SiteCounter™

S-Palmitoylated Protein Kit

Cat no. **K010-312**

A convenient and easy-to-use kit to determine the number of S-Palmitoylation sites and the stoichiometry of S-Palmitoylation.

Please read this manual completely prior to using the product.

FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES

Pack components

Cat. no.		Reagent	Safety	Quantity	Storage
K010-312A		Buffer A		10 mL	4°C
K010-312B		Thiol Blocking Reagent		2 x aliquots	4°C
K010-312C		Binding Buffer		10 mL	4°C
K010-312D		Thioester Cleavage Reagent		2 x aliquots	4°C
K010-312E		Acyl-Preservation Reagent		2 x aliquots	4°C
K010-312F		Mass Tag Reagent (5kDa)		2 x aliquots	-20°C
K010-312G		2 x Laemmli Sample Buffer (with β-mercaptoethanol)		2 x aliquots	4°C
K010-312H		Neutralisation Buffer		1	4°C
K010-312I		Desalting Spin Columns		24	4°C

Why use SiteCounter™ S-Palmitoylated Protein Kit?

- **Enables determination of the number of S-Palmitoylation sites on a protein**
- **Enables stoichiometric determination of protein S-palmitoylation.**
- **Kit designed to process 2 x 6 biological samples with paired negative controls (i.e. 24 samples in total).**
- **Convenient stopping point after blocking treatment.**
- **Assay suitable for downstream western blot analysis.**

Additional Requirements not provided in this kit;

- Ice cold acetone, 1.5ml Eppendorf tubes, general laboratory equipment

WARNING!

If the buffers in the kit appear cloudy, as shown on the left, please gently warm solutions in order to re-dissolve the precipitate prior to use, as shown on the right

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1.0 Background

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 and the residue becomes negatively charged conferring a nucleophilic reactivity (thiolate anion). An important biological manifestation of this property is the ability to form cross links with other cysteine residues within proteins, thus contributing to protein tertiary structure. In addition, the thiolate anion can undergo a variety of adduction reactions including S-nitrosylation[1], S-prenylation[2], and S-acylation[3]. S-palmitoylation (thioester linkage of a 16 carbon fully saturated fatty acid) of cysteine is an important class of S-acylation reaction within mammalian cells (figure 1), modulating protein function and protein localisation[4, 5]. The fate of palmitoylated proteins depends on the protein, its function and the general regulatory environment. There are three 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. Thirdly, palmitoylated proteins can undergo lysosomal degradation, which requires removal of the palmitoyl moiety by the enzyme protein palmitoylthioeterase-1 (PPT-1)[4].

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

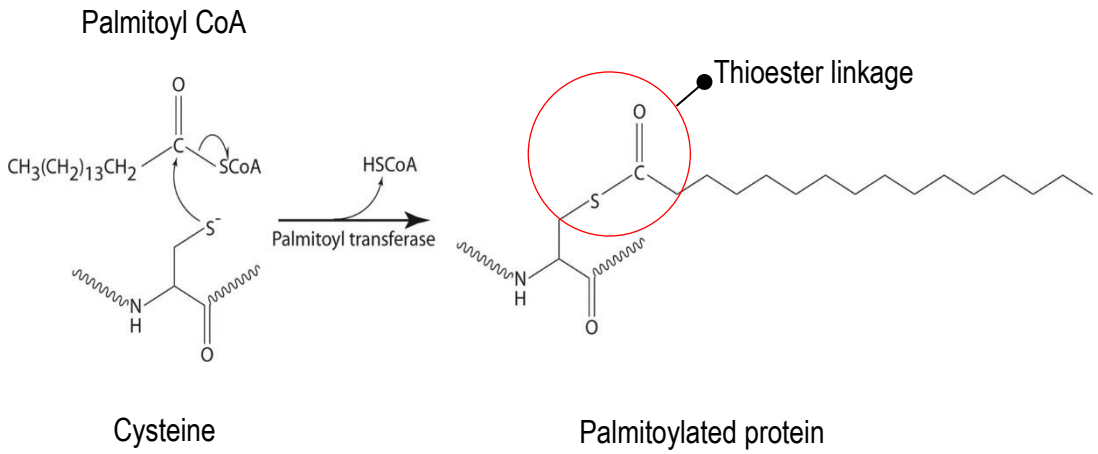


Figure 1. Schematic representation of palmitoyl moiety transfer from palmitoyl CoA to the thiolate side chain of a cysteine residue in a target protein, through formation of a thioester linkage.

