KPL APPLICATION NOTE

SureLINK™ Conjugation Chemistry:

Phosphatase Conjugates and Their Use in Immunodetection

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INTRODUCTION

Immunoassays commonly employ biomolecules conjugated to a reporter or detection molecule. Enzymes are used as reporters because of signal amplification via the catalytic properties of the enzyme, availability of suitable substrates, and ease of detection of reaction products. Enzyme conjugates are detected by reaction with substrates which yield either a chromogenic or chemiluminescent reaction product.

Enzymes most frequently used in immunoassays include β-galactosidase (β-gal), horseradish peroxidase (HRP), and alkaline phosphatase (AP). Each offers unique features that, under the right conditions, makes it the optimal choice for immunodetection. Originally isolated from calf intestine, AP is commonly used in biological assays due to its rapid turnover, sensitivity, and moderate thermostability.

AP CONJUGATION CHEMISTRIES

For use in an immunoassay, AP must be first attached to a biomolecule such as an antibody, streptavidin, or other binding partner. A number of traditional methods are commonly used to make AP conjugates. One employs the homobifunctional linker glutaraldehyde[1] whereas another relies on the use of a heterobifunctional maleimide[1] linker*.

Glutaraldehyde-mediated conjugation of AP is difficult to control whether the one-step or two-step method is used. Due to uncontrolled polymerization, use of either method tends to result in high molecular weight conjugates with reduced solubility and enzyme activity. A second disadvantage is that the Schiff base formed during the reaction must be reduced in order to form a stable conjugate, adding time and steps to the protocol.

In a typical maleimide-based conjugation, AP is first modified with a heterobifunctional linker using primary amines on the enzyme. Conjugation requires a free sulfhydryl group in the target protein, created by reduction of disulfide bonds. Because antibodies have four protein chains held together with disulfide linkages, reduction of disulfide bonds may alter antibody structure and function. In situations where the reduction of integral disulfide bonds is undesirable or where the target protein lacks disulfide bonds, a second heterobifunctional linker, e.g., SATA (N-Succinimidyl-S-acetylthioacetate), can be used to introduce sulfhydryl groups through amine-reactive chemistry. Use of such linkers usually requires deprotection of the sulfhydryl group, adding time and complexity to the conjugation. Additionally, the EDTA (ethylene-diaminetetraacetic acid) used in this protocol chelates the divalent cations in the active site of AP, thus decreasing enzymatic activity.

SureLINK CONJUGATION CHEMISTRY

The SureLINK conjugation method is a two-step protocol that uses two unique, heterobifunctional linkers to couple AP to an antibody, protein, or other biomolecule (See Figure 1). AP is modified with the linker SANH (succinimidyl-6-hydrazinonicotinate acetone hydrazone) and the target protein/biomolecule is modified with a second linker, SFB

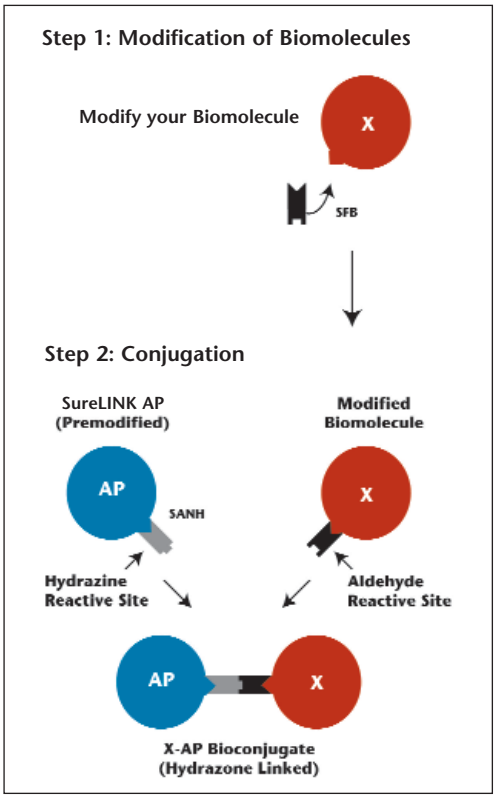

Figure 1. SureLINK Two-step Conjugation Protocol

*Homobifunctional linkers are bi-reactive compounds containing the same functional group at both ends. Heterbiofunctional linkers are bi-reactive compounds with two different reactive groups that can couple to two different functional targets.

