Click S-Palmitoylation Detection-775 Kit

Cat no. **K010-410**

A convenient and easy-to-use kit for the detection of S-palmitoylated proteins via metabolic labelling.

Please read this manual completely prior to using the product.

FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES

Cat No.		Component	Safety	Quantity	Storage
K010-410A		**Lysis Buffer**		20mL	4°C
K010-410B		**Carrier Reagent**		4 aliquots	4°C
K010-410C		**Click Labelling Reagent**		4 aliquots	4°C
K010-410D		**Control Labelling Reagent**		4 aliquots	4°C
K010-410E		**Protein Preservation Reagent**		4 aliquots	4°C
K010-410F		**Detection Reagent**		4 aliquots	4°C
K010-410G		**Catalytic Reagent**		4 aliquots	4°C
K010-410H		**Stabiliser Reagent**		4 aliquots	4°C
K010-410I		**Activator Reagent**		4 aliquots	4°C
K010-410J		**4 x Laemmli Sample Buffer (with β-mercapto-ethanol)**		4 aliquots	4°C

The Click S-Palmitoylation Detection-775 Kit;

- **Enables detection of S-palmitoylated proteins in cultured cells.**
- **Designed to process 4 x 24 well cell culture plates (i.e. 96 samples in total).**
- **Has a Convenient stopping point after labelling step.**

1.0 General Protocol Steps

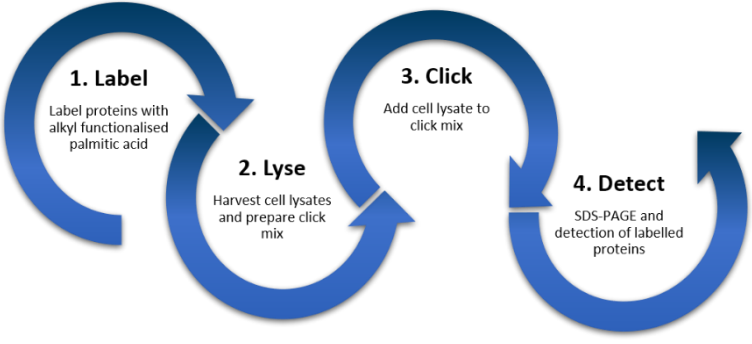


Figure 1. Schematic showing the 4 simple steps comprising Badrilla's Click S-Palmitoylation Detection-775 Kit.

2.0 Background Information

The cysteine amino acid is unique - it is the only amino acid to contain a sulphydryl group. At physiological pH the cysteine sulphydryl is protonated, however, at higher pH levels (above its pKa[a]), ionisation occurs (i.e. it loses a hydrogen) and the residue becomes negatively charged, conferring a nucleophilic reactivity (thiolate anion). S-palmitoylation (thioester linkage of a 16 carbon fully saturated fatty acid) of cysteine is an important post translation modification of proteins (Figure 2), modulating protein function, localisation and trafficking[1, 2, 3]. The fate of palmitoylated proteins depends on the protein, its function and the general regulatory environment. There are a number of potential outcomes for a palmitoylated protein. The protein can be stably palmitoylated by a protein acyltransferase (PAT), remaining in this state throughout its lifetime. Second, palmitoylation can be dynamic and reversible, the fatty acyl moiety being removed by an acyl protein thioesterase (APT) - permitting the possibility of dynamic regulation not dissimilar to that observed for protein phosphorylation[1].

[a] The pKa of cysteine in free solution is 8.37; however, this will vary depending on the local environment within the protein.