2.0 About this assay

The SiteCounter™ S-Palmitoylated Protein Kit provides a robust method to quantify the number of S-palmitoylated sites within the protein of interest. Free thiols are first blocked by treating samples with a Thiol Blocking Reagent. A thioester linkage specific reagent is then used to cleave the thioester bond to release the palmitate group only. Newly liberated thiols are then modified using a Mass Tag Reagent (5kDa) (figure 2). The number of modified sites is determined by identifying the number of mass shifted bands as revealed by western blot analysis of the treated cell lysate.

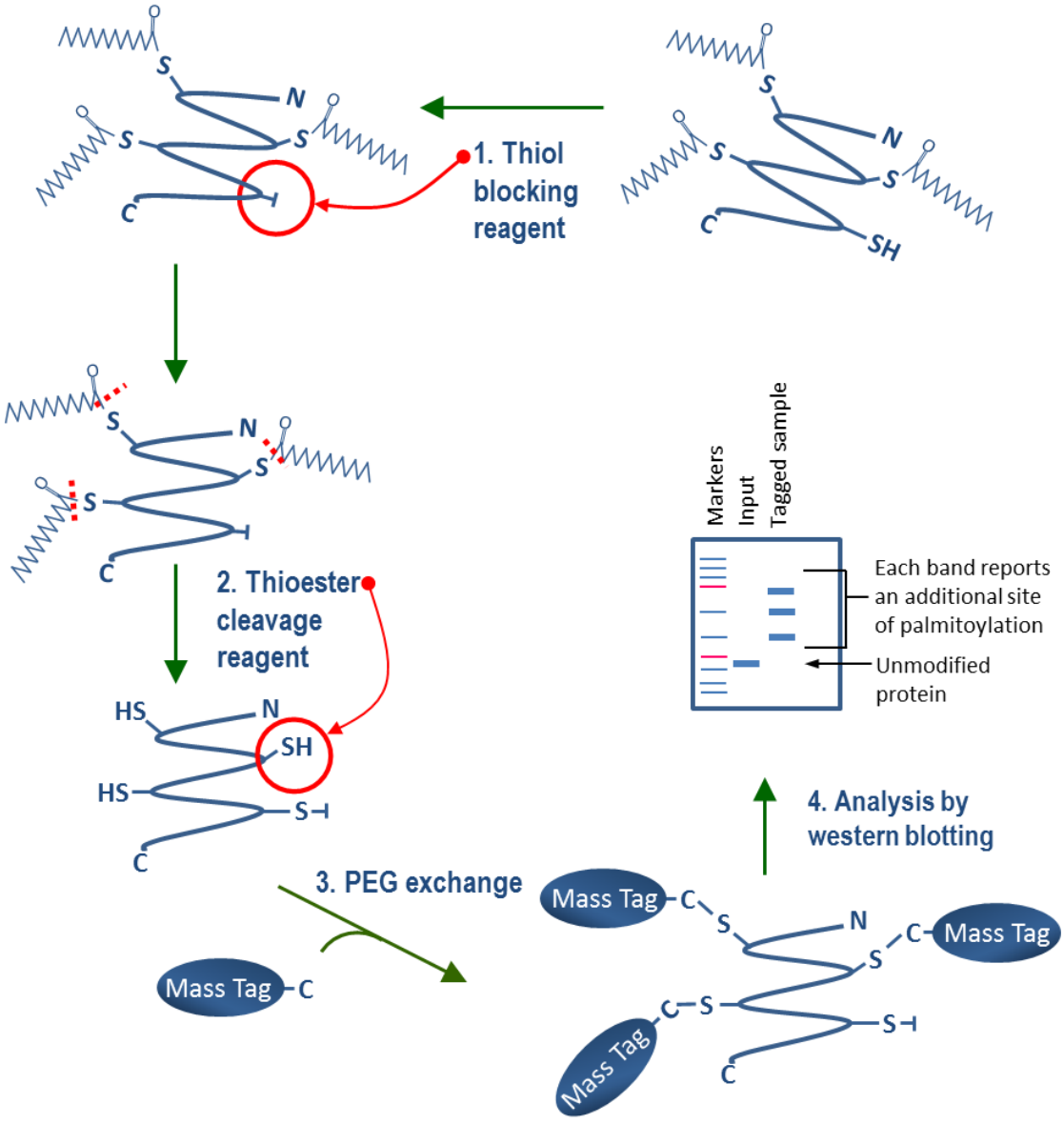
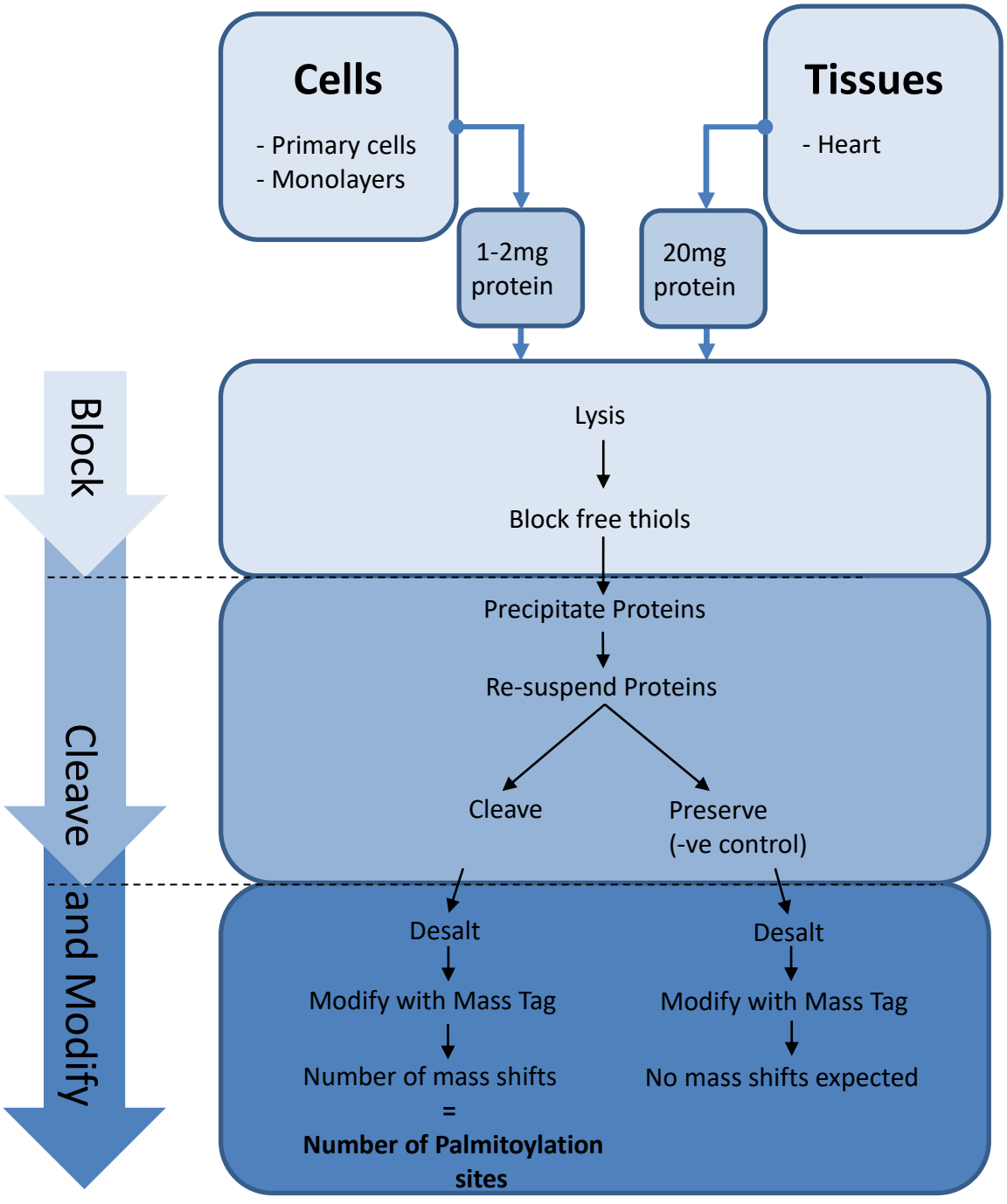


Figure 2. A Schematic of the key steps in the SiteCounter™ S-Palmitoylated Protein kit.

