INSTRUCTIONS

Imject® Immunogen EDC Conjugation Kit with BSA or OVA

77123  77124

Number  Description
77123  Imject® Immunogen EDC Conjugation Kit with BSA, contains sufficient materials for five conjugation reactions

Kit Contents:
Imject® Bovine Serum Albumin (in MES buffer), 5 × 2 mg, supplied lyophilized; when reconstituted with 0.2 ml of ultrapure water, buffer contains 0.05 M MES (2-[N-morpholino]-ethanesulfonic acid); pH 4.7 with proprietary stabilizer
Imject® EDC Conjugation Buffer, 30 ml, 0.1 M MES, 0.9 M NaCl, 0.02% NaN₃; pH 4.7
EDC (1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride), 5 × 10 mg
D-Salt™ Dextran Desalting Columns (5K MWCO), 5 × 5 ml, contains 0.02% sodium azide
Imject® Purification Buffer Salts, 5 × 5 g, upon reconstitution this buffer contains 0.083 M sodium phosphate, 0.9 M NaCl; pH 7.2 with proprietary stabilizer

77124  Imject® Immunogen EDC Conjugation Kit with OVA, contains sufficient materials for five conjugation reactions

Kit Contents:
Imject® Ovalbumin (in MES Buffer), 5 × 2 mg, supplied lyophilized; when reconstituted with 0.2 ml of ultrapure water, buffer contains 0.05 M MES (2-[N-morpholino]-ethanesulfonic acid); pH 4.7 with proprietary stabilizer
Imject® EDC Conjugation Buffer, 30 ml, 0.1 M MES; 0.9 M NaCl, 0.02% NaN₃; pH 4.7
EDC (1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride), 5 × 10 mg
D-Salt™ Dextran Desalting Columns (5K MWCO), 5 × 5 ml, contains 0.02% sodium azide
Imject® Purification Buffer Salts, 5 × 5 g, upon reconstitution this buffer contains 0.083 M sodium phosphate, 0.9 M NaCl, pH 7.2 with proprietary stabilizer

Storage: Upon receipt store kit at 4°C. Kit is shipped at ambient temperature.

Introduction
Antibody production is often used for preparing tools to detect or purify a specific antigen of interest. Imject® Immunogen EDC Conjugation Kits with carrier proteins (see Appendix A for more information on carrier proteins) enable simple hapten conjugations for eliciting an immune response and antibody production against the hapten. Ovalbumin (OVA) and bovine serum albumin (BSA) are often conjugated to haptens and used as a non-relevant carrier in an ELISA for measuring specific anti-hapten antibody titers. Antibodies produced using mcKLH-hapten conjugates will recognize both the hapten and mcKLH. Coupling the hapten to a different carrier protein for the ELISA enables specific measurement of the anti-hapten antibody response.

The Imject® Immunogen EDC Conjugation Kits simplify the production of hapten-carrier conjugates. EDC reacts with carboxyl and amine groups to form stable amide bonds. Because most peptides contain both exposed lysines and carboxyl groups, EDC-mediated immunogen formation may be the simplest method for the majority of hapten-carrier protein conjugations (see Appendix B for more information on EDC chemistry).

Warranty: Pierce Biotechnology (hereafter “Pierce”) products are warranted to meet stated product specifications and to conform to label descriptions when stored and used properly. Unless otherwise stated, this warranty is limited to one year from date of sale when used according to product instructions. Pierce’s sole liability for the product is limited to replacement of the product or refund of the purchase price. Unless otherwise expressly authorized in writing by Pierce, products are supplied for research use only and are intended to be used by a technically qualified individual. Pierce’s quality system is certified to ISO 9001. Pierce makes no claim of suitability for use in applications regulated by FDA. Pierce strives for 100% customer satisfaction. If you are not satisfied with the performance of a Pierce product, please contact Pierce or your local distributor.
Procedure for Hapten-Carrier Conjugation using EDC

This protocol is designed to yield effective immunogens for a wide variety of haptens but is not necessarily optimal for a specific hapten. Differences in size and structure of haptens will affect conjugation efficiencies. Using a molar excess of hapten over the carrier protein ensures efficient conjugation. Generally, reacting equal mass amounts of hapten and carrier protein will achieve sufficient molar excess.

