Product Name: ^{RapidS}**PALM, Protein *S*-Palmitoylation Detection Kit**

Code No. : F017A / F017B

Product Name: ^{RapidS}**PALM, Protein *S*-Palmitoylation Detection Kit**

Code No. : F017A

<Kit components and Storage condition>

Component Name	Quantity and package	Storage
2x Base Buffer	1 bottle	RT
Reduction Reagent	1 vial	<-20°C
Blocking Reagent A	1 vial	<-20°C
Blocking Reagent B	1 vial	<-20°C
Cleavage Reagent	1 vial	<-20°C
Cleavage Control	1 vial	<-20°C
MfTag-labeling Reagent	1 vial	<-20°C
Supplement	1 vial	<-20°C

Product shelf life : 1 year

Product Name: ^{RapidS}**PALM, Additional Components for Affinity Purification**

Code No. : F017B

<Kit components and Storage condition>

Component	Size	Store condition
2x Binding Buffer	1 bottle	RT
10x Wash Additive	2 vials	RT
10x Elution Additive	1 vial	RT
Empty Column	24 columns	RT
Affinity Beads	4 vials	4°C (DO NOT freeze)
10x Reduction Reagent	1 vial	4°C (Before reconstitute) -20°C (After reconstitute)

Product shelf life : 1 year

Introduction Part

1. Abstract

***RapidS*PALM, Protein *S*-Palmitoylation Detection Kit** is a novel and comprehensive analysis kit for the protein *S*-palmitoylation, including *S*-acylation. *S*-palmitoyl/acyl groups on proteins are chemically and rapidly substituted to our unique **m**ulti**f**unctional-**tag** (hereafter MfTag) by multistep chemical reactions. ***RapidS*PALM** kit provides the following information.

1) Quantification of total *S*-palmitoylation amounts in samples by fluorometric measurement
2) Visualization of *S*-palmitoylation protein bands in SDS-PAGE gel by fluorometric detection
3) Estimation of *S*-palmitoyl group number on target proteins by Western blotting
4) Comprehensive purification of *S*-palmitoylated proteins by affinity column
5) Estimation of *S*-palmitoylated ratio of target proteins by affinity column and Western blotting

A wide range of samples, including animal tissues, cultured cells, plant tissues, etc., can be applied to the ***RapidS*PALM** kit. Furthermore, ***RapidS*PALM** is significantly faster and easier than conventional analytic methods for protein *S*-palmitoylation.

2. Overview of the *RapidS*PALM kit

BioDynamics Laboratory Inc., developed a novel chemical strategy called **Rapid** **S**ubstitution of **P**rotein *S*-**A**cy**l**ation for **M**ultifunctional-tag (***RapidS*PALM**, [rˈæpɪzpάːm]). *RapidS*PALM can converts the *S*-palmitoyl group on proteins to our unique MfTag which can semi-quantitate total amount of *S*-palmitoylation, purify comprehensively *S*-palmitoylated proteins, and count number of *S*-palmitoyl group. ***RapidS*PALM, Protein *S*-Palmitoylation Detection Kit** consisting of two kit parts, including a **Reaction Kit** (Cat. No. #F017A) and **Purification Kit** (Cat. No. #F017B) (Figure 1). Firstly, *S*-palmitoyl groups on proteins in any samples are converted to MfTag by the **Reaction Kit**. *RapidS*PALM's MfTag contains three functional units, including a yellow fluorophore (yFL), affinity tag (*Hook*), and a high molecular weight (~5 kDa) backbone and will label to the cysteine residues, which are originally *S*-palmitoylated via a disulfide bond. The *Hook* group on the MfTag can be specifically and quickly isolated by a *Loop* affinity column provided in **Purification Kit**. After purification, the MfTag is easily cleaved by reducing reagents.

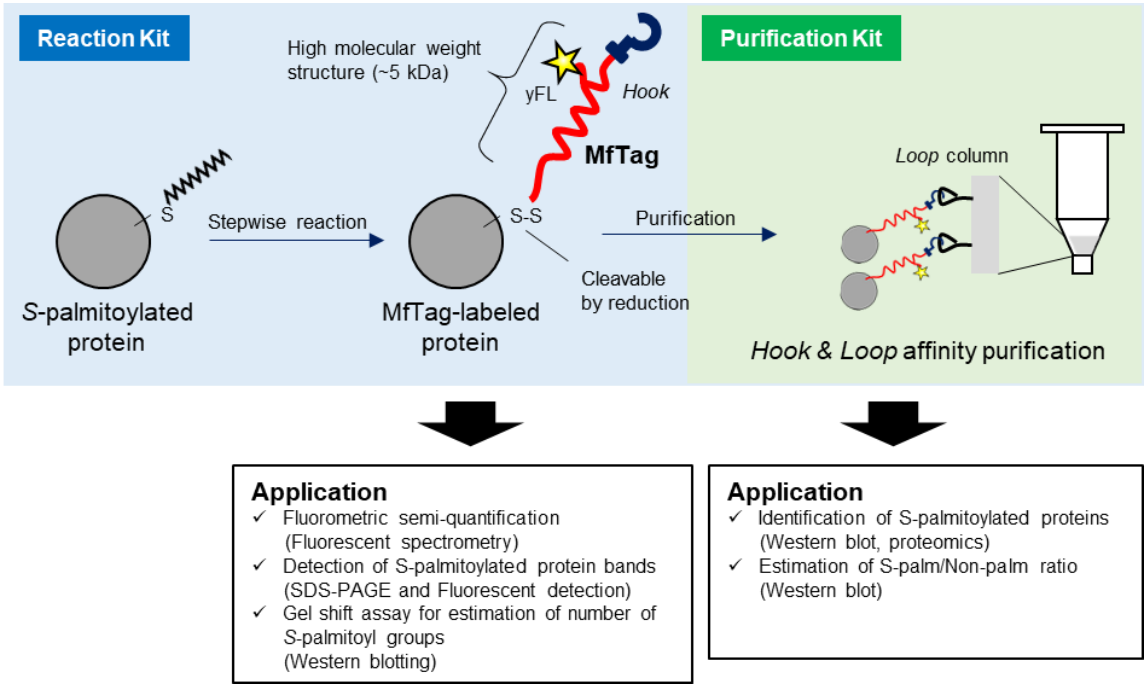


Figure-1 Overview of *RapidS*PALM

As mentioned above, *RapidS*PALM's MfTag has three functions, 1) yellow fluorophore yFL, 2) high molecular weight structure, and 3) affinity tag *Hook*, and it provides a total of 5 experimental outputs (Figure 2). 1) yFL is a thermally and photochemically stable fluorescent dye. By yFL group (Ex 300-370 nm/ Em 450-600 nm), amount of MfTag-labeled proteins (=*S*-palmitoylated proteins) are fluorometrically quantified by any fluorescent spectrophotometer. Furthermore, MfTag-labeled proteins (=*S*-palmitoylated proteins) are visualized in-gel by a fluorescent imager after SDS-PAGE without any additional staining. 2) The high molecular mass (~5 kDa) structure of MfTag, will induce a gel shift of labeled proteins on SDS-PAGE. Counting shifted bands indicates the number of MfTag (=*S*-palmitoyl) on proteins by Western blotting. 3) Owing to the *Hook* group on the MfTag and *Loop* affinity column-specific interaction, MfTag-labeled proteins (=*S*-palmitoylated proteins) are comprehensively isolated by the *Loop* affinity column, and individual proteins are identified by mass spectrometry or Western blotting. Furthermore, *S*-palmitoylated modification ratio can be evaluated by Western blotting after reducing SDS-PAGE.

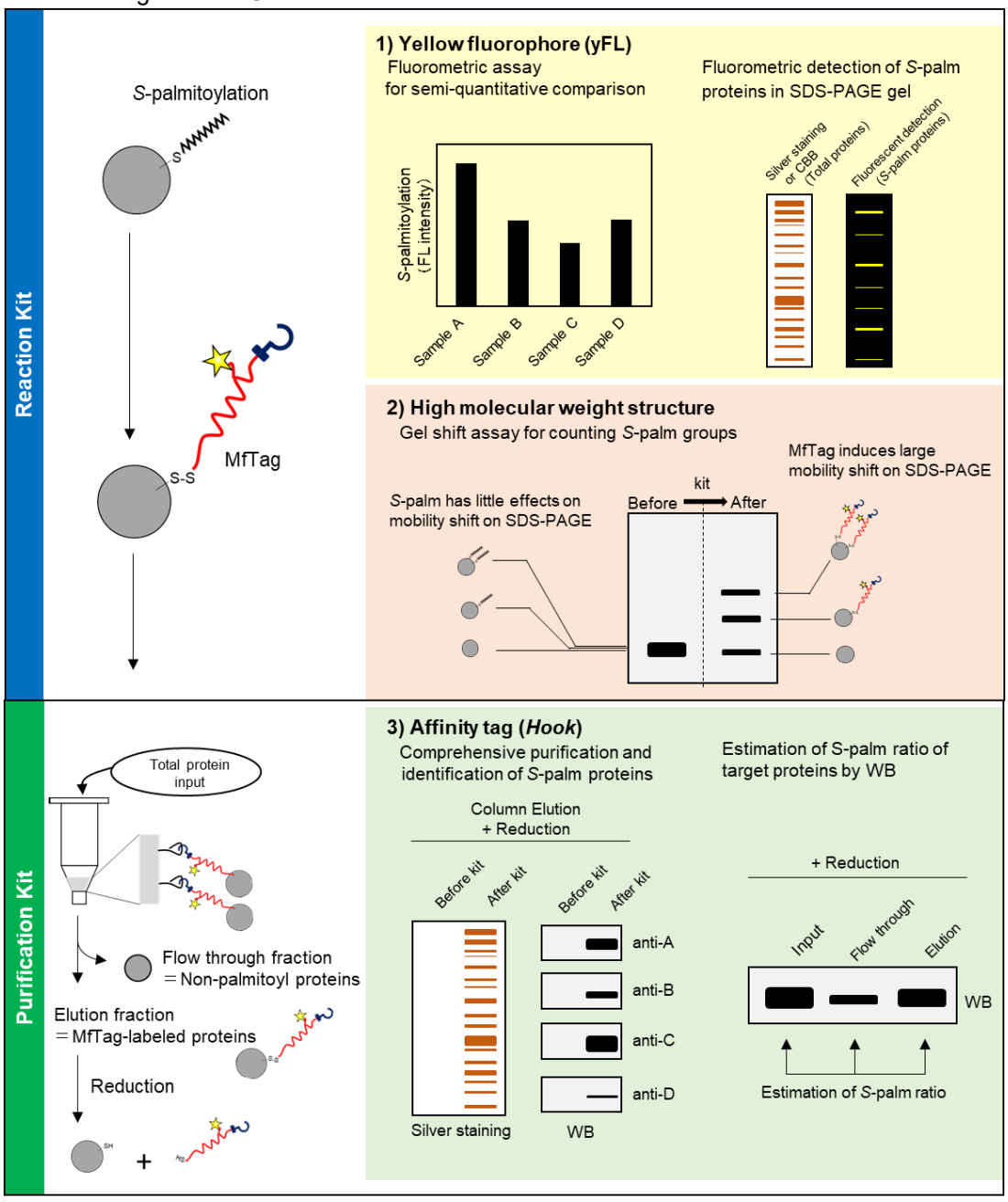


Figure-2 Outputs of *RapidS*PALM

3. Principle of *RapidS*PALM

<The principle of Reaction kit>

Our *RapidS*PALM kit converts *S*-palmitoyl groups on proteins to a unique MfTag by stepwise chemical reactions (Figure-3) similar to conventional *S*-palmitoylation detection methods, including Acyl-Biotin Exchange (ABE) and Acyl-PEGyl Exchange Gel Shift Assay (APEGS). Unlike ABE or APEGS, *RapidS*PALM includes several original and high-performance reagents with a highly optimized protocol and will reduce non-specific labeling reaction and dramatically shorten each reaction time and omit time-consuming steps. Owing to the *RapidS*PALM reagents and protocol, ultra-quick and easy preparation is accomplished.

Step-1 Strong denaturation of proteins

A strong denaturant and heat denature proteins to increase the accessibility of the following reagents.

Step-2 Reduction of disulfide bond

Disulfide bonds are cleaved by a potent reducing reagent.

Step-3 Blocking of free cysteines

Unique and high-performance thiol-specific reagents block free cysteines.

Step-4 Cleavage of *S*-palmitoyl thioester

S-palmitoyl thioesters are cleaved by our **h**igh **p**erformance **h**ydroxyl**a**mine-derivative (hpHA). Then, hpHA(-) sample (Tris-treatment) is also prepared as a negative control for the estimation of non-specific MfTag labeling.

Step-5 MfTag labeling

Free-cysteines newly generated from the hpHA cleavage of *S*-palmitoyl thioester are labeled with MfTag by a cysteine-specific MfTag-labeling reagent. If blocking of non-palmitoyl free-cysteines in Step-3 is insufficient, MfTag non-specifically binds to proteins in the hpHA(-) sample in *S*-palmitoylation independent manner. MfTag(-) sample is also prepared as a completelly negative control.

Step-6 Desalting unreacted reagents by Chloroform/Methanol precipitation (CMppt)

According to reaction procedures above, three control experiments for each sample are required. Hereafter, each control experiment called hpHA(-)/MfTag(-), hpHA(-)/MfTag(+) and hpHA(+)/MfTag(+) in the manual.