3.0 General Protocol Steps



4.0 ASSAY PROTOCOL

[Please also see Appendix I for Quick Reference Guide]

4.1 Phase 1: Blocking Treatment

IMPORTANT NOTE: Both Buffer A and Binding Buffer contain high concentrations of sodium dodecyl sulphate detergent (SDS), which may come out of solution at low temperatures. **Ensure the SDS is fully dissolved by gentle warming and mixing, prior to use.**

This protocol has been optimised for use with both cells and tissue – It is recommended that 1-2mg total cellular protein be used per sample. However, if using tissue as your starting material you will need to scale up the total protein used to obtain a comparable signal. It is recommended that you begin with 20mg of tissue protein.

The following steps have been optimised to process 6 paired samples (i.e. 6 experimental samples with paired negative controls - 12 samples in total).

1. Cell lysis and free thiol blocking steps are combined in this protocol.

   Each sample of 1-2 mg of total cellular protein (note; a 10cm dish of HEK 293 cells at 70-90% confluency will provide approximately 1-2mg of cellular protein) requires addition of 500 µL of blocking buffer and therefore you will need to prepare enough for 6 paired samples. Thus, prepare 3.5 mL of blocking buffer by adding 28 µL Thiol Blocking Reagent (K010-312B: tube with red lid) to 3472 µL of Buffer A, vortex for 10s or longer to ensure thorough mixing. Wash cells in PBS and harvest cells by centrifugation, aspirate PBS and re-suspend pelleted cells in 500 µL of blocking buffer, vortex for 5s.
2. Incubate samples at 40˚C for 4 hours with constant shaking (*e.g.* Eppendorf thermomixer C or equivalent). If not available then use a static heat block, but vortex samples for 5s every 20min for the duration of the incubation.
3. Remove from heat source and add 3 volumes (1.5 mL) of ice-cold acetone to each sample, vortex for 5s, and allow proteins to precipitate at -20˚C (minus 20˚C) for 20min.
4. Centrifuge at 16000 x g for 5min to recover the protein pellet; discard supernatant.
5. Wash pellet 5-times with 1 mL ice cold 70% acetone – vortex for 5s each time then centrifuge at 16000 x g for 1min to recover protein. Allow final pellet to air dry i.e. until the pellet is free from any visible moisture.

   Note: Procedure can be stopped at this point and samples stored at -20˚C overnight.

6. Binding Buffer (K010-312C) is supplied as a 10x concentrate. Prepare 20 mL of 1x binding buffer by adding 2 mL of 10x concentrate to 18 mL of deionised 18MΩ water. Mix with a magnetic stirrer for 1min.
7. Re-dissolve the pellet from step 5 in 250 µL of 1x binding buffer. In order to re-dissolve the proteinaceous pellet you will need to physically disrupt it prior to incubating in a shaking heat block at 40˚C (e.g. Eppendorf thermomixer C or equivalent). **Note**: In order to disrupt the pellet use a clean glass pestle or a micro spatula - you will need to ensure that no proteinaceous material is removed accidentally (please see FAQs: http://badrilla.com/products/residue-modification/tools-for-s-palmitoylation-research/FAQs.html). Continue to heat/shake until the pellet is completely dissolved, this typically takes approximately 20min, but can take up to 60min. The solution should become clear indicating complete dissolution.
8. Centrifuge at 16000 x g for 1min to remove any insoluble material.
9. Remove 30 µL of soluble lysate to a fresh Eppendorf tube. Add 30 µL 2x Laemmli Sample Buffer (K010-312G; tube with blue lid) and store as the total input sample (**Input Fraction, IF**).
10. Divide remaining lysate into 2 x 1.5 mL tubes (100 µL each). One of the pair will be treated with Thioester Cleavage Reagent (experimental sample); the other will be treated with Acyl Preservation Reagent (the negative control sample).

