protocol for endogenous tagging using CRISPR-mediated genome editing (Support Protocol).

NOTE: Most of the plasmids used in these protocols are available from Addgene or the Drosophila Genomics Resource Center (DGRC), and most of the fly stocks are available from the Bloomington Drosophila Stock Center.

NOTE: Experiments involving PCR require extremely careful technique to prevent contamination.

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NANOBODY PRODUCTION IN S2 CELLS

BASIC PROTOCOL 1

Drosophila S2 cells are semi-adherent and can be cultured at room temperature (RT) in culture plates or suspension flasks. They do not require CO_2 and are easily cultured in standard incubators or shaking incubators. Moreover, S2 cells have been used extensively to produce secreted proteins (Zitzmann et al., 2018).

To express nanobodies in S2 cells, we generated inducible nanobody expression plasmids that contain a metallothionein promoter, BiP signal peptide, nanobody, human immunoglobulin Fc domain, TEV cleavage site, Avi tag, and His tag (Table 1). In addition, these plasmids contain a puromycin resistance gene and GFP under the control of the heat-shock protein 70 (*Hsp70*) promoter, which allow for monitoring of transfection efficiency by GFP visualization and generation of stable cell lines. After transfection of a nanobody expression plasmid into S2 cells, expression is induced with $CuSO_4$, as the nanobody sequence is under the control of a metallothionein promoter. After $CuSO_4$ induction, nanobodies are secreted into the culture medium, which can be collected and directly used for most of the experiments described here without further purification. We transfect cells with nanobody expression plasmids using polyethylenimine (PEI), which allows for large-scale transfection at a low cost. PEI results in ~20% transfection efficiency. Alternative transfection reagents can be used to maximize transfection efficiency. Because basic protocols for adherent S2 cell culture are available (Echalier, Perrimon, & Mohr, 2017; Luhur, Klueg, Roberts, & Zelhof, 2019), we will focus on suspension S2 cell culture and collection of nanobody-containing culture medium.

Materials

ESF921-adapted S2 cells (Expression Systems, 94-005S)
ESF921 Insect Cell Culture Medium (Expression Systems, 96-001-01)
NanoTag nanobody expression vectors:
  pMT-HGP-v3-Nb127D01-hIgG (Addgene, 171564, or DGRC, 1550)
  pMT-HGP-v3-NbVHH05-hIgG (Addgene, 171565, or DGRC, 1551)
1 mg/ml PEI, Linear, MW 25,000 (PEI 25000, Polysciences, 23966-1; follow manufacturer's instructions to make 1 mg/ml transfection stock solution; store for years at –80°C or for several weeks at 4°C)
1 M $CuSO_4$ stock solution, 0.2-μm filter-sterilized
5% (w/v) sodium azide (NaN_3; BDH, 7465-2)

Table 1 Components in Nanobody Expression Vectors for *Drosophila* S2 Cells

Component	Comment
Metallothionein promoter	For inducible expression
BiP signal peptide	Secretion signal peptide from ER chaperone BiP
NbVHH05 or Nb127D01	Nanobodies that recognize NanoTags
Human immunoglobulin Fc domain	Detection by anti-human Fc antibodies and purification by Protein A/G
TEV cleavage site	For tobacco etch virus (TEV) protease–mediated cleavage between Nb-hIgG and purification tag
Avi tag	Avidin tag; detected by anti-Avi antibody and used for *in vitro* biotinylation
His tag	6×histidine tag; for purification by Ni^{2+} resin
Hsp70 promoter	For constitutive expression of GFP and puromycin resistance markers
GFP	For transfection efficiency verification
Puromycin resistance gene	For stable cell line generation

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Erlenmeyer flasks with screw caps (125 ml, VWR, 75997-984, or equivalent; 250 ml, VWR, 75997-986, or equivalent; and 1 L, VWR, 75997-990, or equivalent), autoclaved
25°C shaking incubator (with sticky pad; Infors HT, Multitron, or equivalent)
50-ml conical tubes (Fisher Scientific, 06-443-20, or equivalent)
Standard tabletop centrifuge
0.2-µm filter unit (500-ml capacity; Sigma-Aldrich, Z358193, or equivalent)

Additional reagents and equipment for cell counting

Suspension S2 cell culture

1. Subculture ESF921-adapted S2 cells with a 1:2 to 1:10 split ratio in ESF921 Insect Cell Culture Medium using autoclaved Erlenmeyer flasks with screw caps, incubating at 25°C and 130 rpm in a shaking incubator.

   We seed at least 2–4 × 10^6 cells/ml for regular cell culture. At least 1/10 of the total culture volume should be from the previous culture. Too much dilution can lead to cell culture failure. Split cells into at least two ratios (e.g., 1:5 and 1:10) to prevent unexpected cell culture failure. The minimum cell number for subculture should be determined experimentally.

   We use autoclaved Erlenmeyer flasks with screw caps for suspension culture. Caps should be loosened for aeration. We use ≥20% of the flask volume as the culture volume (25 ml for a 125-ml flask, 50 ml for a 250-ml flask, and 200 ml for a 1-L flask).

2. Incubate culture flasks at 25°C (or RT) and 130 rpm until the cell number reaches about 2.5–3.5 × 10^7 cells/ml.

   The color of the suspension cell culture in the flasks will become more yellow over time. If the culture color in the flasks does not change over time and floating aggregates are observed, it means that suspension cell culture failed. Increase the cell ratio.

3. Repeat steps 1 and 2 about 2 to 3 times a week for regular cell maintenance.

PEI transfection and nanobody production in S2 cells

4. Split ESF921-adapted S2 cells 1:2 to 1:5 in Erlenmeyer flasks about 1 to 2 days before transfection.
5. On the transfection day, count cell number and seed 2 × 10^6 cells/ml in a new flask.
6. Mix 0.8 µg NanoTag nanobody expression vector with 50 µl ESF921 medium per 1 ml culture and mix 2.4 µg of 1 mg/ml PEI with 50 µl ESF921 medium per 1 ml culture. Then, combine 50 µl of the DNA-containing medium and 50 µl of the PEI-containing medium by gentle pipetting. Incubate at RT for 5 to 15 min.
7. Add DNA/PEI mixture in ESF921 medium to the flasks with S2 cells and incubate flasks in the shaking incubator.
8. About 2 to 3 days later, add 1 M $CuSO_4$ stock solution directly to flasks to a final concentration of 500 µM.

   Optional: Check the transfection efficiency based on GFP expression if a fluorescent microscope is available. When the GFP expression level reaches its peak (around 40% of cells can be brightly GFP positive), $CuSO_4$ can be added to achieve maximum induction.

   At this point, instead of inducing nanobody expression, puromycin-containing ESF921 medium can be used to make stable cell lines. Subculture cells at a 1:2 to 1:10 ratio in puromycin-containing ESF921 medium. This medium can be made by adding 1000× stock solution of puromycin (Calbiochem, 540411) to ESF921 medium to a final concentration of 5 µg/ml puromycin.

9. Incubate flasks in the shaking incubator for 3 to 5 days to induce protein expression.
10. Collect medium in a 50-ml conical tube by centrifugation for 5 to 10 min at 1000 × *g*.

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11. Filter nanobody-containing medium through a 0.22-µm filter.
12. Add 5% NaN_3 to a final concentration of 0.05% for long-term storage at 4°C or –20°C.

For immunostaining (see Basic Protocol 3), a 1:10 to 1:100 dilution is usually fine. However, it is recommended to test various dilutions to find the most optimal one. Use about 10 times more for immunostaining than immunoblotting.

For immunoblotting (see Basic Protocol 4), a 1:100 to 1:1000 dilution of nanobody-containing medium is usually fine. However, it is recommended to test various dilutions to find the most optimal one. Use about 10 times less for immunoblotting than immunostaining.

Alternatively, the amount of Nb-hIgG in the conditioned medium can be measured by immunoblotting or ELISA. The medium with Nb-hIgG and serially diluted human IgG can be used in immunoblotting or for coating in ELISA. The hIgG portion of Nb-hIgG and human IgG can be detected by anti-human-HRP (Jackson ImmunoResearch, 109-005-170) in both immunoblotting and ELISA.

NANOBODY EXPRESSION AND PURIFICATION IN BACTERIAL CELLS

BASIC PROTOCOL 2

Nanobodies can easily be purified from *E. coli*. Our vectors for nanobody expression in bacteria contain a PelB signal peptide for periplasmic expression, which allows for disulfide bond formation, as well as an ALFA tag or HA tag for detection and a His tag for purification. We describe a protocol for His tag–based purification of nanobodies from the bacterial periplasmic fraction. His tag–purified nanobodies can be used for immunostaining, immunoblotting, and immunoprecipitation without additional purification steps such as size-exclusion chromatography.

Materials

BL21 competent cells (NEB, C2530H, or equivalent)
Nb127D01 or NbVHH05 nanobody expression plasmid with HA or ALFA tag:
  pET-26b-Nb127D01-HA-His (Addgene, 171566, or DGRC, 1552)
  pET-26b-NbVHH05-HA-His (Addgene, 171567, or DGRC, 1553)
  pET-26b-Nb127D01-ALFA-His (Addgene, 171568, or DGRC, 1554)
  pET-26b-NbVHH05-ALFA-His (Addgene, 171569, or DGRC, 1555)
LB broth (Sigma, L3022-1KG, or equivalent)
LB + kanamycin medium: LB broth (Sigma, L3022-1KG, or equivalent) + 1000× (50 mg/ml) stock kanamycin (Sigma-Aldrich, K-1377-25g, or equivalent) solution (final concentration, 50 µg/ml kanamycin)
1× Laemmli sample buffer [diluted from 4× (Bio-Rad, 1510747, or equivalent) supplemented with 5% β-mercaptoethanol (bME; Sigma-Aldrich, M6250-100)]
0.1 or 1 M IPTG I(Goldbio, I2481C100, or equivalent; dissolve in distilled water and sterilize with 0.22-µm filter)
1× Tris-buffered saline (TBS; diluted from 10×; Cell Signaling Technology, 12498S, or equivalent)
B-PER II (B-PER II Bacterial Protein Extraction Reagent, 2×, Thermo Scientific, 78260)
5 M imidazole (BioVision, B1014-100, or equivalent)
Ni^{2+} resin (GE Healthcare, 17531801, or equivalent)
1× TBS (from 10×; Cell Signaling Technology, 12498S, or equivalent) + 10, 20, 100, 250, and 500 mM imidazole (from 5 M; BioVision, B1014-100, or equivalent)
BCA assay kit (Pierce BCA Protein Assay Kit, Thermo Scientific, 23227, or equivalent)
1× TBS (from 10×; Cell Signaling Technology, 12498S, or equivalent) + 0.05% (w/v) NaN_3 (BDH, 7465-2) + 50% (v/v) glycerol (Sigma-Aldrich, G7757-1GA, or equivalent)

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37°C or 42°C heating block (Thermo Scientific, 88870001, or equivalent)
15°C and 37°C shaking incubators (Infors HT, Multitron, or equivalent)
125-ml and 1-L Erlenmeyer flasks 125-ml and 1-L flasks (Pyrex, 4980-125 and 4980-1L, or equivalent)
Spectrophotometer (NanoDrop)
1.5-ml Eppendorf tubes
Microcentrifuge
50-ml conical tubes
Standard tabletop centrifuge, RT and 4°C
Scale
Tube rotator
0.2-μm filter unit (500-ml capacity; Sigma-Aldrich, Z358193, or equivalent)
Purification column (Poly-Prep Columns, Bio-Rad, 7311550, or equivalent)
Dialysis cassette, 3.5K MWCO (Thermo Scientific, 66330, or equivalent)