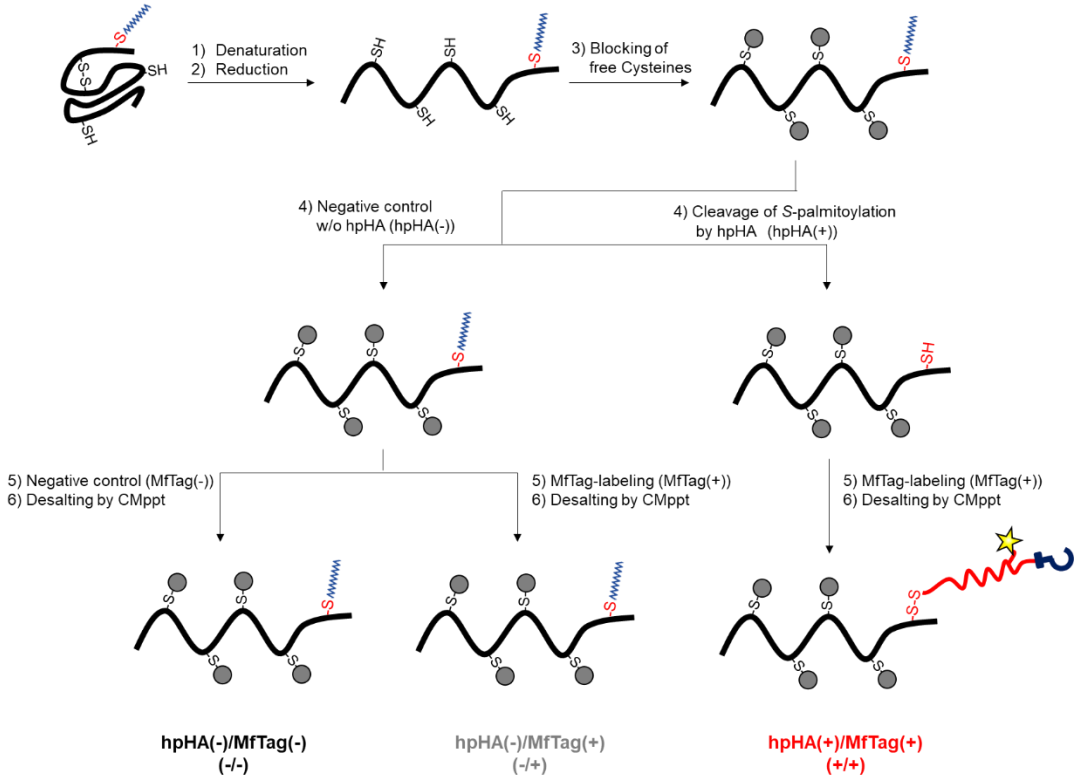


Figure-3 Stepwise chemical reaction of *RapidS*PALM

<The principle of Purification kit>

Hook & *Loop* affinity purification is based on a non-proteinous and small molecular interaction system. Due to a non-protein-based method, all procedures can be performed under completely denatured conditions, and non-specific binding of applied proteins to affinity column are highly suppressed. The spin-column format provides an easy procedure, including only the addition of reagent solutions to the column and spinning-down.

4. Advantages of *RapidS*PALM over conventional ABE method

ABE, the most popular conventional detection method for protein *S*-palmitoylation, requires over one-day procedures from sample preparation to affinity purification. Our *RapidS*PALM kit has ultra-quick procedures and requires only two hours for the MfTag-labeling reaction and an hour for the purification step. Our *RapidS*PALM saves time and labor and provides half-day experiments.

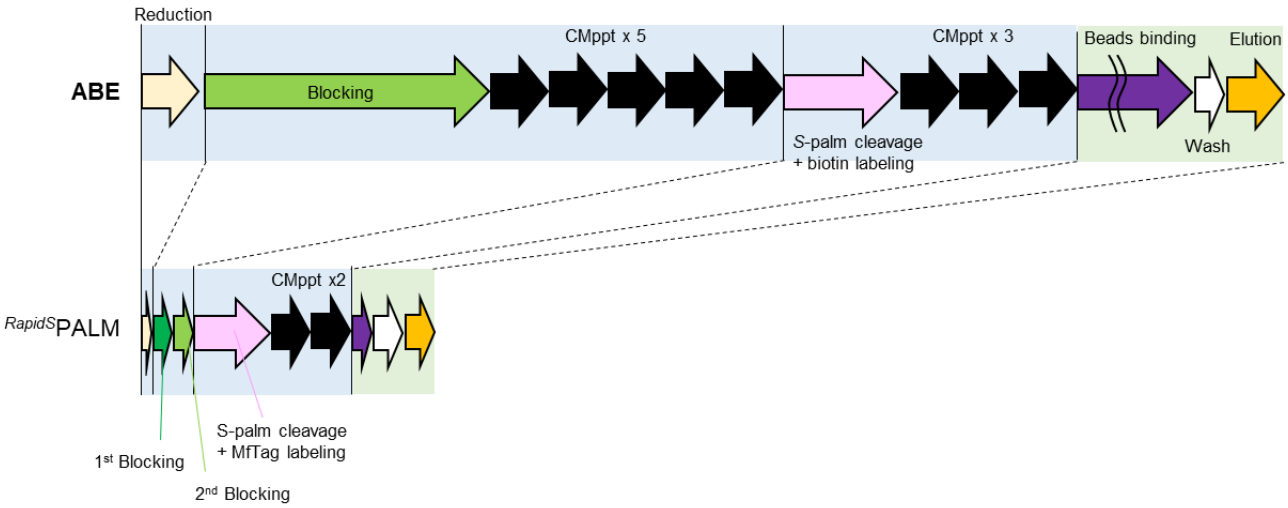


Figure-4 Time course comparison between the conventional ABE and *RapidS*PALM

Protocol Part: Reaction kit (Code : F017A)

Materials Needed but not supplied

<Reagents>

- Ultrapure water
- Dimethyl sulfoxide (DMSO)
- Methanol (MeOH)
- Chloroform
- Phosphate buffered saline (PBS)
- BCA protein assay
- 2x - 5x Laemmli SDS-PAGE sample buffer (w/o any reduction reagents)

<Instruments>

- Dry heat block (95°C)
- Water bath (37-42°C)
- Centrifuge
- Vortex mixer
- Shaker or Rotator
- Probe-type sonicator (Optional)
- Water bath sonicator (Optional)
- Hand UV lamp (Range: 300-370 nm) (Optional)

Materials Supplied and Storage Condition

The kit contains eight components and will arrive packaged as RT; for best results, remove components and store as stated below.

Table 0-1 Kit components

Component Name	Quantity and package	Storage
2x Base Buffer	1 bottle	RT
Reduction Reagent	1 vial	<-20°C
Blocking Reagent A	1 vial	<-20°C
Blocking Reagent B	1 vial	<-20°C
Cleavage Reagent	1 vial	<-20°C
Cleavage Control	1 vial	<-20°C
MfTag-labeling Reagent	1 vial	<-20°C
Supplement	1 vial	<-20°C

0. Preparation of reagents

- Preparation of 1x Base Buffer

Bring **2x Base Buffer** to room temperature. As **2x Base Buffer** contains a high concentration of salts and a detergent, the salts should be completed dissolved. Warm at 37°C is an option to promote dissolving the salts. Dilute an appropriate volume of **2x Base Buffer** with the same volume of ultrapure water. The diluted **1x Base Buffer** can be stored at room temperature for the long term. This kit contains 50 mL of **2x Base Buffer** and enough to prepare 100 mL of **1x Base Buffer**. However, suppose you need a larger volume of 1x Base Buffer (>100 mL) for preparation of tissue lysates, plant lysates, etc. In that case, you can prepare an in-house **1x Base Buffer** according to the following buffer composition.

1x Base Buffer (50 mM sodium phosphate (pH 7.4), 100 mM NaCl, 2% SDS)

- Preparation of kit components

The following components are supplied as lyophilized or dried powders/pellets. Bring to room temperature and dilute the contents with an appropriate volume of solvent mentioned in the list below. If you plan to perform several separate experiments, make aliquots and use fresh for each experiment. Freeze and thaw cycle is not recommended.

Table 0-2 Reconstitution of lyophilized or dried components

Component	Solvent	Volume
Reduction Reagent	Ultrapure water	200 μL
Blocking Reagent A	DMSO	100 μL
Blocking Reagent B	DMSO	50 μL
MfTag-labeling Reagent	Ultrapure water	120 μL

1. Setting of control experiments

In this kit, as described in **Introduction Part, Chapter-3.**,we highly recommend using two or three control experiments for each sample to clarify the specificity of *S*-palmitoylation. Types of control experiments and their purposes are described in Table 1-1 and Table 1-2, respectively. In *RapidS*PALM system, an high-performance hydroxylamine (hpHA) derivative is used as the cleavage reagent of *S*-palmitoylation/acylation instead of normal hydroxylamine. Please note hpHA(-)/MfTag(-), hpHA(-)/MfTag(+) and hpHA(+)/MfTag(+) are abbreviated to **-/-**, **-/+**, and **+/+**, respectively, in the manual.

Table 1-1 Types of control experiment

Experiment name	Abbr.	Reduction	Free-Cys Blocking	*S*-palmitoyl thioester cleavage	MfTag -labeling	Desalting (CMppt)
hpHA(-)/MfTag(-)	**-/-**	✓	✓			✓
hpHA(-)/MfTag(+)	**-/+**	✓	✓		✓	✓
hpHA(+)/MfTag(+)	**+/+**	✓	✓	✓	✓	✓

Table 1-2 Purpose of each control experiment

Experiment name	Abbr.	Purpose
hpHA(-)/MfTag(-)	**-/-**	- Completely negative control - Evaluation of autofluorescence of each sample - Evaluation of influence of each chemical reaction in the kit on proteins in SDS-PAGE or Western blotting
hpHA(-)/MfTag(+)	**-/+**	- Negative control for evaluation of palmitoyl-independent MfTag-labeling - Evaluation of blocking efficiency of non-palmitoyl cysteines
hpHA(+)/MfTag(+)	**+/+**	- *S*-Palmitoylation-specific MfTag-labeled sample

In this kit, as *S*-palmitoyl thioester is cleaved by hpHA, subtraction **-/+** from **+/+** indicates true *S*-palmitoylation. The necessity of each control experiment depends on the output purpose, and a guideline is shown in Table 1-3. To adequately judge *S*-palmitoylation-specificity, negative controls in every experiment are highly recommended.

Table 1-3 Guideline for setting controls

Purpose	**-/-**	**-/+**	**+/+**
Fluorometric assay/detection	✓	✓	✓
Gel shift assay	✓	✓	✓
Affinity purification (*Hook & Loop*)	*	✓	✓

* For affinity purification, **-/-** is not needed. If you confirm non-specific binding of proteins to the column, you can set a **-/-** experiment as a negative control.

2. Preparation of sample lysates

Our kit can be used for a wide range of biological samples, including animal tissues, cultured cells, plant tissues, etc. Before assay, prepare protein lysates with the following guides. In our *RapidS*PALM kit, all reactions are done under strong denatured conditions, do not add protease inhibitors. Protease inhibitors may affect the chemical reaction of the kit; excluding protease inhibitors is highly recommended.

[For cultured cells]

Cultured cells, both floating and adherent cells, are washed with PBS at least twice*1 and lysed with an appropriate volume of **1x Base Buffer**. Prepare over 1 mg/ml total protein lysate. Due to the high concentration of cellular genomic DNA, the lysate will be highly viscous in the heterogeneous solution. To prepare the homogeneous solution, carefully shear the DNA lysate by pipetting repeatingly or probe-type sonication*2. After sufficient DNA shearing of the lysate, the lysate is centrifuged (10,000 xg, 5 min, RT), and the supernatant is collected to remove the insoluble fraction, including cell debris or any waste. The protein concentration of the supernatant is measured by BCA protein assay. Note the **1x Base Buffer** contains a high concentration of detergent, Bradford protein assay is not compatible with the Kit. Over 1 mg/ml total protein concentration is desired for the following reactions*3. Although prepared lysate can be stored at <-20°C, immediate use is recommended.

*1 If cells are cultured in serum-containing media, wash cells minimum of three times. Contamination of serum albumin in the cell lysate may cause background signal in this kit.

*2 If less dispersion of lysate with high viscosity may be a reason of low reproducibility.

*3 If the lysate is very low concentration or large volume, find an optional procedure below. You can concentrate the protein concentration of the lysate.

[For animal tissue]

After mincing, tissues are lysed by appropriate volume of **1x Base Buffer***4 and homogenized by appropriate procedures such as homogenizer and sonicator, etc. Sonication procedures are highly recommended to promote extraction of membrane fraction which contains many *S*-palmitoylated proteins and to prepare homogeneous cell lysate. After sufficient dispersion of the lysate, the lysate is centrifuged (10,000 xg, 5 min, RT), and the supernatant is collected to remove the insoluble fraction, including cell debris or any waste. The protein concentration of the supernatant is measured by BCA protein assay. Over 1 mg/ml total protein concentration is desired for the following reactions. Although prepared lysate can be stored at <-20°C, immediate use is recommended.

*4 If you need over 5 mL/sample of **1x Base Buffer**, 1x Base Buffer supplied in the kit will be insufficient for 12 samples. In this case, prepare in-house **1x Base Buffer** described in “0. Preparation of Reagents”.

*5 If the lysate is very low concentration or large volume, find an optional procedure below. You can concentrate the protein concentration of the lysate.

[For plant samples]

After grinding plant cells or tissues by appropriate procedures, proteins are extracted with appropriate volume of **1x Base Buffer**. Insoluble fraction is removed by centrifugation or appropriate filters. Plant samples contain autofluorescent compounds, including chlorophyll etc., and total protein purification by chloroform/MeOH precipitation (CMppt) described below is recommended to remove non-protein components. The protein concentration of the supernatant is measured by BCA protein assay. Over 1 mg/ml total protein concentration is desired for the following reactions*6. Although prepared lysate can be stored at <-20°C, immediate use is recommended.

*6 If the lysate is very low concentration or large volume, find an optional procedure below to concentrate the protein concentration of the lysate.

[Optional procedure: Chloroform/MeOH precipitation for removing nucleotide and lipids]

Chloroform/Methanol precipitation (CMppt) is a phase separation method to prepare protein fractions and removes nucleotides, lipids, and other biomolecules. CMppt is also useful to concentrate protein and remove color compounds in samples.