A. Conjugation Procedure
1. Reconstitute one vial of BSA or OVA by adding 200 µl of ultrapure water to make a 10 mg/ml solution. Use up to 2 mg of peptide per 2 mg of carrier protein.
2. Dissolve up to 2 mg of the hapten in 0.5 ml of Imject® EDC Conjugation Buffer.
   Note: For haptens with limited solubility, DMSO may be used for solubilization. Use ≤30% DMSO in the final conjugation solution or the carrier protein may irreversibly denature.
3. Add the peptide solution to the carrier protein solution.
4. Add the peptide/carrier solution to one vial of EDC (10 mg) and dissolve by gentle mixing.
5. Allow reaction to proceed for 2 hours at room temperature.

B. Conjugate Purification by Desalting
- Use either desalting or dialysis to remove sodium azide and excess cross-linker. If DMSO was used in the conjugation, add DMSO to the Purification Buffer Salts for desalting to prevent precipitation in the column; dialysis is not compatible with DMSO.
- Desalting or dialysis will not separate non-conjugated protein; however, a large excess of hapten is used in this protocol, making it unlikely that non-conjugated carrier exists in significant quantity.
- If the conjugate is to be used for injection within one week, PBS may be used for purification. If the conjugate will be frozen, use Purification Buffer Salts for purification, which will preserve the product during freeze-thaw cycles.
- If a precipitate formed during conjugation, centrifuge the precipitated material, collect the supernatant and save the precipitate. Purify only the supernatant. Combine the purified conjugate to the precipitate.
1. Dissolve contents of one bottle of Purification Buffer Salts by adding 60 ml of degassed ultrapure water to the bottle. Store excess buffer at 4°C.
2. Sequentially remove the top and bottom caps from a desalting column and allow the storage solution to drain. Use one desalting column for each 0.5 ml of sample.
3. Wash column with 3-5 column volumes (i.e., 15-25 ml) of Purification Buffer Salts.
4. Apply 0.5 ml of the hapten-carrier mixture directly to the center of the column disc. Add 8-10 aliquots of 0.5 ml of Purification Buffer and collect each fraction in a separate tube.
5. Measure absorbance at 280 nm to locate fractions containing the conjugate. The hapten-carrier conjugate will be in the first absorbance peak detected. Pool all fractions that contain acceptable levels of conjugate.
6. After the conjugate-containing fractions have emerged, the non-conjugated hapten can be recovered by continuing to add buffer to the column and collecting additional fractions.
7. If the immunogen is to be stored for more than a few days, sterile filter the conjugate fractions and store in a sterile container at 4°C or -20°C. See Appendix C for immunization suggestions.
   Note: To purify antibodies specific to the peptide, prepare an affinity column by immobilizing the peptide through the same functional group used to prepare the immunogen (see Appendix D).

Appendix
A. Carrier Proteins
Small molecules (haptens) such as peptides, are able to interact with products of an immune response, but cannot stimulate a response alone. Haptens can be made fully immunogenic by coupling them to a suitable carrier molecule. Some of the more common carrier proteins include keyhole limpet hemocyanin (KLH; 4.5 × 10^5-1.3 × 10^7 Da), bovine serum albumin (BSA; 67,000 Da) and ovalbumin (OVA; 45,000 Da).
KLH belongs to a group of non-heme proteins that are present in arthropods and molluscs and is harvested from the hemolymph of giant keyhole limpets (Megathura crenulata). KLH exists as five different aggregate states in Tris-HCl at pH 7.4 and reversibly dissociates to subunits with moderate changes in pH; at pH 8.9 it will completely and irreversibly dissociate to subunits. Subunits contain oxygen-binding sites. The oxygen-containing KLH is blue, while the oxygen-lacking form is colorless. Oxygen removal will dissociate subunits into lower aggregation states. Increased antibody binding occurs when KLH is dissociated to its subunits because of improved availability of antigenic sites.

BSA constitutes approximately half of the protein in plasma and is the most stable and soluble protein in plasma. BSA is much smaller than KLH but is also immunogenic. BSA has an extinction coefficient of 7.16 at 280 nm for a 1% solution. This protein has a total of 59 lysine groups with 30-35 of these capable of reacting with a cross-linker.

Ovalbumin, also known as egg albumin constitutes 75% of the protein in hen egg whites. Ovalbumin contains 20 lysines and is often used as a secondary carrier in ELISA applications. This protein exists as a single polypeptide chain with half of its 400 residues being hydrophobic. Ovalbumin has an acid isoelectric point of 4.63 and is subject to denaturation from electric currents, vigorous shaking, and temperatures above 56°C.

