3050 Spruce Street, St. Louis, MO 63103 USA
Tel: (800) 521-8956 (314) 771-5765 Fax: (800) 325-5052 (314) 771-5757
email: techservice@sial.com sigma-aldrich.com

Product Information

Maleimide Activated BSA, KLH Conjugation Kit

Catalog Number **MBK1**
Storage Temperature 2–8 °C

TECHNICAL BULLETIN

Product Description
Low molecular mass molecules (haptens), such as peptides, are often not sufficiently immunogenic to elicit an immune response alone. Synthetic peptides (1,000–3,000 Da), which are widely used to generate antibodies can be made immunogenic by conjugation to a suitable carrier.

A wide range of proteins, synthetic polymeric carriers, and conjugation methods are available to prepare immunogens from non-immunogenic small haptens. These include carrier proteins such as bovine serum albumin (BSA), keyhole limpet hemocyanin (KLH), ovalbumin (OVA), and thyroglobulin (TG) or synthetic carriers such as multiple antigenic peptides (MAPS). Hemocyanins are multimeric, high molecular mass, oxygen transport metalloproteins. KLH, from the hemolymph of the marine mollusc *Megathura crenulata*, is expressed as two subunit isoforms (KLH1 and KLH2) of 350–400 kDa. The KLH monomers each contain 7 or 8 functional unit domains, each functional unit containing an oxygen binding site carrying two copper atoms. Both KLH isoforms can assemble into multimeric forms containing native decamers of $4–8 \times 10^6$ Da. Higher multimeric forms have also been described. KLH is often used as a carrier protein due to its highly immunogenic properties and the large number of lysine residues available for modification.

Small molecules such as peptides can be coupled to carrier proteins using a wide range of crosslinking reagents such as the water soluble carbodiimide derivative *N*-(3-dimethylamino-propyl)-*N*′-ethylcarbodiimide (EDAC), bifunctional crosslinkers such as glutaraldehyde (GA), bis-succinimidyl esters of dicarboxylic acids (DSS or DST), or heterobifunctional crosslinkers such as *N*-hydroxysuccinimidyl esters of maleimido-alkyl-carboxylate derivatives.

Chemical modification of KLH or BSA with maleimide heterobifunctional crosslinkers results in the selective modification of the protein at the ε-amino group of the lysine side chains to form stable amide bonds. The activated protein has reactive maleimide groups on its surface available for conjugation with a sulfhydryl containing hapten (i.e., cysteine residue in a peptide) to form stable thioether bonds. The reaction of the maleimide group and the sulfhydryl group proceeds rapidly and selectively under mild coupling conditions (pH 6.5–7.5) to yield a stable, covalently-linked peptide-protein conjugate that may be used in immunization protocols or in antibody screening methods.

The Maleimide Activated BSA, KLH Conjugation Kit provides pre-activated KLH and BSA, eliminating the need for time-consuming activation and purification steps of the carrier proteins. The carrier proteins are activated using 3-maleimidopropionic acid *N*-hydroxy-succinimide ester (MPS) as the crosslinking reagent. Maleimide-BSA and KLH contain approximately 15 and 80 maleimide groups per BSA and KLH molecule, respectively. The conjugation reaction with a cysteine-containing peptide or a thiol-containing hapten is performed at pH 6.6–7.0. The amount of unreacted sulfhydryl is then determined by a colorimetric assay with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB or Ellman's reagent). Once the conjugate is formed it can be purified by a single gel filtration step on a Sephadex® G-25M column. The extent of conjugation is determined by calculating the total and unreacted sulfhydryl groups, and determining protein concentration. The conjugates are then ready for immunization and screening of the antibodies.

The immune system will develop antibodies to both hapten (peptide) and carrier protein. Antibody reactions to the carrier protein are not desired during the screening process. Therefore, if KLH is selected as the carrier protein for immunization, then BSA should be used as the carrier protein for screening or vice versa.

In less than 3 hours, the Maleimide Activated BSA, KLH Conjugation Kit performs the coupling of haptens to carrier proteins for immunization and antibody screening. It is designed to prepare hapten-protein conjugates by conjugating sulfhydryl-containing peptides or haptens to maleimide activated KLH or BSA, isolating the conjugates and estimating the level of conjugation.

The Maleimide Activated BSA, KLH Conjugation Kit contains all necessary reagents: pre-activated carrier proteins in a stabilized form; gel filtration columns to isolate the hapten-carrier protein conjugates from unreacted hapten and desalting; and reagents for determining the level of conjugation using the Ellman method. The kit contains sufficient reagents for 6 conjugation reactions with maleimide activated BSA and KLH (3 each).

