SureLINK™

AP Conjugation Manual

For Products:

Product	Catalog No.	Size
SureLINK AP Conjugation Kit	85-00-01	3 x 0.1 mg rxn.
	85-00-02	3 x 0.5 mg rxn.
SureLINK Modified AP	85-01-01	0.2 mg
	85-01-02	1.0 mg

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PRODUCT DESCRIPTION

SureLINK™ AP Conjugation Kits enable labeling of antibody or protein with alkaline phosphatase (AP). Kits contain lyophilized AP and all reagents required to quickly label multiple samples of antibody/protein. The resulting AP conjugates can be used in ELISA, Western blotting, immunohistology, and other protein applications.

BACKGROUND

Alkaline phosphatase (AP) is a ubiquitous dimeric metalloenzyme with a molecular weight of 140 kDa, and is classified as a non-specific phosphomonoesterase with a high rate of substrate turnover.[1,2] The enzyme has been commonly used in enzyme immunoassays (EIA); namely ELISA, Western blotting, and immunohistochemical staining techniques.[3] High substrate turnover rate combined with the commercial availability of several sensitive colorimetric and chemiluminescent substrates have made AP an invaluable tool in biological research. Excellent sensitivity in combination with moderate thermostabilty has also made AP a suitable detection reagent in nucleic acid hybridization assays to replace radioactive labels.[4]

AP conjugation has traditionally focused on using homo- and hetero-bifunctional crosslinkers to prepare conjugates for immunoassay detection.[3,5,6] Glutaraldehyde is the most widely used homobifunctional cross-linking reagent due to its commercial availability, low cost and high reactivity. This method, however, has considerable drawbacks. Changes in antibody to glutaraldehyde ratio may result in low solubility and reduced enzymatic activity. It is difficult to avoid the formation of complex structures with varied levels of activities[7]. The reaction mixture must be quenched and/or purified to remove excess cross-linkers following the conjugation reaction. Finally, reactivity of cross-linkers could vary greatly, resulting in differences of size and efficacies of conjugate preparations. This ultimately requires tedious optimization of downstream applications in which the AP conjugate is used.

Other common AP conjugation is based on maleimide hetero-bifunctional cross-linking reagents where sulfhydryl groups react with maleimide moieties to form stable conjugate. Reactive sulfydryl groups on antibody molecules are introduced by gently reducing the disulfide bonds on the Fc portion of the antibody or by labeling ta

PRODUCT OVERVIEW

SureLINK™ AP Conjugation Kits contain ready-to-use components for the preparation of AP conjugates that are stable for at least 6 months at 4°C. The kit uses a novel chemistry which overcomes many of the limitations mentioned above. The chemistry is based on a coupling reaction which employs hydrazine and carbonyl-based bifunctional crosslinking reagents (USPTO 6800728)[9]. Specifically, the aldehyde group of succinimidyl-P-formyl benzoate (SFB) on the antibody react with the hydrazine group of succinimidyl 4-hydrazinonicotinate (SANH), which has been previously attached to the AP enzyme, hence the term “modified AP”. The reaction begins when provided SFB is coupled to the primary amine group of the antibody, then it reaches the completion through the facilitation of the leaving group, N-hydroxy4h

PRODUCT COMPONENTS

Size: 3 x 0.1 mg rxn.

Kit Component	Part Number	Size	Quantity
SureLINK Modified AP	85-01-01	0.2 mg	3
AP Modification Buffer	85-02-01	5 mL	1
SFB	80-02-01	0.2 mg	3
AP Conjugation Buffer	85-03-01	1.5 mL	1
AP Storage Buffer	85-04-01	5 mL	1

85-00-02, SureLINK AP Conjugation Kit

Size: 3 x 0.5 mg rxn.

Kit Component	Part Number	Size	Quantity
SureLINK Modified AP	85-01-01	1.0 mg	3
AP Modification Buffer	85-02-01	5 mL	1
SFB	80-02-01	0.2 mg	3
AP Conjugation Buffer	85-03-01	1.5 mL	1
AP Storage Buffer	85-04-01	5 mL	1

85-01-01, SureLINK Modified AP

Size: 0.2 mg

Kit Component	Part Number	Size	Quantity
SureLINK Modified AP	85-01-01	0.2 mg	1

85-01-02, SureLINK Modified AP

Size: 1.0 mg

Kit Component	Part Number	Size	Quantity
SureLINK Modified AP	85-01-01	1.0 mg	1

SureLINK Modified AP, 0.2 mg, is ideal for conjugating 0.05-0.1 mg of antibody or protein. The 1.0 mg size Modified AP is ideal for conjugating 0.1-0.5 mg of antibody or protein.