1. Transfer 100 μL of sample lysate to a new 1.5 mL tube
2. Add 400 μL of MeOH and vigorously mix by vortex
3. Add 100 μL of chloroform and vigorously mix by vortex
4. Add 300 μL of ultrapure water and vigorously mix by vortex (Solution will turn cloudy)
5. Centrifuge mixture at 10,000*xg* for 2 min at RT. After centrifugation, phase separation is observed, and proteins precipitate appearing as a thin opaque disk shape between the upper phase and lower phase. There is no protein pellet at the bottom of the tube at this stage.
6. Carefully remove and discard the upper layer without disturbing the precipitated proteins, it's not required to remove the upper phase completely. Leaving a small amount of the upper layer phase is acceptable.
7. Add 300 μL of MeOH carefully to the lower phase and gently mix for a homogeneous single phase solution.
   Note-1 Carefully mix two phases, do not break the protein pellet.
8. Centrifuge mixture at 10,000*xg* for 2 min at RT. The protein pellet will be located at the bottom of the tube. Remove supernatant and discard.
9. Centrifuge tube again at 10,000*xg* for 2 min at R.T. and remove the supernatant completely.
10. Add appropriate volume of 1x Base Buffer and dissolve the protein pellet by pipetting or water-bath sonicator. Combine multiple tubes to concentrate the protein concentration.
    Note-2 CMppt may induce protein aggregation for some proteins and induce less separatability on SDS-PAGE. For our kit, a single CMppt is recommended.

3. Assay Protocol of Reaction kit

3-0 Overview of Assay Protocol

【1】Preparation of protein lysate
(Reagent component: **1x Base Buffer**)
【2】Denaturation of protein and reduction to cleave disulfide bond – 5 min @95°C
(Reagent component: **Supplement**, **Reduction Reagent**)
【3】1st Blocking of free-Cys – 5 min @RT and additionally 5 min @95 °C
(Reagent component: **Blocking Reagent A**)
【4】2nd Blocking of free-Cys – 5 min @RT and additionally 5min @95 °C
(Reagent component: **Blocking Reagent B**)
【5】Cleavage of S-palmitoyl thioester and labeling of MfTag – 40 min@37 °C (~42 °C)
(Reagent component: **Cleavage Reagent**, **Cleavage Control**, **MfTag-labeling Reagent**)
【6】Desalting by Chloroform/MeOH precipitation (CMppt) x 2 times – 15 min x 2

Before experiment
Bring all kit components to RT, vortex well, and check solubility. If there is any precipitation, warm the solutions to 37°C to completely dissolve the salts. Avoid storing components on ice as some components are DMSO stock solutions; until use, store components at RT.

3-1 Palmitoyl-MfTag Exchange Protocol

1) Dilute total protein lysate prepared in Chapter-2 to 1-2 mg/ml concentration with **1x Base Buffer**.
Note-1 Protein concentration over 2 mg/ml is not recommended, there may be insufficient chemical reaction.
Note-2 Protein concentration less than 1 mg/ml is compatible; however, detection sensitivity after the exchange reaction may be low.
2) Pipet 100 μL x number of control of 1-2 mg/ml total protein lysate prepared in step-1). If you use three controls (**-/-**, **-/+** and **+/+**), 300 μL of protein lysate is transferred to a new tube. In the case of two controls (**-/+** and **+/+**), 200 μL of protein lysate is transferred to a new tube.
3) Add 1 μL x number of control (for three controls; 3 μL, for two controls; 2 μL) of **Supplement** and 2.5 μL x number of control (for three controls; 7.5 μL, for two controls; 5 μL) of **Reduction Reagent** to protein lysate and vigorously mix.
Note-3 After the addition of **Supplement**, BCA assay is not compatible. If BCA assay is required, perform after step 17).
4) Quick spin-down tubes and incubate tubes for 5 min at 95°C.
5) Add 2 μL x number of control (for three controls; 6 μL, for two controls; 4 μL) of **Blocking Reagent A** to protein lysate and vigorously mix.
Note-4 Do not allow cool down to RT. You can add **Blocking Reagent A** immediately after the heating step. Be mindful of scalding.
6) Incubate for 5 min with gentle mixing by a rotator or a shaker at RT
7) Quick spin-down tubes and incubate for 5 min at 95°C.
8) Add 1 μL x number of control (for three controls; 3 μL, for two controls; 2 μL) of **Blocking Reagent B** to the protein lysate and vigorously mix.
Note-5 Do not allow cool down to RT. You can add Blocking Reagent B immediately after the heating step. Be mindful of scalding.
9) Incubate for 5 min with gentle mixing by a rotator or a shaker at RT

10) Quick spin-down tubes and incubate for 5 min at 95°C.

11) Centrifuge the lysate at 10,000*xg* for 2 min at RT. After centrifugation, the supernatant is divided into 95 μL/tubes. Hereafter, **-/-**, -/+ and **+/+** are differently treated.

Note-6 After centrifugation, debris or insoluble pellets of reagent may be present at the bottom of tubes. Carefully collect supernatant and avoid contamination of precipitation.

12) Add the following reagents to each control and vigorously mix by vortex

-/- **Cleavage Control** 25 μL

-/+ **Cleavage Control** 25 μL

+/+ **Cleavage Reagent** 25 μL

Note-7 After the addition of **Cleavage Reagent** for **+/+**, the solution may become cloudy, which is normal for the following steps.

13) Add the following reagents to each control and vigorously mix by vortex

-/- ultrapure water 5 μL

-/+ **MfTag-labeling Reagent** 5 μL

+/+ **MfTag-labeling Reagent** 5 μL

14) Quick spin-down tubes and incubate for 40 min at 37-42°C. Every 10 minutes, mix the reaction with a vortex.

Note-8 If difficult to incubate at 37-42°C, incubate for 1 hour at RT.

15) Remove unreacted reagents by CMppt

15-1) Add 400 μL of MeOH and vigorously mix

15-2) Add 100 μL of chloroform and vigorously mix

15-3) Add 300 μL of ultrapure water and vigorously mix. The solution will turn cloudy.

15-4) Centrifuge at 10,000*xg* for 2 min at RT. After centrifugation, phase separation is observed, and proteins precipitate appearing as a thin opaque disk shape between the upper phase and lower phase. There is no protein pellet at the bottom of the tube at this stage. Carefully remove and discard the upper layer without disturbing the precipitated proteins, it's not required to remove the upper phase completely. Leaving a small amount of the upper layer phase is acceptable.

15-5) Add 300 μL of MeOH and mix the two phases by gentle tube inversion. Avoid breaking the protein pellets.

Note-9 If initial protein concentration is very low, protein pellets may not be disc-like precipitation.

15-6) Centrifuge at >10,000*xg* for 2 min at RT to force the protein pellet to the bottom of the tube and carefully remove and discard the supernatant.

15-7) Centrifuge at >10,000*xg* for 30 sec at RT again and remove and discard the supernatant completely.

15-8) Add 100 μL of **1x Base Buffer** to the protein pellet; avoid the protein pellet from drying.

15-9) Resuspend the protein pellet completely by pipetting or water-bath sonication.

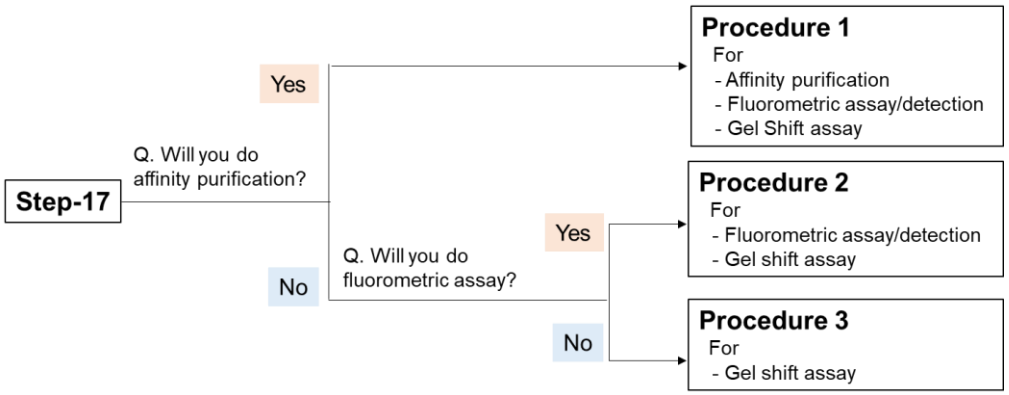
Note-10 Water-bath sonication is recommended to quickly resuspend the protein pellet.

16) Repeat CMppt again from step-15-1) to 15-7)

Note-11 If you will not perform fluorometric assay or affinity purification assay, 2nd CMppt can be skipped.

17) Hereafter, you need to do a different procedure according to your experimental purpose; follow the flowchart below.

Note-12 In all cases, the MfTag-labeling can be visualized as emission of yellow fluorescence using a hand UV lamp in a dark room.



[Procedure 1]

Resuspend the protein pellet with 110 μL of **1x Binding Buffer**. After resuspending the protein pellet in **1x Binding Buffer**, affinity purification, fluorometric assay, and gel shift assay can be performed.

Note **1x Binding Buffer** is a kit component of **Purification Kit**.

[Procedure 2]

Resuspend the protein pellet with 50-100 μL of **1x Base Buffer**. After doing a fluorometric assay, the sample can be used for gel shift assay with concentrated Laemmli SDS-PAGE sample buffer (w/o reducing reagents).

[Procedure 3]

Resuspend the protein pellet with any volume of 1x Laemmli SDS-PAGE sample buffer (w/o reducing reagents) directly.

3-2 Fluorometric assay procedure

For fluorometric assay, measure three controls, **-/-**, **-/+** and **+/+**, by each sample mentioned in Table 1-3.

1) Heat samples prepared in 3-1 for 2 min at 95°C.
   Note-12 This step is essential to measure appropriate fluorescent intensity. As a yellow fluorophore (yFL) group on the MfTag shows great thermal stability, heat step is little effects on yFL decomposition (Figure3-1B).
2) After cooling to RT, centrifuge samples and collect the supernatant.
3) Measure fluorescent intensity at 525 nm excited by 325 nm with any fluorescent spectrophotometer. Three controls, **-/-**, **-/+** and **+/+** should be measured. Figure 3-1A shows spectrum and stability of yFL on the MfTag.
   Optional: Fluorescent spectrum from 400 nm to 600 nm exited by 325 nm is helpful to detect appropriate maximum intensity in each sample and to consider the influence of autofluorescence derived from each sample.
4) Reuse the samples for performing gel shift assay or affinity purification. When reusing samples, carefully collect samples and avoid contamination.

[Important caution]

In *RapidS*PALM fluorometric assay, there is no internal standard to normalize multi-samples, and fluorescent intensity highly depends on total protein concentration. If you desire to compare the fluorescent intensity of multi-samples, normalize total protein concentration by BCA protein assay before measurement of fluorescent intensity. Comparison of multi-samples prepared from different timing is not recommended. The sample group you desire to compare should be prepared with the same timing.

[Analytical methods]

There are two types of normalization methods of fluorescent intensity.

1) Specificity check of each sample

For checking *S*-palmitoylation specificity, background fluorescent intensity of **-/-** is subtracted from one of the -/+ or **+/+** (Eq-1, Figure 3-2 lower left). After normalization, the ratio of **+/+** to -/+ (hpHA**(+)**/(-), Eq-2) indicates the *S*-palmitoylation specificity of the sample preparation. The higher hpHA**(+)**/(-) value indicates the higher *S*-palmitoylation specificity.

Normalized FL intensity = [**+/+** or -/+] - [**-/-**] (Eq-1)

Specificity hpHA**(+)**/(-) = ([**+/+**] - [**-/-**])/([-/+] - [**-/-**]) (Eq-2)

2) Comparison of mulple samples

For comparison of multiple samples, subtracting -/+ from **+/+** indicates “Palmitoylation specific FL intensity” (Eq-3, Figure 3-2 lower right).

Palmitoylation specific FL = [**+/+**]-[-/+] (Eq-3)

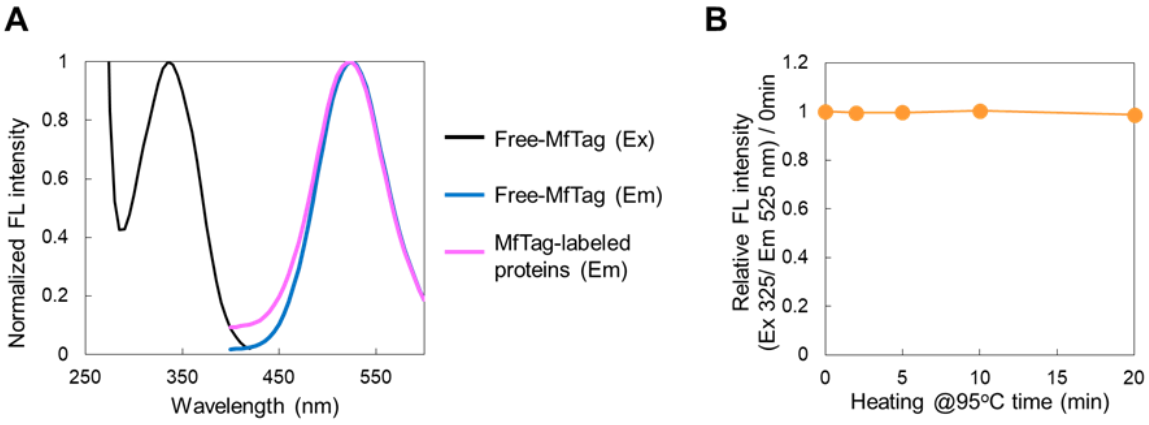


Figure 3-1 Spectrum and stability of a yellow fluorophore (yFL) on MfTag

(A) Excitation and emission spectrum of yFL on free-MfTag and MfTag-labeled proteins.