Additional reagents and equipment for SDS-PAGE (see Current Protocols article: Gallagher, 2012) and Coomassie Blue staining (see Current Protocols article: Sasse & Gallagher, 2009) or stain-free gel imaging (Bio-Rad)

Bacterial culture and periplasmic fraction preparation

1. Transform BL21 competent cells with Nb127D01 or NbVHH05 nanobody expression plasmid with an HA or ALFA tag:
   a. Thaw 50 μl BL21 competent cells on ice.
   b. Add 1 μl miniprepped plasmid to tube with the cells.
   c. Incubate on ice for 10 min.
   d. Incubate in a heating block at 42°C for 45 s or at 37°C for 2 min.
   e. Incubate on ice for >2 min.
   f. Add 450 μl LB broth to cells.
   g. Incubate at 37°C for 1 hr in a shaking incubator with or without shaking.
   h. Inoculate transformed cells into 10 ml LB + kanamycin medium in a 125-ml Erlenmeyer flask.
   i. Incubate in 37°C shaking incubator overnight.
2. Inoculate 2 ml overnight culture into 200 ml LB + kanamycin medium in a 1-L Erlenmeyer flask (1:100 ratio).
3. Grow in 37°C shaking incubator until the OD600 reaches 0.6 to 0.8, as measured using a spectrophotometer.
4. Move culture flask to the induction temperature (15°C) in a shaking incubator.
5. Save 1 ml culture (uninduced sample):
   a. Centrifuge in a 1.5-ml Eppendorf tube for 1 min at 13,000 rpm.
   b. Remove supernatant.
   c. Resuspend pellet in 100 μl of 1× Laemmli sample buffer for the nanobody expression test (see step 23). Use the sample directly for SDS-PAGE or save at −20°C for future use.
6. Add 0.1 or 1 M IPTG to culture from step 4 to a final concentration of 0.3 mM.
7. Incubate in 15°C shaking incubator overnight.
8. Save 0.25 ml culture (induced sample):
   a. Centrifuge in a 1.5-ml Eppendorf tube for 1 min at 13,000 rpm.
   b. Remove supernatant.
   c. Resuspend pellet in 100 μl of 1× Laemmli sample buffer for the nanobody expression test (see step 23). Use the sample directly for SDS-PAGE or save at −20°C for future use.

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9. Centrifuge all induced culture (see step 7) in 50-ml conical tubes for 15 min at 6000 × g.
10. Remove supernatant.
11. Resuspend pellet in 1× TBS.
12. Centrifuge 15 min at 6000 × g.
13. Remove supernatant.
14. Measure weight of the cell pellet, using an empty 50-ml conical tube to tare the scale.
15. Add B-PER II at 2 ml/cell-pellet gram) and resuspend by pipetting.
16. Add 38 ml of 1× TBS (19 times the volume of the B-PER II solution) to resuspended cells.
17. Incubate at RT with rotation for 15 min.
18. Centrifuge 5 min at 15,000 × g, 4°C.

    If a refrigerated centrifuge large enough for a 50-ml conical tube is not available, aliquot the resuspended cells into 1.5- or 2-ml tubes and use a refrigerated microcentrifuge.
19. Collect supernatant (periplasmic fraction).

    If there is any floating material, centrifuge again and try to collect the clear supernatant without disturbing the pellet.
20. Filter supernatant with a 0.2-µm filter unit.
21. Save 50 µl supernatant for filtered periplasmic fraction for SDS-PAGE (see step 23).
22. Store filtered sample at 4°C for short-term storage or at –80°C for long-term storage.
23. Check level of nanobody present in the uninduced sample (step 5), induced sample (step 8), and periplasmic fraction (step 22) by SDS-PAGE and Coomassie Blue staining or stain-free gel imaging.

    If the presence of induced nanobody is confirmed at ~15 kDa in the periplasmic fraction, proceed with His-tag purification. The presence of induced nanobody can be checked with anti-His (Cell Signaling, 2365S, or equivalent; needs anti-rabbit-HRP secondary antibody), anti-ALFA-HRP (NanoTag Biotechnology, N1502-HRP), or anti-HA antibody (Roche, 3F10, or equivalent; needs anti-rat-HRP secondary antibody), depending on the purification and detection tags.

His tag–based purification

24. Add 5 M imidazole to 20 ml filtered sample from step 22 to a final concentration of 10 mM imidazole.
25. Save 50 µl for input for SDS-PAGE (see step 34).
26. Add 1 ml resuspended Ni^{2+} resin to a purification column.
27. Wash resin with 10 ml of 1× TBS in gravity flow.
28. Wash resin with 10 ml of 1× TBS + 10 mM imidazole in gravity flow.
29. Add 10 ml sample (see step 24) to resin.
30. Let sample pass through the resin in gravity flow.
31. Collect flow-through (100 µl) in a 1.5-ml Eppendorf tube. Repeat steps 29 to 31 for the additional 10 ml sample.

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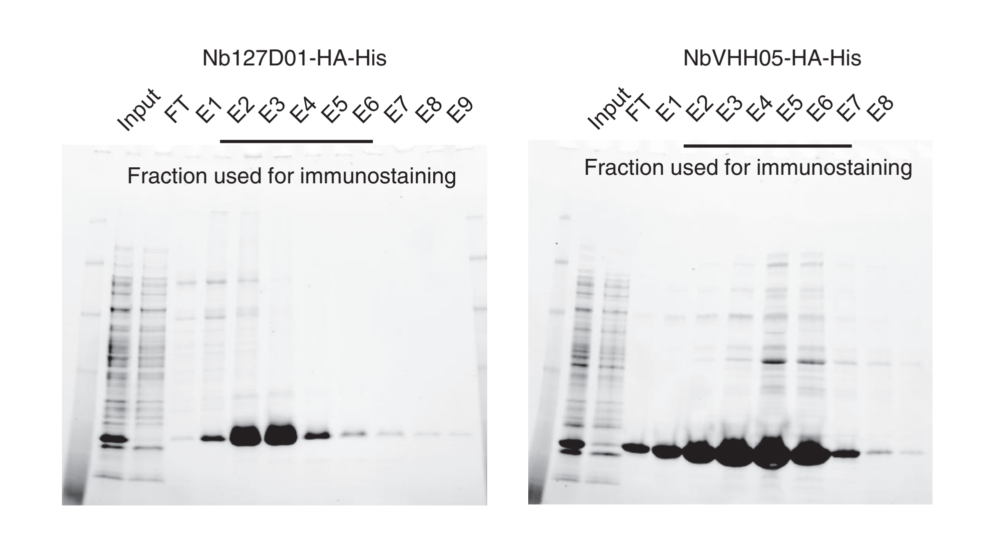

Figure 1 Example gel image of His-tag purification. Nb127D01-HA-His and NbVHH05-HA-His were purified from the periplasmic fraction. Underlined elution fractions were combined and used for dialysis. Stain-free imaging was performed. Input: Periplasmic fraction, FT: Flow-through, E1-E9: Eluate 1-Eluate 9. Red mark indicates saturated signal. From Xu et al. (2022) and used in accordance with the CC-BY license applied by eLife (see *https://creativecommons.org/licenses/by/4.0/*).

32. Wash resin with 10 ml TBS + 20 mM imidazole in gravity flow three times and collect flow-through.

33. Elute His-tagged protein six times in 0.5 ml elution buffer with different imidazole concentrations in gravity flow:

    Eluate 1: 1× TBS + 100 mM imidazole
    Eluate 2: 1× TBS + 250 mM imidazole
    Eluate 3: 1× TBS + 500 mM imidazole
    Eluate 4: 1× TBS + 500 mM imidazole
    Eluate 5: 1× TBS + 500 mM imidazole
    Eluate 6: 1× TBS + 500 mM imidazole.

34. Perform SDS-PAGE with the filtered sample (original sample; step 22), the flow-through (step 31), the wash fraction (step 32), and the eluates (step 33) to check the purified protein.

    Use a protein gel such as 4%-20% Mini-PROTEAN TGX Stain-Free Protein Gels (Bio-Rad, 4568096) or equivalent gels that allow small-size protein detection.

35. Combine eluates that contain purified nanobodies.

    Choose the fractions with more His-tagged proteins but less unwanted proteins. If >50% of the eluates contain His-tagged nanobodies, the eluates can be used as primary antibody reagents despite the relatively poor purity.

    Refer to the selected fractions in Figure 1.

36. Dialyze combined eluates with 1× TBS using a dialysis cassette (>3 kDa).

    Although we use commercially available TBS, other buffers, such as 1× phosphate-buffered saline (PBS), can be used as a storage buffer.

    The dialysis schedule can be flexible (e.g., several hours at RT or overnight at 4°C). We dialyze more than three times.

    After dialysis, a protease inhibitor cocktail (Roche, 4693132001, or equivalent) can be added for long-term storage (see step 39).

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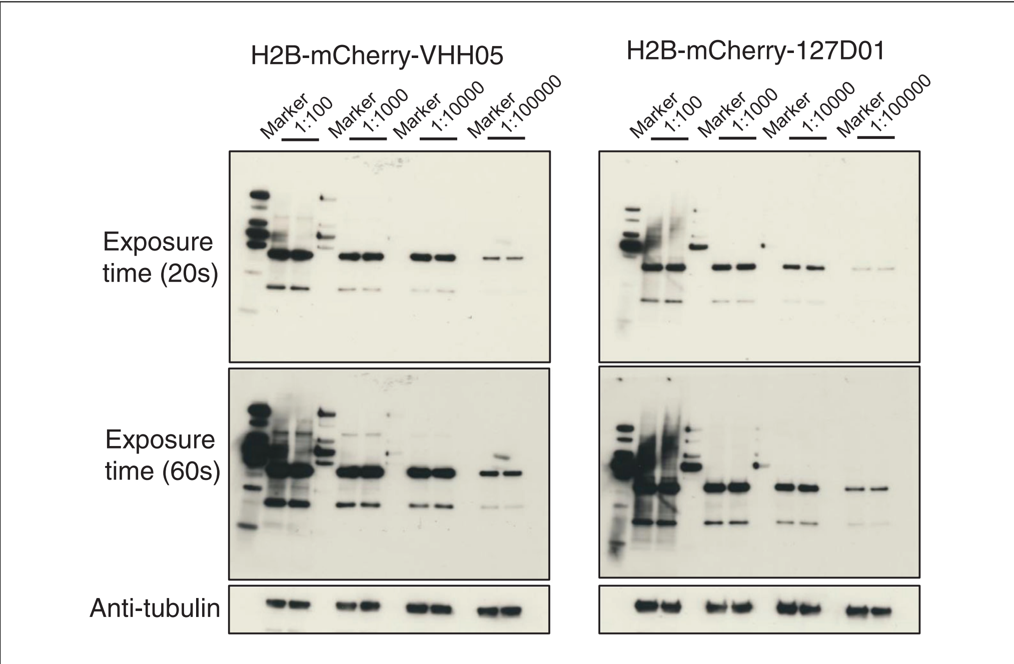

Figure 2 Testing His tag–purified nanobodies. S2R+ cells transfected with H2B-mCherry-VHH05 or H2B-mCherry-127D01 were used to make protein lysates, which were analyzed by immunoblotting. Serially diluted nanobodies (1:100 to 1:100,000) were used to visualize the NanoTagged proteins. The blots were developed with ECL. From Xu et al. (2022) and used in accordance with the CC-BY license applied by eLife (see *https://creativecommons.org/licenses/by/4.0/*).

We avoid using centrifugation-based buffer exchange for dialysis because we find that precipitation often occurs during centrifugation. Refer to the manufacturer's instructions for the dialysis cassette product.

37. Measure protein concentration with a BCA assay kit.
38. Store dialyzed protein sample in 1× TBS + 0.05% NaN_3 + 50% glycerol at –20°C or –80°C.

