KPL SureLINK™ AP Conjugation Manual

Products	Catalog No.	Size
KPL SureLINK AP Conjugation Kit	(5610-0025) 85-00-01	3 x 0.1 mg rxn.
	5610-0026 (85-00-02)	3 x 0.5 mg rxn.
KPL SureLINK Modified AP	5620-0029 (85-01-02)	1.0 mg

TABLE OF CONTENTS

PRODUCT DESCRIPTION

KPL 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 the antibody with a cross-linker that carries a sulfhydryl group. This approach has several drawbacks, including the needs of disulfide bond reduction which could

inactivate the binding ability of antibody to antigen. An additional step is required to produce a reactive sulfhydryl group when using a cross-linker to introduce sulfhydryl moieties. Furthermore, EDTA, required to stabilize the maleimide reaction,[8] inhibits the divalent metal cation-dependent enzymatic activity of the alkaline phosphatase.

PRODUCT OVERVIEW

KPL 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-hydroxysuccinimide (NHS) ester group. The SFB modified antibody/protein can be conjugated with the supplied KPL SureLINK AP. The KPL SureLINK AP is stable in lyophilized form. The conjugation reaction can be completed in 3 hours, with less than 20 minutes of actual hands-on time.

KPL SureLINK™ AP Conjugates can be used in Western blotting, ELISA, and immunohistochemistry applications. Other applications utilizing the AP conjugates may also be possible. See page 12 for recommended dilutions of each application and page 15 for related products to each application.

PRODUCT COMPONENTS

5610-0025 (85-00-01), KPL SureLINK AP Conjugation

Kit Component	Part Number	Size	Quantity
KPL SureLINK Modified AP	5620-0028 (85-01-01)	0.2 mg	3
KPL AP Modification Buffer	5640-0011 (85-02-01)	5 mL	1
KPL SFB	5640-0003 (80-02-01)	0.2 mg	3
KPL AP Conjugation Buffer	5640-0012 (85-03-01)	1.5 mL	1
KPL AP Storage Buffer	5640-0013 (85-04-01)	5 mL	1

5610-0026 (85-00-02), KPL SureLINK AP Conjugation

Kit Component	Part Number	Size	Quantity
KPL SureLINK Modified AP	5620-0029 (85-01-02)	1.0 mg	3
KPL AP Modification Buffer	5640-0011 (85-02-01)	5 mL	1
KPL SFB	5640-0003 (80-02-01)	0.2 mg	3
KPL AP Conjugation Buffer	5640-0012 (85-03-01)	1.5 mL	1
KPL AP Storage Buffer	5640-0013 (85-04-01)	5 mL	1

5620-0029 (85-01-02), KPL SureLINK Modified AP

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

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

STORAGE AND STABILITY

- KPL SureLINK™ AP Conjugation Kits are shipped at 2-8°C.
- Store all components at 2-8° C upon receipt.
- For maximum stability, store KPL SFB under desiccation.
- KPL SFB should be rehydrated just before use. Any excess material should be discarded.
- KPL Modified AP should be rehydrated just before use. Excess material should be discarded.
- All components of the KPL SureLINK AP Conjugation kits are stable for at least one year when stored as directed.