(B) Thermal stability of yFL on MfTag at 95°C (Ex 325 nm/Em 525 nm).

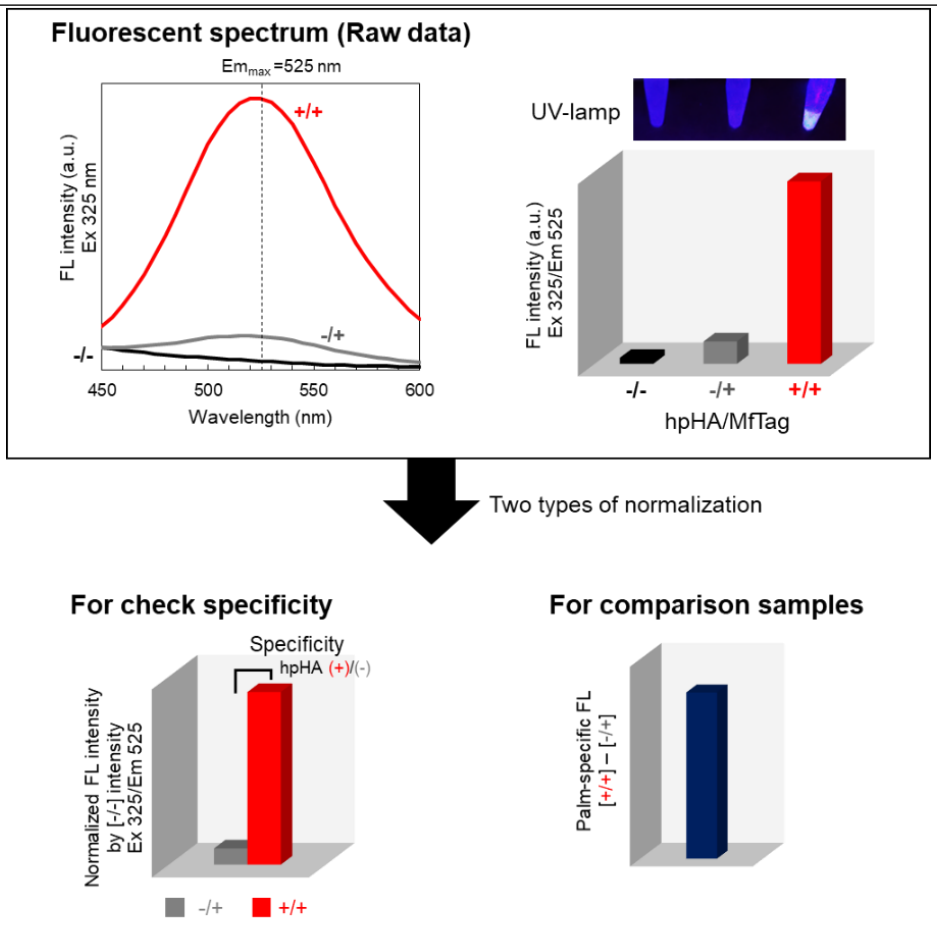


Figure 3-2 Analytic methods of fluorometric assay

3-3. Gel shift assay

For gel shift assay using SDS-PAGE/WB, prepare all three controls (**-/-**, -/+ and **+/+**) by each sample.

1) Add appropriate volume of concentrated Laemmli SDS-PAGE sample buffer (from 2x to 5x concentrated, **w/o reducing reagent**) to each sample prepared in 3-1 above and heat at 95°C for 2 minutes.

Note Under reducing conditions, MfTag will be removed. Performing SDS-PAGE under non-reducing conditions is required.

2) Preparation of appropriate concentration of polyacrylamide gel

Table. Recommended polyacrylamide gel conc.

Molecular weight	Recommended gel conc.
20-30 kDa	13-15%
30-40 kDa	11-13%
40-50 kDa	10-12%
50-70 kDa	8-10%
70-100 kDa	6-8%
>100 kDa*	<6%

*MfTag is about 5 kDa; it may be difficult to estimate the number of MfTag over 100 kDa proteins.

*Separation patterns may be different for each protein. Empirically optimize polyacrylamide gel concentration for your protein of interest.

3) Run SDS-PAGE
4) Transfer to membrane
   Both wet and semi-dry transfers are compatible with MfTag-labeled proteins. The transfer conditions will differ by each protein and require empirically optimizing transfer conditions for your protein of interest.
5) Run Western blotting

[Antibody selection guide]

It is very important to select an antibody for gel shift assay with the following guides.

- Highly specific antibody

  Highly specific antibodies that detect target proteins with a single band in WB are recommended. Because MfTag-labelling induces multiple band splits (*n+1*), where *n= MfTag(s)* from the original band it may be hard to detect MfTag-labeled target proteins by low-specific antibodies.

- Polyclonal antibodies are recommended

  Because proteins are labeled with a large MW MfTag structure, MfTag may inhibit monoclonal antibody-target protein interaction if the MfTag-labeling site (=*S*-palmitoylation site) is located near the epitope of the monoclonal antibody. Notably, multiple MfTag-labeling near the epitope site of monoclonal antibodies induces a dramatic decrease in epitope-antibody interaction. Polyclonal antibodies with wide-range epitopes are recommended with little influence on the MfTag.

- Trial of several antibodies

  When you do the gel shift assay the first time, a trial using several polyclonal antibodies is recommended.

[Evaluation]

1) Evaluation of *S*-palmitoylation-specificity

Likely to Figure 3-3, the protein band of **-/-** is an original band of the target protein in WB. In the left case, only the **+/+** sample shows that several additional bands and no additional band on **-/+** is detected. This data indicates that you successfully prepared **-/+** and **+/+** samples at least the indicated protein and the indicated proteins have “two MfTags (=two *S*-palmitoylation)”. If any additional bands on **-/+** appear likely to the right case, non-specific MfTag-labeling reaction was due to the unblocked free-cysteines. The right data indicates that blocking of free-cysteine is insufficient at least for the indicated protein and you cannot determine the number of *S*-palmitoylation sites.

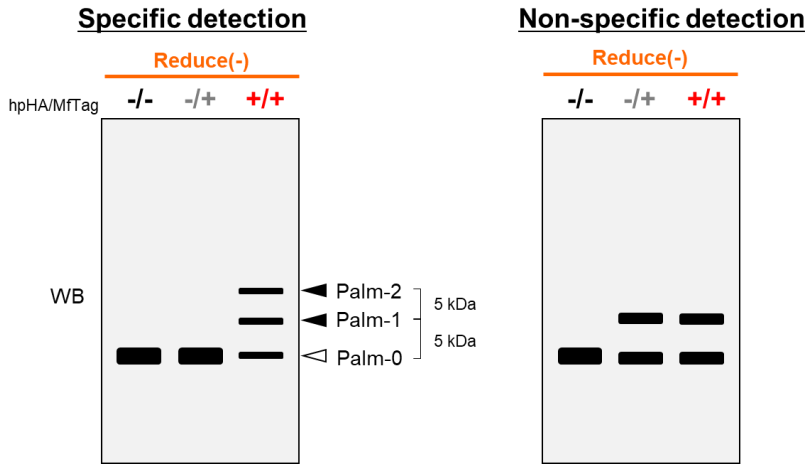


Figure 3-3 Specificity check on gel shift assay

2) Evaluation of each shifted band

As mentioned above, the molecular weight of MfTag is about 5 kDa. If the target protein is labeled with *n* x MfTags, maximum *(n+1)* bands (the original band and *n* x additional bands) will appear. However, in some cases, a certain number of MfTag-labeled bands may not exist in the sample. In these cases, you can consider a number of labeled MfTags on the target protein by mobility distance on the gel and molecular weight plot. Please read the following example.

Example: Ras GTPase family member, Hras and Rap2b

Hras and Rap2b are members of Ras small GTPase protein family and reported two *S*-palmitoylation on the C-terminal region. In the example, the *S*-palmitoylation status of Hras and Rap2b in mouse whole brain was analyzed (Figure 3-4). For Hras (green), as two additional bands appeared only in the **+/+** sample, the data suggests Hras has two MfTags (=*S*-palmitoly group). For Rap2b (pink), the original band disappeared, and a high molecular weight alternative band appeared only in the **+/+** sample. The number of MfTag on both Hras and Rap2b is calculated here.

1) Measure mobility shift (Rf) of each molecular weight (MW) marker proteins and create an MW-Rf plot and fit curve by the exponential function.
2) Measure Rf for each Hras or Rap2b bands and place it on the MW-Rf plot above
   If the original band of **+/+** disappears, use original band of **-/-** or -/+.
3) Calculate the estimated MW of each band shift.

From the MW-Rf plot, for Hras, the second band (●) and the first band (●) were shifted by 10.8 kDa and 5.7 kDa from the original band (○), indicating two, one, and zero *S*-palmitoylated Hras exist. For Rap2b, the alternative band (●) shifted with 11.0 kDa from the site of the original band (○). These results indicate majority of Rap2b has two *S*-palmitoylation in mouse whole brain.

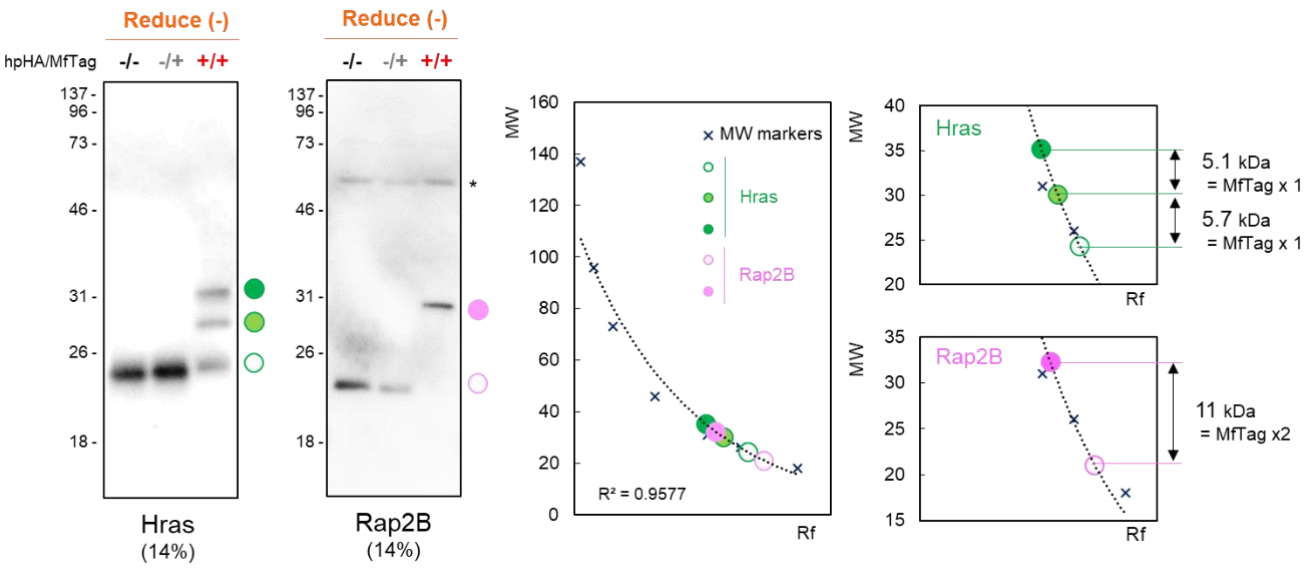


Figure 3-4 Evaluation of MfTag-labeling number of Hras and Rap2b in mouse whole brain tissue
*Non-specific bands of antibody

[Evaluation of the influence of MfTag-labeling on antibody recognition]

As MfTag-labeling may affect the antibody-antigen reaction, our gel shift assay should not recommend comparing each MfTag-labeled band intensity. Figure-3-5 shows two patterns, the upper indicates MfTag-labeled sites are overlapped with the antibody epitope, and the lower indicates MfTag-labeled sites are independent from antibody-epitope interaction. In the upper case, di-MfTag-labeled proteins are not detected well, and the relative signal intensity of di-MfTag-labeled protein is dramatically reduced compared to the true value. On the other hand, in the lower case, the antibody interacts with the epitope independently of MfTag-labeling and provides adequate signal intensity. We strongly recommend the gel shift assay is only for estimation of the number of S-palmitoylation, not for estimation of the existing ratio.

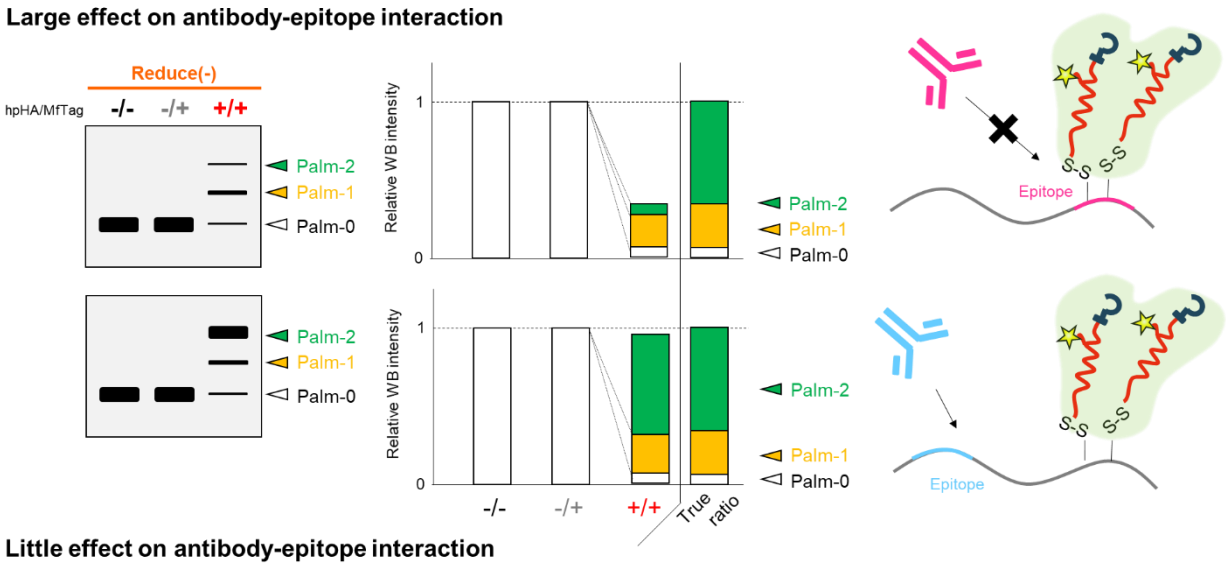


Figure 3-5 Influence of MfTag-labeling on antibody recognition

Protocol Part: Purification kit (Code : F017B)

Materials Required but not supplied

<Reagents>

- Ultrapure water
- 2x - 5x Laemmli SDS-PAGE sample buffer (without any reducing reagent)

<Instrument>

- Dry heat block (95°C)
- Water bath (37-42°C)
- Centrifuge
- Hand UV lamp (300-370 nm) (Optional)

Materials Supplied and Storage Condition

The kit contains six components and will arrive packaged as RT; for best results, remove components and store as stated below.