For general purposes such as immunoblotting or immunostaining, His tag–purified nanobodies do not need further purification.

Usually, we dilute the dialyzed nanobodies to 0.2 mg/ml and aliquot them into several tubes. Because the size of nanobodies (<15 kDa) is smaller than that of conventional antibodies (~150 kDa), 0.2 mg/ml purified nanobodies is enough for standard use as primary antibodies.

The optimal concentration for the following applications (e.g., see Basic Protocols 3 and 4) can vary depending on each purified nanobody and the expression levels of the NanoTagged proteins. For each application, try to use the minimum amount to avoid background signals (Fig. 2).

IMMUNOSTAINING WITH NANOBODIES

BASIC PROTOCOL 3

NanoTagged proteins can be immunostained using NanoTag nanobodies prepared in the form of nanobody-containing medium from S2 cell cultures (Basic Protocol 1) or purified from bacterial culture (Basic Protocol 2). NanoTag nanobodies prepared from S2 cells can specifically bind to NanoTagged proteins in cells or tissues and can be visualized with anti-human Fc secondary antibodies conjugated with fluorophores. Similarly, His tag–purified nanobodies with an ALFA tag (Nb-ALFA-His) or HA tag (Nb-HA-His) prepared from bacteria can specifically bind to NanoTagged proteins in cells or tissues and can be visualized with anti-ALFA or anti-HA secondary antibodies conjugated with fluorophores. For multiplexed immunostaining, it is possible to visualize two different proteins in the same sample if they are tagged with different NanoTags. In this case, one

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nanobody should be purified from S2 cells (Basic Protocol 1) and the other from bacteria (Basic Protocol 2; human IgG vs. ALFA tag or HA tag), or each nanobody should be purified from bacteria and tagged differently (ALFA tag vs. HA tag).

In this protocol, we describe immunostaining using nanobodies purified from bacteria (Basic Protocol 2). A modified version of this approach can be used with nanobodies isolated from S2 cells (Basic Protocol 1; i.e., using an anti-human Fc secondary antibody in place of the anti-HA secondary antibody). Moreover, we use the *Drosophila* protein REPTOR tagged at the N- and C-terminal ends with different NanoTags as an example. This protocol is useful for detection of any single- or double-tagged NanoTagged protein that is expressed at detectable levels.

Materials

Plasmids:
- pAct-Gal4 (from Y. Hiromi, National Institute of Genetics, Mishima, Japan)
- pW10-UAS-VHH05-REPTOR-127D01 (used in Xu et al., 2022)

Effectene (Qiagen, 301427), including EC Buffer and Enhancer
S2R+ cells (DGRC, 150)
Schneider's medium (Thermo Fisher Scientific, 21720–024)
4% (w/v) paraformaldehyde PFA [diluted in 1× PBS (from 10× PBS; Invitrogen, 70011-069) from 16% PFA (Electron Microscopy Sciences, 15710)]
Blocking buffer: 1% (v/v) normal donkey serum (Jackson ImmunoResearch Laboratories, 017-000-121) in 1× PBS (from 10× PBS; Invitrogen, 70011-069)
Purified nanobodies:
- Purified NbVHH05-HA (see Basic Protocol 2)
- Purified Nb127D01-ALFA (see Basic Protocol 2)

1× PBST buffer: 0.3% (v/v) Triton X-100 (Sigma, T9284) and 0.1% (w/v) bovine serum albumin (BSA) in 1× PBS (from 10× PBS; Invitrogen, 70011-069)
1× PBS (from 10× PBS; Invitrogen, 70011-069)
Secondary antibodies:
- NbALFA-Atto647 (NanoTag Biotechnologies, N1502-At647N-L)
- Mouse monoclonal anti-HA-Alexa Fluor 488 (Thermo Fisher Scientific, A-21287)

DAPI (4′,6-diamidino-2-phenylindole; Thermo Fisher Scientific, D1306)
Mounting medium (Vector Laboratories, H-1300)

1.5-ml Eppendorf tubes
Vortex
24-well tissue culture plate (Costar, 29443-952)
Glass microscope slides (Thermo Fisher Scientific, 3050-002), sterilized
6-well tissue culture plate (Corning, 29442-042)
Microscope cover glass (VWR Scientific, 48366205), sterilized
Nail polish
Confocal microscope (Nikon Ti2 Spinning Disk)

1. Transfect S2R+ cells using Effectene:
   a. Add 200 ng plasmid (100 ng pAct-Gal4 and 100 ng pW10-UAS-VHH05-REPTOR-127D01) to 60 μl EC Buffer in a 1.5-ml Eppendorf tube and mix thoroughly by vortexing.
   b. Add 3 μl Enhancer, mix by brief vortexing, and incubate for 3 min.
   c. Add 5 μl Effectene, mix by brief vortexing, and incubate for 10 min.
   d. Add 200 μl S2R+ cells (~5×10^4 cells) in Schneider's medium into each well of a 24-well tissue culture plate.
   e. Add transfection mixture to the cultured cells.
   f. Add another 200 μl medium to each well and then incubate cells at 25°C or RT.

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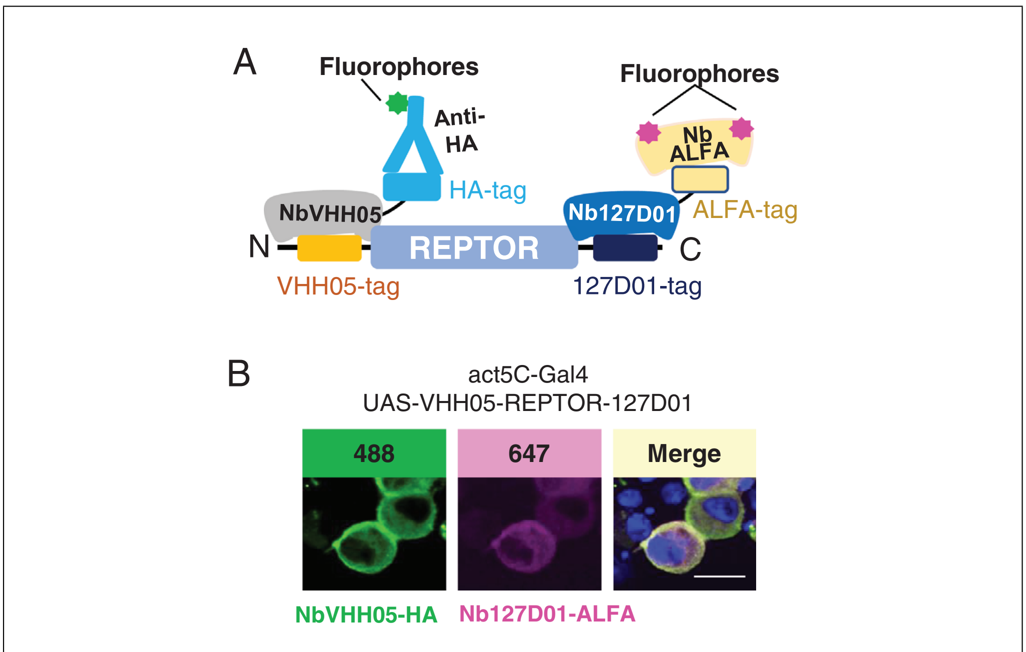

Figure 3 Using NbVHH05 and Nb127D01 for immunofluorescence. (**A**) Schematic of using VHH05 and 127D01 for double tagging. The N- and C-termini of REPTOR contain VHH05 and 127D01. (**B**) Co-staining NbVHH05 and Nb127D01 in S2R+ cells transfected with VHH05-REPTOR-127D01. Scale bar: 10 μm. From Xu et al. (2022) and used in accordance with the CC-BY license applied by eLife (see *https://creativecommons.org/licenses/by/4.0/*).

2. Prepare cells for immunostaining on a glass slide:
   a. Forty-eight hours after addition of the transfection mix, resuspend cells from the transfection plate.
   b. Using a pipet, transfer 50 to 100 μl transfected cells to a sterilized glass microscope slide.
   c. Put slide in a 6-well tissue culture plate.
   d. Incubate for 1 to 2 hr at 25°C or RT.
   e. Remove medium and fix cells with 100 μl of 4% PFA for 20 min.
3. Incubate in 100 μl blocking buffer for 30 min.
4. Dilute purified nanobodies (NbVHH05-HA or Nb127D01-ALFA) 1:500 in 1× PBST buffer. Remove blocking buffer from cells and incubate with 100 μl diluted nanobodies for 2 to 4 hr at RT.
5. Wash with 1× PBS for 5 min. Repeat four times total.
6. Dilute secondary antibodies (NbALFA-Atto647 or mouse monoclonal anti-HA-Alexa Fluor 488) 1:500 and DAPI 1:2000 in 100 μl of 1× PBST. Incubate cells with mixture for 1 to 2 hr at RT.
7. Wash with 1× PBS for 5 min. Repeat four times total.
8. Add mounting medium and then place a sterilized microscope cover glass on slide and seal with nail polish.
9. Image with a confocal microscope.

   Example images were captured with a Nikon Ti2 spinning-disk confocal microscope. Z-stacks of 5 to 20 images covering one layer of the cells were obtained, adjusted, and assembled using NIH Fiji (ImageJ) and are shown as a maximum projection. For sample data, see Figure 3.

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BASIC PROTOCOL 4

IMMUNOBLOTTING WITH NANOBODIES

NanoTag nanobodies in the form of either nanobody-containing medium from S2 cell culture (Basic Protocol 1) or purified nanobodies from bacterial culture (Basic Protocol 2) can be used for immunoblotting. NanoTagged proteins from transfected cells or from flies modified to express one or more NanoTagged proteins *in vivo* can be specifically bound by NanoTag nanobodies, which in turn can be visualized using secondary antibodies with fluorophores or HRP. For multiplexed immunoblotting, proteins tagged with different NanoTags can be visualized simultaneously if the corresponding nanobodies are prepared differently. For example, one nanobody could be prepared from S2 cells (Basic Protocol 1) to generate an Nb-hIgG-format nanobody, and another nanobody could be prepared from bacterial cells (Basic Protocol 2) to generate a nanobody in the Nb-ALFA-His or Nb-HA-His format. Both NanoTag nanobodies can be incubated simultaneously in the primary antibody incubation step. Next, an anti-human Fc secondary antibody plus an anti-ALFA or anti-HA secondary nanobody can be used for detection. Each secondary antibody/nanobody should be conjugated with a different fluorophore (for simultaneous co-detection) or HRP.

