CAPTUREome™

S-Palmitoylated Protein Kit

Cat no. **K010-311**

A convenient and easy-to-use kit for the capture of S-palmitoylated proteins.

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
K010-311A		Buffer A		22 mL	4°C
K010-311B		Thiol Blocking Reagent		4 x aliquots	4°C
K010-311C		Binding Buffer (10x Concentrate)		30 mL	4°C
K010-311D		Thioester Cleavage Reagent		4 x aliquots	4°C
K010-311E		Acyl-Preservation Reagent		4 x aliquots	4°C
K010-311F		CAPTUREome™ Capture Resin		4 x 55 mg	4 - 8°C
K010-311G		2 x Laemmli Sample Buffer (with β-mercapto-ethanol)		4 x 1.5 mL	4°C
K010-311H		Neutralisation Buffer		1 x 1 mL	4°C

Why use CAPTUREome™ S-Palmitoylated Protein Kit?

- **Enables capture of S-palmitoylated proteins.**
- **Kit designed to process 4 x 5 biological samples with paired negative controls (i.e. 40 samples in total).**
- **Convenient stopping point after blocking treatment.**
- **Assay suitable for downstream SDS-PAGE, western blot or mass spectrometry analysis.**

Additional Requirements not provided in this kit;

- Ice cold acetone, general laboratory equipment

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.

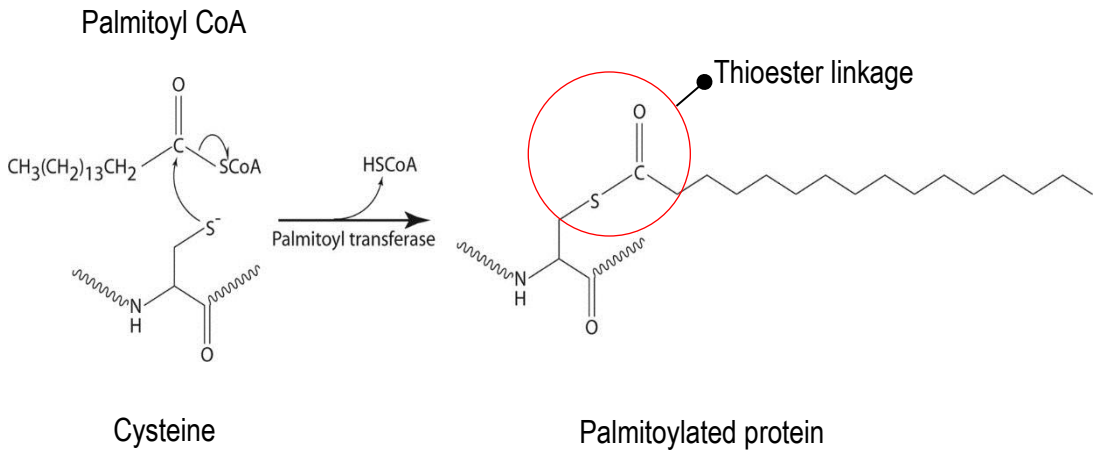


Figure 1. Schematic representation of palmitoyl moiety transfer from palmitoyl CoA to the thiolate side chain of a cysteine residue in a target protein through formation of a thioester linkage.

2.0 About this assay

Badrilla's CAPTUREome™ S-Palmitoylated Protein Kit provides a robust method to identify S-palmitoylated species via resin-assisted capture (acyl-RAC). Free thiols are first blocked by treating samples with a thiol blocking reagent. A palmitoyl thioester linkage specific reagent is then used to cleave the thioester bond to release the palmitate group only. Newly liberated thiols are captured with CAPTUREome™ capture resin (figure 2). After thorough washing, captured proteins are eluted with reductant and analysed by SDS-PAGE with protein staining, immunoblotting (for a specific protein) or by mass spectrometry.

