*RapidS*PALM, Protein *S*-Palmitoylation Detection Kit Application Note Gallery

Application note of *RapidS*PALM, Protein *S*-Palmitoylation Detection Kit are listed in Table. Firstly please find "How to read experimental data of *RapidS*PALM".

Table Application of *Rapids*PALM

*Please click title to link each data.

Purpose	Example	Kit required
Comparison of total *S*-palmitoylation amounts in multiple samples by fluorometric measurement	Semi-quantitative comparison among mouse tissues	**Reaction kit**
	Semi-quantitative comparison among cultured cell lines	
	Semi-quantitative comparison among broccoli sprout	
	Semi-quantitative comparison among broccoli tissue	
Detection of *S*-palmitoylation protein bands in SDS-PAGE gel by fluorometric imager	Fluorescent detection of MfTag-labeled proteins in SDS-PAGE gel	**Reaction kit**
Estimation of *S*-palmitoyl group number on target proteins by gel shift assay	Estimation of number of *S*-palmitoyl group in representative marker proteins in mouse whole brain	**Reaction kit**
Comprehensive purification of *S*-palmitoylated proteins by affinity column	Purification of *S*-palmitoylated proteins of four mouse tissues	**Reaction kit** **Purification kit**
	Purification of *S*-palmitoylated proteins of four cultured cells	
	Purification of *S*-palmitoylated proteins of broccoli sprout	
	Purification of *S*-palmitoylated proteins of broccoli tissue	
Identification of *S*-palmitoylated proteins and estimation of *S*-palm/non-palm ratio of target proteins by affinity column	Detection of major *S*-palmitoylated proteins in mouse whole brain	**Reaction kit** **Purification kit**
Comprehensive analysis	Enrichment of *S*-palmitoylated proteins by brain subcellular fractionation	**Reaction kit** **Purification kit**
	Intracellular distribution of *S*-palmitoylated proteins in cultured Neuro2a cells	
	Drug-induced change of *S*-palmitoylation in Neuro2a cells	
Comparison with conventional ABE method	Comparison between *RapidS*PALM and ABE using mouse whole brain	**Reaction kit** **Purification kit**

Analytic methods for each application are shown below.

- **Analytical Methods of Fluorometric Assay** (Link)
- **Analytical Methods of Gel Shift Assay** (Link)
- **Analytic Methods of Affinity Purification** (Link)

1. How to read experimental data of *RapidS*PALM

1-1. Control experiment guide

In *RapidS*PALM experiments, three control experiments for each sample should be prepared. In this webpage, please note **hpHA(-)/MfTag(-)**, **hpHA(-)/MfTag(+)** and **hpHA(+)/MfTag(+)** are abbreviated to **-/-**, **-/+**, and **+/+**, respectively. Types of control experiments and their purposes are described in Tables 1 and 2.

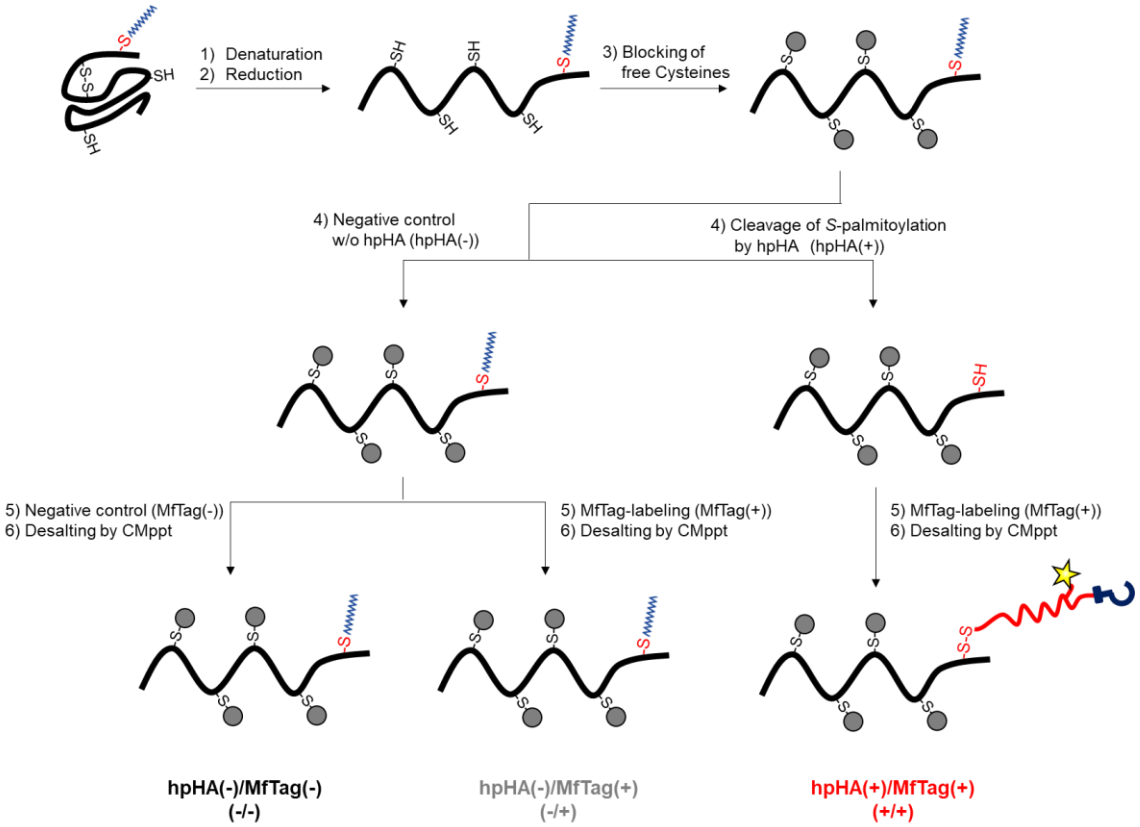


Figure Stepwise chemical reaction of *RapidS*PALM

Table 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-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

1-2. Selection guide for non-reducing or reducing condition on SDS-PAGE

As MfTag is labeled with thiol of cysteine residue via disulfide bond, MfTag can be easily removed by reducing reagent (a Kit component). In the **Purification kit**, Elution buffer will elute intact MfTag-labeled proteins from affinity beads. 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** MfTags were removed from the proteins and protein bands should be a single band. You have to select **non-reducing condition** or **reducing condition** according to the following applications (Table 3).

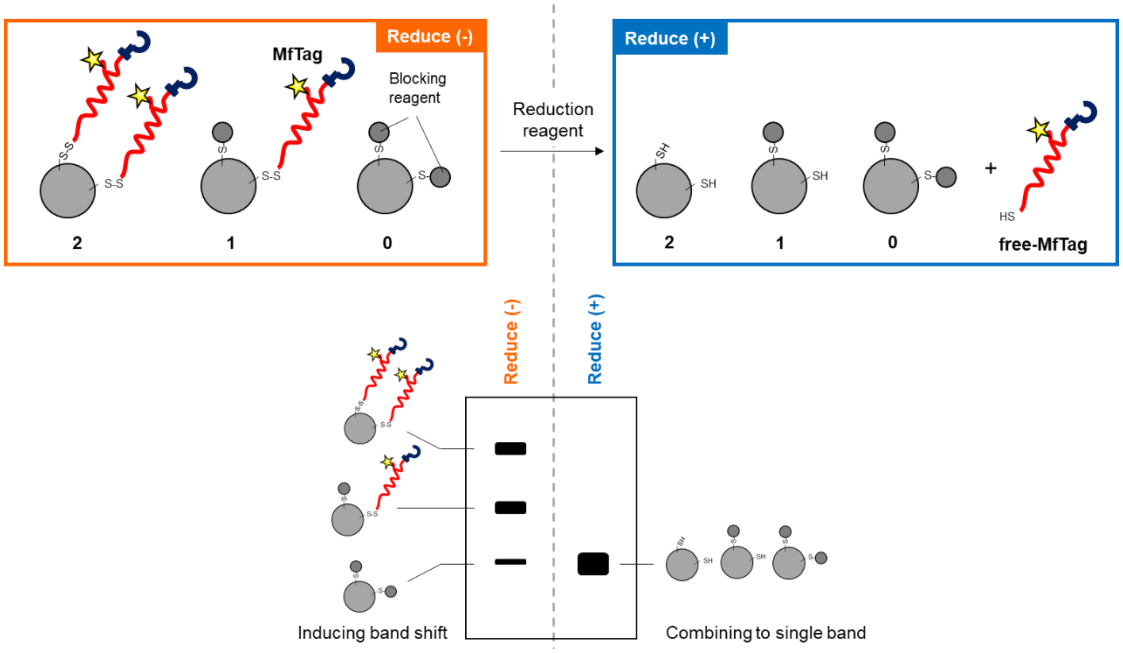


Figure　Model of two MfTag-labeled proteins under Reduce (-) and Reduce (+) condition