4.2 Phase 2: Thioester Cleavage and Site Modification

It is essential that the Thioester Cleavage Reagent (K010-312D; tube with yellow lid) is prepared **immediately prior to use**. It is recommended that dissolution of proteins (step 7) is complete before preparing Thioester Cleavage Reagent.

Note: All the following steps should be carried out at room temperature.

11. Prepare Thioester Cleavage Reagent by addition of 200 µL Neutralisation Buffer (K010-312H: tube with black lid) to one vial of Thioester Cleavage Reagent (K010-312D: tube with yellow lid). Vortex for 5s and stand for 60s to permit full dissolution of reagent - the Thioester Cleavage Reagent must be used within 30min of dissolution.
12. Add 11 µL of Thioester Cleavage Reagent solution (from step 11) to each of the 6 experimental samples, and 11 µL of Acyl-Preservation Reagent (K010-312E: tube with green lid) to each of the 6 negative control samples. Vortex samples for 5s.
13. Incubate reactions for 1hr at room temperature.
14. During the 1hr incubation take 12 Desalting Spin Columns (K010-312I - 6 for the experimental samples and 6 for the negative control samples) and prepare them by first inverting several times to ensure an even slurry within the column. Twist and snap off the seal at the column outlet. Place column in a suitable collection tube (not supplied) – a 1.5 mL Eppendorf tube is recommended for this purpose. Remove storage solution by first loosening the cap on each column without removing it. Spin columns at 6500 x g for 30s. Discard the flow through. You will see that the resin settles on a slant in the column, make a mark on the outside of the column at the top of the slanted resin – in subsequent spins always keep this mark on the outer edge of the centrifuge rotor. Remove cap and add 400 µL of binding buffer dropwise to the

top of the resin in each column, replace cap, but do not tighten. Centrifuge the columns at 6500 x g for 30s, discard flow through. The resin bed needs to be fully dried in this spin step – a dried resin bed appears white – If it appears at all grey, spin for a further 30s. Repeat wash procedure (2 washes in total). Once washing is complete, gently blot the bottom of each spin column to remove any excess liquid. Transfer the columns to fresh collection tubes.

15. Meanwhile make up the Mass Tag Reagent. Add 200 µL of binding buffer to the dry Mass Tag Reagent (Tube with orange lid; K010-312F) and vortex for 10s, allow to stand for 60s and ensure reagent has completely dissolved prior to use.
16. Add 12 µL of Mass Tag Reagent to each fresh collection tube (from step 14).
17. On completion of the 1hr 'cleavage' incubation, add one sample per desalting column and centrifuge at 6500 x g for 1min i.e. desalt directly into the Mass Tag Reagent solution (added to collection tube in step 16).
18. Vortex samples for 5s and incubate for 1hr at RT.
19. Once the 1hr incubation is complete, add an equal volume (123 µL) of 2x Laemmli sample buffer to each tube.
20. Heat samples at 60^0C for 10mins prior to analysis by western blotting. Please note if you protein is liable to aggregation at higher temperatures, some optimisation to find the best solubilisation temperature may be required.

5.0 Exemplar data

The following data were obtained using the SiteCounter™ S-Palmitoylated Protein Kit following the protocol described above. Five putative palmitoylation sites on Caveolin-3 in adult rat ventricular myocytes were identified.