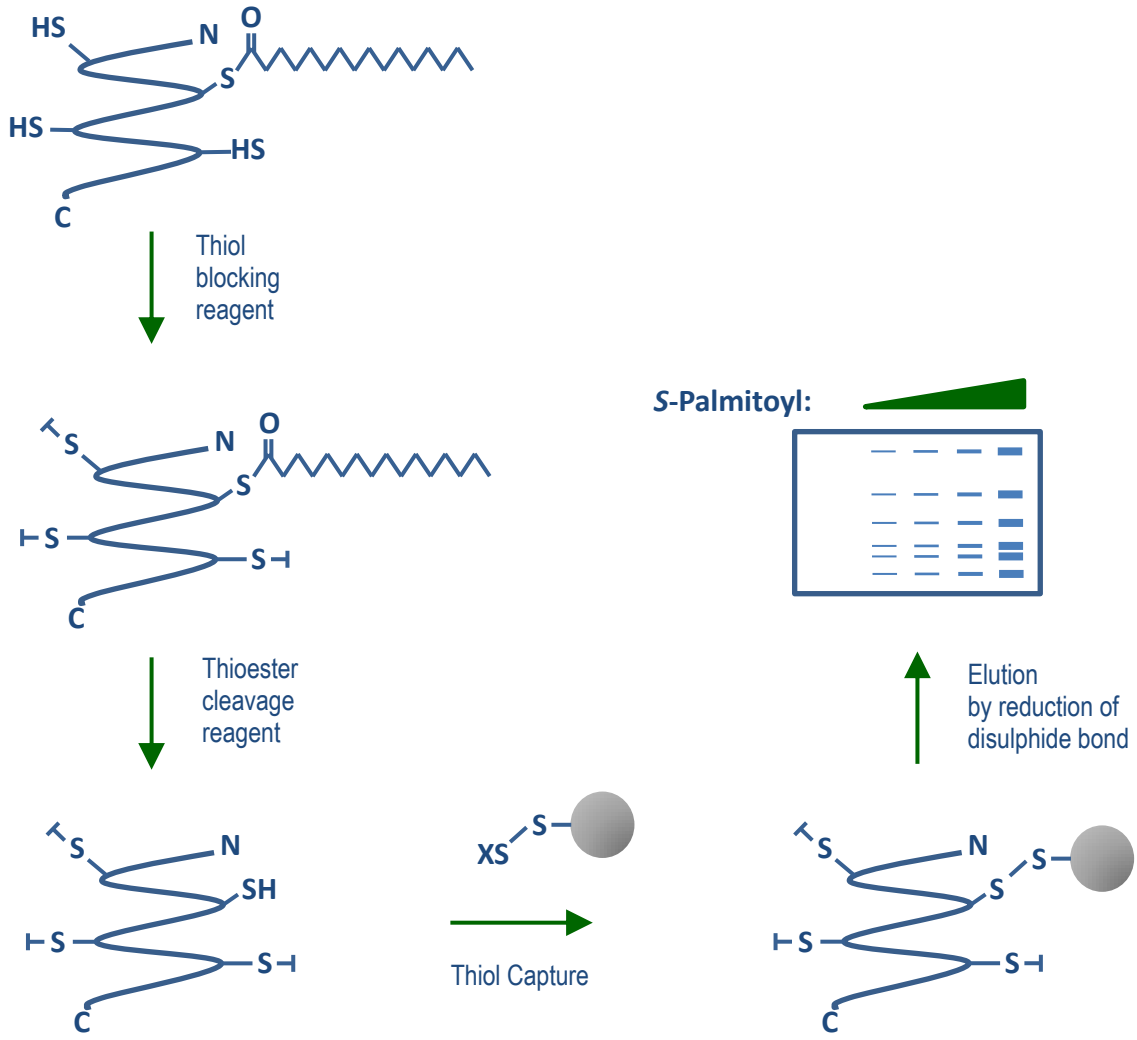