Components

Maleimide Activated KLH 3×5 mg
Catalog Number K0383
Each vial contains 5 mg of Maleimide Activated KLH lyophilized from 10 mM sodium phosphate buffer, pH 6.6, with 0.115 mM NaCl, 1 mM EDTA, and 40 mM sucrose as stabilizer.

Maleimide Activated BSA 3×5 mg
Catalog Number B7542
Each vial contains 5 mg of Maleimide Activated BSA lyophilized from 10 mM sodium phosphate buffer, pH 6.6, with 0.115 mM NaCl, 1 mM EDTA, and 40 mM sucrose as stabilizer.

Conjugation Buffer 1 vial
Catalog Number C3957
Reconsitute with 10 ml of water to yield 20 mM sodium phosphate buffer with 100 mM EDTA and 80 mM sucrose, pH 6.6.

DTNB Buffer 2×1 vial
Catalog Number D4179
Reconstitute each vial with 10 ml of water to yield 0.1 M sodium phosphate buffer, pH 8.0. This buffer is used to dissolve and dilute DTNB (Ellman's reagent), and as reaction buffer in the assay of coupling efficiency.

L-Cysteine hydrochloride monohydrate 500 mg
Catalog Number C7880
Vial contains L-Cysteine for use as a sulfhydryl standard for the assay of coupling efficiency and preparation of cysteine standard curve.

5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) 500 mg
Catalog Number D8130
DTNB is used in the colorimetric determination of free sulfhydryl groups in cysteine containing peptides and any sulfhydryl-containing derivatives.

Gel Filtration Column 2 each
Catalog Number B4783
Each column is prepacked with Sephadex G-25M. The columns are used for the separation of unreacted peptide and buffer exchange. The column is preswollen in water containing 0.1% Kathon® CG/ICP as preservative. The bed volume of the column is 9.0 ml. The maximum sample volume is 2.5 ml.

Reagents and Equipment Required but Not Provided

- Cysteine-containing peptide or sulfhydryl-containing hapten
- Nitrogen gas (optional)
- Magnetic stirrer and mini-stirring bars
- UV/Visible spectrophotometer
- Protein assay reagents
- Phosphate Buffered Saline (PBS)
  0.01 M sodium phosphate buffered saline, pH 7.4. This solution serves as an equilibration and elution buffer for the Sephadex G-25M column, and for final dilution of conjugate.

Precautions and Disclaimer

This product is for R&D use only, not for drug, household, or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

Storage/Stability

Store all reagents at 2–8 °C. **Do not freeze.** For prolonged storage, Maleimide Activated KLH and BSA may be stored lyophilized at –20 °C.

Procedures

This kit can be used only with cysteine-containing peptides or sulfhydryl-haptens in their reduced state. Peptide or haptens in their oxidized form will fail to conjugate to Maleimide Activated KLH or BSA, or will distort the estimation of the conjugation level.

A. Conjugation to Maleimide Activated KLH

The following procedure describes the conjugation of a synthetic peptide containing cysteine to maleimide activated KLH. A molar ratio in the reaction mixture of 800–1000:1 for peptide (1,500 Da) to maleimide-KLH ($4–8 \times 10^6$ Da) is suggested. This ratio usually results in complete conjugation and provides an immunogen with a high hapten density on the carrier surface. This procedure can be modified for a peptide of different molecular mass, for a different amount of peptide, or if a lower molar ratio is desired in the reaction.

Note: The level of peptide conjugation may vary from one peptide to another due to differences in peptide solubility and the varying tendency of the cysteine thiol group toward oxidation, depending on the peptide sequence.

1. Slowly open a vial of Maleimide Activated KLH (Catalog Number K0383) to release the vacuum.
2. Reconstitute the contents of the vial with 1 ml of water to obtain a 5 mg/ml solution of maleimide activated KLH in 20 mM sodium phosphate buffer with 230 mM NaCl, 2 mM EDTA, and 80 mM sucrose, pH 6.6. Do not mix by vortex. Use immediately.
3. Reconstitute the Conjugation Buffer (Catalog Number C3957) with 10 ml of water to obtain a solution of 20 mM sodium phosphate buffer with 100 mM EDTA and 80 mM sucrose, pH 6.6.
4. Dissolve 4 mg of cysteine-containing peptide (~1,500 Da) or hapten in 0.5 ml of Conjugation Buffer or alternatively in water (see Trouble-shooting Guide, number 4). Retain 50 µl of the peptide solution for determination of coupling efficiency (cys-total). Store at 2–8 °C.
5. Immediately mix peptide solution with the Maleimide Activated KLH solution in a reaction vial equipped with stirring bar. De-gas sample, while stirring, under a gentle nitrogen stream for 1–2 minutes.
6. Cap reaction vial and continue stirring for 2 hours at room temperature or overnight at 2–8 °C.
7. Retain 100 µl of the conjugation reaction (cys-free) for determination of coupling efficiency.