Materials

1× PBS (from 10× PBS; Invitrogen, 70011-069), 4°C
Lysis buffer (Pierce IP lysis buffer, Pierce, 87788, or equivalent) supplemented with 1× protease inhibitor cocktail (from 100× Halt Protease Inhibitor Cocktail, Pierce, 87786, or equivalent), 4°C
BCA assay kit (Pierce BCA Protein Assay Kit, Thermo Scientific, 23227, or equivalent)
4× Laemmli sample buffer (Bio-Rad, 1610747, or equivalent), supplemented with 5% bME (Sigma, M6250-100)
Distilled water
Transfer buffer (ProSieve EX Transfer Buffer, 00200309, or equivalent)
Blocking solution: 5% (w/v) non-fat milk in 1× TBST [1× TBS (from 10×; Cell Signaling Technology, 12498S, or equivalent) + 0.05% [w/v] Tween-20)]
Primary antibody (NanoTag nanobodies; see Basic Protocols 1 and 2)
Secondary antibody:
  Anti-human secondary antibodies conjugated with 680- or 800-nm dye or HRP (can be purchased from multiple sources)
  Anti-ALFA secondary nanobodies conjugated with 680- or 800-nm dye or HRP (NanoTag Biotechnologies, *https://nano-tag.com/*).
  Anti-HA secondary antibodies conjugated with 680- or 800-nm dye or HRP (can be purchased from multiple sources)
1× TBS (from 10×; Cell Signaling Technology, 12498S, or equivalent; optional)
ECL substrate (SuperSignal West Pico PLUS Chemiluminescent Substrate, Thermo Scientific, 34580, or equivalent*)*

6-well tissue culture plate (Corning, 29442-042, or equivalent)
1.5-ml Eppendorf tubes
Standard tabletop centrifuge
Microcentrifuge
Pre-cast protein gel (4%-20% Mini-PROTEAN TGX Stain-Free Protein Gels, Bio-Rad, 4568096, or equivalent)
Protein gel electrophoresis system (Mini-PROTEAN System, Bio-Rad, or equivalent)
Power supply
Western blotting filter papers, extra thick (Thermo Scientific, 88605, or equivalent)
Nitrocellulose (NC) membrane, 0.2 µm (Bio-Rad, 1620112, or equivalent)
Transfer system (Trans-Blot Turbo Transfer System, Bio-Rad, or equivalent)

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Immunoblotting boxes
Orbital shaker (Corning LSE Orbital Shaker, or equivalent)
Imaging system (ChemiDoc MP, Bio-Rad, or equivalent) or dark room

Additional reagents and equipment for transfecting S2 cells with plasmids encoding NanoTagged proteins (see Basic Protocol 1, steps 4 to 9, or Basic Protocol 3, step 1)

1. Transfect S2 cells with plasmids encoding NanoTagged proteins in a 6-well tissue culture plate and incubate for 2 to 4 days.

   Either PEI (see Basic Protocol 1, steps 4 to 9) or Effectene (see Basic Protocol 3, step 1) can be used for S2 cell transfection. Usually, Effectene-based transfection is more effective but pricey.

2. Resuspend cells from the cell culture plate by pipetting.

   Because S2 cells are semi-adherent cells, both floating cells in the medium and cells attached to the plate need to be resuspended in the culture medium.

3. Transfer all resuspended cells in culture medium to a 1.5-ml Eppendorf tube.
4. Centrifuge 5 min at 500 × *g*.
5. Remove supernatant.
6. Wash cells with 1 ml cold 1× PBS. Repeat three times total.
7. Resuspend cell pellet in 300 µl cold lysis buffer supplemented with 1× protease inhibitor cocktail.
8. Incubate cells on ice for 15 min.
9. Centrifuge 5 min at 14,000 rpm, 4°C.
10. Collect cleared supernatant in a 1.5-ml Eppendorf tube.
11. Measure protein concentration with a BCA assay kit if necessary.
12. Mix 15 µl protein sample with 5 µl of 4× Laemmli sample buffer.

   The remaining cleared supernatant can be stored at –20°C or –80°C. Avoid repeated freeze/thaw.

13. Load 10 µl protein sample on a pre-cast protein gel with 15 wells.
14. Run at 300 V for 15 min using a protein gel electrophoresis system with a power supply.

   Use the appropriate voltage and time if a different gel is used.

   If a Bio-Rad stain-free protein gel is used, proceed with stain-free imaging to visualize total protein levels.

15. Briefly rinse gel with distilled water and incubate gel in transfer buffer for 5 min.
16. Transfer proteins in the gel to an NC membrane:

   a. Incubate two western blotting filter papers in transfer buffer for >30 s.
   b. Incubate an NC membrane in transfer buffer.

   Alternatively, use a PVDF membrane briefly pre-incubated in methanol for membrane activation.

   c. Place pre-wetted filter paper on the transfer unit of the transfer system.
   d. Place pre-wetted NC membrane on the filter paper.

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e. Place protein gel on the NC membrane.
f. Place pre-wetted filter paper on the protein gel.
g. Transfer proteins at 25 V for 15 min using a transfer system.

Use the appropriate conditions if different kinds of filter paper, membrane, gel, or transfer unit are used.

17. Incubate transferred membrane in blocking solution (5% non-fat milk in 1× TBST) in an immunoblotting box on an orbital shaker at RT for about 30 to 60 min.

18. Wash membrane briefly with 1× TBST. Repeat twice total.

19. Incubate with primary antibody (NanoTag nanobodies) in blocking solution on orbital shaker at RT for 1 hr or at 4°C overnight.

Multiple dilutions of nanobody-containing medium or purified nanobodies in blocking solution should be tested.

For long-term storage of the staining solution, nanobodies can be diluted in 5% BSA/TBST supplemented with 0.05% NaN_3 and stored at 4°C. Alternatively, the nanobody-containing solution can be stored at –20°C. However, avoid repeated freezing/thawing cycles.

20. Wash membrane with 1× TBST on the orbital shaker for 5 min. Repeat four times total.

21. Incubate with secondary antibody in blocking solution at RT for 1 hr or at 4°C overnight.

If a fluorophore-conjugated secondary antibody is used, use a black container or cover the container with aluminum foil to protect it from light. In addition, add sodium dodecyl sulfate (SDS) to a final concentration of 0.01% (w/v) to reduce background signal due to nonspecific binding.

Anti-human secondary antibodies conjugated with 680- or 800-nm dye or HRP can be used. Use the optimal concentration based on the manufacturer's information.

Anti-ALFA secondary nanobodies conjugated with 680- or 800-nm dye or HRP can be used. A dilution of 1:5000 or greater is recommended.

Anti-HA secondary antibodies conjugated with 680- or 800-nm dye or HRP can be used. Use an optimal dilution based on the manufacturer's recommendations.

22. Wash membrane with 1× TBST for 5 min. Repeat four times total.

23. Wash membrane with 1× TBS or PBS for 5 min. Repeat twice total.

24. For fluorophore-based immunoblotting, image using an appropriate imaging system or dark room.

The appropriate exposure time can be different depending on the expression levels of NanoTagged proteins. The automatic exposure setting in the ChemiDoc imaging system usually works fine.

25. For HRP-based immunoblotting, remove excess buffer on membrane by placing the membrane on a clean paper towel.

26. Add ECL substrate to a clean immunoblotting box according to manufacturer's manual and mix well by orbitally shaking by hand.

27. Place membrane in the box and incubate with ECL substrate for 1 to 2 min.

28. Use an appropriate imaging system with an automatic exposure setting to get optimal ECL signal.

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For HRP-based immunoblotting, the appropriate exposure time can be different depending on the ECL sensitivity as well as the expression levels of NanoTagged proteins. The automatic exposure setting in ChemiDoc imaging system usually works fine here as well.

IMMUNOPRECIPITATION WITH NANOBODIES PREPARED FROM S2 CELLS

BASIC PROTOCOL 5

NanoTagged proteins can be immunoprecipitated by NanoTag nanobodies prepared as nanobody-containing medium from S2 cell culture (Basic Protocol 1). Although nanobody-containing medium from S2 cell culture contains unknown secreted proteins, NanoTag nanobodies (Nb-hIgG) in the culture medium can be specifically captured by Protein A/G magnetic beads, which can then be used to immunoprecipitate NanoTagged proteins. Here, we describe a protocol for immunoprecipitation using NanoTag nanobodies prepared from S2 cell culture in Basic Protocol 1.

Materials

Protein A or G magnetic beads (e.g., SureBeads Protein G Magnetic Beads, Bio-Rad, 1614023)
S2 cell culture medium containing NanoTag nanobodies (Nb-hIgG; see Basic Protocol 1)
Lysis buffer (Pierce IP lysis buffer, Pierce, 87788, or equivalent) supplemented with 1× protease inhibitor cocktail (from 100× Halt Protease Inhibitor Cocktail, Pierce, 87786, or equivalent)
Protein samples with NanoTagged proteins (see, e.g., Basic Protocol 4, steps 1 to 11)
2× Laemmli sample buffer [diluted from 4× Laemmli sample buffer (Bio-Rad, 1610747, or equivalent) supplemented with 5% bME (Sigma, M6250-100)]

1.5-ml Eppendorf tubes
Magnetic stand (e.g., 16-Tube SureBeads Magnetic Rack, Bio-Rad, 1614916)
95°C heating block (Thermo Scientific, 88870001, or equivalent)

1. Thoroughly resuspend Protein A or G magnetic beads in storage solution. Wash 20 µl magnetic beads with lysis buffer supplemented with 1× protease inhibitor cocktail in a 1.5-ml Eppendorf tube using a magnetic stand. Repeat three times total.

   The wash buffer can vary based on the lysis buffer used in protein sample preparation.

2. Incubate 1 ml S2 cell culture medium containing Nb-hIgG with washed magnetic beads at RT for 1 hr or at 4°C overnight.

   The ratio between medium and magnetic beads can vary depending on the expression levels of Nb-hIgG in the conditioned medium.

   After incubation of the beads with the medium, the original conditioned medium and the supernatant can be checked by immunoblotting to determine the coating efficiency. If nanobody-hIgG is detected in the supernatant, it is likely that the magnetic beads are fully coated with NanoTag nanobodies, which have the maximized capturing efficiency of NanoTagged proteins. If nanobody-hIgG is not detected in the supernatant, it is likely that the magnetic beads are not fully coated with NanoTag nanobodies. In this case, the amount of magnetic beads can be reduced or the amount of culture medium containing Nb-hIgG can be increased.

3. Wash beads with 1 ml lysis buffer. Repeat three times total, magnetizing beads using a magnetic stand and discarding the supernatant.

4. Add ~200 µl protein sample with NanoTagged proteins to the beads coated with Nb-hIgG.

   The required volume of protein sample can vary based on the expression levels of the NanoTagged proteins.

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5. Incubate at RT for 1 hr or at 4°C for 2 hr or overnight.
6. Wash beads with 1 ml lysis buffer. Repeat four times total.
7. Elute proteins by boiling the beads in 30 µl of 2× Laemmli sample buffer in a heating block at 95°C for 5 min.

Proceed with SDS-PAGE and immunoblotting (see Basic Protocol 4, steps 13 to 28). For co-immunoprecipitation, use additional antibodies to detect co-immunoprecipitated proteins.

BASIC PROTOCOL 6

IMMUNOPRECIPITATION WITH NANOBODIES PREPARED FROM BACTERIA

NanoTagged proteins can be immunoprecipitated by nanobodies purified from bacterial culture (Basic Protocol 2). His tag–purified nanobodies that include an ALFA tag (Nb-ALFA-His) may contain unwanted bacterial proteins due to the lack of an additional purification step such as size-exclusion chromatography, but they can be specifically captured by ALFA resin and unwanted nonspecific proteins can be washed away. Then, NanoTagged proteins can be pulled down by ALFA resin coated with NanoTag nanobody. Here, we describe a protocol for immunoprecipitation using NanoTag nanobodies prepared from bacteria (Basic Protocol 2).

Materials

ALFA selector PE resin (NanoTag Biotechnologies, N1510)
Lysis buffer (Pierce IP lysis buffer, Pierce, 87788, or equivalent) supplemented with 1× protease inhibitor cocktail (from 100× Halt Protease Inhibitor Cocktail, Pierce, 87786, or equivalent)
His tag–purified NanoTag nanobodies (Nb-ALFA-His; see Basic Protocol 2)
Protein samples with NanoTagged proteins (see, e.g., Basic Protocol 4, steps 1 to 11)
ALFA tag peptide
2× Laemmli sample buffer [diluted from 4× Laemmli sample buffer (Bio-Rad, 1610747, or equivalent) supplemented with 5% bME (Sigma, M6250-100)]

1.5-ml Eppendorf tubes
Microcentrifuge
95°C heating block (Thermo Scientific, 88870001, or equivalent)

1. Wash 20 µl ALFA selector PE resin with 1 ml lysis buffer in a 1.5-ml Eppendorf tube, centrifuging 3 min at 500 × *g* and then removing supernatant supplemented with 1× protease inhibitor cocktail. Repeat three times total.