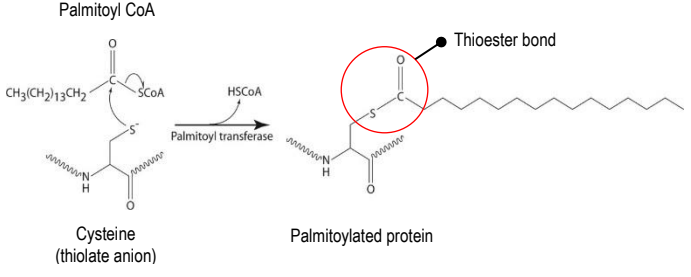


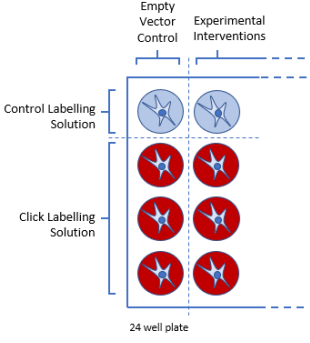
Figure 2. Schematic describing the reaction mechanism for the palmitoylation of a cysteine residue. The nucleophilic thiolate anion of cysteine (-ve) attacks the carbonyl group of the palmitoyl CoA to promote an addition reaction, resulting in the formation of a thioester bond.

3.0 About this assay

One of the potential limitations of studying S-palmitoylation is frequent low levels of endogenous expression of protein S-acyl transferases (PATs) and their substrates in cultured cells. Therefore, current approaches to study this important post translational modification often utilise heterologous expression of both the PAT of interest and its cognate substrate(s)[4]. Badrilla's Click S-Palmitoylation Detection-775 Kit provides a quick, convenient and robust method to identify S-palmitoylation of heterologously expressed proteins in cultured cells (Figure 1). Please note, where expression of endogenous or heterologously expressed protein is low, an immunoprecipitation step may be required to detect palmitoylation. Therefore, to study palmitoylation, a palmitic acid analogue linked to an alkyl group is used to metabolically label cells in culture (step 1). Once labelling is complete, cells are lysed (step 2) and the resulting protein mix is incubated with a fluorophore conjugated to an azide chemical group. Even in this complex mixture the azide group is able to specifically conjugate with metabolically labelled proteins (step 3). Proteins are separated by SDS-PAGE and can be detected by either in gel-fluorescence or western blotting (step 4).

Box 1: The Click Reaction

The 'click' reaction is based on the Cu(I)-catalysed Huisgen cycloaddition mechanism[5]. The reaction involves two small chemical groups, an alkyl ($C\text{-}H_{2n\text{-}2}$) and an azide (N_3) (Figure 4a). The reaction is highly selective, and the small size of these chemical groups makes them ideal for modification of biomolecules and bio-orthogonal reagents for subsequent detection (Figure 4b).



Experimental Design - The exemplar data shown in section 6.0 describes the recommended format for experiments performed using the **Badrilla Click S-Palmitoylation Detection-775 Kit**. Each experimental intervention is accompanied by two controls; 1. Labelling cells with **Control Labelling Reagent** and 2. Transfecting cells with empty enzyme vector in order to distinguish endogenous levels of palmitoylation (Figure 3). The kit is designed around a 24 well cell culture plate in order to maximise productivity. It is recommended that both empty vector and experimental interventions are performed in triplicate.

Figure 3. Recommended experimental format for the **Badrilla Click S-Palmitoylation Detection-775** Kit using 24 well cell culture plates.