B. Conjugation to Maleimide Activated BSA

The following procedure describes the conjugation of a synthetic peptide containing cysteine to maleimide activated BSA. A molar ratio in the reaction mixture of ~35:1 for peptide (MW 1,500) to maleimide activated BSA is recommended. This ratio corresponds to ~2 peptide-Cys moieties available to react with each maleimide activated site. This ratio usually results in complete conjugation and provides an immunogen with a high hapten density on the carrier surface.

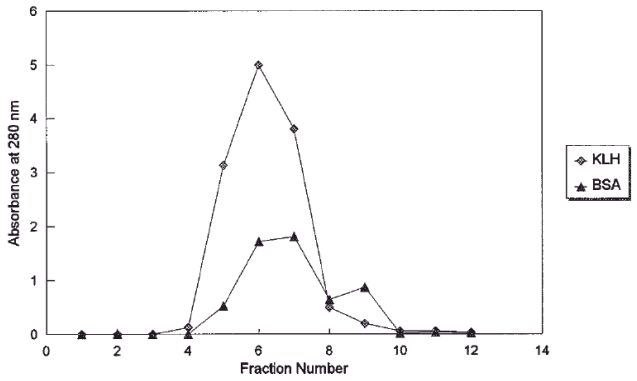
1. Slowly open a vial of Maleimide Activated BSA (Catalog Number B7542) to release the vacuum.
2. Reconstitute the contents of the vial with 1 ml of water to obtain a 5 mg/ml solution of maleimide activated BSA in 20 mM sodium phosphate buffer with 230 mM NaCl, 2 mM EDTA, and 80 mM sucrose, pH 6.6. Do not mix by vortex. Use immediately.
3. Dissolve 5 mg of cysteine-containing peptide (~1,500 Da) or hapten in 0.5 ml of Conjugation Buffer (step A3) or alternatively in water (see Troubleshooting Guide, number 4). Retain 50 µl of the peptide solution for determination of coupling efficiency (cys-total). Store at 2–8 °C.
4. Immediately mix peptide solution with the Maleimide Activated BSA solution in a reaction vial equipped with a stirring bar. De-gas sample, while stirring, under a gentle nitrogen stream for 1–2 minutes.
5. Cap reaction vial and continue stirring for 2 hours at room temperature or overnight at 2–8 °C.
6. Retain 100 µl of the conjugation reaction (cys-free) for determination of coupling efficiency.

C. Isolation of KLH or BSA Conjugates

1. Dissolve the contents of a PBS package (Catalog Number P3813) in 1 liter of water. For prolonged storage of this buffer, add 0.01% sodium azide.
2. Support the Sephadex G-25M gel filtration column (Catalog Number B4783) over a suitable (100 ml) beaker.
3. Remove cap from the top of the column, cut open lower tip of column, and let excess of liquid flow through. The column will not run dry.
4. Equilibrate the column with 30 ml of PBS. If the column is not immediately used, close with top and bottom caps and store the column at 2–8 °C.
5. Apply reaction mixture to the column and collect flow through (fraction 1).
6. Elute column with PBS, using a total volume of 10 ml and collect fractions of 0.5–1.0 ml. Monitor protein presence by measuring absorbance at 280 nm.

7. Pool fractions containing protein. When collecting fractions of 0.5 ml, the labeled protein is present in fractions 5–10. Do not pool fractions after fraction 10. A typical elution profile is shown in Figure 1.
8. Store the pooled fractions of immunogen in small aliquots frozen at –20 °C.
9. Wash the column with 50 ml of PBS (10 × 5 ml). The gel filtration columns can be regenerated at least 5 times.
10. For prolonged storage, wash the column with 50 ml of PBS containing 0.05% sodium azide and store capped, with 2 ml of storage solution above the gel, at 2–8 °C.
11. Determine coupling efficiency by the cysteine assay and protein concentration of conjugates by protein assay prior to use as immunogens.