The wash buffer can vary based on the lysis buffer used in protein sample preparation.

2. Incubate >5 µg Nb-ALFA-His with ALFA selector PE resin in a 1.5-ml Eppendorf tube at RT for 1 hr or at 4°C overnight.

The ratio between His tag–purified nanobodies and ALFA selector PE resin can vary depending on the expression and purity levels of Nb-ALFA-His in the conditioned medium.

After incubation of the ALFA resin and His tag–purified nanobodies, the supernatant can be checked by immunoblotting to determine the coating efficiency. If ALFA-tagged nanobodies are found in the supernatant, it is likely that the ALFA resin is fully coated with NanoTag nanobodies. If ALFA-tagged nanobodies are not found in the supernatant, it is likely that the ALFA resin is not fully coated with NanoTag nanobodies. To have ALFA resin fully coated with ALFA-tagged nanobodies, the amount of Nb-ALFA-His can be increased.

3. Wash resin with 1 ml lysis buffer. Repeat four times total.

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4. Add protein samples with NanoTagged proteins to the resin coated with Nb-ALFA-His.
5. Incubate at RT for 1 hr or at 4°C for 2 hr or overnight.
6. Wash resin with 1 ml lysis buffer. Repeat four times total.
7. Elute proteins with ALFA-tag peptide or by boiling in 30 µl of 2× Laemmli sample buffer in a heating block at 95°C for ≥5 min.

   The NanoTagged protein complex can be eluted by ALFA peptide incubation. Nb-ALFA-His will be co-eluted by ALFA peptide. This will minimize the elution of nonspecific binders to the resin. Follow the instructions provided with the ALFA selector PE resin for peptide elution.

   The NanoTagged protein complex can also be eluted by boiling. However, nonspecific proteins bound to the resin in addition to Nb-ALFA-His will be co-eluted.

   Use SDS-PAGE and immunoblotting to check immunoprecipitated proteins (see Basic Protocol 4, steps 13 to 28). For co-immunoprecipitation, use additional antibodies to detect co-immunoprecipitated proteins.

NbVHH05 AND Nb127D01 USED AS CHROMOBODIES

BASIC PROTOCOL 7

Both NbVHH05 and Nb127D01 can be genetically encoded as fluorescent protein fusions (i.e., CBs), enabling the detection of target proteins tagged with NanoTags. Here, we provide a detailed protocol for constructing NanoTagged proteins or new CBs and for performing cell transfection for detecting CB binding. The pAW vector (cloning vector) is first linearized, and PCR fragments are then joined together with the digested pAW backbone by Gibson assembly. Miniprepped plasmids are transfected into S2R+ cells, and the cells are incubated for 48 hr. NanoTagged proteins are then detected by the CBs.

Materials

pAW cloning vector (DGRC, 1127)
QIAquick Gel Extraction Kit (Qiagen, 28704), including Buffers QG, PE, and EB and QIAquick columns (Qiagen, 28115)
Nuclease-free water (Thermo Fisher Scientific, AM9930)
Chemically competent TOP10 *E. coli* (Invitrogen, C404010)
LB broth (Sigma, L3022-1KG, or equivalent)
LB plate containing 100 µg/ml ampicillin
LB broth (Sigma, L3022-1KG, or equivalent) containing 100 µg/ml ampicillin
QIAprep Spin Miniprep Kit (Qiagen, 27104)
4% (w/v) paraformaldehyde PFA [diluted in 1× PBS (from 10× PBS; Invitrogen, 70011-069) from 16% PFA (Electron Microscopy Sciences, 15710)]
1× PBS (from 10× PBS; Invitrogen, 70011-069)
Mounting medium (Vector Laboratories, H-1300)

42°C and 60°C heating blocks (Thermo Scientific, 88870001, or equivalent)
Microcentrifuge
Spectrophotometer (NanoDrop)
37°C incubator
96-well plate
1.5-ml Eppendorf tubes
Microscope cover glass (VWR Scientific, 48366205), sterilized
Glass microscope slides (Thermo Fisher Scientific, 3050-002), sterilized
Confocal microscope (Nikon Ti2 Spinning Disk)

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Table 2 Restriction Enzyme Double Digestion[a]

Component	Per reaction
NheI-HF (NEB, R3131)	1 µl
XbaI (NEB, R0145)	1 µl
Vector	1 µg
10× rCutSmart buffer (NEB, B6004S)	5 µl
Nuclease-free water	to 50 µl

[a] Digest at 37°C for 2 hr.

Table 3 PCR Fragments and Backbones

Insert (PCR product)	PCR backbone	Comment[a]
NbVHH05-GFP	pW10-NbVHH05-GFP	Cloning for VHH05-tag chromobody
Nb127D01-GFP	pW10-Nb127D01-GFP	Cloning for 127D01-tag chromobody
CD8-mCherry	pQUASp-mCD8mCherry (Addgene, 46164)	Cloning for CD8-mCherry with VHH05-tag or 127D01-tag
H2B-mCherry	pBac (3×P3-gTc'v; pUb:lox-mYFP-lox-H2BmCherry) (Addgene, 119064)	Cloning for H2B-mCherry with VHH05-tag or 127D01-tag
mito-mCherry	pcDNA4TO-mito-mCherry-10×GCN4_v4 (Addgene, 60914)	Cloning for mitochondrial-mCherry with VHH05-tag or 127D01-tag

[a] The purpose of amplifying each PCR fragment is described in the comment.

Table 4 PCR Reaction (Phusion Polymerase) Mix

Component	Per reaction
5× Phusion buffer (NEB, M0530)	10 µl
Phusion polymerase (NEB, M0530)	0.5 µl
Dimethyl sulfoxide (DMSO)	1.5 µl
dNTP mix (NEB, M0530)	1 µl
Vector	100 ng
Nuclease-free water	to 50 µl
Primers[a]	2 × 1 µl

[a] The primer concentration is 10 µM. Detailed primer information listed in Table 5.

Additional reagents and equipment for linearization of pAW vector (see Table 2), amplification of cloning inserts by PCR with backbone plasmids (Tables 3–6), agarose gel electrophoresis (see Current Protocols article: Voytas, 2000), Gibson assembly (Table 7), colony PCR (Tables 8 and 9), Sanger sequencing, and transfection with Effectene (see Basic Protocol 3, step 1)

1. Linearize pAW cloning vector with NheI-HF and XbaI (Table 2).

   After digestion, run the reaction mixture on an agarose gel (step 2), excise the large fragments from the gel (step 2), and purify by gel purification (step 3).

2. Amplify cloning inserts (PCR fragments) by PCR with backbone plasmids (Tables 3 to 6). After the PCR reaction, proceed with electrophoresis of reaction mixture in a 1% agarose gel at 170 V for 20 min.

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Table 5 Primer Information

Name	Sequence	Purpose
pAW-nb-F	TACCATCCAGCCTCCGGACTCTAGAGAATTGGGAATTCC AAAATG	Cloning VHH05-GFP or 127D01-GFP PCR fragment to make pAW-NbVHH05-GFP or pAW-Nb127D01-GFP
pAW-nb-R	TCCTTCACAAAGATCCTGCTAGCTTACTTGTACAGCTCGT CCA	
H2B-mCherry-F	TACCATCCAGCCTCCGGACTCTAGAatggctccgaaaactagtgg	Cloning H2B-mCherry-VHH05 fragment to make pAW-H2B-mCherry-VHH05
VHH05-H2B-R	TCCTTCACAAAGATCCTGCTAGCTCAGGAAATCTGCCGT GCCAATTCTTTAGCCTCTTGATCTGCCTGtccggatcccttgtaca gctcgtccatgc	
H2B-mCherry-F	TACCATCCAGCCTCCGGACTCTAGAatggctccgaaaactagtgg	Cloning H2B-mCherry-127D01 fragment to make pAW-H2B-mCherry-127D01
127D01-H2B-R	TCCTTCACAAAGATCCTGCTAGCTCAATCCTCGCCTTTC CAGAAATCTTCAAAACTtccggatcccttgtacagctcgtccatgc	
CD8-mCherry-F	TACCATCCAGCCTCCGGACTCTAGAatggcctcaccgttgacccg	Cloning CD8-mCherry-VHH05 fragment to make pAW-CD8-mCherry-VHH05
VHH05-H2B-R	TCCTTCACAAAGATCCTGCTAGCTCAGGAAATCTGCCGT GCCAATTCTTTAGCCTCTTGATCTGCCTGtccggatcccttgtaca gctcgtccatgc	
CD8-mCherry-F	TACCATCCAGCCTCCGGACTCTAGAatggcctcaccgttgacccg	Cloning CD8-mCherry-127D01 fragment to make pAW-CD8-mCherry-127D01
127D01-H2B-R	TCCTTCACAAAGATCCTGCTAGCTCAATCCTCGCCTTTC CAGAAATCTTCAAAACTtccggatcccttgtacagctcgtccatgc	
mito-mCherry-F	TACCATCCAGCCTCCGGACTCTAGAatgagtctgacttccagttc	Cloning mito-mCherry-VHH05 fragment to make pAW-mito-mCherry-VHH05
VHH05-H2B-R	TCCTTCACAAAGATCCTGCTAGCTCAGGAAATCTGCCG TGCCAATTCTTTAGCCTCTTGATCTGCCTGtccggatcccttgta cagctcgtccatgc	
mito-mCherry-F	TACCATCCAGCCTCCGGACTCTAGAatgagtctgacttccagttc	Cloning mito-mCherry-127D01 fragment to make pAW-mito-mCherry-127D01
127D01-H2B-R	TCCTTCACAAAGATCCTGCTAGCTCAATCCTCGCCTTTC CAGAAATCTTCAAAACTtccggatcccttgtacagctcgtccatgc	
mCherry-VHH05-F	ctctcatctgctaccacagcggatccggaCAGGCAGATCAAGAGGCTA AAGAATTGGCACGGCAGATTTCCggatccggaatggtg agcaagggcgagga	Cloning VHH05-mCherry fragment to make pAW-CD8-VHH05-mCherry
mCherry-VHH05-R	GTTCCTTCACAAAGATCCTGCTAGCTCActtgtacagctcgtcca tgc	
mCherry-127D01-F	ctctcatctgctaccacagcggatccggaAGTTTTGAAGATTTCTGGAA AGGCGAGGATggatccggaatggtgagcaagggcgagga	Cloning 127D01-mCherry fragment to make pAW-CD8-127D01-mCherry

Table 6 PCR Program

Initial step:	30 sec	98°C
30 cycles:	10 sec	98°C
	30 sec	(Tm – 5)°C
	30 sec/Kb	72°C
Final step:	hold	10°C

3. Purify pAW backbone and PCR fragments in the gel from steps 1 and 2 using a QIAquick Gel Extraction Kit and QIAquick columns following the manufacturer's protocol with modification:

    a. Incubate gel in Buffer QG (three times the volume of the gel) at 60°C in a heating block.

    b. Filter dissolved gel via the spin columns for 30 s at 7000 rpm.

    c. Wash the spin columns using 300 µl Buffer QG for 30 s at 7000 rpm.

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Table 7 Gibson Assembly Reaction Mix[a]

Component	Per reaction
2× Gibson assembly mix (NEB, E2611)	5 μl
pAW backbone (NheI and XbaI linearized)	20 ng
PCR fragments	50 ng
Nuclease-free water (Thermo Fisher Scientific, AM9930)	to 10 μl

[a] Incubate the mixture at 50°C for 1 hr.

d. Wash the spin columns using 700 μl Buffer PE for 30 s at 7000 rpm.
e. Spin-dry columns for 1 min at 12,000 rpm.
f. To elute DNA, add 40 μl Buffer EB or nuclease-free water and spin for 1 min at 12,000 rpm.
g. Measure DNA concentration of the pAW backbone and PCR fragments using a spectrophotometer.