Table Additional components for Affinity Purification

Component	Size	Store condition
2x Binding Buffer	1 bottle	RT
10x Wash Additive	2 vials	RT
10x Elution Additive	1 vial	RT
Empty Column	24 columns	RT
Affinity Beads	4 vials	4°C (DO NOT freeze)
10x Reduction Reagent	1 vial	4°C (Before reconstitute) -20°C (After reconstitute)

0. Preparation of reagents

0-1. Preparation of Binding, Wash, and Elution buffers

In this purification kit, there are three buffers Binding buffer, Wash buffer, and Elution buffer. These buffers are prepared from **2x Binding buffer** and **10x Wash** or **Elution additive** according to the following table. Salts from the **2x Binding Buffer** are easily precipitated at lower temperatures; pre-heat the bottle at 37°C and confirm the complete dissolving of salts before use. All 1x Buffers should be prepared fresh just before use.

Preparation of **1x Binding Buffer**

	x 1 column	x 2 column	x 24 column
2x Binding buffer	200 μL	400 μL	4.8 mL
H_2O	200 μL	400 μL	4.8 mL
Total	400 μL	800 μL	9.6 mL

Preparation of **1x Wash Buffer**

	x 1 column	x 2 column	x 24 column
2x Binding buffer	250 μL	500 μL	6.0 mL
10x Wash additive	50 μL	100 μL	1.2 mL
H_2O	200 μL	400 μL	4.8 mL
Total	500 μL	1000 μL	12 mL

Preparation of **1x Elution Buffer**

	x 1 column	x 2 column	x 24 column
2x Binding buffer	75 μL	150 μL	1,800 μL
10x Elution additive	15 μL	30 μL	360 μL
H_2O	60 μL	120 μL	1,440 μL
Total	150 μL	300 μL	3,600 μL

1. Purification protocol

1-0. Overview protocol of column purification

【1】 Pre-heat samples — @95°C for 2 min and @RT for 5 min
【2】 Preparation of spin column — 5 min
(Reagents: **Empty Column**、**Affinity Beads**、**1x Binding Buffer**)
【3】 Centrifug pre-heated samples — @RT for 2 min
【4】 Apply samples to spin column — @RT for >10 min
【5】 Wash spin column — ~15 min
(Reagents: **1x Binding Buffer**, **1x Wash Buffer)**
【6】 Elution — ~15 min
(Reagents: **1x Elution Buffer**)

1-1. Protocol for column purification

For column purification of MfTag-labeled proteins, the pair of **-/+** and **+/+** prepared by our reaction kit is used. You can optionally use **-/-** as a complete negative control to check the non-specific purification of non-MfTagged protein.

1) Protein pellets of **-/+** and **+/+** samples prepared in the Reaction kit (step 3-1-17) are resuspended with 110 μL of **1x Binding Buffer** using pipetting or water bath sonicator.
2) After complete resuspension of protein pellets, samples are heated at 95°C for 2 min
3) Samples are cooled down to RT for at least 5 min
Note-1 These samples can be used for fluorometric assay before column purification. Protocol for the fluorometric assay is described in the Reaction kit manual 3-2.
4) Spin columns are prepared with the following procedures.
4-1) Empty Columns are placed onto either 1.5 mL or 2 mL tubes
4-2) **Affinity Beads** (50% slurry) are suspended, invert tube several times to resuspend **Affinity Beads**, pipet 150 μL of **Affinity Beads** (50% slurry) and transfer to empty column prepared in step-4-1 using a wide-bore tip or cut tip.
4-3) Centrifuge the column at 3,000*xg* for 1 min at RT with open cap and separate Affinity Beads and beads suspension solution.
4-4) Add 150 μL of **1x Binding Buffe**r to the Affinity Beads and incubate for at least 1 min at RT
Note-2 Affinity Beads Column can be stored for a maximum of a half day. Avoid Affinity Beads from drying.
4-5) Centrifuge the column at 3,000*xg* for 1 min at RT with open cap and separate Affinity Beads and 1x Binding Buffer.
4-6) Transfer the equilibrated Affinity Beads Column to a new 1.5 mL or 2 mL tube. The column is read-to-use.
5) Centrifuge protein samples prepared in step 3 at 10,000*xg*, 2 min, RT to remove insoluble debris.
Note-3 If insoluble debris contaminates the column in the next step, background signal may increase. Avoid contamination of insoluble debris into the column.
6) Pipet 50 μL of the centrifuged supernatant sample into the equilibrated Affinity Beads Column and mix the column well by tapping.
7) Incubate for at least 10 min at RT with an open cap.
During incubation, 50 μL of the remaining supernatant is transferred to a new 1.5 mL tube. The supernatant is diluted with 50 μL of 1x Binding Buffer to prepare 100 μL of **"Input"** Fraction.
8) Centrifuge the column at 3,000*xg* for 1 min at RT. A new tube exchange is not required.
Note-4 After separation of Affinity Beads and flow-through solution, visually check bead-binding of MfTag-

labeled proteins as emission of yellow fluorescence with a hand UV lamp. In a successful case, the flow-through solution has little yellow fluorescence, while the affinity beads emit strong fluorescence. If the flow-through solution shows yellow fluorescence, the bead-binding efficiency of MfTag-labeled proteins is insufficient. In this case, you can increase binding efficiency by re-applying the flow-through solution to the Affinity Beads and repeating step-6 and step-7.

9) Add 50 μL of **1x Wash Buffer** to the column and incubate for 2 min with an open cap.
10) Centrifuge the column at 3,000*xg* for 1 min at RT. A total of 100 μL of the flow-through solution is stored as **"Flow through (FT)"** fraction.
11) Transfer the column to a new 1.5 mL or 2 mL tube.
12) Add 100 μL of **1x Wash Buffer** to the column, mix the beads by tapping with an open cap, and centrifuge at 3,000*xg* for 1 min at RT.
13) Repeat step-12 again and collect a total of 200 μL of **"Wash-1"** fraction.
14) Transfer the column to a new 1.5 mL tube and repeat step-11 and step-12 twice. Collect a total of 200 μL of **"Wash-2"** fraction.

Note-5 If analyzing all wash fractions, exchange tube each time.

15) Transfer the column to a new 1.5 mL tube for the elution sample.
16) Add 25 μL of **1x Elution Buffer** to the column, incubate for 2 min with an open cap, and centrifuge at 3,000*xg* for 1 min at RT.
17) Repeat step-15 three times and collect a total of 100 μL of **"Elution"** fraction.

Note-6 After the elution procedure, Affinity Beads are highly denatured and cannot be reused.

Note-7 **Elution** fraction cannot be applied in a fluorometric assay. The fluorescent intensity of a yellow fluorophore (yFL) is dramatically decreased in Elution Buffer.

Note-8 As **1x Elution Buffer** has a chemical that inhibits BCA assay, Elution fraction cannot be applied to BCA assay. If checking for protein concentration of the Elution fraction, must perform CMppt, acetone ppt, or TCA ppt, etc., to remove salts.

18) Add 2x-5x Laemmli SDS-PAGE Sample Buffer to the **"Input"**, **"FT"** and **"Elution"** fractions. Optionally add **10x Reduction Reagent** to remove MfTag moiety from purified proteins. After adding **10x Reduction Reagent**, the sample is heated at 95°C for 5 min.

Note-9 **Elution Buffer** will elute intact MfTag-labeled proteins. Under non-reducing conditions, **"Elute"** fraction proteins will show a gel shift on SDS-PAGE. Under reducing conditions, MfTags are cleaved and proteins will show no gel shift on SDS-PAGE.

Note-10 In our protocol, the volumes of **"Input"**, **"FT"** and **"Elution"** are the same 100 μL, allowing for the comparison of the band intensity with WB.

1-2 Analytic methods

[Selection guide for non-reducing or reducing condition on SDS-PAGE]

In this kit, **Elution Buffer** will elute intact MfTag-labeled proteins. When SDS-PAGE runs under **non-reducing condition**, MfTag-labeled proteins show mobility shift on SDS-PAGE depending on it number of MfTag. On the other hand, under **reducing condition** using **10x Reduction Reagent** as a kit component, MfTags were removed from the proteins and protein bands should be a single band (Figure 1-1). After purification procedures, you have to select **non-reducing condition** or **reducing condition** accoding to the following applications (Table 1-1).

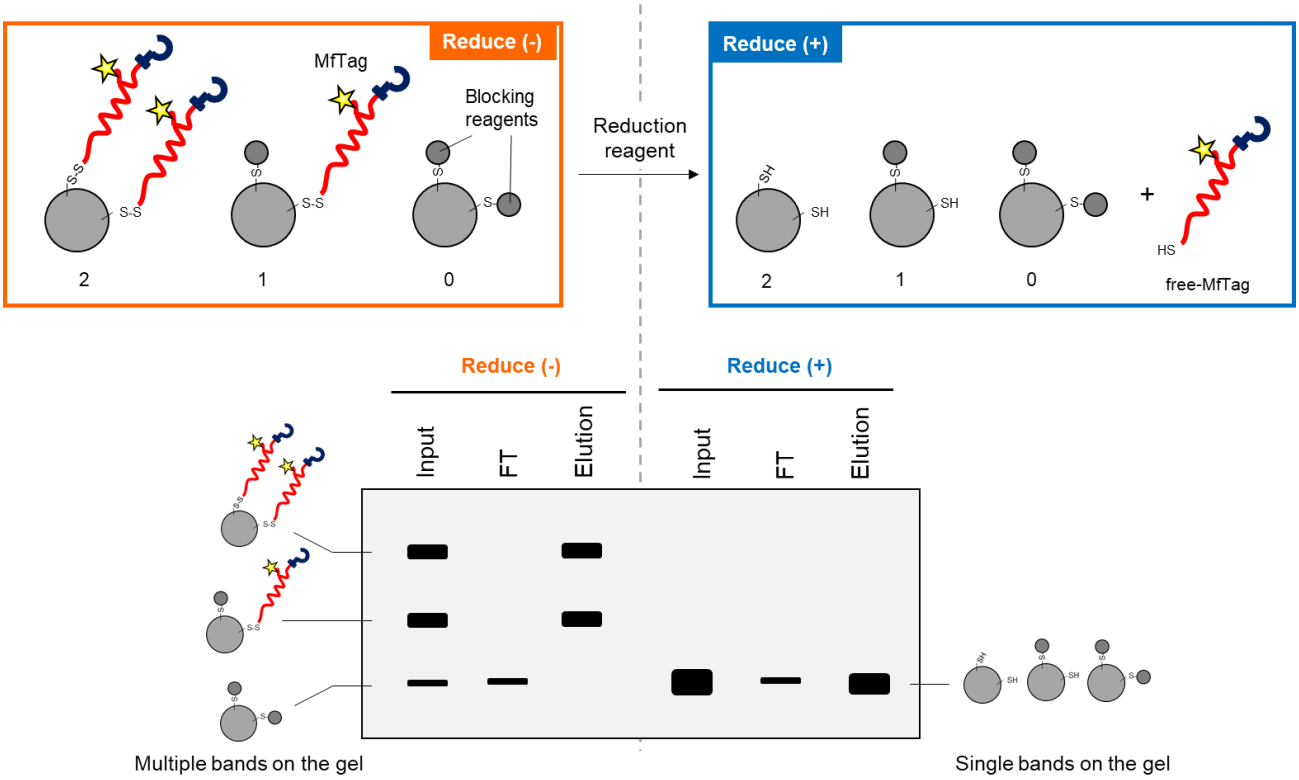


Figure 1-1 Model of two MfTag-labeled proteins under Reduce (-) and Reduce (+) condition

Table 1-1 Selection guide for non-reducing and reducing condition on SDS-PAGE

Condition	Application
Non-reducing condition	Gel shift assay Fluorescent detection of MfTag-labeled proteins in SDS-PAGE gel
Reducing condition	Comprehensive detection of purified proteins by silver staining Western blotting detection of purified target proteins
Both non-reducing and reducing conditions required	Estimation of S-palmitoylated ratio of target proteins by affinity column and Western blotting

[Check specificity of each purified protein]

"Elution" fractions of both **-/+** and **+/+** are separated by SDS-PAGE under **reducing condition** for cleaving MfTag and stained by general silver staining. Likely to the left of Figure1-2A, if only **+/+** shows protein signals, the exchange reaction was successfully achieved. In this condition, each protein band can be considered as *S*-palmitoylated proteins. On the other hand, likely to the right of Figure1-2A, if **-/+** has some protein bands (white arrows), white arrow-indicated proteins were non-specifically labeled by MfTag independently *S*-palmitoylation. These proteins should be excluded from identified *S*-palmitoylation proteins.