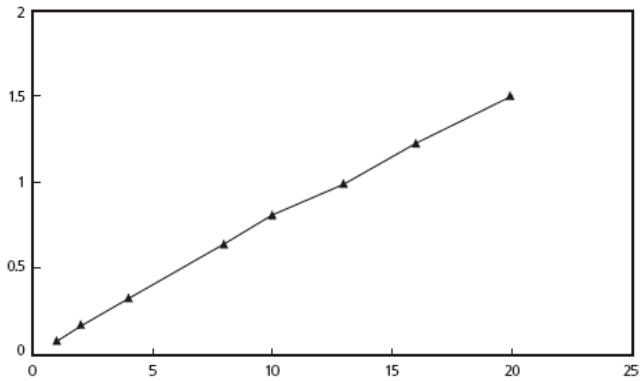
Figure 1.
Typical Gel Filtration Elution Profile



D. Cysteine Assay/Coupling Efficiency Determination

1. Cysteine Standard Assay - To estimate the coupling efficiency of the cysteine peptide, prepare a standard curve using known concentrations of cysteine. 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB or Ellman's reagent) reacts with sulfhydryl groups at pH 8.0 to produce a chromophore with maximum absorption at 412 nm.
   a. Dissolve the contents of the vial of the DTNB Buffer (Catalog Number D4179) in 10 ml of water.
   b. Dissolve 5 mg of the DTNB Reagent (Catalog Number D8130) in 5 ml of DTNB buffer.
   c. Immediately before use, prepare cysteine solution by dissolving 32 mg of L-Cysteine hydrochloride monohydrate (Catalog Number C7880) in 1 ml of water. Do not store this solution. Make serial stock dilutions in water in the range of 0.4–0.04 mg/ml. Use immediately.
   d. Add 50 µl of the prediluted cysteine standards to labeled test tubes. Use 50 µl of water as a blank.
   e. Add to all tubes, 0.1 ml of water, 0.75 ml of DTNB buffer, pH 8.0, and immediately 0.1 ml of DTNB reagent solution (1 mg/ml), to final assay volume of 1 ml.
   f. Mix assay tubes.
   g. Determine absorbance of samples at 412 nm. If the absorbance is above 1.4, dilute samples and repeat assay.
   h. Plot absorbance at 412 nm versus cysteine concentration (µg/ml). A typical standard curve for cysteine is shown in Figure 2. Use the linear part of the plot with cysteine concentrations ranging from 2–20 µg/ml, (final concentration in the assay).

Figure 2.
Typical Standard Curve for Cysteine



2. Cysteine Assay - Determination of Coupling Efficiency - If samples generate values higher than the highest cysteine standard in the cysteine/DTNB assay, dilute the samples and repeat the assay.
   a. Dilute an aliquot (50 µl) of peptide solution (cys-total) (Conjugation steps A4 or B3) in 0.1 ml of water.
   b. Add 50 µl of the following solutions to appropriately labeled test tubes:
      - DTNB Buffer (Blank)
      - Diluted peptide sample (cys-total, KLH conjugation, step A4)
      - Peptide-KLH (cys-free, KLH conjugation, step A7)
      - Diluted peptide sample (cys-total, BSA conjugation step B3)
      - Peptide-BSA (cys-free, BSA conjugation, step B6)

c. Add to all tubes 0.1 ml of water, 0.75 ml of DTNB buffer, pH 8.0, and immediately 0.1 ml of DTNB reagent solution (1 mg/ml), to final assay volume of 1 ml.
d. Mix assay tubes.
e. Determine absorbance of samples at 412 nm. If the absorbance is above 1.4, dilute samples and repeat assay.
f. Determine the concentration of total and residual cysteine-peptide in the assay mixture from the standard curve obtained in Section D1, step h (see Figure 2). Calculate peptide concentration in undiluted solution and coupling efficiency as described under Calculations.
g. Determine protein concentration of the conjugates with standard protein assay reagents prior to immunization or screening.