(succinimidyl 4-formylbenzoate). A stable AP conjugate is formed when the two modified proteins are subsequently mixed.

Figures 2a and 2b depict the chemical reactions whereby each biomolecule (AP and the target biomolecule) is separately modified using primary amines (in proteins, the N terminus and R groups of lysine residues supply the primary amino groups) and one of two different heterobifunctional modification reagents - SANH or SFB. The primary amine groups are located on the surface of the protein and are easily accessible for reaction without affecting the stability or structure of the protein. SANH introduces a reactive hydrazine group on AP, whereas SFB introduces an active aldehyde group on the target biomolecule. When the two modified biomolecules are mixed, the hydrazine and aldehyde groups react in a specific manner to form a stable hydrazone bond (Figure 3).

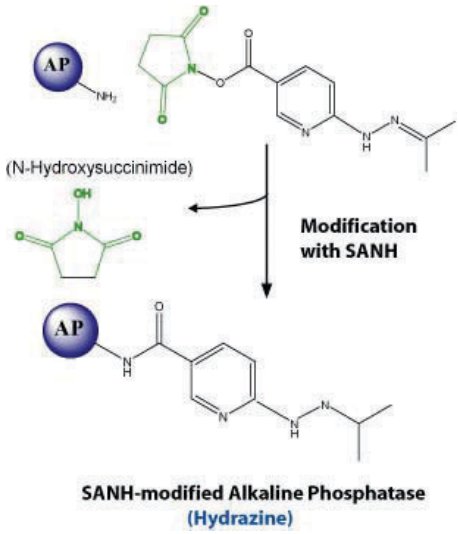

Figure 2a. AP Modification: AP is modified with SANH at the site of primary amines. In this modification reaction, a N-hydroxysuccinimide leaving group from the SANH is removed as part of the chemical reaction. The modification introduces a hydrazine, protected as the acetone hydrazone form.

The SureLINK conjugation chemistry offers several advantages over traditional methods. First, use of this chemistry assures formation of heteroconjugates, *e.g.* AP-Ab, with no formation of homoconjugates, *e.g.* AP-AP or Ab-Ab. Nonspecific interactions and polymerization are precluded by virtue of the reactive moieties on the modified biomolecules. Second, the method provides two points of control, *i.e.*, the number of modifications per target biomolecule in the first step and the ratio of the two modified molecules in the second step. Third, no hazardous reducing agents are required to stabilize conjugates, thereby eliminating reduced functionality of biomolecules. Finally, biomolecules modified with SureLINK heterobifunctional linkers are stable for extended periods.

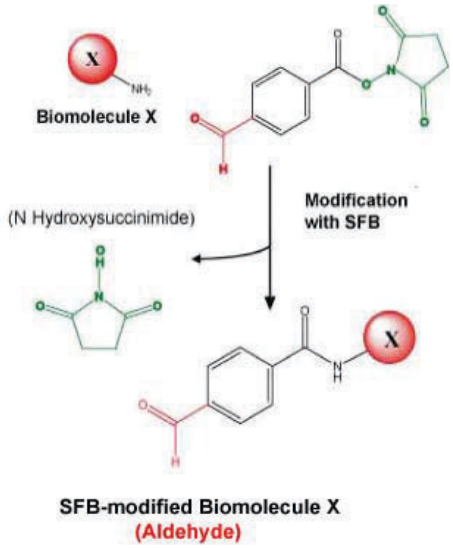

Figure 2b. Target Biomolecule Modification: The primary amines of a protein or biomolecule (X) are reacted with SFB to introduce reactive aldehyde groups at the sites of primary amines. An N-hydroxysuccinimide leaving group is removed as part of the chemical reaction.