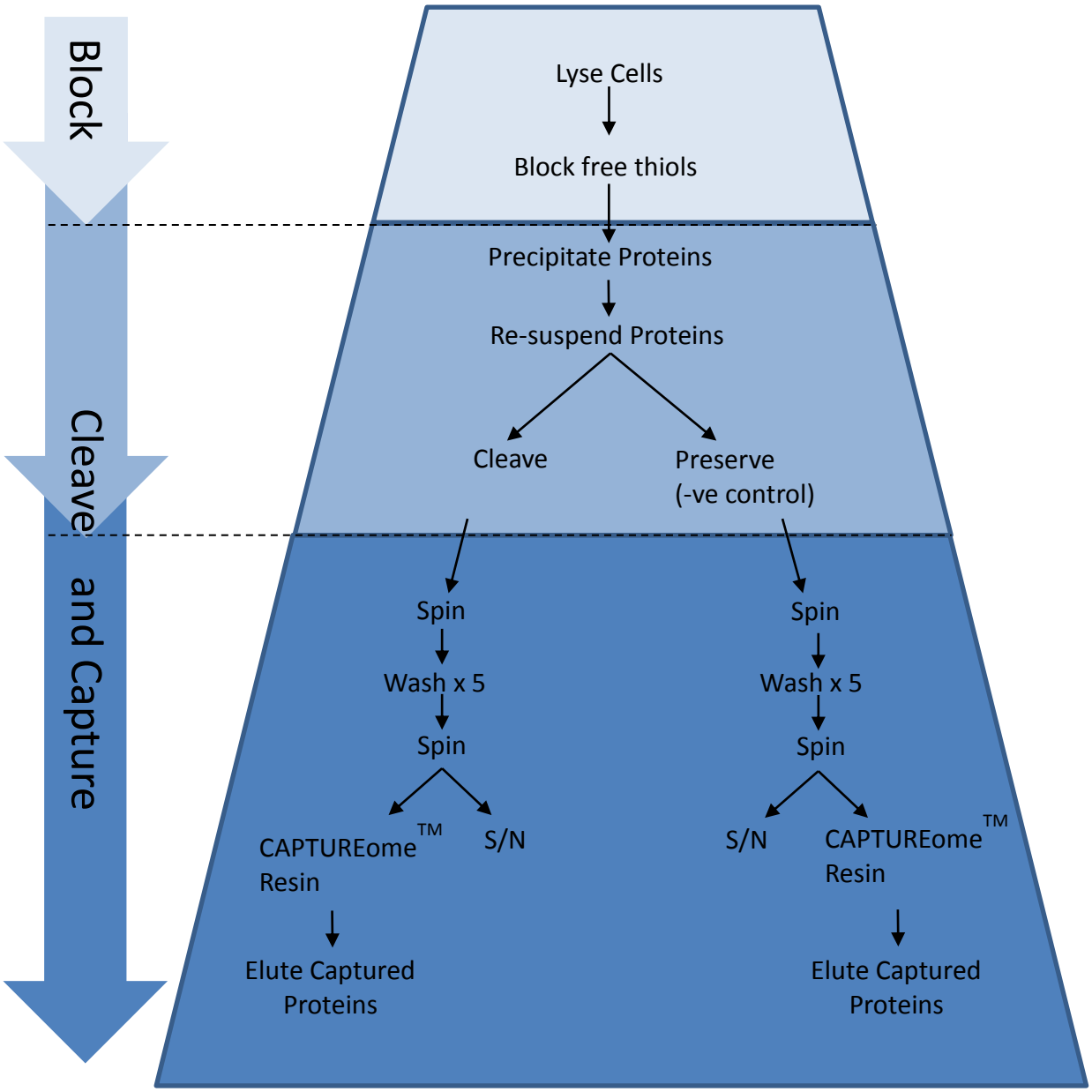