BEFORE YOU BEGIN

SAFETY AND HANDLING

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

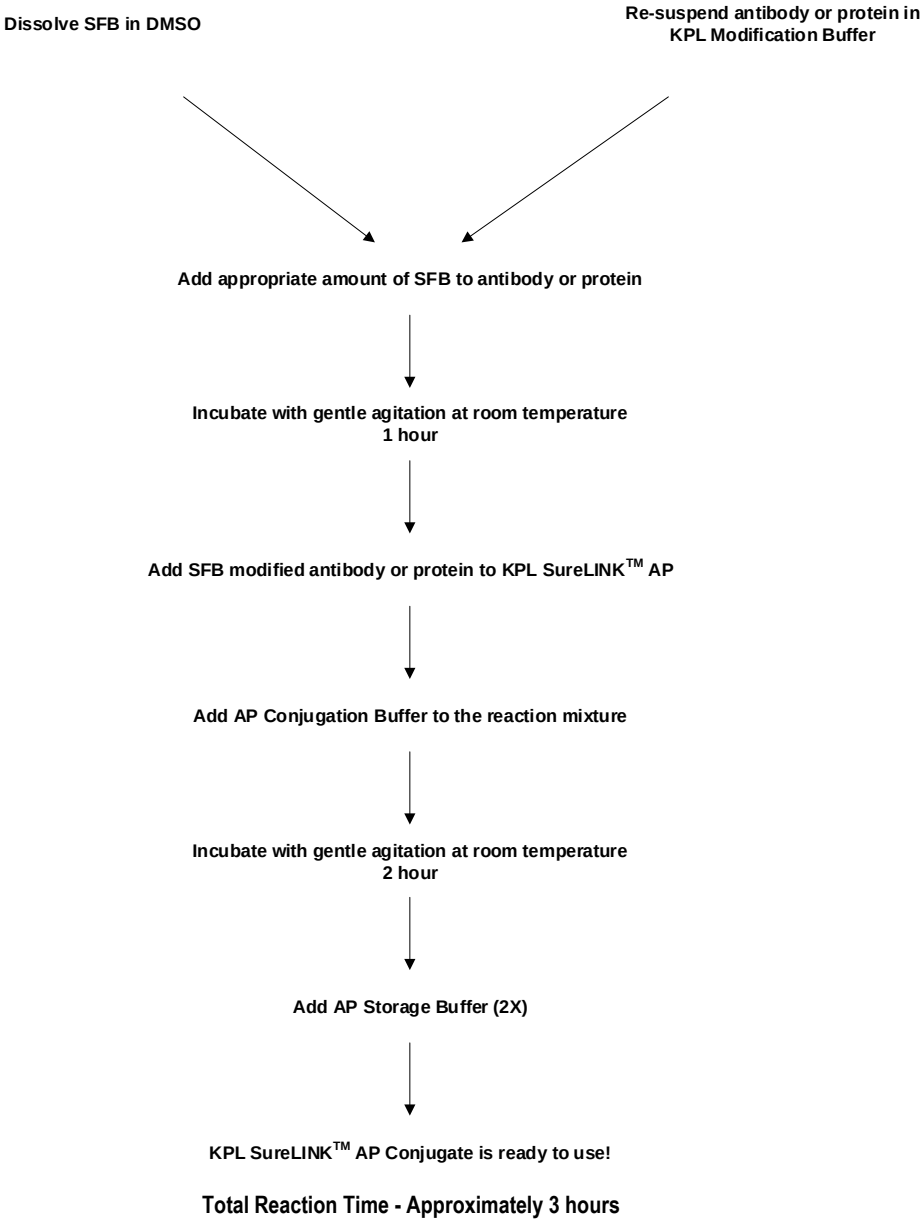
OTHER REQUIRED SUPPLIES AND EQUIPMENT

- 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

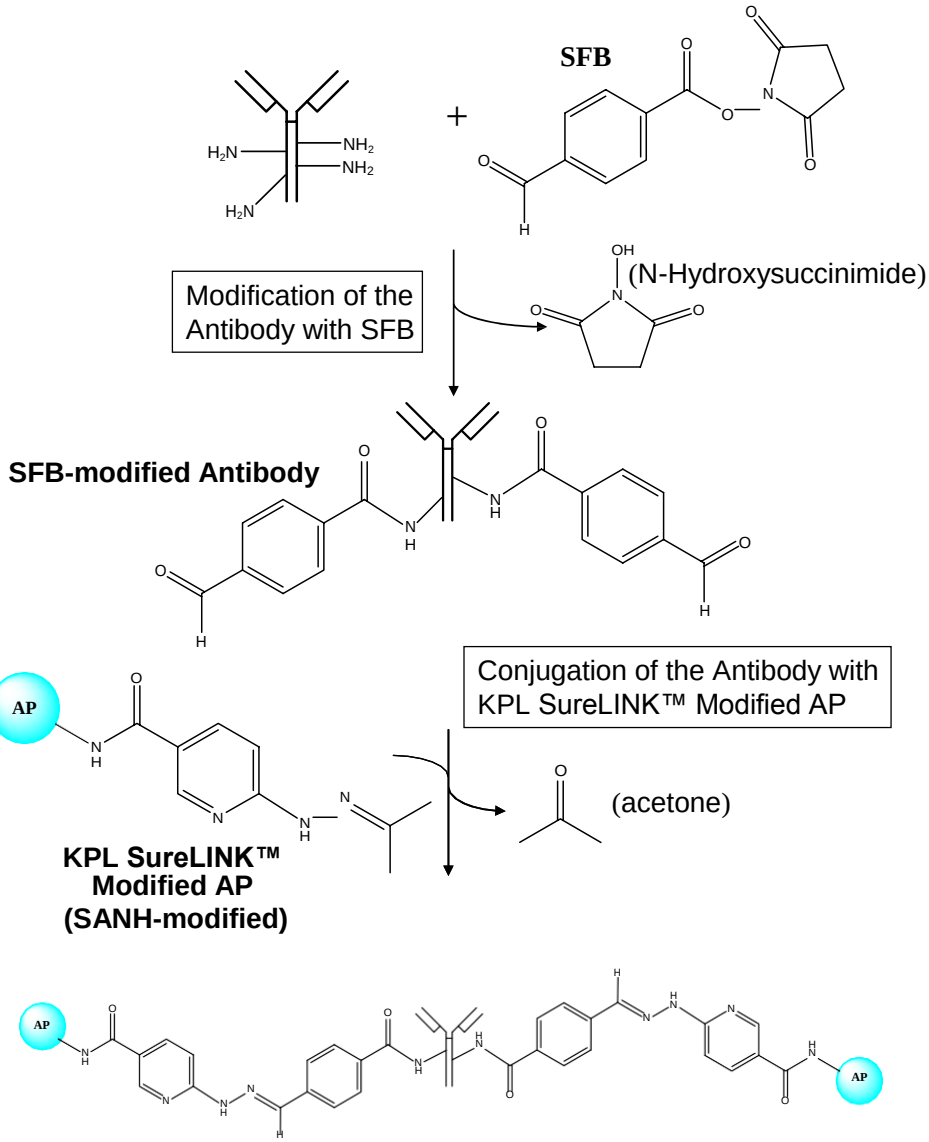
- Equilibrate each buffer to be used in modification and conjugation reactions to room temperature. If precipitations are visible in the buffers following the storage at 4°C, incubate the buffers in a water bath (50°C to 60°C) to dissolve the precipitate.
- 5X Conjugation Buffer will be diluted to 1X 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



Alkaline Phosphatase-Antibody Conjugate

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 KPL AP Modification Buffer and KPL AP Conjugation Buffer at room temperature before use.**

2. **Rehydrate the lyophilized antibody (free of salts) with KPL 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 0.1 M sodium phosphate buffer, pH 7.2 in 0.15 M 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 KPL 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 KPL SFB to the antibody or protein sample. Recommended molar ratio of KPL SFB:Antibody is 25:1. Incubate for 1 hour at room temperature or overnight at 4°C with gentle agitation.**

See Table 1, page 10 for guidelines on KPL SFB amounts needed to modify specific quantities of antibody. Titrating the amount of KPL SFB may enhance the efficiency of the antibody or protein labeling reaction, depending on the availability of reactive amine moieties. If excess KPL 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 KPL 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 10 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 4°C. 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 KPL 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 KPL 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 KPL AP Storage Buffer (2X) to the reaction and mix gently. The conjugate is now ready to use.**

See page 12 for recommended starting dilutions of the conjugates for ELISA, Western blotting, and immunohistology assays.

