

Keyhole Limpet Hemocyanin (KLH)

Cat.Number: 992335, 50mg 992337, 250mg 992337, 1 g
Product: **Hemocyanin Keyhole Limpet (KLH), in BES**
from *Megathura crenulata*, lyophilized with a stabilizing buffer.
Supplied as a Blue-Gray lyophilized powder from MgSO₄, BES, pH 6.5 buffer, containing more than 60% protein
CAS : 9013-72-3 ; MW :High molecular mass (4.5 x 10⁵ - 1.3 x 10⁷ M.W.)

Cat.Number: FN077A, 20mg
Product: **KLH SubUnits** (from *Megathura crenulata*)
Supplied as 1ml solution 20mg/ml sterilized by filtration, endotoxin-free.
MW : 350 and 390KDa (2 main characteristic bands co-migrating with ferritin by Native PAGE analysis)
Copper/protein ratio: 18-22 mg/ml; EC(279nm): 1.4 ml/cm/mg; EC(347nm): 0.4 ml/cm/mg
Endotoxin level: ≤ 11.7 USP-EU/mg

Storage: +4°C (L)

Solubility : 5-10 mg/ml (proteins) in aqueous buffer. See solubility information below.

Technical and Scientific Information

Keyhole Limpet Hemocyanin (KLH) is one of the most commonly used carriers in the conjugation of antigens (peptides, haptens) for antibody production. Our KLH is high grade quality to ensure excellent solubilisation (a common bottleneck) and immunization response.

- **Structure, MW**

KLH is a copper-containing protein that belongs to a group of non-heme proteins called hemocyanins, which are found in the hemolymph of many arthropods and mollusca. KLH is isolated from the mollusc *Megathura crenulata*.

KLH is usually associated with divalent cations in aggregates of 350 and 390 kDa subunits. As a result, MW ranges typically from 6 000 to 7 500 KDa, and below (as subunits) and above (as a didecamer, multidecamers, or flexible tubules of varying length). Unlike other gastropod hemocyanins, this protein does not dissociate simply by removing divalent cations from a hemocyanin suspension, although divalent cations aid in the formation of larger aggregates.

With moderate changes in pH, or ionic strength, it will reversibly dissociate to subunits, and at pH8.9 will completely dissociate. Each subunit contains oxygen binding sites and for every two atoms of copper in KLH, one molecule of oxygen can be found. The oxygen-containing KLH is blue, while the oxygen-lacking form is colorless. Removal of oxygen will also dissociate the protein to lower aggregate states. KLH is dissociated into subunits is expected to have more available antigenic sites, hence in some applications to give increased antibody titer or binding. KLH subunits are available as product # .

Our KLH is purified and lyophilized to optimize its stability and solubility. After reconstitution, it yields an opalescent blue colored suspension-solution, that is characteristic of highly pure and nondenatured protein (intact heme groups).

KLH SubUnits are useful for obtaining more controlled material (MW) than KLH. Our product is a research grade material. Formulation does not contain any proprietary stabilizers or preservatives, is packaged sterile and is low in endotoxin. It is designed for animal studies and should not be used for human, unlike cGMP formulation of KLH subunits that are used in human clinical vaccine trials.

- **Solubility**

KLH is an extremely large complex protein and has the tendency to aggregate and precipitate from solutions.



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KLH of improper quality in particular suffers from poor water solubility. Freezing will also cause aggregation and precipitation. A slightly acidic pH (~pH 6.5) and the concentration of $MgSO_4$ (≥ 10 mM $MgSO_4$ is recommended for most procedures) are essential for facilitating and maintaining solubility. While aggregation may not affect KLH immunogenicity by itself, it affects the real available protein quantity. Hence it affects the process of conjugation (solubility, unproper amount of present KLH, lower coupling yield).

▪ Suggested procedure for solubilization :

Add gently 2 mL deionized water per 20mg vial under agitation yields an opalescent solution of KLH at 10 mg/mL. Do not vortex or agitate vigorously. For further dilutions , use appropriate buffer (i.e. PBS) and maintain the magnesium concentration at a level higher than 10 mM.

Reconstituted protein solution may be stored up to 2 months at -20 °C.

▪ Suggested procedure for protein quantitation:

BC Assay #UP4084A. KLH solution is turbid so 280 nm absorbance readings are inaccurate.

▪ Solubility of haptens is also need in the conjugation protocol!

For highly insoluble peptides, one may increase concentrations of solvents (typically $\leq 30\%$ of the aqueous reaction) or solubilizing agents (i.e., non-ionic detergents at 0.05%-1.0%) to determine optimal conjugation conditions.

• Immunogenicity

Low size substances such as peptides or steroids, are generally not or weakly immunogenic. Hence, for immunization applications, these haptens are conjugated to large molecules (carriers) that aid to stimulate an immune response. The carrier-hapten conjugate carry many epitopes to which T lymphocytes can respond and subsequently provide the necessary help for antibody-producing B cells. Confering antigenicity, it is called antigen.

KLH is one of the most commonly used carriers in the conjugation of haptens (peptides) for antibody production. Thanks its large mass, KLH has numerous primary amines available for coupling haptens, and combined with its complex structure, KLH is well presented to the immune system, hence usually elicits a stronger immune response than other carrier proteins (BSA, Ovalbumin). It less likely produces antibodies that could cross-react with typical target samples (from mammals), because it is phylogenetically not related.

An immune response to a hapten-carrier conjugate results in antibodies that are specific for both the hapten and the carrier. Hence, it is a common practice to conjugate a given hapten to more than one carrier protein, one for immunization and another for screening, purification, etc.

Directions for use

Protocol of coupling peptide to KLH with glutaraldehyde (homobifunctional)⁽¹⁾

This recipe is for about 2 rabbits with five injections each.