[Check purification efficiency and estimate ratio of *S*-palm/non-palm form]

After specific detection of MfTag-labeled proteins (=S-palmitoylated proteins) only observed in the **+/+** sample mentioned above (Figure 1-2A), an equal volume of **"Input"**, **"FT"** and **"Elution"** fractions of **+/+** are separated by SDS-PAGE under both **reducing condition** (w/o MfTag), and **non-reducing condition** (with MfTag) and proteins of interest are detected by WB (Figure 1-2B). First, see the **non-reducing condition** in Figure 1-2B. If affinity purification is successfully done, MfTag-labeled forms (=*S*-palmitoylated forms) will disappear in the "**FT**" fraction (see upper Good case). In this condition, estimating a ratio of MfTag-labeled and non-labeled forms indicates the ratio of *S*-palmitoylated and non-palmitoylated forms under the **reducing condition**. On the other hand, if affinity purification is insufficiently done, some portion of MfTag-labeled forms remain in the **"FT"** fraction under **non-reducing condition** (see lower Bad case). Estimating a ratio of MfTag-labeled and non-labeled forms cannot be determined under **reducing condition**.

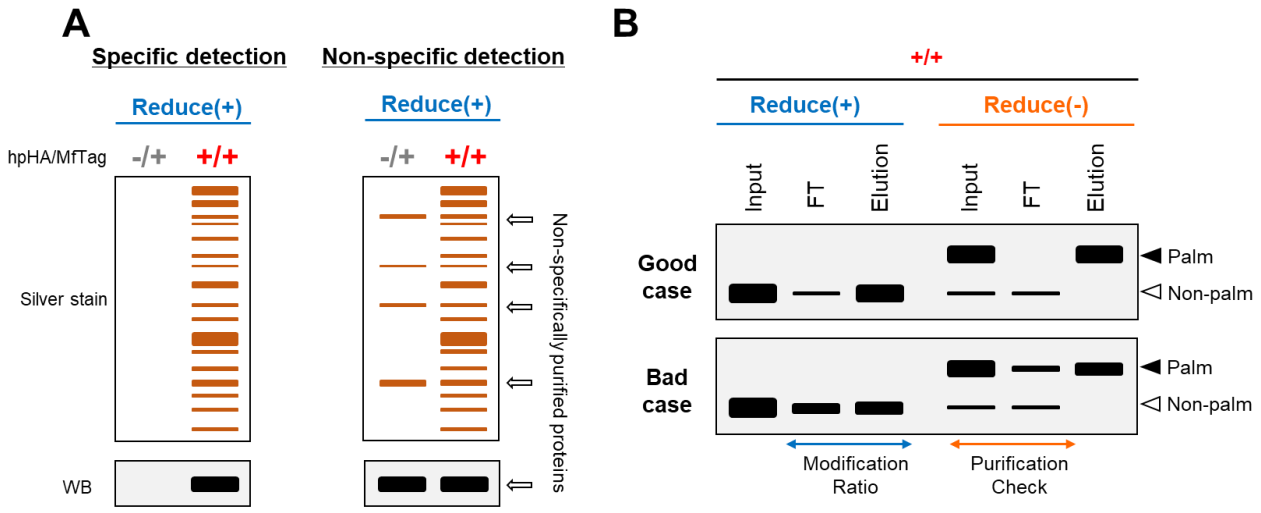


Figure 1-2 Check specificity (A) and check purification efficiency and estimate the *S*-palm ratio (B)

Application Note Part

NOTE

In the manual, hpHA(-)/MfTag(-), hpHA(-)/MfTag(+) and hpHA(+)/MfTag(+) are hereafter shown as **-/-**, -/+ and **+/+**, respectively.

Fluorometric assay

Three types of control experiments **-/-**, -/+ and **+/+** for each sample are prepared and measured fluorescent intensity (Ex 325 nm/Em 525 nm) by fluorescent spectrometer. All experiments are analyzed by the two methods below.

A Specificity test of each sample (-/+ and **+/+** are normalized by **-/-**)

B Comparison of different samples (**+/+** is normalized by -/+)

NOTE Please refere to the Protocol part (Reaction Kit) 3-2. Fluorometric Assay for detail information of each analysis method.

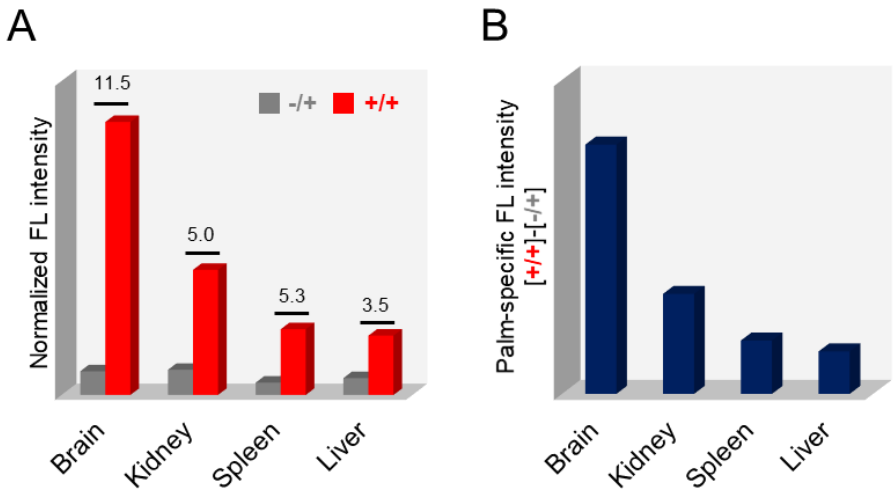
Example 1 Semi-quantitative comparison among mouse tissues

Samples: Adult mouse-derived whole brain, kidney, spleen, and liver tissues

Lysate preparation: Whole tissue lysate

Protein amount: 200 μg/experiment

Kit used in this experiment: Reaction Kit



(A) Specificity test of each sample

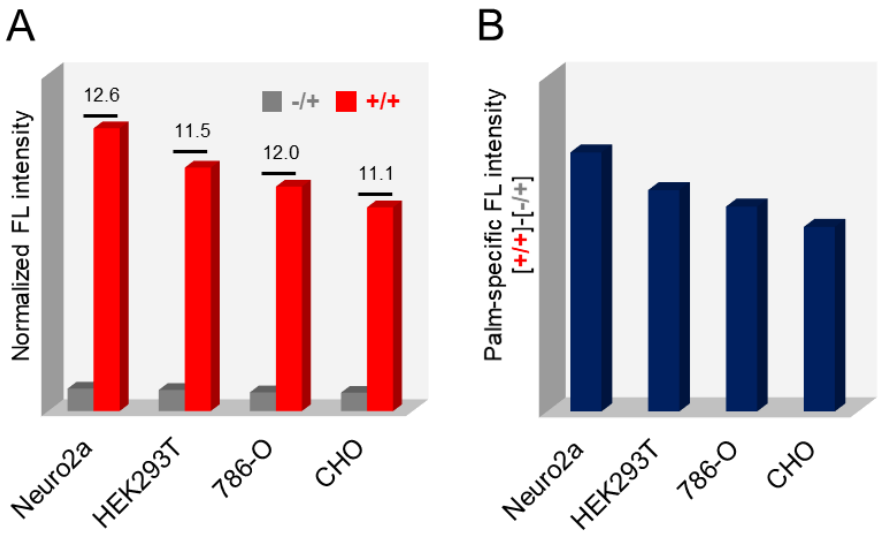
In all samples, high S/N ratio (hpHA(+)/(-) = 3.5-11.5) was observed. These ratio indicates *S*-palmitoyl-specific signal are successfully and preferentially detected.

(B) Semi-quantitative comparison among four mouse tissues

Among four tissues tested in this experiment, brain tissue has the largest amount of total *S*-palmitoylated proteins.

Example 2 Semi-quantitative comparison among cultured cell lines

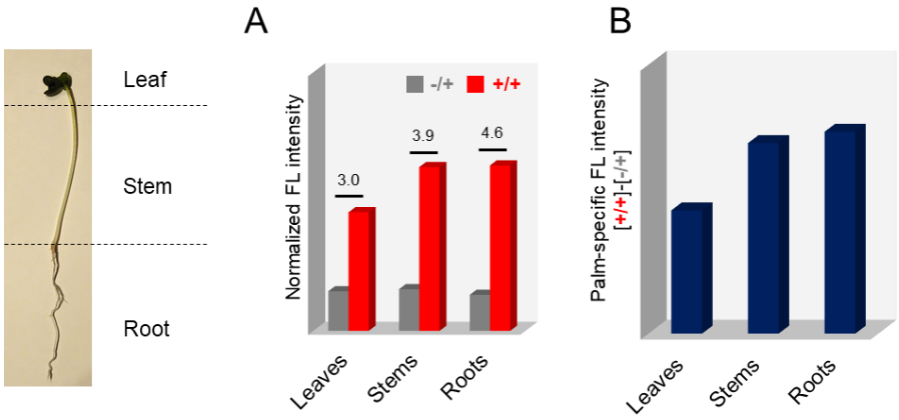
Samples: Cultured cell lines, Neuro2a, HEK293T, 786-O, and Chinese hamster ovary(CHO)
Lysate preparation: Whole cell lysate
Protein amount: 200 μg/experiment
Kit used in this experiment: Reaction Kit



(A) Specificity test of each sample
In all samples, high S/N ratio (hpHA(+)/(-) >11) was observed. These ratio indicates *S*-palmitoyl-specific signal are successfully and preferentially detected.
(B) Semi-quantitative comparison among four cultured cell lines
Among four cultured cell lines tested in this experiment, Neuro2a cells has the largest amount of total *S*-palmitoylated proteins.

Example 3 Semi-quantitative comparison among broccoli sprout tissues

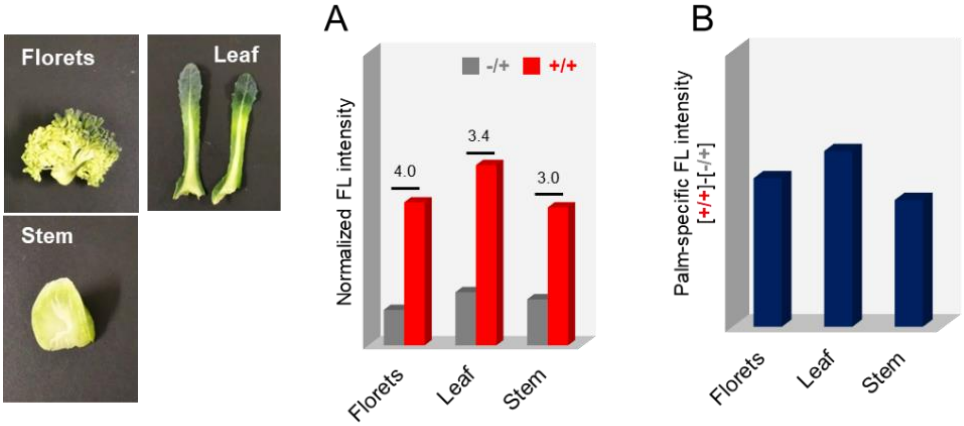
Samples: Broccoli sprout-derived leaves, stems, and roots
Lysate preparation: Soluble fraction from whole tissue lysate
Protein amount: 200 μg/experiment
Kit used in this experiment: Reaction Kit



(A) Specificity test of each sample
In all samples, high S/N ratio (hpHA(+)/(-) >3) was observed. These ratio indicates *S*-palmitoyl-specific signal are successfully and preferentially detected.
(B) Semi-quantitative comparison among three sprout tissues
Among three tissues tested in this experiment, roots have the largest amount of total *S*-palmitoylated proteins.

Example 4 Semi-quantitative comparison among Broccoli tissues

Samples: Broccoli-derived florets, leaves, stem
Lysate preparation: Soluble fraction from whole tissue lysate
Protein amount: 200 μg/experiment
Kit used in this experiment: Reaction Kit

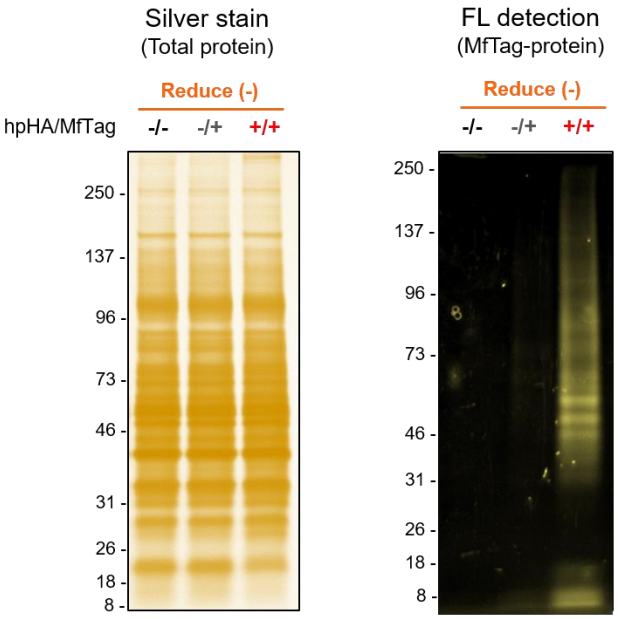


(A) Specificity test of each sample
In all samples, high S/N ratio (hpHA(+)/(-) >3) was observed. These ratio indicates *S*-palmitoyl-specific signal are successfully and preferentially detected.
(B) Semi-quantitative comparison among three broccoli tissues
Among three tissues tested in this experiment, leaf has the largest amount of total *S*-palmitoylated proteins.