STORAGE AND STABILITY

BEFORE YOU BEGIN

SAFETY AND HANDLING

- Read MSDS and all instructions thoroughly before using SureLINK AP Conjugation Kits or Modified AP.
- Wear appropriate personal protective equipment when handling reagents.

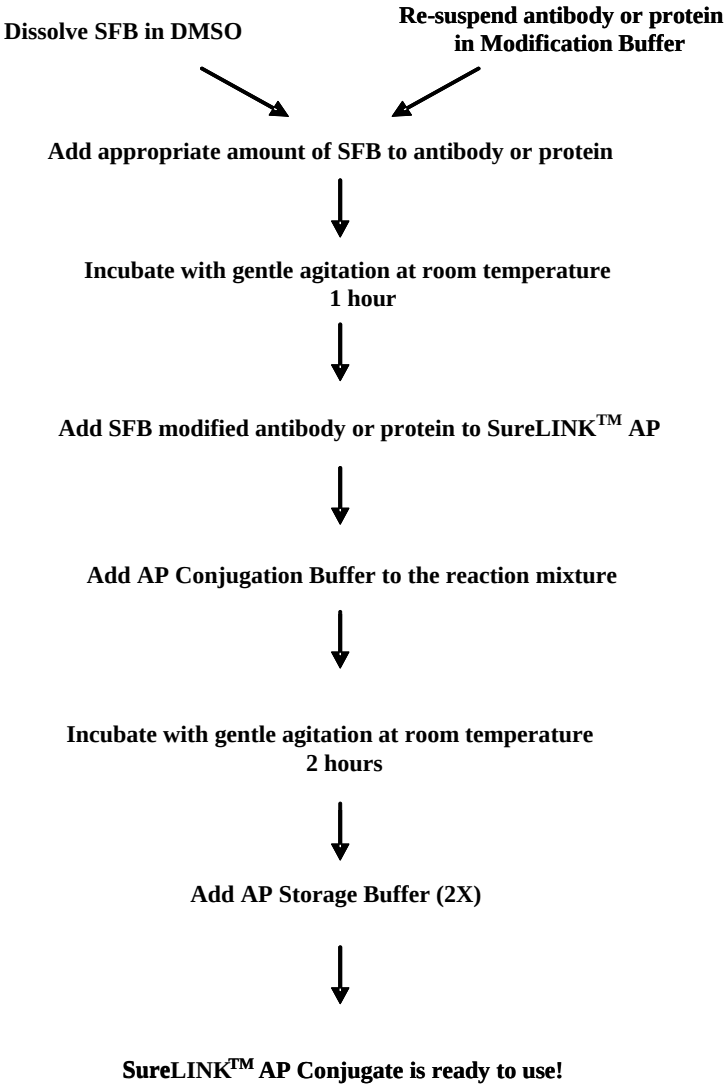
OTHER REQUIRED SUPPLIES AND EQUIPMENT

- Antibody or protein, free of salts or contaminants (see Troubleshooting section for removing salts or other contaminants.)
- Dimethyl sulfoxide (DMSO) or Dimethyl formamide (DMF)
- Molecular biology grade water
- Shaker
- Vortex
- Microcentrifuge