4. Perform a Gibson assembly reaction by ligating pAW backbone and PCR fragments by three enzymatic activities (Table 7).

The resultant NbVHH05 chromobody and VHH05-tagged protein expression plasmids are pAW-NbVHH05-GFP (NbVHH05 chromobody, Addgene, 171570, or DGRC, 1556); pAW-VHH05-H2B-mCherry (VHH05-tagged nuclear protein; used in Xu et al., 2022); pAW-CD8-VHH05-mCherry (VHH05-tagged membrane protein; used in Xu et al., 2022); and pAW-mito-mCherry-VHH05 (VHH05-tagged mitochondrial protein; used in Xu et al., 2022).

The resultant Nb127D01 chromobody and 127D01-tagged protein expression plasmids are pAW-Nb127D01-GFP (Nb127D01 chromobody, Addgene, 171571, or DGRC, 1557); pAW-127D01-H2B-mCherry (Nb127D01-tagged nuclear protein; used in Xu et al., 2022); pAW-CD8-127D01-mCherry (Nb127D01-tagged membrane protein; used in Xu et al., 2022); and pAW-mito-mCherry-127D01 (Nb127D01-tagged mitochondrial protein; used in Xu et al., 2022).

5. Perform transformation:

a. After the Gibson assembly reaction, transfer 5 μl reaction mix into 50 μl chemically competent TOP10 *E. coli* for 30 min on ice.
b. Heat-shock at 42°C for 90 s in a heating block.
c. Add 400 μl LB broth for recovery at 37°C for 15 min.

The plasmids with AmpR do not require full incubation, such as 45 min to 1 hr.

d. Plate 200 μl resuspended cells on an LB plate containing 100 μg/ml ampicillin for selection and incubate at 37°C overnight.

6. Pick single bacterial colonies from the LB plate and inoculate them into a 96-well plate with 50 μl LB broth containing 100 μg/ml ampicillin in each well. After 2 hr of growth, use bacterial culture as the template for colony PCR (Tables 8 and 9).

7. Inoculate colonies with desired insert into 3 ml LB broth containing 100 μg/ml ampicillin. Use a QIAprep Spin Miniprep Kit to isolate plasmid DNA according to the manufacturer's instructions. Verify insert sequence by Sanger sequencing.

8. To test the CBs and their binding to the corresponding tag (each has a prediction of the subcellular compartment), transfect plasmids encoding the CB and a compatible NanoTagged protein (100 ng:100 ng) into S2R+ cells in a 24-well plate using Effectene (see Basic Protocol 3, step 1). For CB testing in the S2R+ cells, transfect plasmids encoding the NanoTag CB and the NanoTagged protein.

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Table 8 PCR Reaction (Takara Taq Polymerase) Mix

Components	Per reaction
10× Takara Taq buffer (Clontech, TAKR001C)	5 µl
Takara Taq polymerase (Clontech, TAKR001C)	0.25 µl
Primers[a]	2 × 1 µl
dNTP mix (Clontech, TAKR001C)	1 µl
Template	1 µl
Nuclease-free water (Thermo Fisher Scientific, AM9930)	15.75 µl

[a] The primer concentration is 10 µM. The primers are as follows:
Primer 1: ACACAAAGCCGCTCCATCAG
Primer 2: CTCATTAAAGGCATTCCACCA

Table 9 PCR Program (Takara Taq Polymerase)[a]

Initial step:	10 sec	98°C
30 cycles:	10 sec	98°C
	30 sec	55°C
	30 sec/Kb	72°C
Final step:	hold	10°C

[a] Run the PCR reactions on a 1% agarose gel at 170 V for 20 min. Identify the correct colonies by the size of the PCR product.

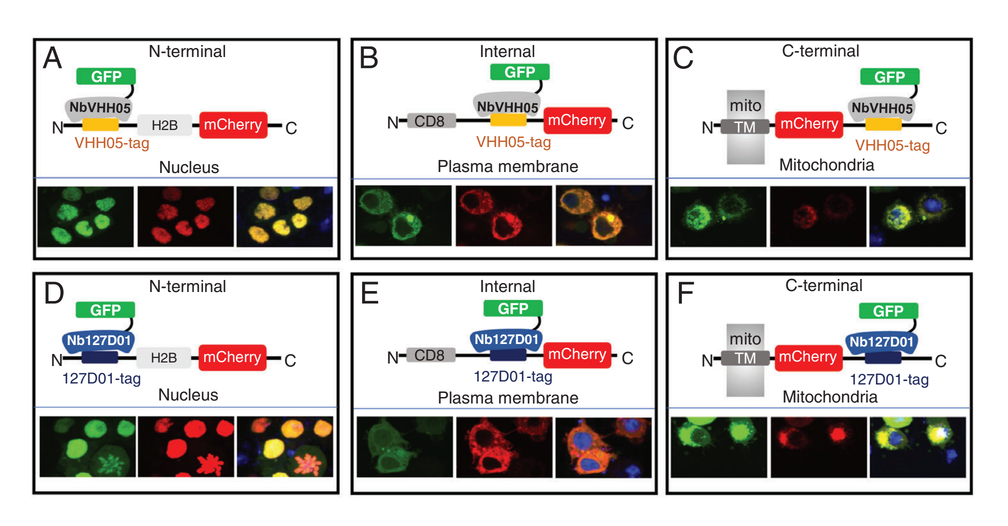

Figure 4 VHH05 and 127D01 NanoTags and their corresponding nanobodies used as chromobodies. (**A**) Co-transfection of pAW-NbVHH05-GFP and pAW-VHH05-H2B-mCherry into S2R+ cells. H2B is a nuclear protein. The left-hand panel is GFP, the center panel is mCherry, and the right-hand panel is the merged image. 4′,6-Diamidino-2- phenylindole (DAPI) staining shows the nuclei. (**B**) Co-transfection of pAW-NbVHH05-GFP and pAW-CD8-VHH05-mCherry into S2R+ cells. CD8 is a cell membrane protein. (**C**) Co-transfection of pAW-NbVHH05-GFP and pAW-mito-mCherry-VHH05 into S2R+ cells. Mito-mCherry-VHH05 contains a localization signal peptide for mitochondrial outer membrane targeting. (**D-F**) Experiments are as in (A), (B), and (C), except that pAW-Nb127D01-GFP and pAW-127D01-H2B-mCherry were co-transfected. From Xu et al. (2022) and used in accordance with the CC-BY license applied by eLife (see *https://creativecommons.org/licenses/by/4.0/*).

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NanoTagged proteins do not need to be tagged with fluorescent proteins, but to visualize the same localization between NanoTag CB and NanoTagged proteins as examples, mCherry proteins with NanoTags and different localization signals are shown with NanoTag CB with GFP (Fig. 4). The example plasmids used in Figure 4 can be found in the Materials list above.

9. Forty-eight hours after transfection, fix cells to visualize NanoTagged protein–CB complexes:

   a. Place 50 to 100 µl transfected cells onto a sterilized microscope cover glass for 1 to 2 hr.
   b. Fix cells with 4% PFA for 20 min.
   c. Wash with 1× PBS three times.
   d. Place cover glass on a sterilized glass microscope slide and mount with ~20 µl mounting medium.

   Alternatively, the cells can be live-imaged because CBs and their corresponding NanoTagged proteins are tagged with fluorescent proteins.

10. Capture confocal images with a confocal microscope.

    For sample data, see Figure 4.

BASIC PROTOCOL 8

NANOTAG TRAP AS A METHOD TO ALTER PROTEIN LOCALIZATION

NanoTag "traps" use fusions of the NanoTag nanobody to localization signals or domains in order to alter the localization of the nanobody and, along with it, any NanoTagged proteins to which it is bound. Thus, a NanoTag trap can be used to determine whether protein localization is important for protein function. In this protocol, we introduce some examples of altering protein localization using endoplasmic reticulum (ER) NanoTag traps and cell membrane NanoTag traps.

Materials

See Basic Protocols 3 and 7.

1. Prepare NanoTag trap plasmids and NanoTagged protein expression plasmids (see Basic Protocol 7, steps 1 to 7).

   Secreted NanoTagged GFP proteins can be captured by ER NanoTag traps.

   Mitochondrial NanoTagged mCherry proteins can be mislocalized by cell membrane NanoTag traps.

   All plasmids can be constructed as described in Basic Protocol 7 or can be obtained from Addgene, DGRC, or the Perrimon lab. To construct your own plasmids, linearize a destination vector with the appropriate restriction enzyme. PCR fragments with cloning homology arms can then be joined together with the linearized backbone by Gibson assembly. The sequence-verified plasmids are used for the following cell transfection.

   The resultant NanoTagged protein expression plasmids are pAW-mito-mCherry-127D01 (mitochondrial 127D01-tagged mCherry; used in Xu et al., 2022); pAW-BiP-GFP-127D01 (secreted 127D01-tagged GFP; used in Xu et al., 2022); pAW-BiP-GFP-VHH05 (secreted VHH05-tagged GFP; used in Xu et al., 2022); and pAW-mito-mCherry-VHH05 (mitochondrial VHH05-tagged mCherry; used in Xu et al., 2022).

   The resultant VHH05 NanoTag trap plasmids are pAW-BiP-NbVHH05-mCherry-KDEL (ER NanoTag trap; Addgene, 171574, or DGRC, 1560) and pAW-CD8-NbVHH05-GFP (cell membrane NanoTag trap; Addgene, 171676, or DGRC, 1562).

   The resultant 127D01 NanoTag trap plasmids are pAW-BiP-Nb127D01-mCherry-KDEL (ER NanoTag trap; Addgene, 151575, or DGRC, 1561) and pAW-CD8-Nb127D01-GFP (cell membrane NanoTag trap; Addgene, 171577 or DGRC, 1563).

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2. Perform transfection in S2R+ cells (see Basic Protocol 3, step 1).

   Transfect plasmids for expressing NanoTag Traps and NanoTagged proteins into S2R+ cells using Effectene. Transfection is described in detail in Basic Protocol 3, step 1.

3. Fix and image transfected cells (see Basic Protocol 3, steps 2 to 9).

   After 48 hr, fix and image the transfected cells using a confocal microscope. To do so, resuspend the transfected cells in the plate by pipetting, transfer 50 to 100 μl suspension to a microscope cover glass, and incubate for 1 to 2 hr. Fix the cells with 4% PFA in 1× PBS. Later, the microscope cover glass can be placed on microscope slides with mounting medium. Because proteins with secretion signal peptides are actively secreted, the expression levels of secreted proteins inside the cells are weak when ER NanoTrap is absent. However, when transfected with ER NanoTraps, cells have more secreted proteins inside compared to control cells.

4. Perform immunoblotting with ER NanoTag traps (see Basic Protocol 4).

   For an ER NanoTag trap, trapping efficiency can be determined by immunoblotting. Cells are resuspended in medium by pipetting and transferred into a 1.5-ml Eppendorf tube. After centrifugation for 2 min at 500 × g, 4°C, separate the supernatant (medium) and the cell pellet into new tubes. Boil them in SDS sample buffer for 10 min at 100°C for immunoblotting. A detailed protocol for immunoblotting can be followed as in Basic Protocol 3, steps 2 to 9. Primary antibodies are rabbit polyclonal anti-GFP antibody (Molecular Probes, A-6455; 1:10,000) and mouse monoclonal anti-α-tubulin antibody (Sigma-Aldrich, T5168; 1:10,000), and secondary antibodies are anti-rabbit-HRP (1:10,000) and anti-mouse-HRP (1:10,000). Because proteins with secretion signal peptide are actively secreted, the secreted proteins from cellular lysates without ER NanoTraps have weaker bands on the immunoblot compared to the secreted proteins prepared from the cells with ER NanoTraps.