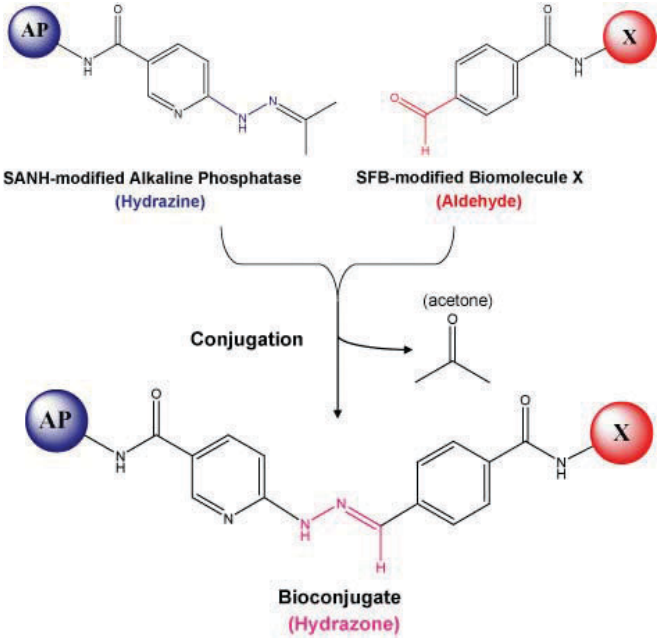

Figure 3. Conjugation: At mildly acidic pH, the acetone protecting group on the hydrazine of SANH is not stable. There is a rapid exchange with an aromatic aldehyde during conjugation to yield a stable bis-aromatic hydrazone. Acetone is removed as a byproduct. AP is covalently linked to biomolecule X.

This application note will demonstrate the performance of SureLINK AP conjugates of a monoclonal antibody, a polyclonal antibody, and streptavidin as immunodetection reagents in ELISA, Western blot and immunohistology applications.

MATERIALS AND METHODS

Materials: The SureLINK AP Conjugation Kit (Catalog.No. 85-00-01) was obtained from KPL. The following materials were also obtained from KPL: polyclonal Goat Anti-Mouse IgG (Catalog No. 01-18-06), Biotin-labeled Goat Anti-Mouse IgG (Catalog No. 16-18-06), Normal Goat Serum (10%) (Catalog No. 71-00-27), 10% BSA Diluent/Blocking Solution Concentrate (Catalog No. 50-61-00), Detector Block (5X) (Catalog No. 71-83-00), Wash Solution Concentrate (Catalog No. 50-63-00), BCIP/NBT Phosphatase Substrate (Catalog No. 50-81-07), BluePhos Microwell Phosphatase Substrate 2-Component (Cat. No. 50-88-02), BluePhos Stop Solution Concentrate (Catalog No. 50-89-00), PhosphaGLO AP Substrate (Catalog No. 55-60-03), HistoMark RED Staining System (Catalog No. 55-69-00) and Contrast GREEN (Catalog No. 71-00-11).

Monoclonal Mouse Anti-Goat IgG was obtained from OEM Concepts (Catalog No. M2-GG16), Streptavidin from Roche (Catalog No. 115 20679 SQ), Mouse IgG from Stellar Biosystems (Catalog No. MIGG), Goat IgG from Cappel (Catalog No. 55926), ELISA plates from VWR (Catalog No. 62409-289), formalin-fixed paraffin-embedded (FFPE) human breast tissue (Catalog No. T1360) and mouse anti-human Epithelial Membrane Antigen Clone E29 (Cat. No. M0613) from DakoCytomation, Permount from Fisher (Catalog No. SP15), Xylene Substitute from Shandon (Catalog No. 9990507), and 100% Alcohol Reagent from VWR (Catalog No. VW0470-3). Precast SDS PAGE (Tris-HCl, 4-20% gradient) gels (Catalog No. 345-0034), protein sample buffer (Laemmli) (Catalog No. 161-0737), buffers for electrophoresis and transfer (Catalog Nos. 161-0771 and 161-0772), nitrocellulose membrane (0.45 µm pore size) (Catalog No. 162-0235), and the blotting apparatus (PowerPac HC) were obtained from Bio-Rad.