REAGENT PREPARATION

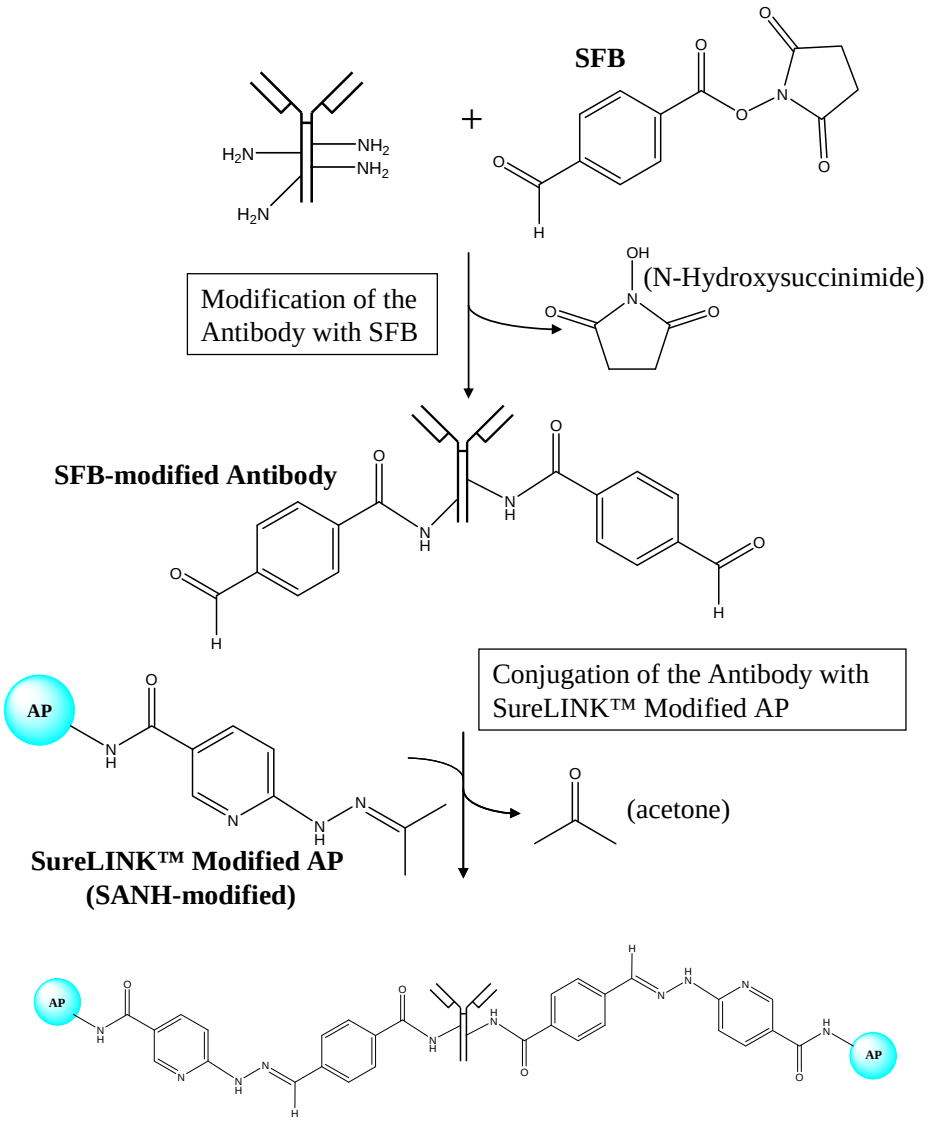
- Equilibrate each buffer to be used in modification and conjugation reactions to room temperature. If precipitations are visible in the buffers following the storage at 4ûC, incubate the buffers in a water bath (50ûC to 60ûC) to dissolve the precipitate.
- 5X Conjugation Buffer will be diluted to 1X during the conjugation reaction.
- All other buffers are ready-to-use; dilution or mixing prior to use is not required.

QUICK REFERENCE PROTOCOL



TOTAL REACTION TIME
Approximately 3 hours

SURELINK™ AP CONJUGATION CHEMISTRY



CONJUGATION PROTOCOL

This protocol describes the experimental conditions for IgG conjugation. Other antibodies and proteins can also be labeled by following recommendations in the protocol. Optimal conditions occur if antibody concentration is maintained at 0.25 –2.0 mg/mL in the conjugation reaction.

1. **Equilibrate the AP Modification Buffer and AP Conjugation Buffer at room temperature before use.**

2. **Rehydrate the lyophilized antibody (free of salts) with AP Modification Buffer to a final concentration of 0.5 – 2.0 mg/mL.**

   A minimum volume of 0.2 mL antibody is required to perform the labeling reaction. If liquid antibodies are used, the protein concentration should be in the range of 0.5-2.0 mg/mL in 0.1M sodium phosphate buffer, pH 7.2 in 0.15M NaCl. If the buffer of antibody or protein is different or contains contaminants, see Troubleshooting Guide for recommendations prior to performing conjugation with this kit.

3. **Dissolve SFB (0.2 mg) in 200 µL of DMSO or DMF. Mix thoroughly by vortexing. The concentration of SFB is approximately 4 nmole/µL.**

4. **Initiate modification of the antibody to attach the carbonyl moeities by adding the appropriate amount of SFB to the antibody or protein sample. Recommended molar ratio of SFB:Antibody is 25:1. Incubate for 1 hour at room temperature or overnight at 4°C with gentle agitation.**

   See Table 1, page 11 for guidelines on SFB amounts needed to modify specific quantities of antibody. Titrating the amount of SFB may enhance the efficiency of the antibody or protein labeling reaction, depending on the availability of reactive amine moieties. If excess SFB is added, dialyzing the reaction mix following the modification step may be required (against 0.1 M citric/citrate pH 6.0 buffer in 0.15 M NaCl).