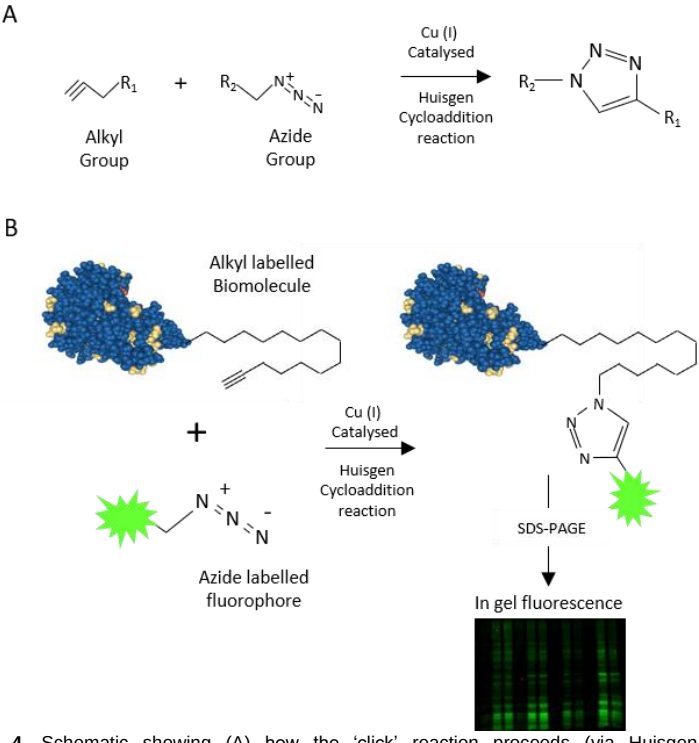


Figure 4. Schematic showing (A) how the 'click' reaction proceeds (via Huisgen cycloaddition) between an alkyl (R1:palmitic acid) and an azide (R2: fluorophore) group. (B) How the click reactions are used to detect biomolecules in complex protein mixtures.

4.0 ASSAY PROTOCOL

Additional requirements include:

- Phosphate Buffered Saline (PBS: pre-warmed (37^{0}C) for washing cells prior to labelling and ice cold (4^{0}C) prior to cell lysis)
- Serum free cell culture media.
- DMSO (dimethylsulphoxide)
- Universal and Eppendorf tubes
- Vortex
- Microcentrifuge
- Rotary wheel
- Fluorescence detection apparatus able to detect fluorescence with an excitation/ emission maxima of 753nm and 775nm respectively e.g. Licor ®, Syngene G-box series® etc.

Please also see Appendix I for Quick Reference Protocol Guide.

Protocol

1. Pre-warm PBS and Serum free media to 37^{0}C.
2. To two separate universal tubes (30 mL size) add 7.5 mL of pre-warmed serum free media.
3. To a **Carrier Reagent** tube (tube with red lid – K010-410B) add of 1 mL of pre-warmed serum free media – gently mix until white solid has completely dissolved. Once dissolution is complete add 484 µL of **Carrier Reagent** to each universal tube of pre-warmed serum free media. Label them **Carrier Solution 1** and **Carrier Solution 2.**
4. Add 20 µL of DMSO to **Click Labelling Reagent** (tube with violet lid - K010-410C) – vortex for 5 seconds and allow 1 minute for complete dissolution. Spin tube briefly in a microcentrifuge.

[b] Licor ® and G-box series® are registered trademarks of Licor Inc. and Synoptics respectively.

5. Next, add 16 µL of **Click Labelling Reagent** to **Carrier Solution 1** (made in step 3) in order to make **Click Labelling solution –** label tube clearly.
6. Add 20 µL of DMSO to **Control Labelling Reagent** (tube with white lid - K010-410D) – vortex for 5 seconds and allow 1 minute for complete dissolution. Spin tube briefly in a microcentrifuge.
7. Next, add 16 µL of **Control Labelling Reagent** to **Carrier Solution 2** (made in step 3) in order to make **Control Labelling solution –** label tube clearly.
8. Gently remove media from cells.
9. Add 500 µL of warmed PBS, gently agitate in order to wash cells - remove PBS.
10. Add 300 µL **Control Labelling solution** to wells intended as controls and 300 µL of **Click Labelling solution** to experimental wells. Incubate for a minimum of 4 hours under conditions appropriate to your cells and experiment (commonly 37^0C & 5% CO_2).

    Note: This incubation time may require further optimisation.

