INSTRUCTIONS

Imject™ mcKLH Subunits High Purity Research Grade

77649

1371.2

Number	Description
77649	**Imject mcKLH Subunits, High Purity Research Grade,** 1mL, 20mg/mL in 0.22µm filtered water pH: 7-9 Molecular Weight: 350K and 390K Appearance: Clear dark blue liquid, which may contain some particulate and fibers Source: *Megathura crenulata*, giant keyhole limpet Purity (MPLC-SEC): ≥ 95% Copper/protein ratio: 18.0-22.0mg/mL Extinction Coefficient: 279nm ($\varepsilon = 1.4 cm^{-1} \times mg^{-1} \times mL$) and at 347nm ($\varepsilon = 0.4 cm^{-1} \times mg^{-1} \times mL$) Native PAGE analysis: Two main characteristic bands co-migrating with ferritin (MW standard) Endotoxin level: ≤ 11.7 USP-EU/mg **Storage:** Upon receipt store at 2-8°C. Product is shipped at ambient temperature.

Introduction

Keyhole limpet hemocyanin (KLH) is an extremely effective carrier protein for generating antibodies. KLH produces a strong immune response because of its large size (6-8 million daltons) and because it is isolated from a non-mammalian source the giant keyhole limpet (*Megathura crenulata*). KLH is an oxygen carrier and therefore is a hemocyanin. KLH also contains copper molecules that appear opalescent to dark blue when in solution.[1-6]

Mariculture KLH (mcKLH) is isolated from the hemolymph of giant keyhole limpets that are maintained in marine tanks. These limpets are not sacrificed during hemolymph collection and can therefore continue to supply mcKLH for an extended period. Previously, limpets were harvested and then destroyed. Giant Keyhole Limpets inhabit shorelines and continual harvesting from the wild threatens their population and results in the disruption of the local marine habitat.

Thermo Scientific™ Imject™ mcKLH Subunits consist of a mixture of two predominant subunits (350kDa and 390kDa). This highly defined product allows researchers to use this formulation as an alternative to the cGMP formulation of KLH subunits that are used in human clinical vaccine trials. This research grade material is designed for animal studies and should not be used for human applications. Thermo Scientific™ Pierce™ Protein Biology can provide access to cGMP-grade material of the KLH Subunits upon request. The mcKLH Subunits formulation does not contain any proprietary stabilizers or preservatives, is packaged sterile and is low in endotoxin.

Antibodies can be generated by coupling haptens to the mcKLH Subunits using heterobifunctional cross-linkers, homobifunctional cross-linkers, the Mannich reaction or many other methods.[7-9] Sulfo-SMCC (Product No. 22322) and Sulfo-MBS (Product No. 22312) are two of the most frequently used crosslinkers and a general protocol is described in this document. For analytical purposes, the same peptide may be conjugated to maleimide activated BSA (Product No. 77116) and used to measure specific anti-peptide antibody response in an ELISA. This method eliminates the interference of anti-mcKLH subunit antibodies when determining the success of a humoral response to the peptide.

After the haptens are cross-linked to the mcKLH Subunits, the material is typically mixed with an adjuvant. The most popular and powerful adjuvant is Freund's Complete Adjuvant (Product No. 77140), which is an emulsion of attenuated mycobacterium and mineral oil. When mycobacteria must be avoided, use Imject Alum Adjuvant (Product No. 77161), which is a water-soluble adjuvant that mixes easily with the coupled mcKLH Subunits and does not cause tissue necrosis at the injection site.

Procedure for Conjugation using Sulfo-SMCC or SMCC

The following is a general protocol for coupling peptides to mcKLH Subunits. Coupling conditions may require optimization for specific peptides depending on it solubility of and the immune response to the conjugate. For highly insoluble peptides, test various concentrations of solvents (typically ≤ 30% of the aqueous reaction) or solubilizing agents (i.e., non-ionic detergents at 0.05%-1.0%) to determine optimal conjugation conditions.

A. Additional Materials Required

- Desalting column such as Polyacrylamide Plastic Desalting Columns (Product No. 43426) or Thermo Scientific™ Slide-A-Lyzer™ Dialysis Cassettes
- Sulfo-SMCC (Product No. 22322) or SMCC (Product No. 22360)
- Ellman's Reagent (Product No. 22582) to test for the presence of free sulfhydryl groups (-SH)
- Phosphate Buffered Saline (e.g., BupH™ Phosphate Buffered Saline Packs: 0.1M phosphate, 0.15M NaCl; pH 7.2; Product No. 28372)

B. Maleimide Activation of mcKLH Subunits

1. Add 200µL (4mg) of mcKLH Subunits to 300µL Phosphate Buffered Saline (PBS).
2. Add approximately 1mg sulfonated crosslinker to the mcKLH Subunit solution, or 50µL of 10-60mM non-sulfonated cross-linker dissolved in DMSO or DMF (e.g., 1mg in 50µL of solvent).
3. Incubate for 60 minutes at room temperature or 30 minutes at 37°C.
4. Remove nonreacted cross-linker from the mcKLH Subunits using a desalting column. Use PBS as the desalting buffer, and collect 500µL fractions. Locate the protein peak by measuring the absorbance at 280nm or by using the Pierce BCA Protein Assay Reagent (Product No. 23225) in the microplate format (use 5µL sample per well).
5. Add EDTA to a final concentration of 5mM and store the maleimide-activated mcKLH at 4°C for up to one month.