   See Figure 5 for sample data.

CRISPR-MEDIATED TAGGING OF ENDOGENOUS GENES WITH NANOTAGS

SUPPORT PROTOCOL

Studying proteins expressed at endogenous levels is essential to understand their physiologically relevant functions. In this protocol, we describe how to insert VHH05 or 127D01 NanoTags at the C-terminus of the *H2Av* gene using CRISPR knock-in (KI) technology. This scarless CRISPR editing strategy can also be adapted to introduce other small or large sequences into the endogenous genes, similar to other CRISPR KI technologies (see Current Protocols article: Bosch et al., 2020; Kanca et al., 2019).

Materials

Drosophila strains:
- yw; nos-Cas9/CyO (originally from Bloomington Drosophila Stock Center, 78781)
- w; TM3/TM6B, Tb (Bloomington Drosophila Stock Center, 3720)
- w; Sp^1/CyO, tub-piggyBac; l(3)*/ TM6B, Tb (Bloomington Drosophila Stock Center, 8285)

Quick-gDNA Miniprep Kit (Zymo Research, D3025), containing Genomic Lysis Buffer, Zymo-Spin Columns, Collection Tubes, DNA Pre-Wash Buffer, and g-DNA Wash Buffer
Nuclease-free water (Thermo Fisher Scientific, AM9930)
pScarlessHD-2×HA-DsRed (Addgene, 80822)
Injection buffer: 100 μM $NaPO_4$ and 5 mM KCl

1.5-ml Eppendorf tubes
Pellet pestles (Kimble Kontes pellet pestles, Millipore, Z359947)

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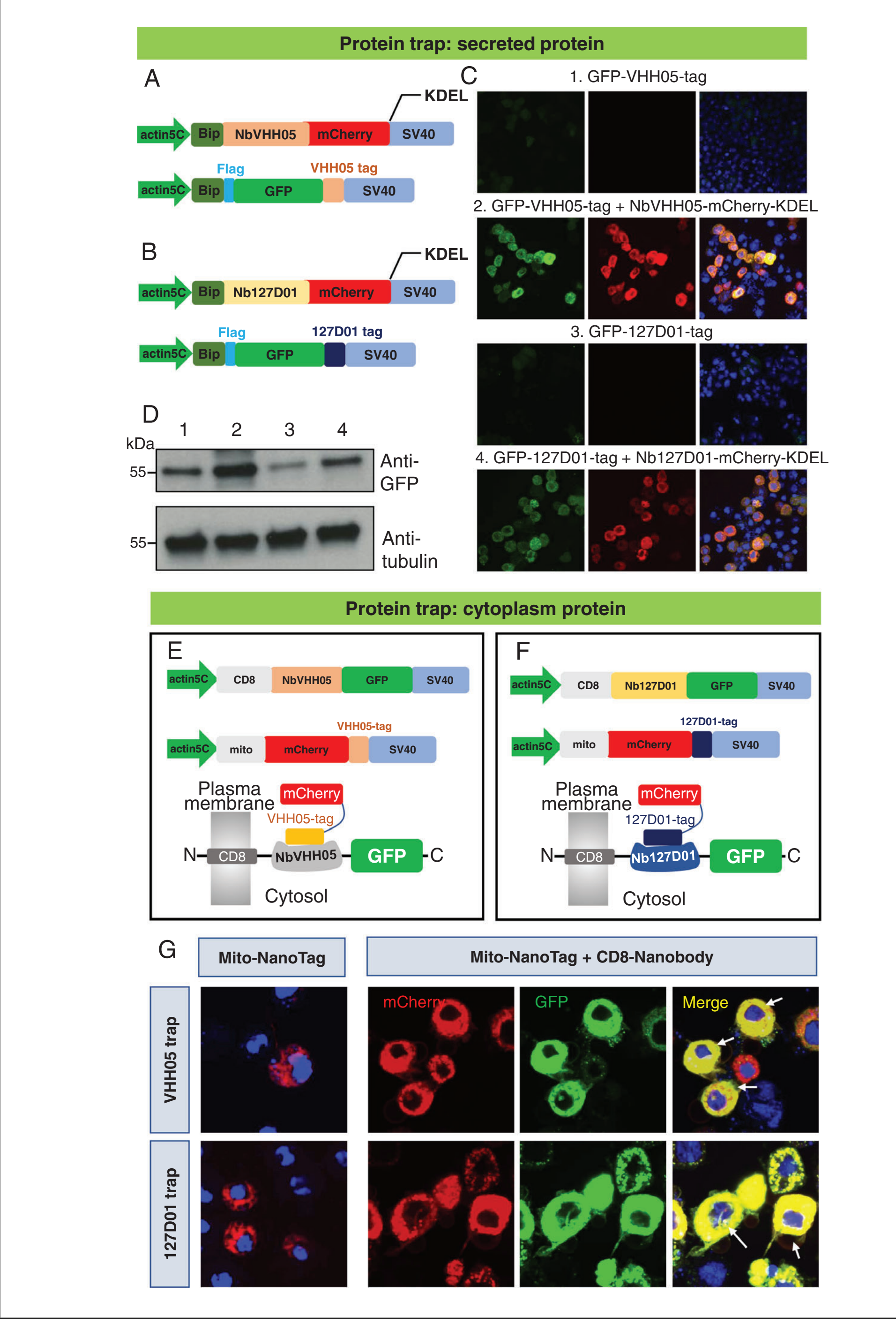

Figure 5 Nanobody-based system for altering the localization of NanoTagged proteins. (**A and B**) Diagram showing the vectors used for the secreted protein trapping method. NbVHH05/Nb127D01 fused to mCherry contains KDEL and BiP signal peptide and is driven by the actin5C promoter. (**C**) Four independent cell transfection experiments were performed. In 1 and 3, only GFP-VHH05 or GFP-127D01 was transfected. In 2 and 4, NbVHH05-mCherry-KDEL was co-transfected with GFP-VHH05 or Nb127D01-mCherry-KDEL was co-transfected with GFP-127D01, respectively. Images show the GFP and mCherry signals 48 hr after transfection. Nuclei are stained with DAPI. (**D**) Immunoblots of GFP and tubulin in cell lysates from transfections 1 to 4. (**E and F**) Diagrams showing the vectors used for cytoplasmic protein trapping. NbVHH05/Nb127D01 is fused to GFP and CD8 and driven by the actin5C promoter. Target proteins are mCherry containing VHH05-tag or 127D01-tag at the C-terminus and mito signal at the N-terminus. (**G**) Results of transfection of mito-mCherry-NanoTag or co-transfection of CD8-NbVHH05-GFP/mito-mCherry-VHH05 and CD8-Nb127D01-GFP/mito-mCherry-127D01 in S2R+ cells. GFP, mCherry, and merged channels show protein expression levels with antibody staining. From Xu et al. (2022) and used in accordance with the CC-BY license applied by eLife (see *https://creativecommons.org/licenses/by/4.0/*).

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Table 10 Restriction Enzyme Reaction Mix[a]

BbsI-HF® (NEB, R3539)	1 µl
pCFD3 (Addgene, 49410)	1 µg
10× rCutSmart buffer (NEB, B6004S)	5 µl
Nuclease-free water (Thermo Fisher Scientific, AM9930)	to 50 µl

[a] Digest at 37°C for 2 hr.

Table 11 Phosphorylation and Annealing Reaction Mix[a]

Sense oligo (100 µM)	1 µl
Anti-sense oligo (100 µM)	1 µl
10× T4 ligation buffer (NEB, M0201)	1 µl
T4 PNK (NEB, M0201)	0.5 µl
Nuclease-free water (Thermo Fisher Scientific, AM9930)	6.5 µl

Table 12 Reaction Program

30 min	37°C
5 min	95°C
5°C/min	ramp down to 25°C

Vortex
Microcentrifuge
Microinjection handle (Joystick Micromanipulator, NARISHIGE, MN-151) with pressure control (FemtoJet, Eppendorf, LV41365120)
18°C injection room

Additional reagents and equipment for plasmid construction; restriction enzyme digestion of pCFD3 (see Table 10); gel purification (see Basic Protocol 7, step 3); phosphorylation of sgRNA oligos (see Table 11); sgRNA oligo annealing (see Table 12); ligation of linearized vector and oligos (see Table 13); transformation, colony PCR, and plasmid miniprep (see Basic Protocol 7, steps 5 to 7); PCR amplification of right and left homology arms; restriction enzyme digestion of vector (see Table 14); Gibson assembly (see Basic Protocol 7, step 4); and isolation of transformed lines using DsRed marker

1. Perform sgRNA design and plasmid construction.

   Select sgRNA sites at the target site based on the presence of NN$_{(19)}$NGG.

   sgRNA primers:

   Sense: 5’-GTCG-N19/20-3’

   Anti-sense: 5′-AAAC-N19/20-3′ reverse complement

   For the H2Av gene, the following sgRNA primers were used:

   5′-GTCGCGATTGCCGACTGGGTTAGT-3′

   5′-AAACACTAACCCAGTCGGCAATCG-3′.

2. Digest pCFD3 with BbsI restriction enzyme (Table 10) and perform gel purification (see Basic Protocol 7, step 3).

3. Phosphorylate sgRNA oligos (Table 11).

4. Anneal sgRNA oligos (Table 12).

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Table 13 Ligation Reaction Mix[a]

BbsI-digested pCFD3 (use 50 ng)	x µl
Annealed oligos diluted 1:100	1 µl
10× T4 ligation buffer (NEB, M0202)	1.5 µl
T4 DNA ligase (NEB, M0202)	1 µl
Nuclease-free water (Thermo Fisher Scientific, AM9930)	to 15 µl

[a] Incubate the mixture at RT for 1 hr.

Table 14 Restriction Enzyme Reaction Mix[a]

EcoRI-HF (NEB, R3101)	1 µl
Antarctic phosphatase (NEB, M0289S)	0.5 µl
pScarlessHD-2×HA-DsRed	1 µg
10× rCutSmart buffer (NEB, B6004S)	5 µl
Nuclease-free water (Thermo Fisher Scientific, AM9930)	to 50 µl

[a] Digest at 37°C for 1 hr and proceed to gel purification.

5. Ligate linearized vector and oligos (Table 13).
6. Perform transformation, colony PCR, and plasmid miniprep as described in Basic Protocol 7, steps 5 to 7.
7. Perform donor design and plasmid construction.

   The following primers are used to amplify the right and left homology arms of H2Av.

   For the right homology arm:

   5′-GACTATCTTTCTAGGGTTAAAGTCGGCAATCGGACGCCTT-3′

   5′-GTTTAAACGAATTCGCCCTTTCGCTCCGTCTCGCGCCACGA-3′

   For the left homology arm:

   5’-TTGGTGGATCTGGAGGTTCCTGGTTGGTTGGGGTTGACAA-3′

   5′-CCGGAGCCGCTGCCACCTCCGTAGGCCTGCGACAGAATGA-3′

8. Extract genomic DNA from flies to be injected using Quick-gDNA Miniprep Kit:
   a. Collect 6 to 8 flies (specifically yw; nos-Cas9/CyO females) in a 1.5-ml Eppendorf tube.
   b. Add 200 µl Genomic Lysis Buffer.
   c. Homogenize flies using pellet pestles.
   d. Vortex for 4 to 6 s.
   e. Incubate at RT for 10 min.
   f. Transfer mixture into a Zymo-Spin Column in a Collection Tube.
   g. Centrifuge 1 min at 7000 rpm.
   h. Wash column with DNA Pre-Wash Buffer.
   i. Wash column with g-DNA Wash Buffer.
   j. Elute genomic DNA with Nuclease-free water (Thermo Fisher Scientific, AM9930).
9. Perform PCR amplification of right and left homology arms (see Basic Protocol 7, step 2, and Tables 4 and 6).