Preparation of conjugates: AP conjugates were prepared according to the protocol provided in the SureLINK AP Conjugation Kit. Prior to conjugation, monoclonal mouse anti-goat IgG (0.2 mg), polyclonal goat anti-mouse IgG (0.4 mg), and streptavidin (0.1 mg) were modified with SFB using a 25 molar excess of SFB. AP conjugates of the SFB-modified antibodies were then prepared using 0.2 mg of SANH-modified AP and 0.1 mg each of SFB-modified antibody to give a final ratio of 2:1 AP to antibody. SFB-modified streptavidin was conjugated at 0.03 mg to give a final ratio of AP to streptavidin of 2.5:1. Prior to conjugation, Monoclonal Mouse Anti-goat IgG was dialyzed for 2 hours at 4° C against 0.1M phosphate buffer, pH 7.2, containing 0.15M sodium chloride to remove sodium azide.

Antigen used to test performance of AP conjugates: For demonstration purposes in ELISA and Western blot, mouse IgG and goat IgG were used as antigens for AP conjugates of goat anti-mouse IgG and anti-goat IgG, respectively. Biotinylated goat anti-mouse IgG was used in conjunction with AP-conjugated streptavidin.

Determination of AP conjugate activity in ELISA: Microplate wells were coated in duplicate using 2-fold serial dilutions of antigen starting at a dilution of 10 µg/mL. Wells were blocked with 1% BSA Diluent/Blocking Solution. A 100-µL aliquot of AP conjugate, diluted in 1% BSA Diluent/Blocking Solution, was added to each well and the plate incubated for 30 minutes at room temperature. After washing to remove excess conjugate, 100 µL of BluePhos Microwell Substrate (2-Component) was added to each well and incubated for 15 minutes at room temperature. The reactions were stopped with 100 µL of BluePhos Stop Solution and the optical density at 630 nm was determined using a 96-well plate reader.

Determination of AP conjugate activity in Western Blotting: Mouse or goat IgG was prepared in 1X TBS. Each was then diluted 1:3 in Laemmli buffer without 2-mercaptoethanol to preserve the disulfide bonds in the antibodies. Samples were heated at 95° C for 3 minutes. Ten microliters of the sample were then loaded onto wells of precast SDS PAGE (Tris-HCl, 4-20% gradient) gels. Proteins in the gel were separated by electrophoresis using the procedure recommended by the manufacturer. Proteins in the gel were then transferred to nitrocellulose membranes using manufacturer's suggested settings. Membranes were blocked in 20 mL of Detector Block solution for 1 hour at room temperature with gentle agitation. Conjugates were then added directly to the blocking solution and the gels incubated for 1 hour at room temperature with gentle agitation. Subsequently, membranes were washed in 1X Wash Solution three times for 5 minutes each. For detection with a chromogenic substrate, the blots were incubated for 15 minutes with BCIP/NBT, washed with distilled water for 10 - 20 seconds, and then dried. For detection with a chemiluminescent substrate, the blots were incubated for 1 minute with PhosphaGLO, placed in a hybridization bag, and then exposed to Kodak BioMax film for various time intervals.