Table 3　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

2. Application Note Gallery

■ **Comparison of total *S*-palmitoylation amounts in multiple samples by fluorometric measurement**

Analytical Methods of Fluorometric Assay

Figure 1 shows excitation and emission spectrum of yellow fluorophore (yFL) on the MfTag. Fluorescent intensity at 525 nm excited by 325 nm is recommended. As fluorescent spectrum may be varied by solvent, please use specific buffer in the kit.

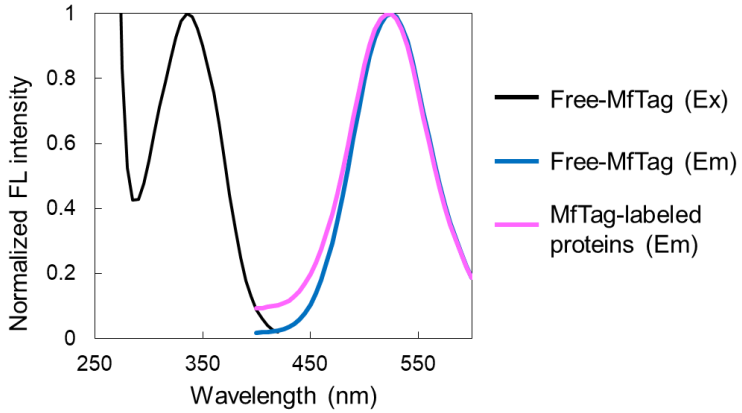


Figure 1 Excitation and emission spectrum of yFL on free-MfTag and MfTag-labeled proteins

For fluorometric assay, measure fluorescent intensity (Ex 325 nm/ Em 525 nm) of three controls, **-/-**, -/+, and **+/+**, by each sample.

[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 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 multiple samples

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

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

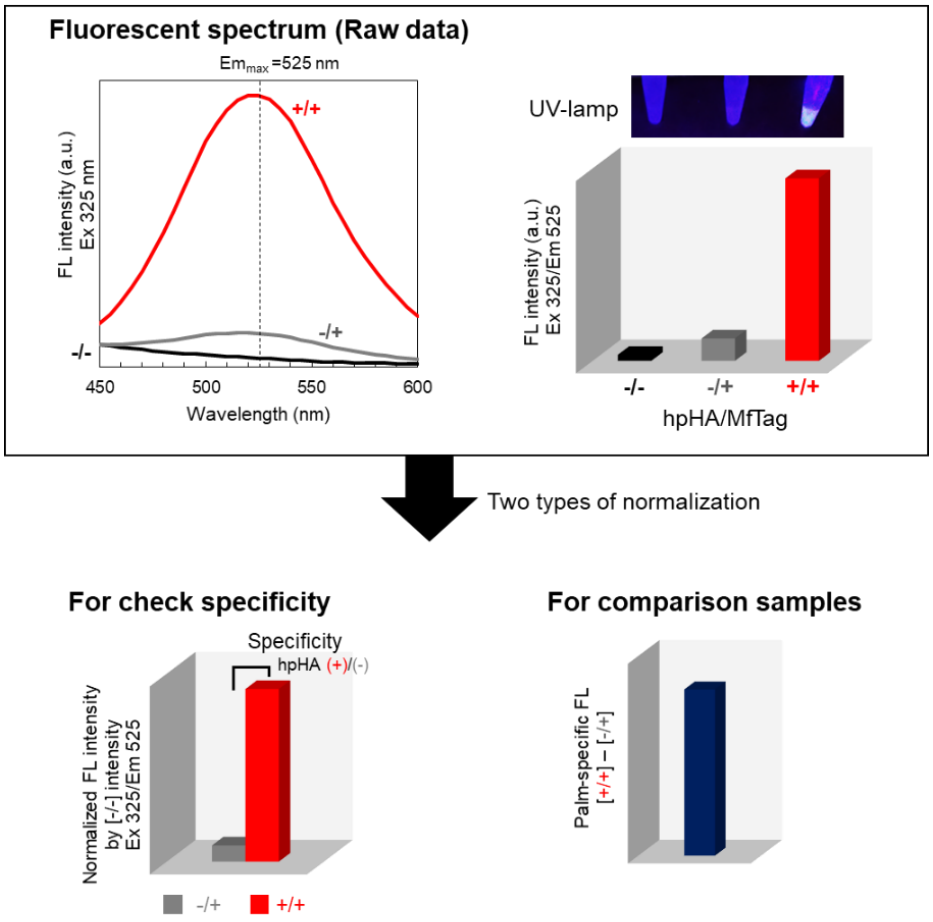


Figure 2 Analytic methods of fluorometric assay

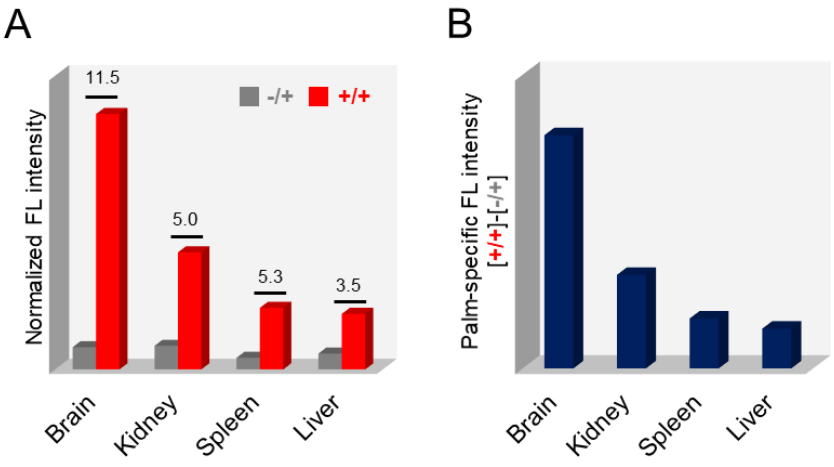
Example 1　Semi-quantitative comparison among mouse tissues

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 (hpHP**(+)**/(-) =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.

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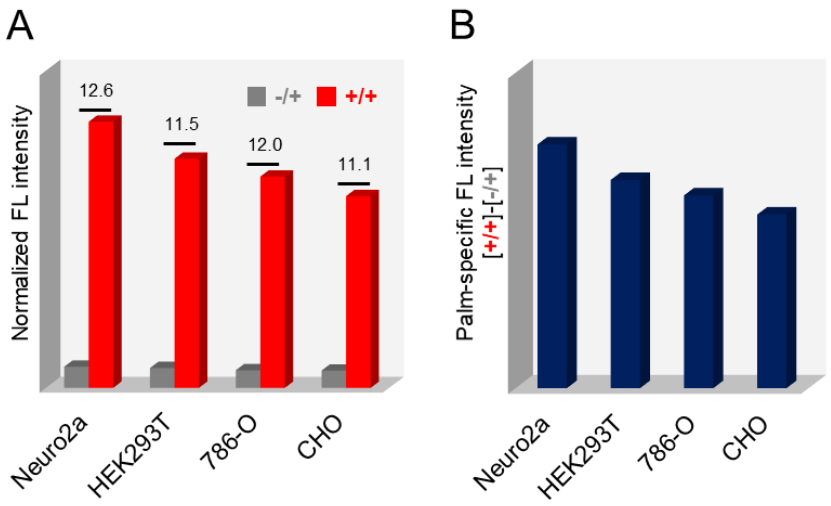
Example 2　Semi-quantitative comparison among cultured cells

Samples: Cultured 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 cell lines

Among four cultured cell lines tested in this experiment, Neuro2a cells has the largest amount of total *S*-palmitoylated proteins.