   Use the designed primers from step 7 and the genomic DNA from step 8 for PCR amplification.

10. Perform restriction enzyme digestion of vector (Table 14).

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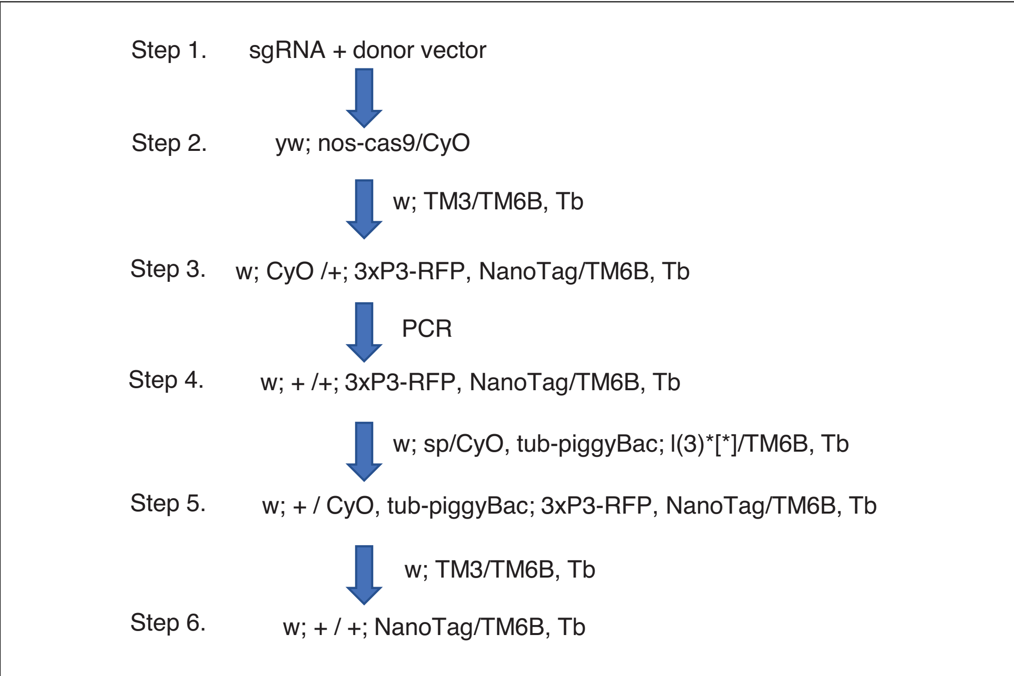

Figure 6 Example workflow for introducing tags via CRISPR knock-in (KI) into a site on the third chromosome.

11. Assemble 3×P3-DsRed (from pScarlessHD-2×HA-DsRed) right and left homology arms into the linearized vector by Gibson cloning to generate the donor plasmids (pScarlessHD-2×HA-DsRed).

    Gibson assembly, transformation, colony PCR, and plasmid miniprep are followed as described in Basic Protocol 7, steps 4 to 7.

12. Proceed to germline transformation to generate KI flies:

    a. Mix donor (see step 10) and sgRNA (see step 6) plasmids (final concentration, ∼200 ng/μl in injection buffer).
    b. Inject mixture into ∼100 fertilized embryos (yw; nos-Cas9/CyO) through a microinjection handle with pressure control in an 18°C injection room.

13. Screen transformants with DsRed marker:

    a. Keep injected embryos at 18°C to the pupal stage and then transfer to 25°C.
    b. Cross adults to w; TM3/TM6B, Tb flies.
    c. Isolate transformed lines using DsRed marker.

    To improve the success rate, injected males can be crossed with w; TM3 /TM6, Tb flies several times.

14. To remove the DsRed cassette, cross transformed lines to a line expressing PBac transposase (w; Sp^1/CyO, tub-piggyBac; l(3)*/ TM6B, Tb).

    A detailed workflow is shown in Figure 6.

15. Sequence-verify resulting lines to confirm the insertion of NanoTags at the C-terminus of the *H2Av* gene.

COMMENTARY

Background Information

Tag binders [such as single-chain variable fragment (scFv) and nanobody (Nb)] have emerged in recent years as powerful tools to study protein function. GFP-tagged proteins can be captured and trapped using a GFP nanobody or degraded by deGradFP (Harmansa, Alborelli, Bieli, Caussinus, & Affolter, 2017; Caussinus, Kanca, & Affolter, 2011). Despite the usefulness of GFP

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nanobody–based applications, adding GFP to proteins may affect the target protein structure, resulting in the generation of nonfunctional proteins. An alternative is to tag proteins with small epitopes, as these are less likely to interfere with the overall protein structures.

Thus, proteins of interest can be tagged with small peptide epitopes so that antibodies recognizing the peptides, such as Myc, HA, and FLAG, can be used for immunostaining and immunoprecipitation. However, such peptide-recognizing antibodies cannot be expressed intracellularly. To overcome this drawback of antibodies, intracellularly expressible protein binders have gotten attention. One way to overcome the drawback of conventional antibodies is engineering them into an intracellularly expressible format. For example, recently reported frankenbodies against HA and FLAG tags (Liu et al., 2021; Zhao et al., 2019) can help to visualize proteins tagged with HA or FLAG intracellularly.

The other way to overcome the drawback is a nanobody, which can be easily expressed intracellularly. Nanobodies recognizing small epitope tags have been successfully used to visualize and manipulate proteins tagged with the epitopes in mammalian cells (Boersma et al., 2019; De Genst et al., 2010; Götzke et al., 2019; Tanenbaum et al., 2014; Traenkle et al., 2015, Cabalteja, Sachdev, & Cheloha, 2022). The two nanobodies/NanoTags that we characterized, NbVHH05 and Nb127D01 (Xu et al., 2022), allow similar manipulations as those shown for GFP nanobodies. However, because the corresponding tags, VHH05 and 127D01, are much smaller than GFP, these tags are less likely to affect the activity of a targeted protein.

Critical Parameters and Troubleshooting

Choice of NanoTag location within a protein

Although NanoTags work well when fused at an N-terminal, internal, or C-terminal site in a target protein in most cases, in some cases, the function of a tagged protein can be compromised. We recommend checking prediction algorithms for the presence of a protein secretion signal, protein cleavage site, or transmembrane domain before deciding where to insert the tag. We also recommend that you avoid inserting the NanoTag into a functional domain and test multiple tagging locations.

Failure to isolate knocked-in tags

Sometimes, NanoTagged protein–expressing flies cannot be obtained after CRISPR-based editing (Support Protocol). This may be due to failure of DNA cleavage at the sgRNA-targeted endogenous locus, as is observed in other CRISPR-based genome engineering. It may be possible to troubleshoot with a different sgRNA with a better cutting efficiency. An alternative is to use prime editing to insert sequences into any position in the gene (Anzalone et al., 2019; Bosch, Birchak, & Perrimon, 2021). Because the sizes of the two NanoTags are small enough for pegRNA designs, it should be possible to insert single copies of a NanoTag using this system (Anzalone et al., 2019).

In rare cases, a NanoTag inserted into an endogenous locus can produce a dominant lethal allele. Here, we advise altering the position of the tag (e.g., from the N-terminus to the C-terminus).

Understanding Results

NanoTag nanobody preparation in both fly cells and bacteria and NanoTagging protocols have been used to visualize and manipulate a protein of interest. Correctly NanoTagged proteins will enable study of localization and function by using purified nanobodies or intracellularly expressed nanobodies along with appropriate tools.

Time Considerations

Preparation of nanobodies expressed from S2 cells (Basic Protocol 1): Cell culture medium that contain nanobodies can be collected within 7 days upon transfection.

Preparation of nanobodies purified from bacterial culture (Basic Protocol 2): Purified nanobodies from bacteria can be obtained within 7 days upon bacterial transformation.

Immunostaining with nanobodies (Basic Protocol 3): It will take several days to visualize NanoTagged proteins.

Cloning of plasmids encoding NanoTagged proteins: It will take ∼1 week to clone the plasmids. They can be fluorescent protein free and visualized by either immunostaining (Basic Protocol 3) or live imaging with CBs (Basic Protocol 7).

Immunoblotting of NanoTagged proteins (Basic Protocol 4): Immunoblotting can be completed within 2 days if nanobodies are already available. If NanoTagged protein–containing sample preparation is needed, additional time will be required.

Immunoprecipitation of NanoTagged proteins (Basic Protocols 5 and 6): Immunoprecipitation can be finished within 2 days. To see the results, immunoblotting will be needed,

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which takes ~2 days. If protein samples with NanoTagged proteins and anti-NanoTag nanobodies are not ready, the additional time will be needed.

Live imaging of NanoTagged proteins with NanoTag CBs (Basic Protocol 7): If all plasmids are ready, it will take 2 days to 1 week to see the results.

Manipulation of protein localization by NanoTag traps (Basic Protocol 8): To see the mislocalized proteins with NanoTags, cells need to be transfected with multiple plasmids that encode NanoTagging and NanoTag traps. A negative control without NanoTag trap should be tested as well to see how NanoTagged proteins are normally localized. All processes will take ~1 week.

Generation of transgenic flies with NanoTagging at the endogenous locus (Support Protocol): The plasmid construction will take ~1 week. The transgene process will take about 3 to 4 months.

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Author Contributions

Jun Xu: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Resources, Software, Validation, Visualization, Writing – original draft, Writing – review and editing. **Ah-Ram Kim**: Conceptualization, Data curation, Investigation, Methodology, Resources, Software, Validation, Visualization, Writing – original draft, Writing – review and editing. **Ross Cheloha**: Conceptualization, Methodology, Resources. **Stephanie E. Mohr**: Methodology, Project administration, Writing – review and editing. **Jonathan Zirin**: Methodology, Project administration, Resources, Writing – review and editing. **Hidde L. Ploegh**: Conceptualization, Resources, Supervision. **Norbert Perrimon**: Conceptualization, Data curation, Funding acquisition, Project administration, Resources, Supervision, Validation, Writing – review and editing.

Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

All data generated or analyzed in this article are from Xu et al. (2022). See the details and additional information in Xu et al. (2022).

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Key Reference

Xu et al. (2022). See above.

Original article about NbVHH05 and Nb127D01.

Internet Resources

http://www.addgene.org/

Addgene is a non-profit organization that operates a plasmid repository for the research community. All vectors for expressing nanobodies and NanoTagged proteins described in the article can be purchased from Addgene.

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http://flystocks.bio.indiana.edu/

The Bloomington Drosophila Stock Center (at Indiana University) collects, maintains, and distributes Drosophila melanogaster strains for research.

https://dgrc.bio.indiana.edu/

Description for the Drosophila Genomics Resource Center (DGRC). All vectors for expressing nanobodies and NanoTagged proteins described in the article can be purchased from the DGRC.

http://flybase.org

Flybase is the leading database and Web portal for genetic and genomic information on Drosophila melanogaster and related fly species.

https://services.healthtech.dtu.dk/service.php?SignalP-5.0

The SignalP 5.0 server predicts the presence of signal peptides and the location of their cleavage sites in proteins from Archaea, Gram-positive bacteria, Gram-negative bacteria, and Eukarya.

https://services.healthtech.dtu.dk/service.php?ProP-1.0

The ProP 1.0 server predicts arginine and lysine propeptide cleavage sites in eukaryotic protein sequences using an ensemble of neural networks. Furin-specific prediction is the default. It is also possible to perform a general proprotein convertase (PC) prediction.

https://dtu.biolib.com/DeepTMHMM

DeepTMHMM is a deep learning model for transmembrane topology prediction and classification.

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