E. Calculations

1. To estimate peptide concentrations and coupling efficiency, prepare a standard curve using known concentrations of cysteine as described in Procedures, Section D1 (Cysteine Standard Assay). In this calculation, one mole of cysteine is equivalent to one mole of cysteine-containing peptide.

$$\% \text{ Coupling Efficiency} = \frac{\text{Cys (conjugated)}}{\text{Cys (total)}} \times 100$$

$$= \frac{\text{Cys (total)} - \text{Cys (free)}}{\text{Cys (total)}} \times 100$$

Cys (total) = Peptide (total) μmole/ml
Cys (free) = Peptide (free) μmole/ml
Cys (conjugated) = Cys (total) – Cys (free)

2. Example of conjugation of cysteine-containing peptide to maleimide-KLH:

Calculate cysteine/peptide concentration [Cys (total)]. Extrapolation from standard curve:

A_{412} = 1.14 is equivalent to 14.5 μg/ml of cysteine

Cysteine (mg/ml) = mg/ml × 20 (dilution factor)
= 0.0145 mg/ml × 20
= 0.29 mg/ml

Cysteine (μmole/ml) = Peptide (μmole/ml)
$= \frac{0.29 \text{ mg/ml}}{175.6 \text{ (MW Cys)}}$
= 1.65 μmole/ml

Note: The cysteine concentration (μmole/ml) is equivalent to the cysteine-containing peptide solution. The estimated initial peptide concentration is 1.65 μmole/ml. The expected initial peptide (MW 1,500, peptide content 80%) concentration in the conjugation is:

$$\frac{4 \text{ mg}}{1.5 \text{ ml} \times 1{,}500} = 1.4 \text{ μmole/ml}$$

3. Calculate cysteine/peptide concentration (cys-free) as described. Extrapolate concentration from standard curve.

4. A value of A_{412} = 0.3 was obtained for the KLH conjugate. From the standard curve this corresponds to 3.5 μg/ml of cysteine or peptide:

Cysteine (mg/ml) = (sample) mg/ml × 20
= 0.0035 mg/ml × 20
= 0.07 mg/ml

Cysteine (μmole/ml) = Peptide (μmole/ml)
$= \frac{0.07 \text{ mg/ml}}{175.6 \text{ (MW Cys)}}$
= 0.4 μmole/ml

5. The estimated level of peptide conjugation to KLH or coupling efficiency is:

Peptide conjugated = Peptide (total) - Peptide (free)
= 1.65 – 0.4
= 1.25 μmole/ml

% Coupling Efficiency
$= \frac{\text{Peptide (total)} - \text{Peptide (free)}}{\text{Peptide (total)}} \times 100$
$= \frac{1.25 \text{ μmole/ml}}{1.65 \text{ μmole/ml}} \times 100$
= 76%

Troubleshooting Guide

1. Synthetic peptides used in the conjugation reaction should be of the highest possible purity (HPLC purified, >90%) to avoid unwanted side reactions.
2. Determine the extent of peptide solubility and stability in aqueous solutions before proceeding to conjugation. Some cysteine peptides do not readily dissolve in aqueous solutions and/or tend to rapidly oxidize their cysteine residues to form disulfide-containing dimers.
3. Peptide solutions should not be prepared in buffers containing thiols, sodium azide, or amines such as Tris or glycine, since they compete with the conjugation reaction. Do not dissolve peptide directly in PBS buffers. Predissolve in water or in conjugation buffer.
4. Do not dissolve cysteine-containing peptides in dimethyl sulfoxide (DMSO). This may lead to extensive oxidation of the sulfhydryl groups. Highly purified dimethylformamide (DMF) can be used as an organic solvent to predissolve slightly insoluble peptides. Do not exceed 10% of this organic solvent in the conjugation reaction.
5. Avoid prolonged storage of the peptide in solution due to the tendency to form aggregates and oxidize even when stored frozen.
6. Oxidized cysteine-peptides of sulfhydryl haptens must be reduced with 2-mercaptoethanol, dithiothreitol (DTT), or sodium borohydride ($NaBH_4$), and purified by HPLC prior to use for conjugation.
7. Do not store reconstituted Maleimide Activated KLH or Maleimide Activated BSA solutions since they are not stable. Use immediately upon reconstitution. When mixing protein solutions, always avoid foaming.
8. For prolonged storage of the PBS and DTNB buffers, add 0.1% sodium azide as a preservative, and store solutions at 2–8 °C.
9. Prepare cysteine solutions immediately prior to use.
10. The immunogens prepared can be used in immunizations in combination with the following adjuvants, according to their product specifications:
    - Freund's Adjuvant, Incomplete (Catalog Number F5506)
    - Freund's Adjuvant, Complete (Catalog Number F5881)
    - TiterMax® Classic Adjuvant (Catalog Number H4397)
    - TiterMax Gold Adjuvant (Catalog Number T2684)
    - Sigma Adjuvant System® (Catalog Number S6322)

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