Preparation of slides for immunohistochemistry: Slides of formalin-fixed, paraffin-embedded (FFPE) human breast tissue were deparaffinized for 5 minutes each in: Xylene Substitute (Shandon), 100% Reagent Alcohol (VWR), 80% Reagent Alcohol, 40% Reagent Alcohol, 20% Reagent Alcohol, Distilled water, and then 0.1 M Tris HCl, pH 7.6. The slides were then incubated in Normal Goat Serum (KPL) for 10 minutes. A positive control slide was prepared by incubating a slide with mouse anti-human Epithelial Membrane Antigen Clone E29 diluted 1:50 in 1% Normal Goat Serum for 30 minutes at room temperature. A negative control slide was prepared by incubating a slide in Tris HCl for 30 minutes at room temperature. Slides to demonstrate the use of AP conjugates of streptavidin were prepared as follows: slides were incubated in 1 mL of biotinylated goat anti-mouse (2 mg/mL) for 30 minutes, in Tris HCl for 5 minutes, in 1 mL of AP-streptavidin (2 µg/mL in PBS) for 30 minutes, and again in Tris HCl for 5 minutes. Slides to demonstrate the use of AP conjugates of antibodies were prepared as follows: slides were incubated with 1 mL of AP-conjugated polyclonal goat anti-mouse (20 µg/mL in PBS) for 5 minutes followed by incubation in Tris HCl for 5 minutes.

Immunohistochemical staining: Each slide was stained using the HistoMark RED Staining System. Contrast GREEN was used for counterstaining. For HistoMark RED staining, each slide was incubated for 10 minutes in 1 mL of substrate prepared according to the package insert. Slides were then washed in distilled water for 2 minutes. One mL of Contrast GREEN was subsequently added to each slide and each slide was incubated for 10 minutes. Slides were then washed in distilled water for 2 minutes. Finally, the slides were incubated in ammonia water solution (2 mL of concentrated ammonium hydroxide in 1L water) for 10 seconds and rinsed in distilled water for 2 minutes. Slides were briefly rinsed in 80% alcohol, 100% alcohol, and then Xylene Substitute. Slides were then air dried and mounted with Permount.

Determination of enzyme activity of conjugates in solution: The activity of AP conjugates in solution was measured at various substrate concentrations. Serial dilutions (1:2) of BluePhos Microwell Substrate Solution A were prepared in BluePhos Microwell Substrate Solution B. A 100-µL aliquot of each dilution was added to each of 8 wells of a 96-well plate. The reaction was initiated by addition of 10 µL (1 ng/well) of conjugate to each well. After 15 minutes at room temperature, the reactions were stopped with 100 µL of BluePhos Stop Solution and the optical density of each reaction mixture at 630 nm was then read. Each sample was run in duplicate and the average of the optical density responses were plotted and analyzed using Eadie-Hoftsee kinetics. Results were reported as Vmax/Km.

Stability testing of SureLINK AP: Two lots of SureLINK AP (0.2 mg) were stored lyophilized at 4° C. At various times, samples were conjugated to 0.1 mg of polyclonal goat anti-mouse IgG according to the directions in the kit. The resulting AP conjugates were subsequently stored at 4° C and enzyme activity measured as described above.

RESULTS AND DISCUSSION

SureLINK Modified AP was used to prepare conjugates of three frequently used protein binding partners in immunoassays: a polyclonal antibody, a monoclonal antibody and streptavidin. Results presented below show the functionality of the conjugates in ELISA, Western blot, and immunohistology (IHC) applications. For the ELISA and Western Blot assays, a test system was used with IgG as the antigen and an anti-IgG secondary antibody as the antibody. The IHC slides were prepared with an anti-EMA antibody bound to normal human breast tissue. Also shown are the results of stability studies of SureLINK AP conjugates.

Use of SureLINK AP Conjugates in ELISA

Experiments were performed to determine the functionality and sensitivity of SureLINK AP conjugates of goat anti-mouse IgG (polyclonal), mouse anti-goat IgG (monoclonal) and streptavidin in an ELISA format. The antibody conjugates were used to detect antigen that had been serially diluted and coated onto the surface of microwells. The results shown in figure 4a illustrate that the titration curves for both the AP-labeled polyclonal and AP-labeled monoclonal antibodies were similar. The range of detection was between 8 and 500 ng of the IgG antigen demonstrating the sensitivity of the antibody conjugates prepared with SureLINK Modified AP.