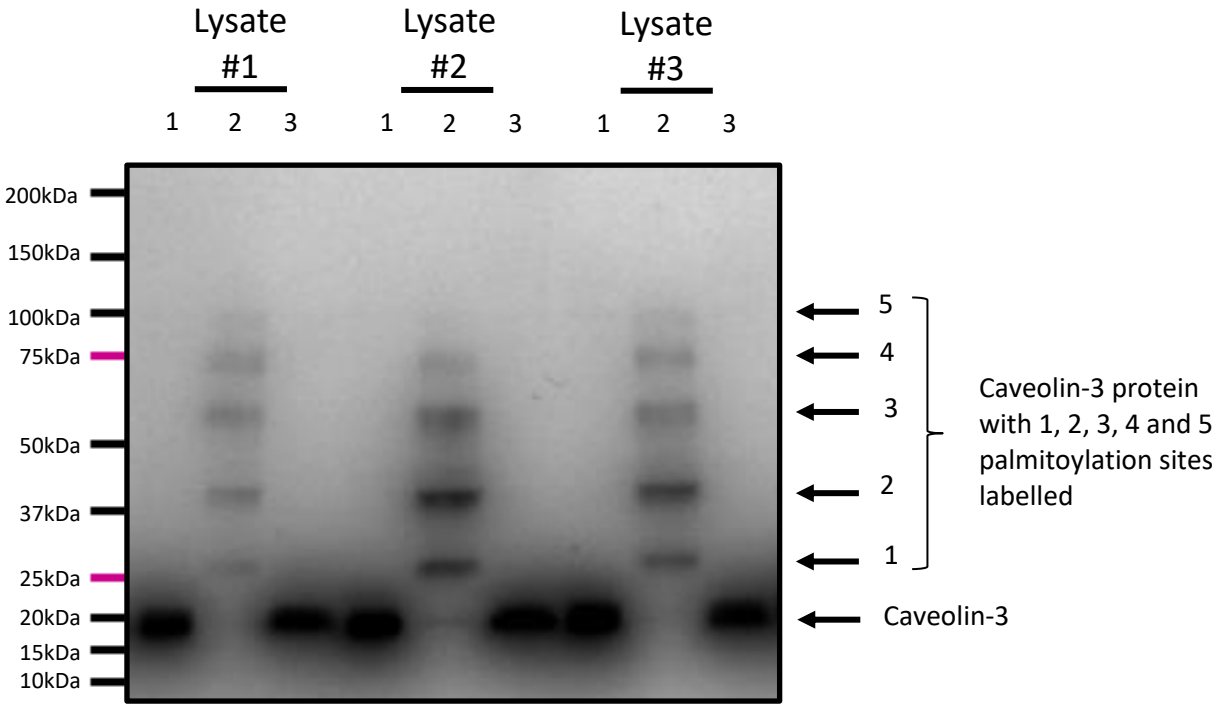


Figure 3. Five putative palmitoylation sites on Caveolin-3 as detected using the SiteCounter™ Palmitoylated Protein Kit.

Adult rat ventricular myocytes (1.5mg total cellular protein) were subjected to the SiteCounter™ S-Palmitoylated Protein Kit assay. Samples were subjected to SDS-PAGE using 4-20% gradient gels prior to immunoblotting for Caveolin-3 using an antibody specific for caveolin-3 (1:5000 BD Transduction Labs product 610420). In triplicate; Lane 1: **Input Fraction** (**IF)** post-block (5 µL loaded). Lane 2: Cleaved and Mass Tag Reagent (5kDa) modified sample (5 µL loaded). Lane 3: Preserved and Mass Tag Reagent (5kDa) modified sample (5 µL loaded).

6.0 References

1. Benhar, M., M. T. Forrester, and J. S. Stamler . 2009. Protein denitrosylation: Enzymatic mechanisms and cellular functions. Nat. Rev.Mol. Cell Biol. 10: 721 – 732

2. Zhang, F. L., and P. J. Casey. 1996. Protein prenylation: molecular mechanisms and functional consequences. Annu. Rev. Biochem. 65: 241 – 269.

3. Smotrys, J. E., and M. E. Linder. 2004. Palmitoylation of intracellular signalling proteins: regulation and function. Annu. Rev. Biochem. 73: 559 – 587.

4. Linder, M. E., and R. J. Deschenes. 2007. Palmitoylation: policing protein stability and traffic. Nat. Rev. Mol. Cell Biol. 8: 74 – 84.

5. Resh, M. D. 2006. Trafficking and signalling by fatty-acylated and prenylated proteins. Nat. Chem. Biol. 2: 584 – 590.

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.

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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 SiteCounter™ Palmitoylation 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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7.0 Appendix I : Quick Reference Protocol Guide