5. **Add appropriate amount of SureLINK™ Modified AP to the antibody-SFB mix. Recommended molar ratio of modified AP:Antibody-SFB is 2.0:1 to 2.5:1.**

   See Table 2, page 11 for guidelines on amounts of modified AP to add to specific quantities of antibody-SFB. The Modified AP should be rehydrated with molecular biology grade water. The rehydrated Modified AP is stable for at least 24 hrs at 4ûC. Reducing the Modifed AP:Antibody-SFB ratio (using less Modified AP) may

favor the production of lower molecular weight conjugates, enhancing the ability of the conjugates to penetrate through cell membranes.

6. **Add AP Conjugation Buffer (5X) to the antibody-AP reaction to reach the final concentration of 1X.**

   If needed, adjust reaction volume with molecular biology grade water. For example, reaction volume of 500 µL will need 140 µL AP Conjugation Buffer (5X) and 60 µL H_20. Incubate for 2 hours at room temperature or overnight at 4°C with gentle agitation.

7. **Finally, add equal reaction volume of AP Storage Buffer (2X) to the reaction and mix gently. The conjugate is now ready to use.**

   See page 13 for recommended starting dilutions of the conjugates for ELISA, Western blotting, and immunohistology assays.

CALCULATIONS

Table 1: Amount of SFB Needed to Modify Various Quantities of Antibody

Calculations are based on IgG and SFB with the molecular weight of 160 kDa and 247.1g/mole respectively. Optimal molar ratio SFB:IgG is 25:1 0.2 mg SFB is rehydrated in 200 µL DMSO at 4 nmole/µL. Titrating the amount of SFB may enhance the efficiency of the antibody labeling reaction, depending on the availability of amine groups.

IgG Amount	IgG (nmoles)	SFB (nmoles)	Amount of 4 nmole/

Example Protocol Using SureLINK™ AP Conjugation Kit:

Conjugation of IgG Antibody (0.2 mg) with SureLINK Modified AP

1) Re-hydrate 0.2 mg IgG (1.25 nmole) sample with 200 µL of AP Modification Buffer.
2) Dissolve SFB (0.2 mg) with 200 µL DMSO. SFB is now at 4 nmole/µL.
3) To achieve the 25:1 molar ratio of SFB:IgG, add 7.5 µL of SFB to the entire 200 µL IgG solution. Incubate for 1 hour with gentle agitation at room temperature.
4) Re-hydrate SureLINK Modified AP (1.0 mg, catalog number 85-01-02) with 200 µL H_2O. Modified AP is now at 0.035 nmoles/µL.
5) Initiate the conjugation reaction by adding 80 µL of the reconstituted Modified AP sample to the IgG-SFB mix.
6) Add 80 µL of AP Conjugation Buffer (5X) and 40 µL H_2O to the reaction mix. Final reaction volume is now 400 µL. Incubate for 2 hours with gentle agitation at room temperature.
7) Add 400 µL AP Storage Buffer (2X) to the reaction. AP conjugate is now ready to use. The final concentration of the AP-labeled IgG preparation is approximately 0.25 mg/mL based on the starting quantity of IgG. Store conjugate at 4^oC.

RECOMMENDED USE OF CONJUGATES

SureLINK™ AP Conjugates can be used in a variety of immunoassays. Recommended conjugate concentrations for several common immunoassays are listed below. The conjugate concentration that will provide the best signal to background ratio in your specific assay, may vary and should be determined for each conjugate. Include positive and negative controls in each immunoassay for proper review of experimental results and successful troubleshooting.