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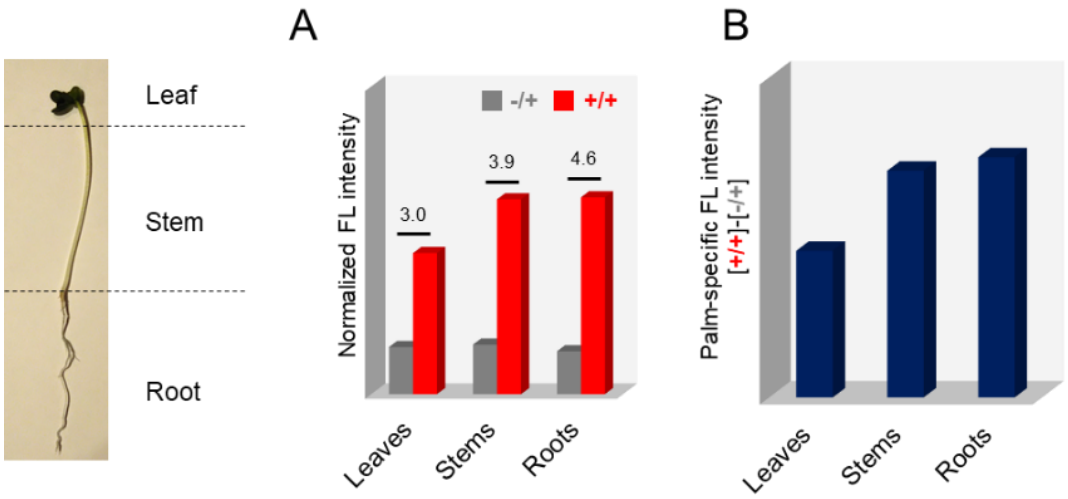
Example 3　Semi-quantitative comparison among broccoli sprout

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*-palimtoyl-specific signal are successfully and preferentially detected.

(B) Semi-quantitative comparison among three broccoli sprout tissues

Among three tissues tested in this experiment, roots have the largest amount of total *S*-palmitoylated proteins.

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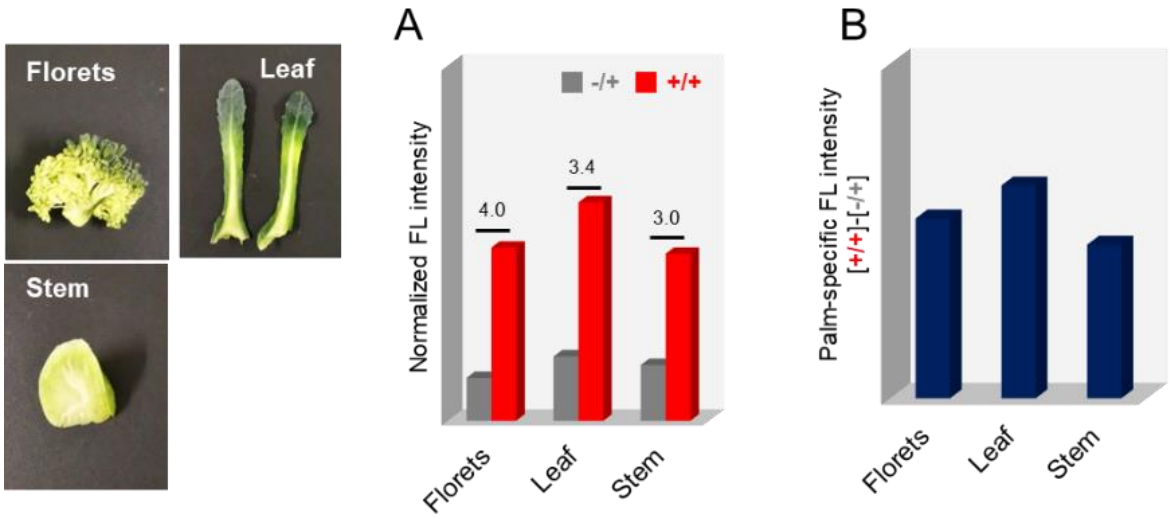
Example 4　Semi-quantitative comparison among broccoli tissue

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.

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Detection of S-palmitoylation protein bands in SDS-PAGE gel by fluorometric imager

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

Sample: Adult mouse-derived whole brain

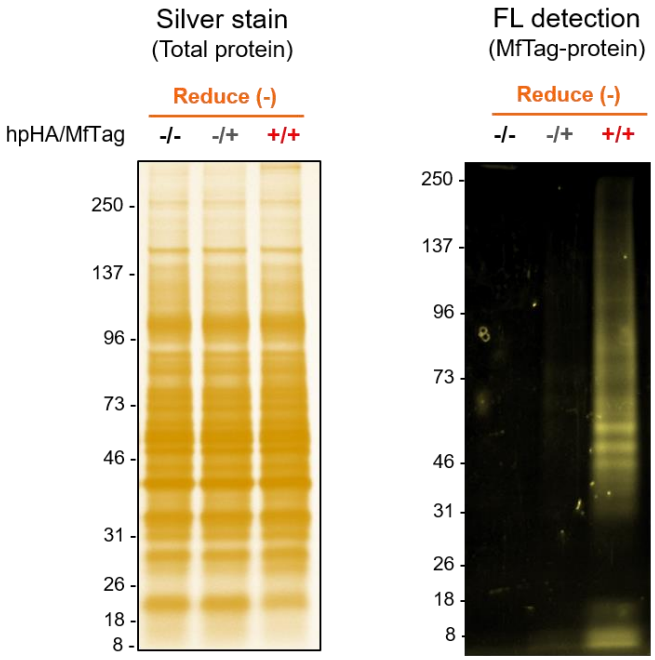
Lysate preparation: Whole brain lysate

Kit used in this experiment: **Reaction kit**

SDS-PAGE condition: **Non-reducing condition** (Keep MfTag-labeling)

*Please click here to find selection guide for non-reducing or reducing condition.

Detection: Silver staining and fluorescent imaging (Ex 312 nm/Em>560 nm)



Three types of control experiments **-/-**, **-/+**, **+/+** 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 **+/+**.

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Estimation of *S*-palmitoyl group number on target proteins by gel shift assay

Analytic Methods of Gel Shift Assay

After conversion of *S*-palmitoylation to MfTag by **Reaction kit**, separation of proteins by SDS-PAGE under **non-reducing condition**, and detection of individual proteins by Western blotting using antibodies of interest, you can estimate the number of *S*-palmitoyl group on the target protein.

[Evaluation]

1) Evaluation of *S*-palmitoylation-specificity

Likely to Figure 1, 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 can 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 the least for the indicated protein and you cannot determine the number of S-palmitoylation sites.