C. Confirmation of Free Sulfhydryl (-SH) Groups on Peptide

1. Determine the amount of free -SH groups in the peptide preparation by performing the Ellman's assay (see instructions included with the Ellman's Reagent, Product No. 22582).

   Note: If free sulhydryls are not present, the peptide must be reduced to dissociate disulfide bonds using a solid-phase reductant (Immobilized TCEP, Product No. 77712) or a free sulfhydryl group can be added using a modification reagent such as Traut's Reagent (Product No. 26101) or SATA (Product No. 26102). The instructions for these products will provide information on the procedures.

D. Conjugation of Peptide to Maleimide Activated mcKLH Subunits

1. Dissolve up to 2mg of sulfhydryl-containing peptide in 200-500µL of PBS. Alternatively, if the peptide is freely soluble add it as a solid to the activated mcKLH in solution. DMSO may be used to help solubilize peptides that have limited solubility in aqueous systems.

   Note: For best results use a 40-fold molar excess of the peptide (as a minimum) to the mcKLH Subunits. **For example:** when using 5mg of mcKLH Subunits (4×10^5 Da) and the peptide has a molecular weight of 2000, then a 40-fold excess = 1mg of peptide.
2. Add up to 2mg of peptide to 2mg of the maleimide activated mcKLH Subunits in PBS and incubate for 2 hours at room temperature.

   Note: To quantify the degree of conjugation, perform an Ellman's assay on the peptide solution from step 1. For accurate results, this initial assay of the non-conjugated peptide must be performed as soon as possible after reconstitution because disulfide bonds can form even during short-term storage of the sulfhydryl-containing peptide.
3. Reserve a portion (20-50µL) of the solution to estimate the degree of conjugation.

   Note: For injection purposes, it is not necessary to remove non-conjugated peptide from the preparation. Removal of the non-conjugated peptide may be accomplished using a desalting column or by dialysis.

E. Estimate Degree of Peptide Coupling to mcKLH Subunits

Perform the Ellman's assay on the peptide sample before and after coupling to determine conjugation efficiency. Ellman's Reagent (5,5´-dithiobis-[2-nitrobenzoic acid]) reacts with sulfhydryl groups to produce a chromophore with maximum absorbance at 412 nm ($\varepsilon_{412} = 1.44 \times 10^4 cm^{-1} M^{-1}$). Prepare a standard curve with known quantities of cysteine. The cysteine produces a similar response to a peptide containing one free sulfhydryl group.

Related Thermo Scientific Products

77140	**Imject Freund's Complete Adjuvant,** 5 × 10mL
77145	**Imject Freund's Incomplete Adjuvant,** 5 × 10mL
77161	**Imject Alum Adjuvant,** 50mL
66382	**Slide-A-Lyzer Dialysis Cassette Kit**
45212	**Melon™ Gel IgG Purification Kit**
45206	**Melon Gel IgG Spin Purification Kit**
44999	**SulfoLink™ Immobilization Kit for Peptides**
77666	**Imject Maleimide-Activated mcKLH Spin Kit**

Cited References

1. Harlow, E. and Lane, D. (1988). *Antibodies: A Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor: New York, 56-100.
2. Male, D., *et al.* (1987). *Advanced Immunology*. London: J.B. Lippincott Co., Gower Medical Publishing 8.1-8.8.
3. Sell, S. (1987). *Immunology, Immunopathology and Immunity*. Elsevier, N.Y. 69-78.
4. Senozan, N.M. and Landrum, J. (1981). Hemocyanin of the giant keyhole limpet, *Megathura crenulata*. In *Invertebrate Oxygen Binding Proteins: Structure, Active Sites and Function*. Lamy J. and Lamy J., Editors, Marcel Dekker, New York. 703-717.
5. Hersckovits, T. (1988). Recent aspects of the subunit organization and dissociation of hemocyanins. *Comp Biochem Physiol* **91B:**597-611.
6. Bartel, A. and Campbell, D. (1959). Some immunochemical differences between associated and dissociated hemocyanin. *Arch Biochem Biophys* **82:**2332.
7. Yoshitake, S., *et al.* (1979). Conjugation of glucose oxidase from *Aspergillus niger* and rabbit antibodies using *N*-hydroxysuccinimide ester of N-(carboxycyclohexyl-methyl)-maleimide. *Eur J Biochem* **101:**395-9.
8. Partis, M.D., *et al.* (1983). Cross-linking of protein by w-maleimido alkanoyl *N*-hydroxysuccinimido esters. *J Protein Chem* **2(3):**263-77.
9. Hermanson, G.T. (1996). Bioconjugate Techniques. Academic Press.