B. EDC Coupling Chemistry

EDC-mediated immunogen formation is a simple method for hapten-carrier protein conjugations. The carbodiimide initially reacts with available carboxyl groups on either the protein carrier or peptide to form an active O-acylurea intermediate. This unstable intermediate reacts with a primary amine to form an amide bond with the release of a soluble urea derivative.

EDC coupling is an efficient, one-step method. Like most coupling reagents, EDC is subject to hydrolysis and moisture must be avoided until used. The hydrolysis of the EDC intermediate is a major competing reaction during coupling and is independent of temperature, pH and buffer concentration. Conjugation may occur at either the C- or N-terminal of the peptide or at any carboxyl- or amine-containing side chains. Therefore, do not use this method if the interested area of the peptide contains groups that may be blocked when coupled. If using peptides rich in lysine, glutamic acid or aspartic acid, hapten polymerization may occur upon conjugation, changing the antigenic structure of the resulting immunogen; however, some peptide polymerization on the carrier surface may enhance the immune response.

C. Immunization for Mice and Rabbits

After preparing the immunogen with an adjuvant (see Related Pierce Products section), the following protocol may be used for immunization. This schedule is a general protocol for immunization and may be customized as needed.

**CAUTION:** Only qualified personnel should perform these procedures. Individuals unfamiliar with these techniques should consult an experienced investigator for training before attempting to immunize and bleed animals.

**Day 0:** Collect non-immune serum to be used as a blank for ELISAs and store frozen.

For mice, make initial injection of 50-100 µg of immunogen per mouse in one to two injections. Intraperitoneal and subcutaneous injection sites are the most common for mice.

For rabbits, make an initial injection of 100 µg of immunogen into 6-10 subcutaneous sites on the animal’s back.

**Day 14:** Boost with same sample size as the initial injection. If Freund’s Complete Adjuvant was used for the initial injection, use Freund’s Incomplete Adjuvant for boosts to avoid animal stress.

**Day 21:** Test bleed and screen for antibody response by ELISA. For mice, 200-400 µl samples from the tail vein or the retro orbital plexis are appropriate. For rabbits, 5-10 ml samples from the ear vein may be obtained.

**Day 28:** Boost again if necessary. Continue to periodically bleed and boost until a satisfactory immune response occurs.

D. Purification of Peptide-specific Antibodies

Using a carrier protein for antibody production also results in the production of antibodies against the carrier protein. To purify peptide-specific antibodies, immobilize peptide through the same functional group used to prepare the immunogen. The CarboxyLink™ Kit (Product No. 44899) contains an amine-containing gel support that will couple peptides via carboxyl groups using EDC. The peptide affinity column can then be used to specifically bind anti-peptide antibodies from serum, allowing antibodies against the carrier protein to flow through the column. Peptide-specific antibodies can then be eluted with a buffer such as ImmunoPure® IgG Elution Buffer (Product No. 21009) or ImmunoPure® Gentle Elution Buffer (Product No. 21027).
Information Available from the Web

Please visit the Pierce website for additional information relating to this product including the following items:

- Tech Tip protocol: Remove air bubbles from columns
- Tech Tip protocol: Degas solutions for use in affinity columns
- Tech Tip: Protein stability and storage
- Tech Tip protocol: Block amino groups to prevent polymer formation in peptide-carrier protein conjugations

Related Pierce Products

- 77140 Imject® Freund’s Complete Adjuvant, 5 × 10 ml
- 77145 Imject® Freund’s Incomplete Adjuvant, 5 × 10 ml
- 77161 Imject® Alum, 50 ml
- 77138 AdjuPrime™ Immune Modulator, 20 mg
- 66382 Slide-A-Lyzer® Dialysis Cassette Kit, 10 dialysis cassettes, each appropriate for 0.5-3.0 ml samples
- 45212 Melon™ Gel IgG Purification Kit, sufficient reagents to purify IgG from up to 50 ml of serum
- 45206 Melon™ Gel IgG Spin Purification Kit, sufficient reagents to purify up to 3 ml of serum

Cited Reference


General References


Current versions of product instructions are available at www.piercenet.com. For a faxed copy, call 800-874-3723 or contact your local distributor.