Figure 2. Schematic representation of the key steps in the Badrilla CAPTUREome™ S-Palmitoylated Protein Kit assay.

3.0 General Protocol Steps



4.0 ASSAY PROTOCOL

[Please also see Appendix I for Quick Reference Guide]

4.1 Phase 1: Blocking Treatment

It is recommended that 1-2mg total cellular protein be used per sample.

> **IMPORTANT NOTE**: Both the 10x binding buffer concentrate and Buffer A 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.**

1. Cell lysis and free thiol blocking steps are combined in this protocol. Each sample of 1-2 mg of total cellular protein (cells) requires addition of 500 µL of blocking buffer and therefore you will need to scale up the amount of blocking buffer you prepare in line with the number of samples you want to analyse. Prepare blocking buffer by addition of 4 µL Thiol Blocking Reagent (K010-311B: tube with red lid) per 500 µL of Buffer A (K010-311A). Add 500 µL of blocking buffer to 1-2 mg of cells, 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. 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 each time – vortex for 5s each time then centrifuge at 16000 x g for 1min. Air dry completely after final wash.

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

The following steps have been optimised to process 5 paired samples (i.e. 5 experimental samples with paired negative controls - 10 samples in total).

6. Binding Buffer (K010-311C) is supplied as a 10x concentrate. Prepare 60 mL of 1x binding buffer by adding 6 mL of 10x concentrate to 54 mL of deionised 18MΩ water. Mix with a magnetic stirrer for 1 minute.
7. Re-dissolve the pellet from step 5 in 300 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 could take up to 60 minutes. The solution should become clear indicating complete dissolution.

8. Centrifuge at 16000 x g for 1 min to remove any insoluble material.
9. Remove 30 µL of soluble lysate to a fresh Eppendorf tube. Add 30 µL 2x Laemmli Sample Buffer (K010-311G; 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 (120 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 Resin Capture

It is essential that the thioester cleavage reagent (K010-311D; 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 CAPTUREome™ Capture Resin (K010-311F: tube with orange lid) by adding 2 mL of 1x binding buffer to one tube of K010-311F (the tube contains 55 mg dry resin) and incubating on a rotary wheel for 30 min. Centrifuge the washed resin at 16000 x g for 1min. Aspirate all the buffer and re-suspend resin in 275 µL of 1x binding buffer at a ratio of 1:1 settled resin to buffer (55 mg beads ≈ 275 µL bed volume, providing 550 µL slurry) by vortexing for 5s.
12. Add 50 µL of the resin slurry to each sample (from step 10).
13. Prepare Thioester Cleavage Reagent by addition of 200 µL Neutralisation Buffer (K010-311H: tube with black lid) to one vial of Thioester Cleavage Reagent (K010-311D: 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 30 minutes of dissolution.
14. Add 19 µL of Thioester Cleavage Reagent solution (from step 13) to each experimental sample, and 19 µL of Acyl-Preservation Reagent (K010-311E: tube with green lid) to each negative control sample.
15. Incubate reactions for 2.5 hours at room temperature with constant agitation (e.g. on a rotating wheel mixer).
16. Centrifuge each sample for 1min at 16000 x g. Remove the supernatant and retain 50 µL of supernatant: add 50 µL 2x Laemmli Sample Buffer (K010-311G: tube with blue lid). In the case of experimental samples this fraction is referred to as the cleaved **Unbound Fraction** (c**UF**). For negative control samples this fraction is referred to as the preserved **Unbound Fraction** (p**UF**). Wash resin 5 times in 1 mL 1x binding buffer (1x 30s, then 4 x 5 min on a rotating wheel mixer and centrifuge for 1 min at 16000 x g each time to recover resin).
17. After removing the final wash, elute captured proteins from the resin using 50 µL of 2x Laemmli Sample Buffer (K010-301G: tube with blue lid). In the case of experimental samples this fraction is referred to as the cleaved **Bound Fraction** (c**BF**). For negative control samples this fraction is referred to as the preserved **Bound Fraction** (p**BF**). Heat to 60˚C for 10 min (NB some multi-pass transmembrane proteins are not compatible with 60˚C for 10min – an incubation at 37˚C for 30min could be used as an alternative in these circumstances).