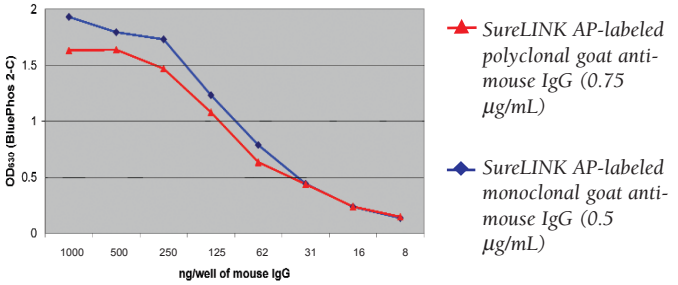

Figure 4a. *Detection of mouse IgG (1000 to 8 ng) in an ELISA format using SureLINK AP-labeled polyclonal goat anti-mouse IgG or SureLINK AP-labeled monoclonal mouse anti-goat IgG and BluePhos Microwell Phosphatase Substrate.*

To test the functionality and sensitivity of SureLINK AP-labeled streptavidin, a constant amount of mouse IgG was used to coat the surface of microwells. Serial dilutions of biotinylated goat anti-mouse IgG ranging from 20 to 0.2 ng were then added to the microwells, and the biotinylated goat anti-mouse IgG was detected with the SureLINK AP-streptavidin conjugate. The results in figure 4b demonstrate detection of biotinylated IgG with the AP-labeled streptavidin over a broad range of biotinylated antibody. In summary, SureLINK conjugates of a polyclonal and a monoclonal antibody as well as streptavidin can be used effectively in an ELISA format to detect antigen.

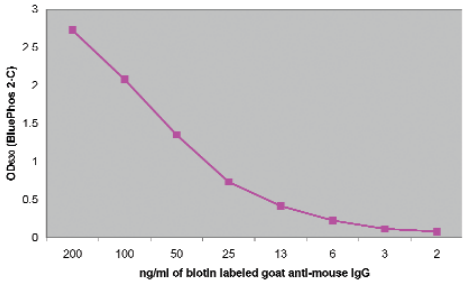

Figure 4b. *Detection of mouse IgG (1 µg/well) in an ELISA format using biotinylated goat anti-mouse IgG (20 to 0.2 ng), SureLINK AP-labeled streptavidin (0.3 µg/mL), and BluePhos Microwell Phosphatase Substrate.*

Use of SureLINK AP Conjugates in Western Blotting

The same three SureLINK AP conjugates were used to detect antigens in Western Blot assays. Antigen preparations were separated on SDS PAGE gels and then transferred to nitrocellulose membranes. Antigens on the membranes were then detected with the AP conjugates and either a chromogenic or chemiluminescent substrate. The results in figure 5 a-d show that SureLINK AP conjugates of a polyclonal or monoclonal antibody or of streptavidin can be used effectively in Western blots. With a chemiluminescent substrate, significantly lower concentrations of AP conjugates were used to detect comparable levels of antigen versus that used with a chromogenic substrate. This is consistent with results typically obtained using chemiluminescent substrates as compared to chromogenic systems.

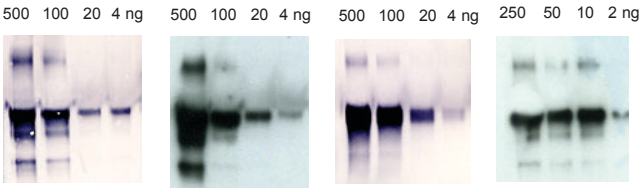

Figure 5a. *Figure 5b.* *Figure 5c.* *Figure 5d.*

Figure 5a: *Western blot prepared using a SureLINK AP conjugate of polyclonal goat anti-mouse IgG (0.1 µg/mL) and BCIP/NBT Phosphatase Substrate to detect mouse IgG ranging from 500 to 4 ng per lane.*

Figure 5b: *Western blot prepared using a SureLINK AP conjugate of polyclonal goat anti-mouse IgG (2.5 ng/mL) and PhosphaGLO AP Substrate to detect mouse IgG ranging from 500 to 4 ng per lane. The film was exposed to the blot for 10 minutes.*