In-gel fluorescent detection of labeled proteins

Example　Fluorescent detection of MfTag-labeled proteins in SDS-PAGE gel

Sample: Adult mouse-derived whole brain
Lysate preparation: Whole brain lysate
Kit used in this experiment: Reaction kit
Protein amount: 200 μg/experiment
SDS-PAGE condition: **Non-reducing condition** (Keeping MfTag-labeling)
Detection: Silver staining and fluorescent imaging (Ex 312 nm LED/Em >560 nm)

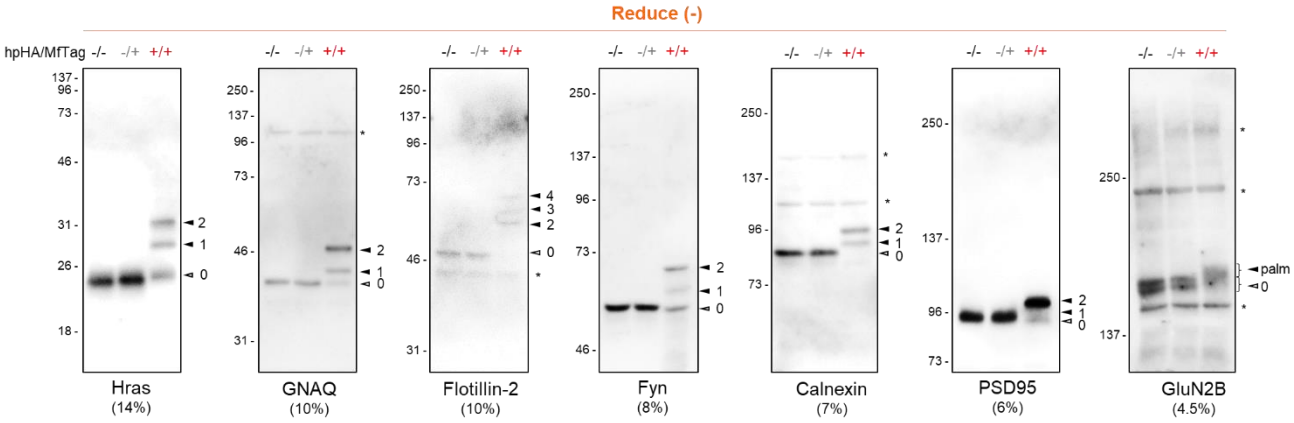


Three types of control experiments **-/-**, **-/+** and **+/+** are prepared by Reaction Kit. Each sample was separated by SDS-PAGE under **non-reducing condition** to keep MfTag-labeling. Total proteins were detected by silver staining and MfTag-labeled proteins were visualized by fluorescent imager with 312 nm UV excitation and >560 nm detection. Although there are no change of total protein amount among three experimental samples, majority of fluorescent signals are specifically detected in **+/+**.

Gel shift assay

Example　Estimation of number of *S*-palmitoyl group in representative marker proteins in mouse whole brain

Sample: Adult mouse-derived whole brain
Lysate preparation: Whole brain lysate
Kit used in this experiment: Reaction Kit
Protein amount: 200 μg/experiment
SDS-PAGE condition: **Non-reducing condition** (Keeping MfTag-labeling)
Gel concentration is refered under each protein name
Detection method: Western Blotting analysis



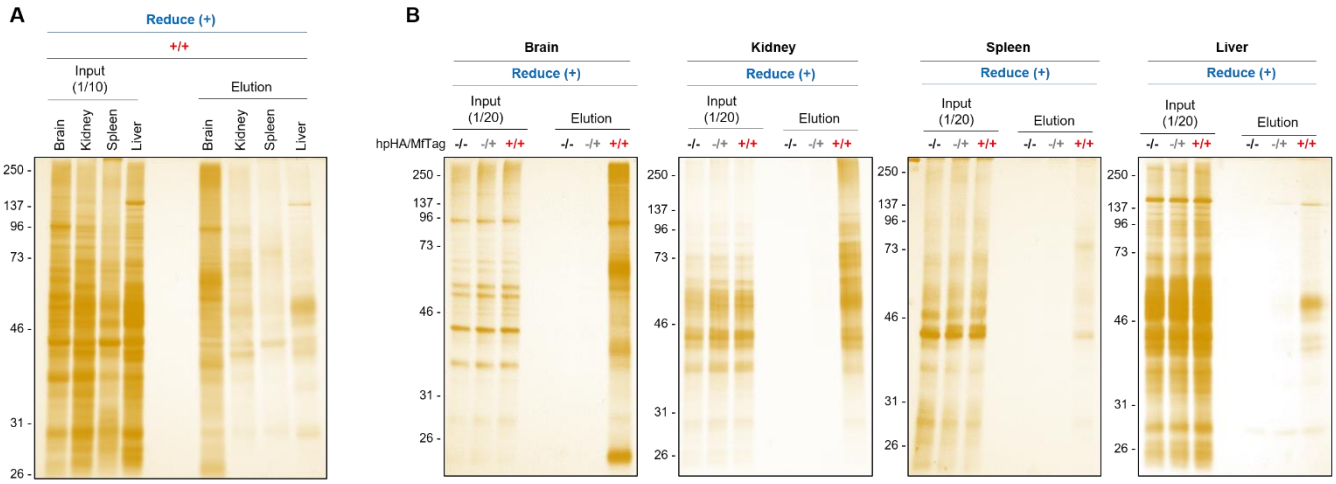
Note: * marks in membranes showed non-specific detection of antibodies.

Three types of control experiments **-/-**, **-/+** and **+/+** are prepared by Reaction Kit. Each sample was separated by SDS-PAGE under **non-reducing condition** to keep MfTag-labeling with appropriate concentration of polyacrylamide gel. After SDS-PAGE, proteins on the gel were transferred to PVDF membranes. The membranes were probed by antibodies agains representative seven *S*-palmitoylated proteins. All proteins shows band shifts in only **+/+** and no additional bands in **-/+**. This indicates exchange reaction was successfully done by Reaction Kit at least seven proteins tested here. In the adult whole mouse brain, these WB data suggest Hras has two, GNAQ has two, Flotillin-2 has four, Fyn has two, Calnexin has two and PSD95 has two MfTag (=*S*-palmitoyl group). As GluN2B is about 150 kDa molecular weight, **+/+**-specific band shift of GluN2B was smear and it was challenging to identify the labeled number of MfTag.

Affinity purification

Example 1 Purification of *S*-palmitoylated proteins of four mouse tissues and specificity check

Sample: Adult mouse-derived brain, kidney, spleen, liver
Lysate preparation: Whole tissue lysate
Kits used in this experiment: Reaction kit and Purification kit
Protein amount in Reaction kit: 200 μg/experiment
Protein amount in Purification kit: 100 μg/column
SDS-PAGE condition: **Reducing condition** (Removing MfTag)



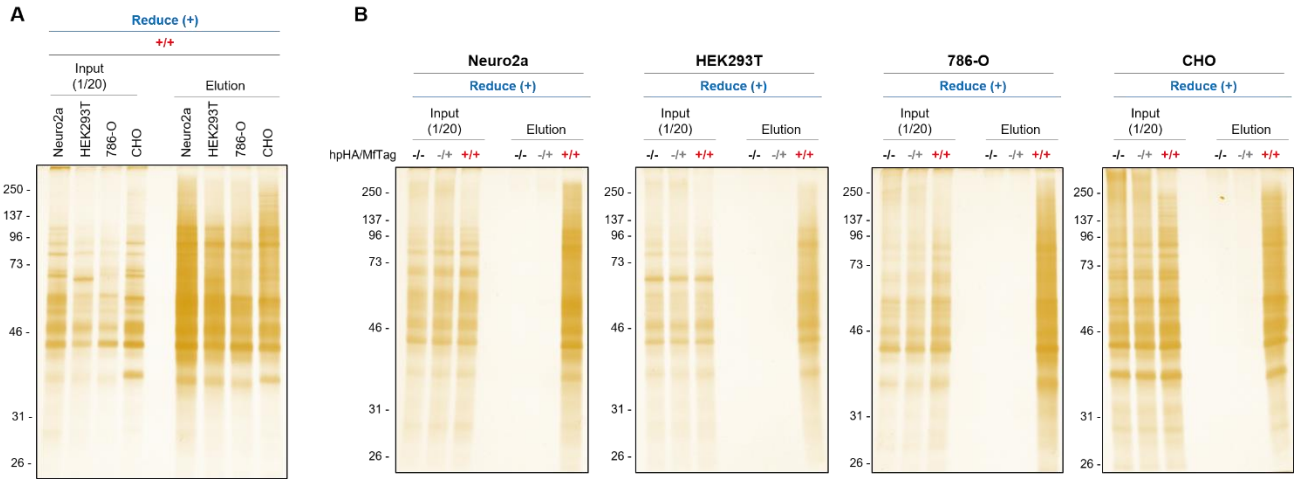
A After purification of MfTag-labeled proteins by the *Loop* affinity column, **Input** (10 fold dilution) and **Elution** fractions of **+/+** samples were separated by SDS-PAGE under **reducing condition** and the gel was silver stained. Brain shows the highest amount of purified proteins among four tissues tested.

B After purification of MfTag-labeled proteins by the *Loop* affinity column, **Input** (20 fold dilution) and **Elution** fractions of **-/-**, **-/+**, **+/+** were separated by SDS-PAGE under **reducing condition** and the gel was silver stained. Purified proteins were specifically observed in **+/+** treatment samples for all tissues.

NOTE In the liver sample, a protein around 30 kDa was detected in the **Elution** fraction of **-/-** sample. This protein binds to the Affinity Beads potentially without MfTag and should be considered a non-specific protein.

Example 2 Purification of *S*-palmitoylated proteins of four cultured cells and specificity check

Sample: Cultured Neuro2a, HEK293T, 786-O, CHO cells
Lysate preparation: Whole cell lysate
Kits used in this experiment: Reaction kit and Purification kit
Protein amount in Reaction kit: 200 μg/experiment
Protein amount in Purification kit: 100 μg/column
SDS-PAGE condition: **Reducing condition** (Removing MfTag)



A After purification of MfTag-labeled proteins by the *Loop* affinity column, **Input** (20 fold dilution) and **Elution** fraction of **+/+** samples were separated by SDS-PAGE under **reducing condition** and the gel was silver stained. Neuro2a cells show a relatively high amount of purified proteins among four cell lines tested.

B After purification of MfTag-labeled proteins by the *Loop* affinity column, **Input** (20 fold dilution) and **Elution** fractions of **-/-**, **-/+** and **+/+** were separated by SDS-PAGE under **reducing condition** and the gel was silver stained. Majority of purified proteins were specifically observed in **+/+** samples for all cell lines.

Example 3 Detection of major *S*-palmitoylated proteins in mouse whole brain

Sample: Adult mouse-derived whole brain

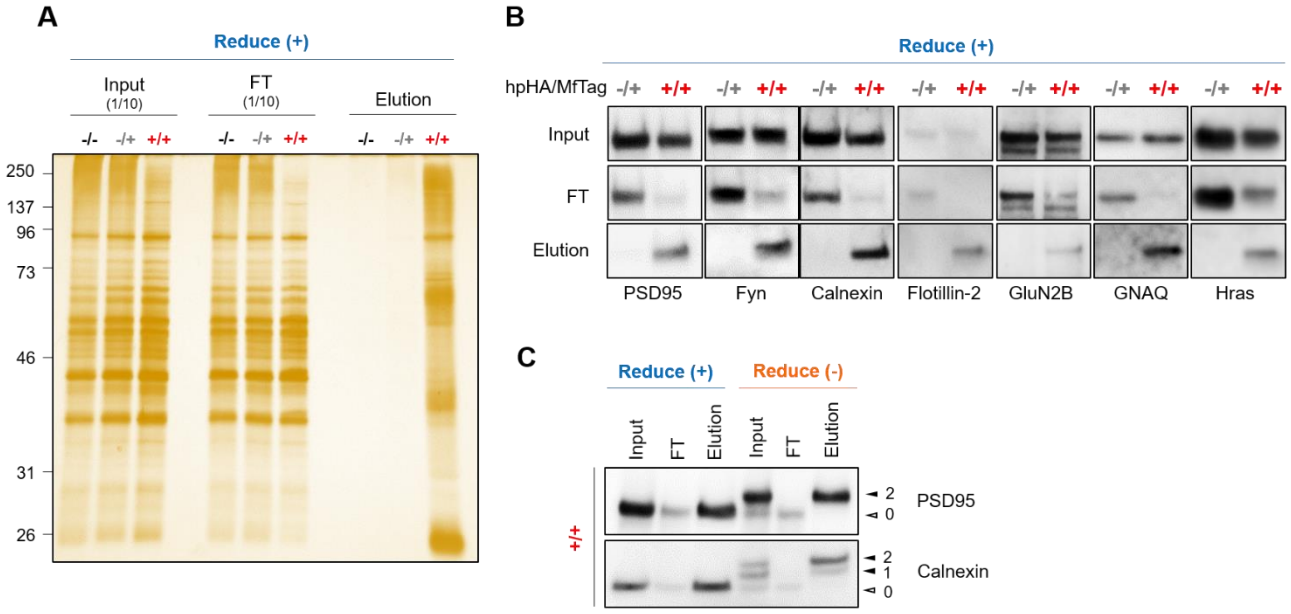
Lysate preparation: Whole brain lysate

Kits used in this experiment: Reaction kit and Purification kit

Protein amount in Reaction kit: 200 μg/experiment

Protein amount in Purification kit: 100 μg/column

SDS-PAGE condition: **Reducing condition** (A, B, C-left) and **Non-reducing condition** (C-right)



A After purification of MfTag-labeled proteins by the *Loop* affinity column, **Input** (10 fold dilution), **FT** (10 fold dilution), and **Elution** fractions were separated by SDS-PAGE under **reducing condition**, and the gel was silver stained. Majority of the purified proteins were specifically observed in **+/+**.

B Same volume of **Input**, **FT** and **Elution** fractions were applied to SDS-PAGE under **reducing condition**, separated proteins were transferred to PVDF membranes, and probed indicated antibodies against seven representative *S*-palmitoylated proteins. The WB of **Input**, **FT,** and **Elution** samples ran in the same membrane; the band intensities can be compared. All seven proteins were specifically detected in **+/+** in **Elution** fraction. These results indicate this kit successfully detects representative *S*-palmitoylated proteins.