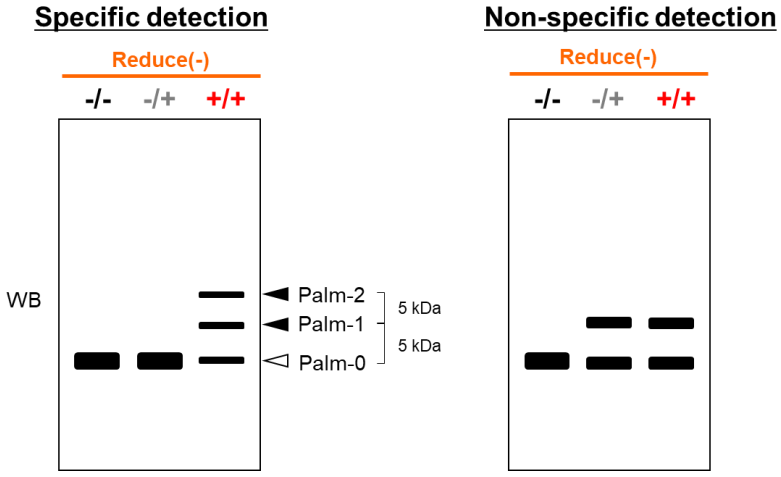


Figure 1　Specificity check on gel shift assay

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 2). For Hras (green), as two additional bands appeared only in the **+/+** sample, the data suggests Hras has two MfTags (=*S*-palmitoyl 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 band 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.

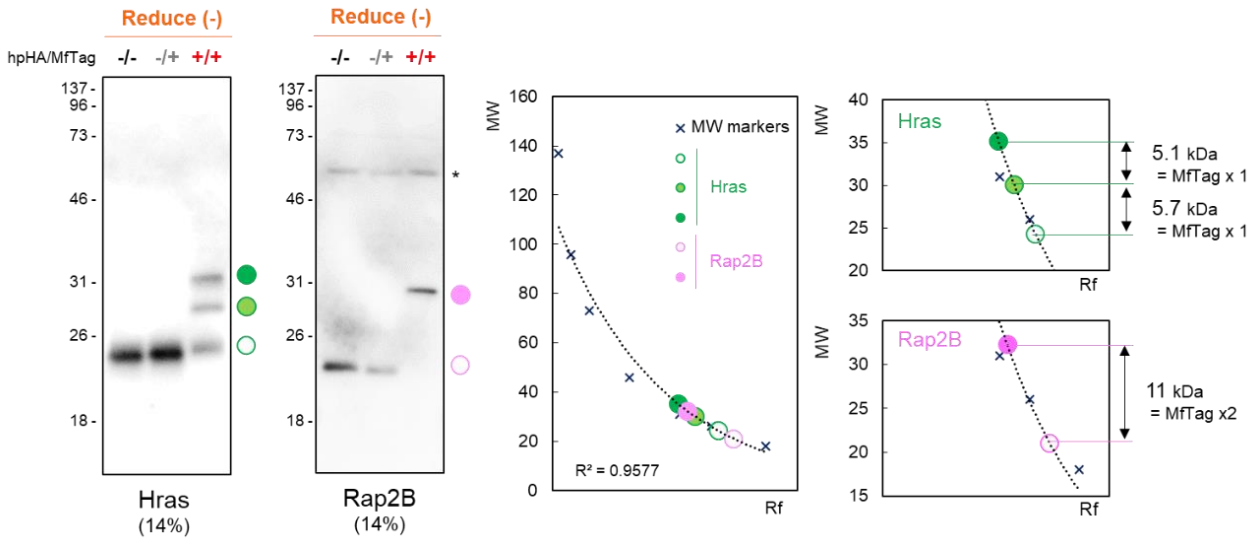


Figure 2 Evaluation of MfTag-labeling number of Hras and Rap2b in mouse whole brain tissue

*Non-specific bands of antibody

3) 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 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.

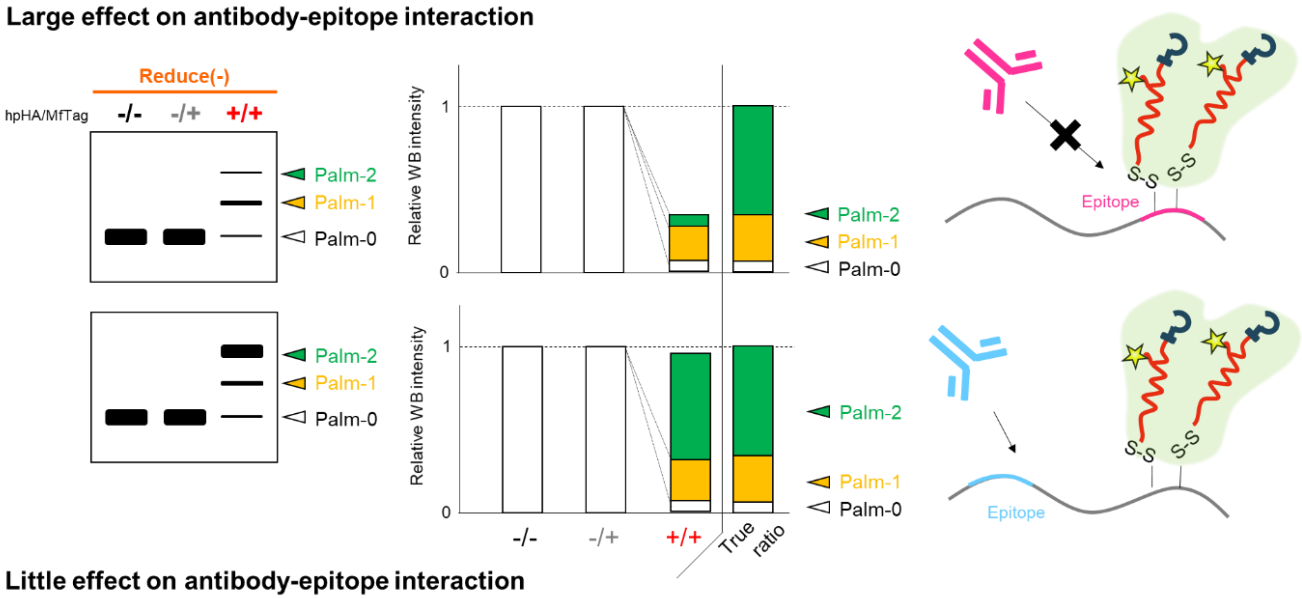


Figure 3　Influence of MfTag-labeling on antibody recognition

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

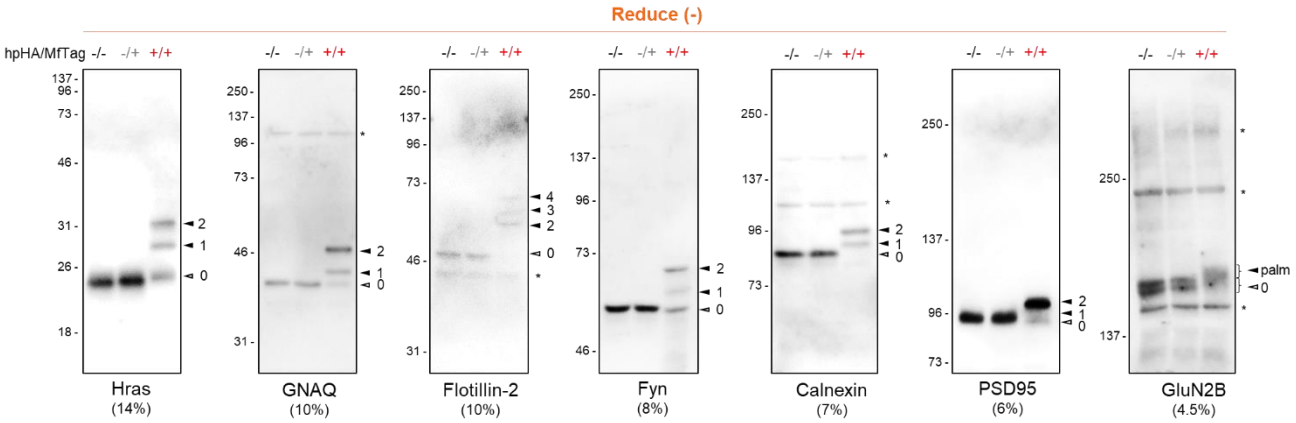
Protein amount: 200 μg/experiment

SDS-PAGE condition: **Non-reducing condition** (Keeping MfTag-labeling)

Gel concentration is referred under each protein name

*Please click here to find selection guide for non-reducing or reducing condition.