    One hour before the incubation time is complete you will need to prepare the following reagents;

11. Add 35 µL of DMSO to the **Protein Preservation Reagent** (tube with green lid - K010-410E), mix thoroughly.
12. In order to prepare **Lysis Buffer Mix** add 2970 µL of **Lysis buffer** to a clean universal tube (or other appropriately sized tube). Then add 30 µL of **Protein Preservation Reagent** (prepared in step 11), mix thoroughly**.**
13. Prepare **CLICK Mix** components:

    a. Add 5 µL of DMSO to reconstitute **Detection Reagent** (Black tube - K010-410F), vortex for 5 seconds and allow to stand for 1 minute to enable complete dissolution. Briefly spin tube in a microcentrifuge.
    b. The **Catalytic Reagent** (tube with Yellow lid - K010-410G) is already in solution.
    c. Add 15 µL DMSO to **Stabiliser Reagent** (tube with orange lid - K010-410H), vortex for 5 seconds and allow to stand for 1 minute to enable complete dissolution. Briefly spin in a microcentrifuge.

14. Prepare **Click Mix:** To a clean Eppendorf tube, add the volumes of **Click Mix** components shown in table 1 including H_2O (18MΩ) to complete. Invert the tube several times to mix. The **Click mix** should be used within 2 hours.

    A clean Eppendorf tube will be required for each separate **Click reaction**, so 24 tubes will be required per 24 well plate. To each clean Eppendorf add 80 µL of **CLICK Mix**.

Kit Component	***µL required for 1 x 24 well plate***
Detection Reagent (Black tube)	3.75
Catalytic Reagent (tube with Yellow lid)	300
Stabiliser Reagent (tube with orange lid)	12
H_2O	2085
Total volume	**2400 *µL***

Table 1 : Volumes of Click Components required for the **Click Mix**

15. Prepare **Activator Reagent** by adding 1 mL of H_2O (18MΩ) to **Activator Reagent** (tube with brown lid - K010-410I), mix to ensure complete dissolution.

Cell Lysis

During cell lysis the 24 well plate(s) should be kept on ice.

16. Remove labelling media.
17. Wash cells with 500 µL of ice-cold PBS per well. Remove PBS from wells at the end of the wash.
18. Lyse cells by adding 100 µL **Lysis buffer mix** (prepared in step 12) to each well. Whilst keeping the plate on ice, 'harvest' the cell lysate. You may find that the consistency of the lysate is thick and gloopy, therefore, to aid collection of the lysate, cut the end of the pipette tip and use it to vigorously scrape and mix cells. Harvest cell lysate with the same tip and add to appropriate **CLICK mix** tube. Repeat for all samples, using a fresh tip each time.
19. To each **CLICK reaction** (containing 80 µL of **Click Mix** and 100 µL of cell lysate) add 20 µL of **Activator Reagent** (prepared in step 15) and vortex for 5 seconds.
20. Incubate on a rotary wheel for 1hr at RT.
21. Once incubation is complete add 67 µL of 4 x Laemmli sample buffer (tube with blue lid - K010-410J) to each reaction and vortex for 5 seconds. **Note**: You may need to warm the Laemmli sample buffer prior to use as the SDS tends to precipitate at low temperatures.
22. Heat samples at 95^0C for 5 minutes prior to gel electrophoresis.
    Note: Some membrane proteins are incompatible with this regime, instead try incubating sample at 37^0C for 30 minutes.
23. Once electrophoresis is complete, the gel can be scanned directly (775nm; LICOR®, Syngene G:Box series®) in order to visualise the **Click** signal i.e. palmitoylated proteins. The gel can then be transferred to nitrocellulose in the standard way for a western blot experiment. As shown in the exemplar data (section 6.0), the membrane can be probed with target protein specific antibodies to determine the total amount of your protein of interest.

    Note on Interpreting Data

    To determine the extent of palmitoylation of your protein of interest, It is standard practice to quantify data by expressing the click signal (775nm) relative to the corresponding total protein signal. This is then normalised to the empty vector control[4].