1-Weigh out 100mg of keyhole limpet hemocyanin (KLH). Dissolve in 2ml water. It generally takes about 4 hours to dissolve by vortexing. Be patient and put on a rotator at 4 deg C. You may need to sonicate. Dialyze against 2l of 0.1M NaPhosphate pH 7.8 overnight. This is to remove any contaminating thiols or amino compounds.

Spin 10 minutes at full speed in microfuge to remove aggregates (don't be surprised to see a substantial pellet).

2-For -NH₂ coupling, add to KLH 10mg NH₂-peptide, followed by glutaraldehyde to 0.1% final. Add the peptide as a solid if it is soluble, otherwise from a 100 mg/ml stock in DMSO. Precipitation does not matter and often happens. After adding the glutaraldehyde, check the pH with pH paper, and adjust to 7.8 if necessary using NaOH. Incubate 8-12 hrs at 4 degrees, rotating gently.

3-Add a tiny pinch of NaBH₄ to kill remaining glutaraldehyde. Make sure the sample is in a large tube since it tends to fizz up. Incubate 8-12 hrs at 4 degrees. This is the glut-conjugate.

4-Dilute to 5ml with 0.15M NaCl. If there is a precipitate, sonicate vigorously to break it up. Split the immunogen into 1 ml aliquots (each aliquot will immunize two rabbits) and freeze it.

Protocol of coupling peptide to KLH through Bis-diazo-tolidine (homobifunctional)⁽¹⁾

Diazotize o-tolidine by dissolving 0.23 g of o-tolidine-HCl in 45 ml of 0.2 M HCl and then adding 0.175 g NaNO₂ in 5ml of H₂O. Prepare a 5 mg/ml stock solution of peptide in 0.16M sodium borate buffered saline, pH 9. Mix

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carrier protein (15 mg/ml stock) with the peptide stock solution, followed by the addition of 0.6 ml of bis-diazotized o-tolidine to a final volume of 4 ml, pH 7.4. Incubate at +4°C for 2 hours, in the dark with occasional shaking. Dialyze against H₂O.

Protocol of coupling peptide to KLH with EDC (heterobifunctional) ^(*)

Dilute the peptide stock solution to 1 mg/ml, add EDC to a final concentration of 10 mg/ml, and adjust the pH to 5. Incubate at room temperature for 5 min. Add an equal volume of carrier protein to the desired molar ratio and incubate for 4 hours at room temperature. Stop the reaction by adding sodium acetate, pH 4.2, to a final concentration of 100 mM and incubating at room temperature for 1 hour. Dialyze against PBS.

More information in the technical sheet of EDC (#UP52005)

Protocol of coupling peptide to KLH with MBS (heterobifunctional) ^(*)

This method is generally used with peptides that contain a cysteine. It employs M-Maleimidebenzoic acid N-hydroxy succinimide (MBS) ester (heterobifunctional)

Dissolve the peptide at 20 mg/ml in 1 mM NaOH. Dialyze against 10 mM potassium phosphate buffer, pH 7.2. Add 55 µl of 10 mM phosphate buffer to 4 mg of KLH solution, then add 85 µl of MBS (3 mg/ml stock in DMF). Stir at room temperature for 30 min. The MBS-activated KLH is added to the peptide and the pH is adjusted to 7.4. The coupling reaction is incubated at room temperature for 3 hours, with stirring.

Protocol of coupling peptide to KLH through thiols ^(*)

1- Check Peptide Thiol Groups

Peptide thiol groups have a tendency to oxidize after synthesis during storage, making peptide dimers. Free SH should be measured with DTNB reagent (#UP01566)

2- For the -SH coupling, warm the KLH to room temp. Add 1/9th volume of SATA (#UP84235) or Iodoacetic acid N-hydroxysuccinimide ester at 100mg/ml in DMSO. Make the DMSO stock fresh, and protect the reagent from light. After 10 minutes at room temp the KLH will start to get a little cloudy. Load it onto a desalting column (#UP84874) equilibrated with 0.1M NaPhosphate pH 7.8. Pool the KLH containing fractions by color (it will be sort of greyish green).

3- The SH-peptide can then be reacted as above.

4- Incubate the SH-peptide and the SH-KLH at least 8 hrs at 4°C, rotating gently.

5- Dilute to 5ml with 0.15M NaCl. If there is a precipitate, sonicate vigorously to break it up. Split the immunogen into 1 ml aliquots (each aliquot will immunize two rabbits) and freeze it.

Related / associated products and documents

See [BioSciences Innovations catalogue](#) and [e-search tool](#).

•Inquire for KLH in other grades (raw extract #835992, in PBS #885021, in MES #88503A, in glycerol #FH3380, endotoxin-free #FH3360 and depyrogenated).

•Other carrier proteins: [BSA](#), [OVA](#), MaxiBind™, Activated KLH [86734A](#).

•Buffer: PBS #[68723A](#)

•Adjuvants: [Freund's Adjuvant](#), [TiterMax](#)

•Desalting tools, i.e [CelluSep dialysis tubings](#), Desalting gel filtration columns #[UP84874](#)

-Crosslinkers

•Heterobifunctional crosslinkers: MAL-NHS reagents, i.e. [MAL-PEO-NHS](#) #[AL6580](#), EDC #[B11361](#), Glutaraldehyde,...

•Homobifunctional crosslinkers: NHS-NHS reagents, i.e. [NHS-PEO-NHS](#) and SMCC #[54940A](#)

•Homobifunctional crosslinkers: MAL-MAL reagents, i.e. [MAL-PEO-MAL](#) #L7736A

•PhotoActivable (PA) crosslinkers: SH and PA reactive i.e. SCBP #[B11361](#),...

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