Detection method: Western Blotting analysis



Note Asterisk(*) marks indicate non-specific signal of each antibody.

Three types of control experiments **-/-**, **-/+**, **+/+** 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 against 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, Fluotillin-2 has four, Fyn has two, Calnexin has two and PSD95 has two MfTag (=S-palmitoylated group). As GluN2B is about 150 kDa molecular weight, **+/+** specific band shift of GluN2Bwas smear and it was challenging to identify the labeled number of MfTag.

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Comprehensive purification of *S*-palmitoylated proteins by affinity column

Analytic Methods of Affinity Purification

After purification of MfTag-labeled proteins by the **Purification Kit**, total purified proteins can be visualized by silver staining following to SDS-PAGE under **reducing condition**. Furthermore, western blot analysis allows us to identify individual *S*-palmitoylated protein. Combination of both **reducing condition** and **non-reducing condition** SDS-PAGE/western blotting provides quantitative information about *S*-palm/non-palm ratio.

1. 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 Figure 1, 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 Figure 1, 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*-palmitoylated proteins.

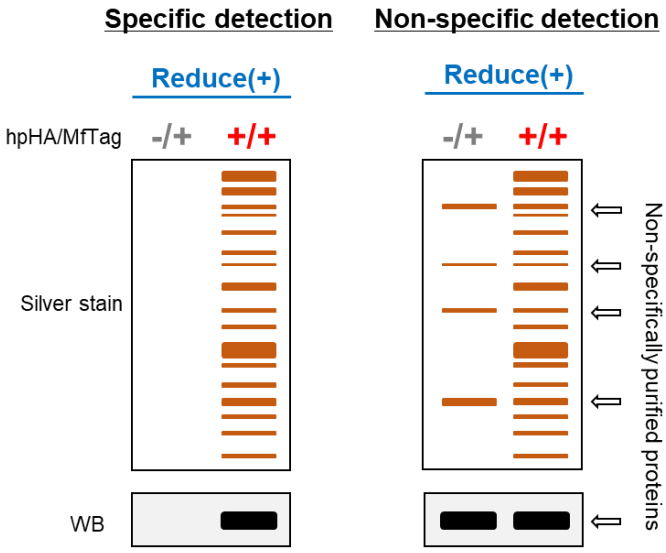


Figure 1 Check specificity of affinity purification

2. 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), 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 2). First, see the **non-reducing condition** in Figure 2. 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**.

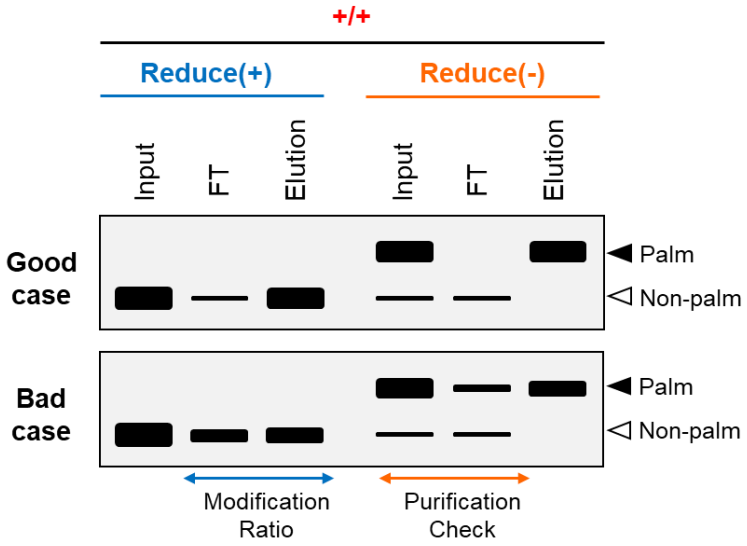


Figure 2 Check purification efficiency and estimate the *S*-palm ratio

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

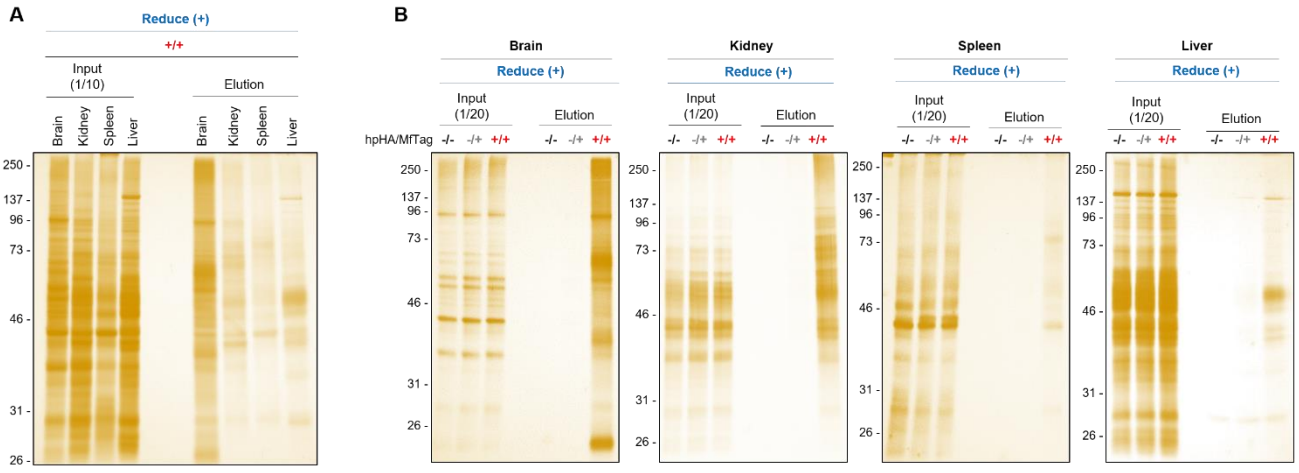
Kits used in this experiment: **Reaction kit** and **Purification kit**

Protein amount in Reaction kit: 200 μg/experiment

Protein amount in Purification kit: 100 μg/column

SDS-PAGE condition: **Reduction condition** (Removing MfTag)

*Please click here to find selection guide for non-reducing or reducing condition.



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.

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

Sample: Cultured Neuro2a, HEK293T, 786-O, Chinese hamster ovary(CHO)

Lysate preparation: Whole cell lysate

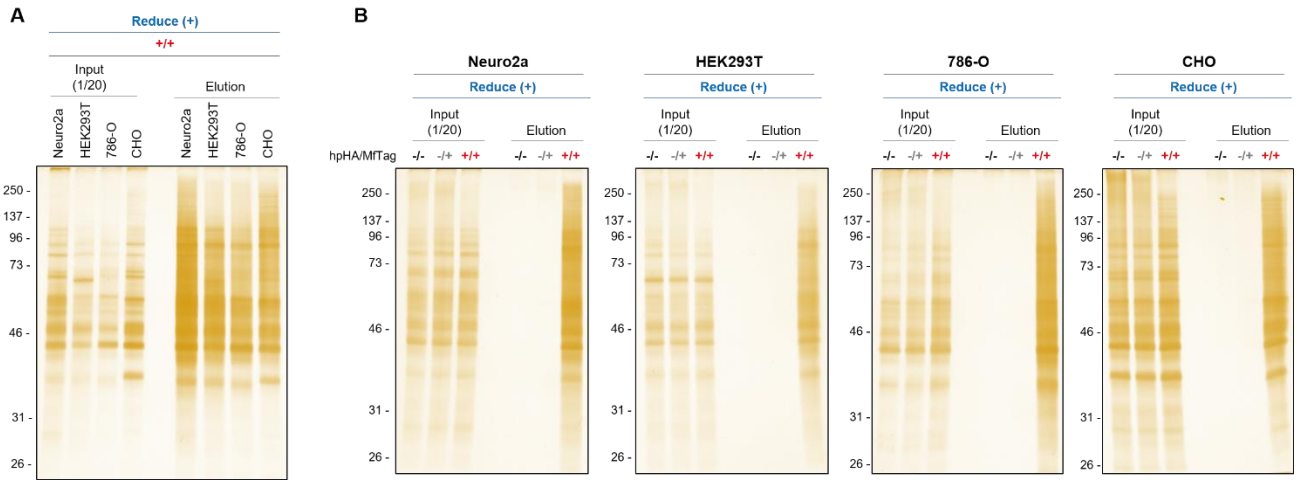
Kits used in this experiment: **Reaction Kit** and **Purification Kit**

Protein amount in Reaction kit: 200 μg/experiment

Protein amount in Purification kit: 100 μg/column

SDS-PAGE condition: **Reducing condition** (Removing MfTag)

*Please click here to find selection guide for non-reducing or reducing condition.