6.0 Exemplar data

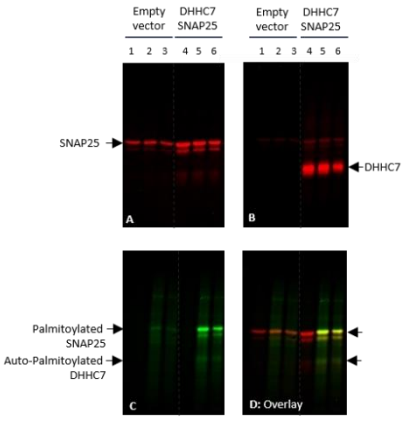


Figure 5. SNAP25 is palmitoylated by DHHC7 in HEK293T cells as detected by the **Badrilla Click S-Palmitoylation Detection-775 Kit.**
HEK 293T cells, either heterologously expressing GFP-SNAP25 and HA-DHHC7 (lanes 4-6) or containing GFP-SNAP25 plus empty vector (lanes 1-3), were grown to 90% confluency in a 24 well format. Cells were subjected to the steps outlined in the above protocol (section 4.0), proteins were separated by SDS-PAGE (10% bis-acrylamide) prior to electo-transfer on to nitrocellulose membrane. Membranes were treated with; panel A, anti-GFP antibody (SNAP25); panel B, anti-HA antibody (DHHC7); panel C, blot scanned at 775nm in order to detect incorporation of fluorescently labelled palmitic acid (i.e. palmitoylation of SNAP25 and auto-palmitoylation of DHHC7); panel D, overlay of SNAP25 and CLICK-palmitate fluorescent signal.
Lane 1, **Control Labelling Reagent**; Lane 2 and 3, **Click Labelling Reagent**; Lane 4, **Control Labelling Reagent**; lane 5 and 6, **Click Labelling Reagent**.

7.0 References

1. Linder, M. E., and R. J. Deschenes. 2007. Palmitoylation: policing protein stability and traffic. Nat. Rev. Mol. Cell Biol. 8: 74 – 84.
2. Resh, M.D.2006. Trafficking and signalling by fatty-acylated and prenylated proteins. Nat. Chem. Biol. 2: 584 – 590.
3. Gao X. and Hannoush R.N. 2017. A Decade of Click Chemistry in Protein Palmitoylation: Impact on Discovery and New Biology. Cell Chemical Biology 25: 236-246
4. Greaves, J., Munro, K. R., Davidson, S C., Riviere, M., Wojno, J., Smith, T. K., Tomkinson, N. C. O. and Chamberlain L. H. 2017. Molecular basis of fatty acid selectivity in the zDHHC family of S-acyltransferases revealed by click chemistry. Proc. Nat. Acad. Sci. 114: 1365-1374
5. Wang, Q., Chan, T.R., Hilgraf, R., Fokin, V.V., Sharpless, K.B., and Finn, M.G. 2003. Bioconjugation by copper(I)-catalyzed azide-alkyne [3 + 2] cycloaddition. J. Am. Chem. Soc. 125, 3192–3193.

Use of Product

Unless otherwise stated in our catalogue or other company documentation accompanying the product(s), our products are intended for **Research Use Only** (RUO) and are not to be used for any other purpose, which includes, but is not limited to, unauthorised commercial uses, *in vitro* diagnostic uses, *ex vivo* or *in vivo* therapeutic uses or any type of consumption or application to humans or any living organisms.

Warranty

The seller, 'Badrilla Ltd', warranties product performance limited to the description set forth in the product documentation. Warranty will cover the duration of the product 'shelf life', in this case a period of 6 months, which will take effect from date of sale, PROVIDED THAT the product has been handled and stored as directed and limited to use by suitably trained individuals.

The seller shall be under no liability in respect of any defect arising from fair wear and tear, inappropriate storage, wilful damage, negligence, abnormal working conditions, failure to follow the Click S-Palmitoylation Detection-775 Kit instructions, misuse or alteration or repair of the product without the Seller's written approval.

Buyer's exclusive remedy for non-conforming products is limited to replacement of or refund for the non-conforming product(s).

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8.0 Appendix I : Quick Reference Protocol Guide