IMPORTANT: If proteins are to be analysed by mass spectrometry, see protocol detailing 'on bead trypsinisation for subsequent mass spectrometry analysis', Journal of Lipid Research, 52, 393-398 (2011) or an appropriate method of your choosing.

5.0: Notes on Interpreting Data

1. A protein palmitoylated at least once on every polypeptide chain should be completely depleted from the thioester cleavage reagent treated (cleaved **Unbound Fraction** (c**UF**)) and quantitatively recovered in the cleaved **Bound Fraction** (c**BF**).
2. A palmitoylated protein should be ~5x enriched in the cleaved **Bound Fraction** (c**BF**) - thus 10µL **Input Fraction (IF)** = 2µL cleaved **Bound Fraction** (c**BF**) on a western blot.
3. The appearance of proteins in the preserved **Bound Fraction** (p**BF**) indicates incomplete cysteine blocking in phase 1, step 1, or non-specific binding of proteins to the resin. (For further information please refer to the frequently asked questions (FAQ) page on the Badrilla website - : http://badrilla.com/products/residue-modification/tools-for-s-palmitoylation-research/FAQs.html).

6.0 Exemplar data

The following data were obtained using CAPTUREome™ S-Palmitoylated Protein Kit following the protocol described above. Caveolin-3 was identified as S-palmitoylated in adult rat ventricular myocytes.

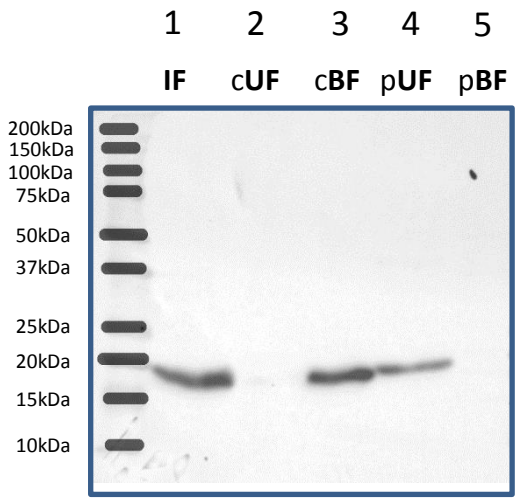


Figure 3. Caveolin-3 is identified as a palmitoylated protein in adult rat ventricular myocytes.

Adult rat ventricular myocytes (1mg total cellular protein) were subjected to the Badrilla CAPTUREome™ S-Palmitoylated Protein Kit assay and samples immunoblotted for caveolin-3 using an antibody specific for caveolin-3 (1:5000 BD Transduction Labs product 610420). Lane 1: **Input Fraction** (**IF)** post-block (60 µL loaded). Lane 2: Cleaved **Unbound Fraction** (c**UF**), after treatment with thioester cleavage reagent (60 µL loaded). Lane 3: Cleaved **Bound Fraction** (c**BF**), proteins recovered from the resin after treatment with thioester cleavage reagent (37.5 µL loaded). Lane 4: Preserved **Unbound Fraction** (p**UF**), after treatment with thiol preservation reagent (60 µL loaded). 5: Preserved **Bound Fraction** (p**BF**), proteins recovered from the resin after treatment with acyl preservation reagent **(**37.5 µL loaded). Caveolin 3 was depleted from the thioester cleavage reagent treated, cleaved **Unbound Fraction** (c**UF** - lane 2) and quantitatively recovered in the cleaved **Bound Fraction** (c**BF** - lane 3) representing S-palmitoylated caveolin-3.

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

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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 fare wear and tear, inappropriate storage, wilful damage, negligence, abnormal working conditions, failure to follow the CAPTUREome^{TM} 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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6.0 Appendix I : Quick Reference Protocol Guide