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.

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Example 3　　Purification of *S*-palmitoylated proteins of broccoli sprout and specificity check

Samples: Broccoli sprout-derived leaves, stems, and roots

Lysate preparation: Soluble fraction from whole tissue lysate

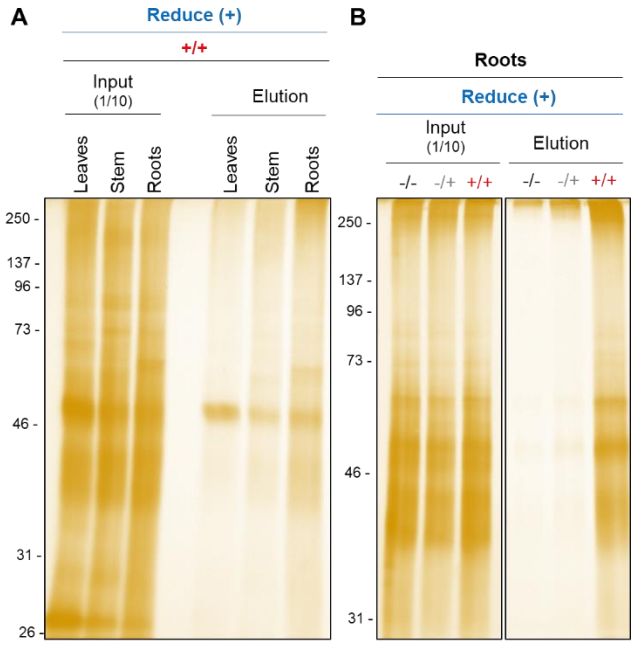
Kits used in this experiment: **Reaction Kit** and **Purification Kit**

Protein amount in Reaction kit: 200 μg/experiment

Protein amount in Purification kit: 100 μg/column

SDS-PAGE condition: **Reducing condition** (Removing MfTag)

*Please click here to find selection guide for non-reducing or reducing condition.



A: After purification of MfTag-labeled proteins by the *Loop* affinity column, **Input** (10 fold dilution) and **Elution** fraction of **+/+** samples were separated by SDS-PAGE under **reducing condition** and the gel was silver stained. Roots cells show a relatively high amount of purified proteins among three broccoli sprout tissues tested.

B: After purification of MfTag-labeled proteins by the *Loop* affinity column, **Input** (10 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 the **+/+** roots sample.

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Example 4　Purification of *S*-palmitoylated proteins of broccoli tissue and specificity check

Samples: Broccoli-derived florets, leaves, and stem

Lysate preparation: Soluble fraction from whole tissue lysate

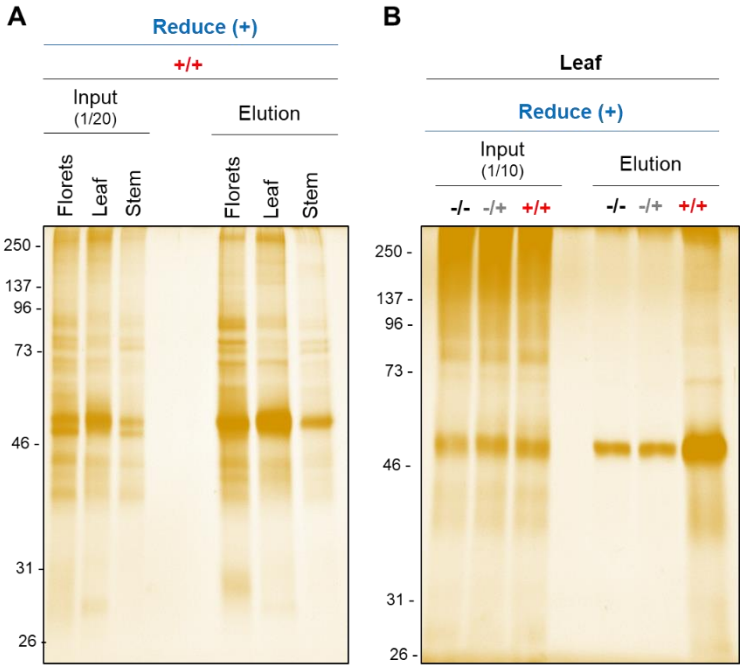
Kits used in this experiment: **Reaction kit** and **Purification kit**

Protein amount in Reaction kit: 200 μg/experiment

Protein amount in Purification kit: 100 μg/column

SDS-PAGE condition: **Reducing condition** (Removing MfTag)

*Please click here to find selection guide for non-reducing or reducing condition.



A: After purification of MfTag-labeled proteins by the *Loop* affinity column, **Input** (10 fold dilution) and **Elution** fraction of **+/+** samples were separated by SDS-PAGE under **reducing condition** and the gel was silver stained. Florets and leaf show relatively high amount of purified proteins than stem.

B: After purification of MfTag-labeled proteins by the *Loop* affinity column, **Input** (10 fold dilution) and **Elution** fractions of **-/-**, **-/+** and **+/+** of leaf were separated by SDS-PAGE under **reducing condition** and the gel was silver stained. Majority of purified proteins were specifically observed in the **+/+** roots sample. A protein around 50 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.

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Identification of *S*-palmitoylated proteins and estimation of *S*-palm/non-palm ratio of target proteins by affinity column

Example　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**

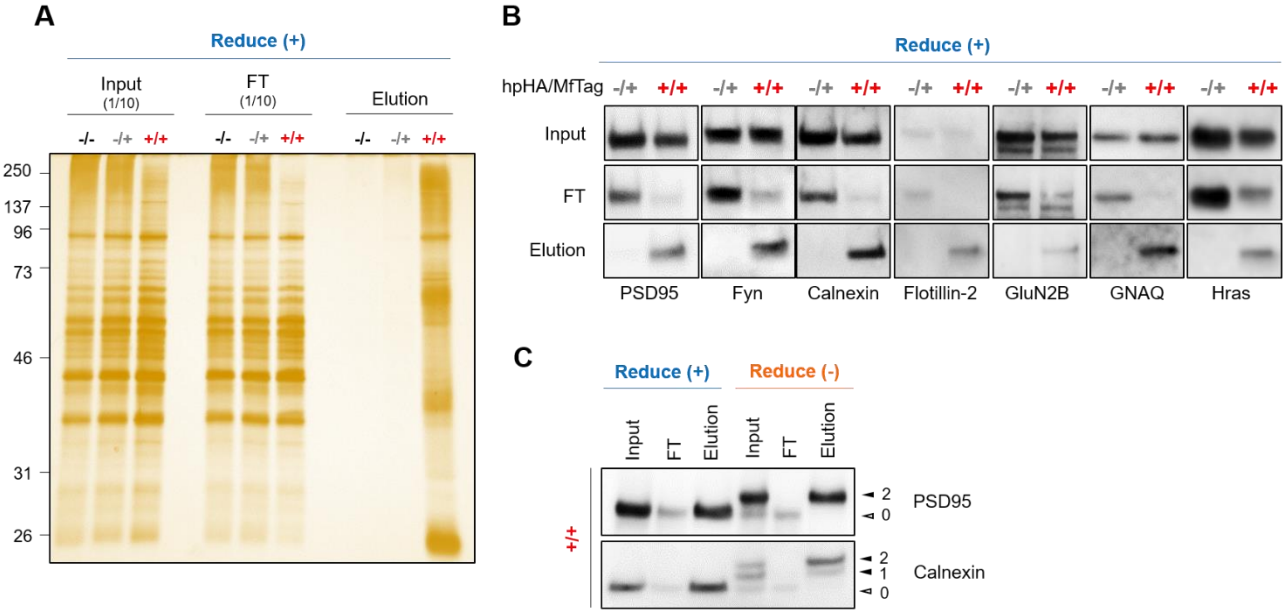
Protein amount in Reaction kit: 200 μg/experiment

Protein amount in Purification kit: 100 μg/column

SDS-PAGE condition: **Reduction condition** (Removing MfTag, A, B, C-left)

and **Non-reducing condition** (Keeping MfTag, C-right)

*Please click here to find selection guide for non-reducing or reducing condition.