Figure 5c: *Western blot prepared using a SureLINK AP conjugate of monoclonal mouse anti-goat IgG (0.1 µg/mL) and BCIP/NBT Phosphatase Substrate to detect Goat IgG ranging from 500 to 4 ng per lane.*

Figure 5d: *Western blot prepared using a SureLINK AP conjugate of Streptavidin (0.5 ng/mL), biotinylated goat anti-mouse IgG (25 ng/mL) and PhosphaGLO AP Substrate to detect mouse IgG ranging from 250 to 2 ng per lane. The film was exposed to the blot for 2 minutes.*

Use of SureLINK AP Conjugates in Immunohistology

Epithelial membrane antigen (EMA) in human breast tissue was detected using mouse anti-human EMA and either 1) SureLINK AP conjugated polyclonal goat anti-mouse IgG or 2) biotinylated goat anti-mouse IgG and SureLINK AP-conjugated streptavidin. HistoMark RED was used as substrate and tissue was counterstained with Contrast GREEN. EMA is a normal protein in secretory and ductal epithelial cells of the skin, breast and other glandular organs; it may be a marker for adenocarcinoma in other organs.[2]

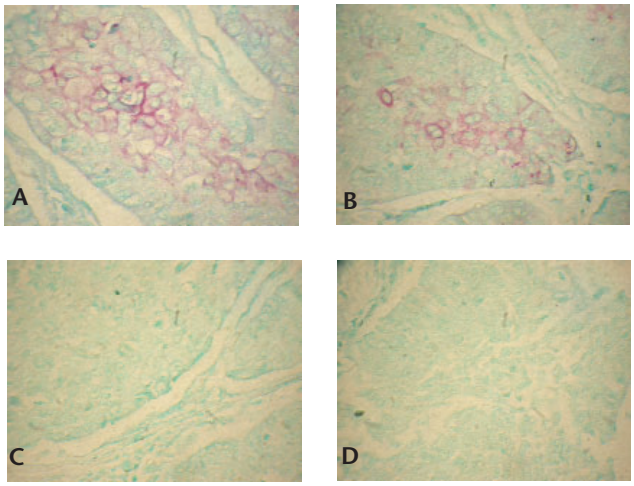

Figure 6. Detection of EMA in human breast tissue using mouse anti-EMA primary antibody and either SureLINK AP-conjugated goat anti-mouse IgG, 20 µg/mL (A) or biotinylated goat anti-mouse IgG and AP-conjugated streptavidin, (B). HistoMark RED was used as the AP substrate and Contrast GREEN was used as the counterstain. No primary antibody was added to the control slides (C and D).

The results shown in figure 6 demonstrate detection of EMA with both conjugates (figures 6A and 6B); results were similar for both. EMA in the breast tissue was stained brilliant pink whereas nuclei were counterstained blue-green. EMA was not detected in the control slides where the primary antibody was omitted. Only the blue-green color of the counterstain was visible. As expected, the use of a biotin/streptavidin system decreased the amount of AP conjugate needed for detection. Based on the results described above, SureLINK AP conjugates of polyclonal antibodies and streptavidin can be used to facilitate the detection of tissue-specific markers in IHC applications.

Stability of SureLINK AP-Antibody Conjugates

The results depicted in figure 7 demonstrate the stability of conjugates made with SureLINK AP Conjugation Kits. Conjugates of goat anti-mouse IgG were prepared with SureLINK Modified AP and then stored at 4° C. AP activity was determined at intervals during an 8-month period. The results demonstrate the stability of the conjugates over at least an 8-month period SureLINK AP conjugates are kinetically active even after prolonged storage at 4° C.