C Same volume of **Input**, **FT,** and **Elution** fractions of **+/+** were applied to SDS-PAGE under both **reducing** and **non-reducing** conditions, separated proteins were transferred to PVDF membranes, and probed antibodies against PSD95 or calnexin. Under the **non-reducing condition**, both PSD95 and Calnexin show that only MfTag-labeled bands were detected in the **Elution** fraction, and the non-labeled band was completely eluted in the **FT** fraction. This data indicates that MfTag-labeled PSD95 and Calnexin were successfully purified in this experiment. Under the condition, the data of the **reduced condition** indicates the majority of PSD95 and Calnexin are *S*-palmitoylated form in adult mouse brain.

Comprehensive application

Example 1 Enrichment of *S*-palmitoylated proteins by brain subcellular fractionation

Sample preparation

Subcellular fractionation was performed by scheme A. P2 membrane fraction and P2-TritonX100 insoluble fraction were prepared from whole brain homogenate.

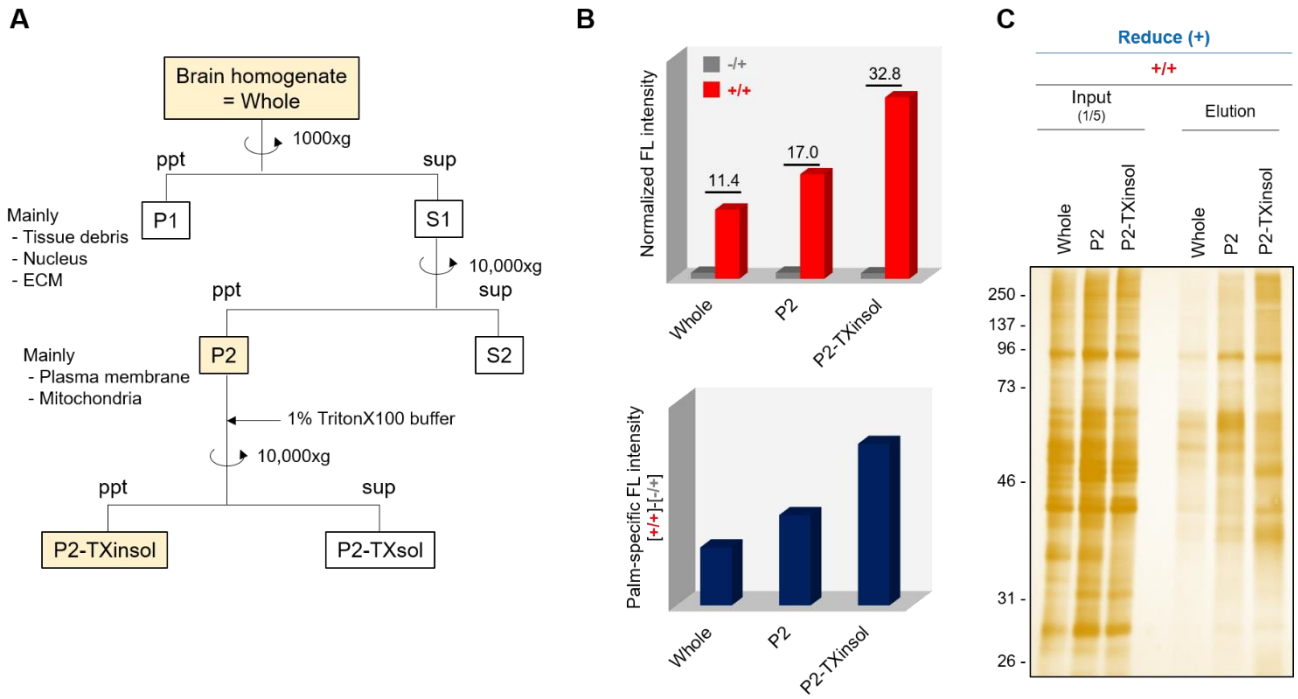
Kits used in this experiment: Reaction kit and Purification kit

Methods: Fluorometric assay, Silver staining of purified proteins

Protein amount in Reaction kit: 100 μg/experiment

Protein amount in Purification kit: 50 μg/column

SDS-PAGE condition: **Reducing condition**



A Subcellular fractionation protocol. Whole mouse brain was homogenized in homogenate buffer (50 mM phosphate (pH 7.4), 150 mM NaCl, 320 mM sucrose, w/o any detergent) with a dounce-type homogenizer. The whole brain homogenate was initially separated by slow-speed centrifugation (1,000*xg*) to P1 and S1 fractions. S1 fraction was further separated by middle-speed centrifugation (10,000*xg*) to P2 and S2 fractions. P2 fraction was divided into two batches, and one batch was treated with 1% TritonX100 Buffer, separated by middle-speed centrifugation (10,000*xg*) to P2-TritonX insoluble fraction (P2-TXinsol) and P2-TritonX soluble fraction (P2-TXsol). Whole brain homogenate, P2, and P2-TXinsol were solubilized in 1x Base Buffer and applied to the Reaction kit.

B Fluorometric assay. Specificity check (above) and relative comparison (below). These results indicate that *S*-palmitoylated proteins were highly accumulated in P2 and P2-TXinsol fractions.

C After purification of MfTag-labeled proteins by the *Loop* affinity column, **Input** (5 fold dilution) and **Elution** fractions of **+/+** were separated by SDS-PAGE under **reducing condition**, and the gel was silver stained. Consistent with a fluorometric assay, *S*-palmitoylated proteins were highly enriched in P2 and P2-TXinsol fractions.

Example 2　Intracellular distribution of *S*-palmitoylated protein in cultured Neuro2a cells

Sample preparation

Rough subcellular fractionation was performed by scheme A. Whole cell lysate, P_{10k} fraction (mainly plasma membrane), and S_{10k} fraction (mainly endomembranes and cytosol) were prepared.

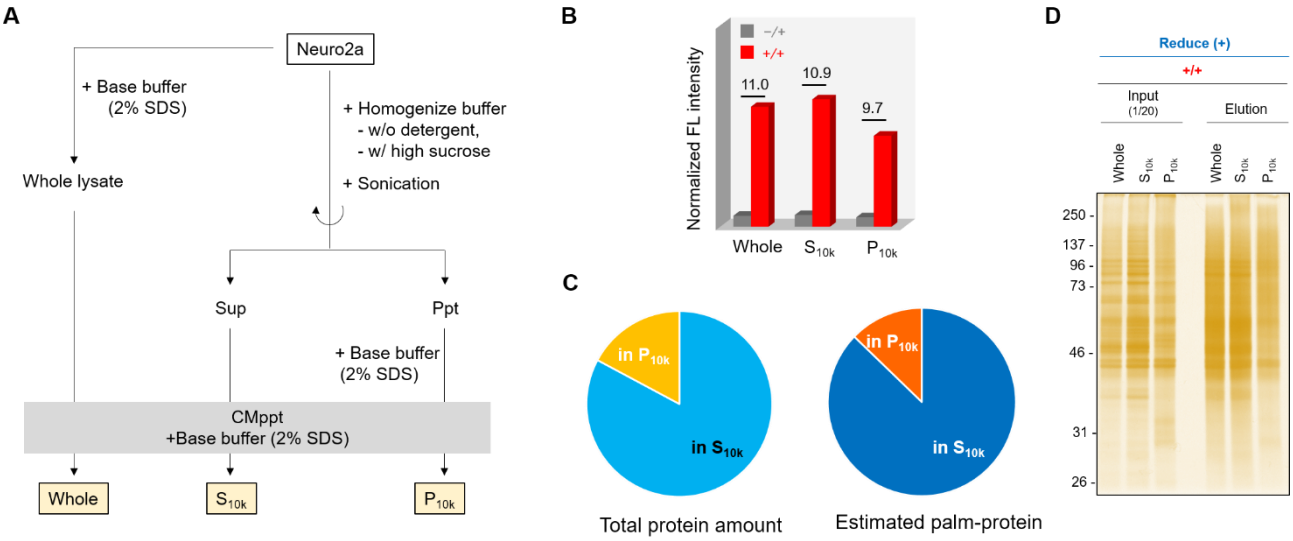
Kit: Reaction Kit and Purification Kit

Methods: Fluorometric assay, Silver staining of purified protein

Protein amount in Reaction kit: 100 μg/experiment

Protein amount in Purification kit: 50 μg/column

SDS-PAGE condition: **Reducing condition**



A　Rough subcellular fractionation protocol. “Whole” was prepared by direct lysis of Neuro2a cell with 1x Base Buffer. For the preparation of S_{10k} and P_{10k}, cells were homogenized by homogenate buffer (50 mM phosphate (pH 7.4), 150 mM NaCl, 320 mM sucrose, w/o any detergent) with sonication and separated by middle-speed centrifugation (10,000*xg*, 20 min) to P_{10k} and S_{10k} fractions. All fractions were done with CMppt, the pellet resolubilized by 1x Base Buffer, and applied to the Reaction kit.

B　Fluorometric assay (Specificity check)

C　Total protein amount (Left) and estimated *S*-palmitoylated protein amount (right) calculated from B. S-palmitoylated proteins are mainly enriched in S_{10k} fraction.

D　After purification of MfTag-labeled proteins, **Input** (20 fold dilution) and **Elution** fractions of **+/+** were separated by SDS-PAGE under **reducing condition**, and the gel was silver stained. Consistent with a fluorometric assay, *S*-palmitoylated proteins were enriched in S_{10k} fractions.

Example 3　Drug-induced change of *S*-palmitoylation in Neuro2a cells

Sample preparation

Neuro2a cells were treated with four drugs for 24 hours. After the treatment, cells were homogenized by homogenization buffer, and rough plasma membrane fractions (P_{10k}) were separated by the same procedure as Example 2.

- Control: Serum-free DMEM
- Protein acyl transferase (PAT) inhibitor: 10 μM 2-Bromopalmitate + 10 μM Cerulenin in DMEM
- Nitric oxide (NO) stress: 1 mM SNAP DMEM
- ER stress: 1 μg/ml Tunicamycine in DMEM
- Membrane depolarization: 50 mM KCl in DMEM

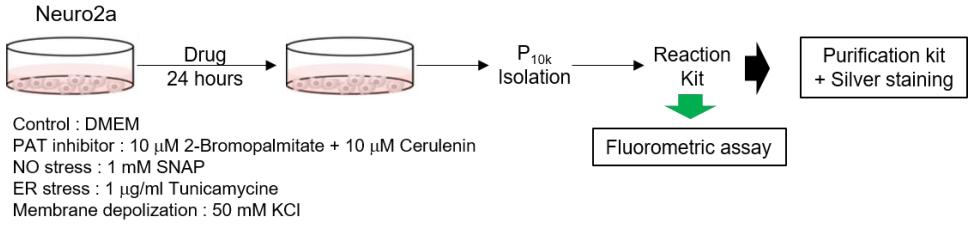
Protein amount in Reaction kit: 100 μg/experiment

Protein amount in Purification kit: 50 μg/column

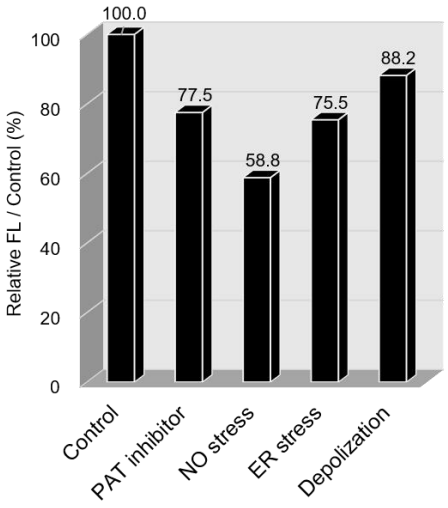
Kits used in this experiment: Reaction kit and Purification kit

Methods: Fluorometric assay, Silver staining of purified proteins

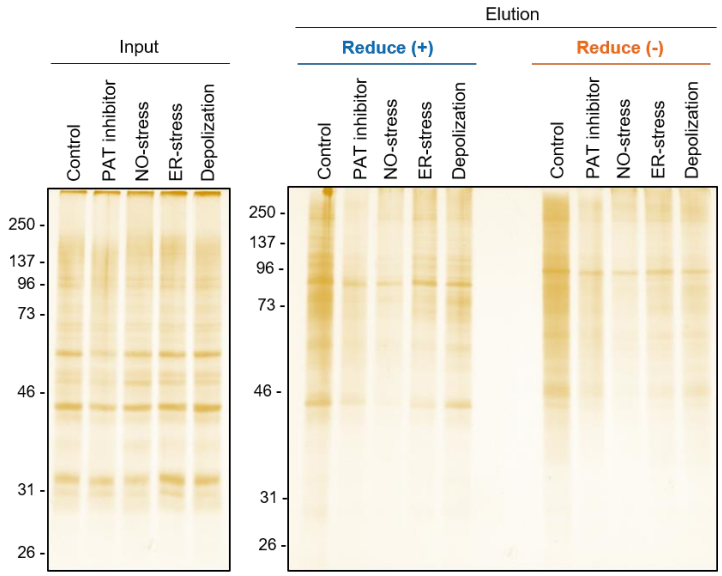
A



B



C



A　Overview of the experimental protocol

B　After measurement of fluorescent intensities of each **+/+** sample, fluorescent intensities were normalized by the control experiment. In all cases, after drug treatment the total *S*-palmitoylation level was reduced and, especially, NO stress dramatically reduced *S*-palmitoylation level in the PM.

C　After purification of MfTag-labeled proteins, **Input** and **Elution** samples of **+/+** were separated by SDS-PAGE under both **reducing** and **non-reducing** conditions, and gels was silver stained. Consistent with a fluorometric assay, purified proteins of NO stress sample were clearly reduced.