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

PSDS95 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.

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Comprehensive analysis

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**

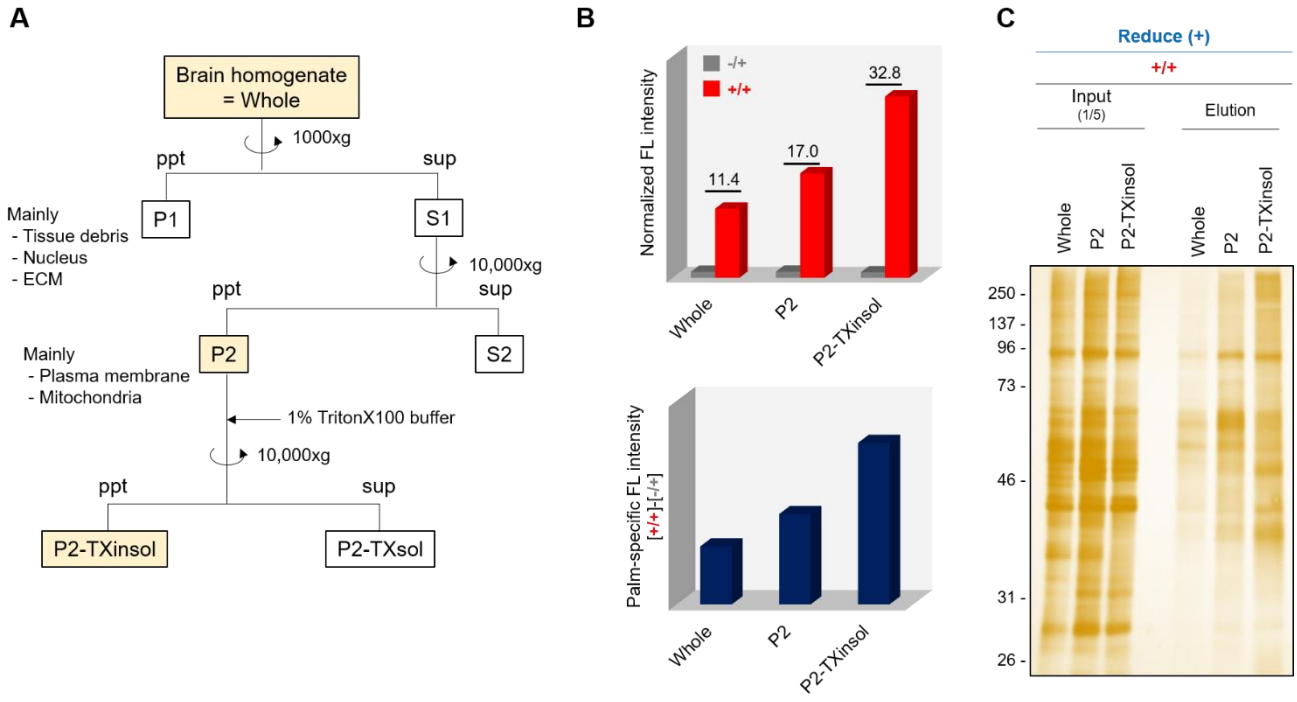
Methods: Fluorometric assay, Silver staining of purified proteins

Protein amount in Reaction kit: 100 μg/experiment

Protein amount in Purification kit: 50 μg/column

SDS-PAGE condition: **Reducing condition** (Removing MfTag)

*Please click here to find selection guide for non-reducing or 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 (1,000xg) to P1 and S1 fractions. S1 fraction was further separated by middle-speed centrifugation (10,000xg) to P2 and P2 fractions. P2 fraction was divided into two batches, and one batch was treated with 1% TritonX100 Buffer, separated by middle-speed centrifugation (10,000xg) 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 Basal 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.

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Example 2　Intracellular distribution of S-palmitoylated proteins 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

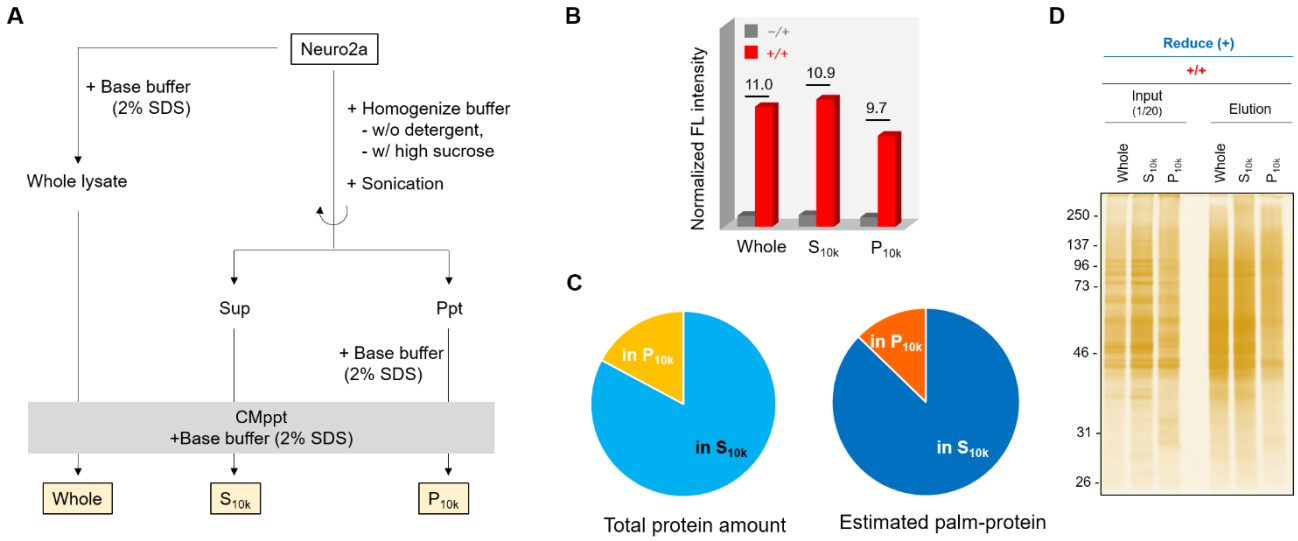
Methods: Fluorometric assay, Silver staining of purified protein

Protein amount in Reaction kit: 100 μg/experiment

Protein amount in Purification kit: 50 μg/column

SDS-PAGE condition: **Reducing condition**

*Please click here to find selection guide for non-reducing or reducing condition.



A: Rough subcellular fractionation protocol. “Whole” was prepared by direct lysis of Neuro2a cell with 1x Basal 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 (10,000xg, 20 min) to P_{10k} and S_{10k} fractions. All fractions were done with CMppt, the pellet resolubilized by 1x Basal 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.