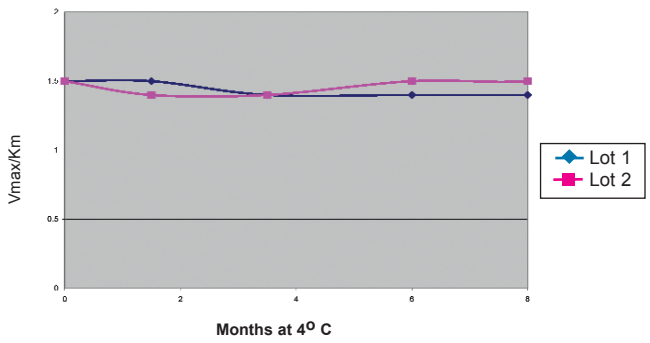

Figure 7. AP activity (Vmax/Km) of two lots of SureLINK AP-conjugated Goat anti-Mouse IgG after storage at 4° C for the time intervals indicated.

CONCLUSIONS

SureLINK AP conjugation chemistry enables the facile preparation of AP conjugates. The resultant SureLINK AP conjugates are broadly useful in immunoassays. These include formats such as ELISA, Western blotting, and IHC applications as described herein but could well be expanded beyond the realm of such formats to other applications where AP is a suitable reporter.

As demonstrated in this application note, SureLINK chemistry can be used to prepare conjugates of both monoclonal and polyclonal antibodies and other proteins such as streptavidin. Although the chemistry has been optimized for use with proteins, it can readily be used with any biomolecules containing primary amines or aldehydes as reactive sites. Furthermore, its use can be expanded to biomolecules lacking either amine or aldehyde reactive groups by derivatizing such molecules, e.g., amine modifications of nucleic acids.

SureLINK AP conjugation chemistry overcomes many of the limitations of other conjugation methods. The method is based on reactions of the heterobifunctional linkers SANH and SFB with the amino groups of two proteins. Unwanted cross-linking is virtually eliminated by the formation of highly specific hydrazone linkages. No reducing agents are required to stabilize the bond. Disulfide bonds are not reduced and proteins are not denatured during conjugation. A forthcoming KPL application note will compare the performance of SureLINK AP conjugates with those produced using glutaraldehyde and maleimide/thiol chemistry.

The fast, reliable, and reproducible protocol also facilitates broad application of AP as a reporter enzyme. In 3 hours with less than 20 minutes of 'hands on' lab time, the reaction is complete and conjugates are ready for immediate use, as no further purification is required. Moreover, once prepared, SureLINK AP conjugates remain stable, *i.e.*, functionally active, in solution at 4° C for extended periods.

REFERENCES

1. Hermanson, Greg T., (1996) Bioconjugate Techniques.

2. ten Berge, R. L., Snijdewint, F. G., von Mensdorff-Pouilly, S. et al. MUC1 (EMA) is preferentially expressed by ALK positive anaplastic large cell lymphoma, in the normally glycosylated or only partly hypoglycosylated form. *J Clin Pathol* 2001; 54:933-939.

Related Products

Description	Size	Catalog No.
SureLINK™ AP Conjugation Kits Each kit includes SureLINK Modified AP, AP Modification Buffer, SFB, AP Conjugation Buffer and AP Storage Buffer.		
SureLINK AP Conjugation Kit	3 x 0.5 mg rxn.	85-00-02
SureLINK AP Conjugation Kit	3 x 0.1 mg rxn.	85-00-01
SureLINK HRP Conjugation Kits Each kit includes SureLINK Activated HRP, HRP Conjugation Buffer, Reducing Reagent and HRP Storage Buffer.		
SureLINK HRP Conjugation Kit	6 x 1 mg rxn.	84-00-02
SureLINK HRP Conjugation Kit	6 x 0.1 mg rxn.	84-00-01
SureLINK HRP Conjugation Kit	2 x 0.1 mg rxn.	84-00-03
SureLINK Activated HRP and Modified AP		
SureLINK Activated HRP	1.5 mg	84-01-02
SureLINK Activated HRP	0.3 mg	84-01-01
SureLINK Modified AP*	1.0 mg	85-01-02
SureLINK Modified AP*	0.2 mg	85-01-01
SFB Linker	100 mg	80-02-04
SFB Linker	20 mg (4 x 5 mg)	80-02-02

*SureLINK Modified AP must be used with SFB Linker for successful conjugation of proteins.

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