Application	**AP Conjugate Concentrations**
ELISA	0.25 µg/mL to 2.5 µg/mL
Western-Blot	0.5 µg/mL to 5 µg/mL
Immunohistology	2.0 µg/mL to 5.0 ug/mL

Most reagents required for performing ELISA, Western Blot, and Immunohistology assays are available from KPL. A listing of products is
desa.s ise1.oy96(5710(o)4.e Rela)-4.8(e96(Produ.8(e96c-2(sect)6.6(h)4u)5-2.1(od)4od)4Vi5(n www.kp6(h)4ul.4.8(c

TROUBLESHOOTING GUIDE

Problem 1: Weak Level of Detection in Immunoassay

	Causes and/or Observations	Possible Solutions
x	Inactive SureLINK AP samples	Check the expiration date and follow the storage condition of each component in the kit.
x	Low signal and/or high background levels	Titrate the amount of the conjugate in the assay and optimize the signal to noise ratio.
x	Other proteins are present in the Ab sample—compromising the preparation of the desired AP conjugate.	Fractionate the antibody sample over an acrylamide gel electrophoresis and stain using Coomassie dye. Depending on the level of impurity, an affinity protein purification may be required.
x	The NHS-ester bond of the SFB cross-linker has been hydrolyzed during storage, compromising the labeling reaction.	Upon receipt, store the SFB in dessicator. Use anhydrous DMSO to dissolve the SFB powder and store in a dessicator. Otherwise, dissolve the SFB powder immediately prior to each use.
x	Contaminants are present in the antibody sample that carry an amine or a nucleophilic group (ex. Tris, glycine and azide), reducing the efficiency of the modification reaction.	Dialyze the antibody sample thoroughly against a 0.1 M sodium phosphate buffer at pH 7.2 (+ 0.15 M NaCl) prior to the modification step.
x	The level of SFB labeling of the antibody sample needs to be quantitated.	Determine the level of carbonyl labeling (SFB) by following the protocol described elsewhere (USPTO 6,800,728)[1], using the quantitation reagents supplied in SureLINK™ Bioconjugation kit (catalog no. 80-00-01).
x	The concentration of the antibody in the conjugation mix may have been under-estimated, resulting in a significant level of unlabeled antibody in the final conjugate mix.	Estimate the antibody concentration using techniques such as the Bradford since the SFB labels contribute to the absorbance measurements at 280 nm.
x	The concentration of the antibody in the modification reaction may have been over-estimated, resulting in excess amount of SFB that can readily competes in the	Use triplicate absorbance measurements to determine the Ab sample before the addition of SFB. Dialyze the Ab-SFB sample against a 0.1 M

conjugation reaction.	citric/citrate buffer at pH 6.0 (+ 0.15 M NaCl) following the modification step.

Problem 2: High level of Background on Immunoassay

Causes and/or Observations	Possible Solutions
x The conjugate size is too large.	Optimize the molar ratio of the SFB:Antibody and/or the time of the modification reaction. Optimize the molar ratio of the Modified AP to Antibody-SFB, and/or the time of the conjugation reaction.
x The amount of the AP conjugate is much higher than optimal amount in a Western blot application.	Titrate and optimize the amount of conjugate required for each immunoassay.

If you are having problems regarding ELISA and Western Blot assays, visit our website at www.KPL.Com. For additional assistance, contact KPL Technical Services at 800-638-3167 (USA), 301-948-7755 or techserv@kpl.com.

RELATED PRODUCTS

Product/Application Group	Product Name	Size	Catalog Number
Protein Labeling Kits & Reagents	**SureLINK™ HRP Conjugation Kit** **SureLINK™ HRP Conjugation Kit** **SureLINK™ Activated HRP** **SureLINK™ Activated HRP** **SureLINK™ Bioconjugation Kit**	**6 x 0.1mg rxn** **6 x 1.0 mg rxn** **0.3 mg** **1.5 mg** **kit**	**84-00-01** **84-00-02** **84-01-01** **84-01-02** **80-00-01**
ELISA Products	**BluePhos™ AP Microwell Substrate** **BluePhos™ Stop Solution** **pNPP AP Microwell Substrate**	**600 mL** **200 mL** **500 mL**	**50-88-00** **50-89-00** **50-80-00**
Western Blot	**PhosphaGLO™ AP Substrate** **PhosphaGLO Reserve™ AP Substrate** **BCIP/NBT Phosphatase Substrate**	**100 mL** **100 mL** **100 mL**	**55-60-04** **55-60-02** **50-81-18**
Immunohistochemistry	**HistoMark™ RED AP Substrate** **HistoMark™ BLUE AP Substrate**	**1000 slides** **1000 slides**	**55-69-00** **55-70-00**
Support Reagents	**Wash Solution Concentrate** **Coating Solution Concentrate (10X)** **AP Stabilizer** **BSA Blocking Solution** **Milk Diluent Blocking Solution** **Detector Block Solution**	**800 mL** **50 mL** **200 mL** **200 mL** **200 mL** **240 mL**	**50-63-00** **50-84-00** **55-15-00** **50-61-00** **50-82-01** **71-83-00**

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