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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 (P10k) 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
- Membrane depolarization: 50 mM KCl in DMEM

Protein amount in Reaction kit: 100 μg/experiment

Protein amount in Purification kit: 50 μg/column

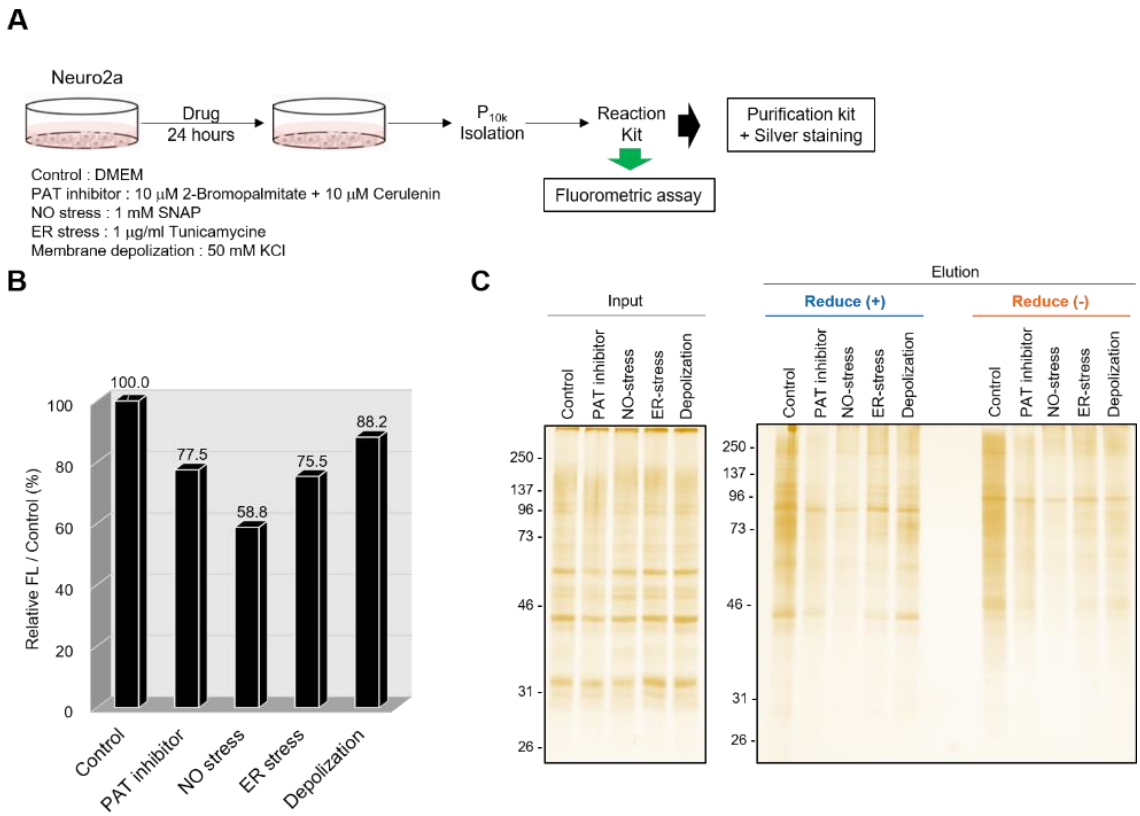
Kits used in this experiment: Reaction kit and Purification kit

Methods: Fluorometric assay, Silver staining of purified proteins

SDS-PAGE condition: **Reducing condition** (Removing MfTag)

and **Non-reducing condition** (Keeping MfTag)

*Please click here to find selection guide for non-reducing or reducing condition.



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 fluoremetric assay, purified proteins of NO stress sample were clearly reduced.

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Reference: Comparison with conventional ABE method

Example Comparison between *RapidS*PALM and ABE using mouse whole brain

Sample: Adult mouse-derived whole brain

Lysate preparation: Whole brain lysate

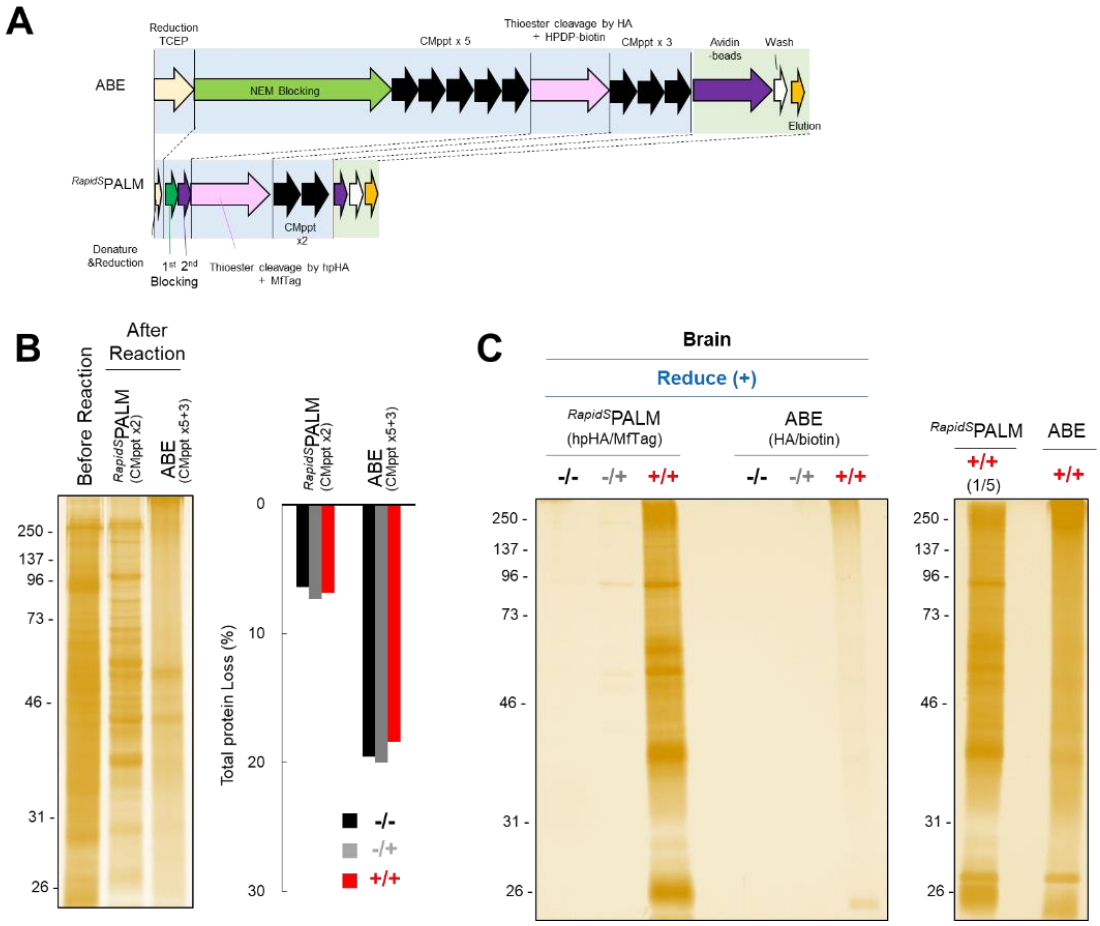
Kits used in this experiment: **Reaction kit** and **Purification kit**

Protein amount in Reaction kit: 200 μg/experiment

Protein amount in Purification kit: a half amount of each reacted total proteins

SDS-PAGE condition: **Reduction condition** (Removing MfTag or biotin)

*Please click here to find selection guide for non-reducing or reducing condition.



A Protocol of both methods. Standard ABE method contains 8 times of CMppt (5 times after blocking and 3 times after biotin-labeling). *RapidS*PALM contains only 2 times of CMppt.

B Comparison of total protein loss. Silver staining (left) and BCA protein assay (right). *RapidS*PALM clearly reduced protein loss and protein aggregation.

C Comparison of total amount of purified proteins. *RapidS*PALM dramatically increases purification amount of proteins. To accurate comparison in silver-staining, 5 fold dilution of *RapidS*PALM sample shows same level with ABE on silver staining.

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