CALCULATIONS

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

*Calculations are based on IgG and SFB with the molecular weight of 160 kDa and 247.1 g/mole respectively. Optimal molar ratio of **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)	KPL SFB (nmoles)	Amount of 4 nmole/µL KPL SFB to Add to IgG Solution
0.05 mg	0.3	7.5	1.9 µL
0.1 mg	0.6	15	3.8 µL
0.2 mg	1.25	30	7.5 µL
0.5 mg	3	75	19 µL

Table 2: Amount of KPL SureLINK Modified AP to Add to IgG-SFB Sample

*Calculations are based on SureLINK Modified AP with the molecular weight of 140 kD. Optimal molar ratio of **Modified AP:Antibody-SFB is 2.0-2.5:1**. Titrating the modified AP may be required to enhance efficacy of the conjugates. Reducing the modified AP:antibody 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.*

IgG-SFB Amount	IgG-SFB (nmoles)	KPL SureLINK Modified AP (nmoles)	KPL SureLINK Modified AP Amount	Product Choices and Preparation of KPL SureLINK Modified AP	Volume of KPL SureLINK Modified AP to Add to Reaction
0.05 mg	0.3	0.7	0.1 mg	Rehydrate 1 vial of 0.2 mg AP (5620-0028) in 200 µL water	100 µL
0.1 mg	0.6	1.3	0.2 mg	Use 1 vial of 0.2 mg AP (5620-0028) in powder form	Add Antobody-SFB mix directly to AP
0.2 mg	1.2	2.6	0.4 mg	Rehydrate 1 vial of 1 mg AP (5620-0029) in 200 µL water	80 µL
0.5 mg	3	6.6	1 mg	Use 1 vial of 1 mg AP (5620-0029) in powder form	Add Antibody- SFB mix directly to AP

Example Protocol Using KPL SureLINK™ AP Conjugation Kit:

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

1) Re-hydrate 0.2 mg IgG (1.25 nmole) sample with 200 µL of KPL AP Modification Buffer.
2) Dissolve KPL 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 KPL 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 KPL 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 KPL 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°C.

RECOMMENDED USE OF CONJUGATES

KPL 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 SeraCare. A listing of products is described in the Related Products section. Visit www.seracare.com for a complete listing or for additional information contact KPL Technical Services at kpltechserv@seracare.com.

TROUBLESHOOTING GUIDE

Problem 1: Weak Level of Detection in Immunoassay

Causes and/or Observations	Possible Solutions
• Inactive KPL SureLINK AP samples	Check the expiration date and follow the storage condition of each component in the kit.
• Low signal and/or high background levels	Titrate the amount of the conjugate in the assay and optimize the signal to noise ratio.
• Other proteins are present in the Ab sample, thus 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.
• The NHS-ester bond of the SFB cross-linker has been hydrolyzed during storage, compromising the labeling reaction.	Upon receipt, store the KPL SFB in a dessicator. Use anhydrous DMSO to dissolve the KPL SFB powder and store in a dessicator. Otherwise, dissolve the KPL SFB powder immediately prior to each use.
• 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.
• 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.
• The concentration of the antibody in the modification reaction may have been over-estimated, resulting in excess	Use triplicate absorbance measurements to determine the Ab sample before the addition of KPL SFB.

Causes and/or Observations	Possible Solutions
amount of KPL SFB that can readily competes in the conjugation reaction	Dialyze the Ab-SFB sample against a 0.1 M 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
• 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.
• 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.seracare.com. For additional assistance, contact KPL Technical Services at kpltechserv@seracare.com.

RELATED PRODUCTS

Product/Application Group	Product Name	Size	Catalog Number
Protein Labeling Kits & Reagents	KPL SureLINK™ HRP Conjugation Kit	6 x 0.1mg rxn	5610-0022 (84-00-01)
	KPL SureLINK™ HRP Conjugation Kit	6 x 1.0 mg rxn	5610-0023 (84-00-02)
	KPL SureLINK™ HRP Conjugation Kit	2 x 0.1 mg rxn	5610-0024 (84-00-03)
	KPL Spin-Pure Filters (10K MWCO)	5 per pack	5640-0001 (60-00-53)
ELISA Products	KPL BluePhos™ AP Microwell Substrate	600 mL	5120-0059 (50-88-00)
	KPL APStop™ Solution	200 mL	5150-0026 50-89-00)
	KPL *p*NPP AP Microwell Substrate	500 mL	5120-0056 50-80-00)
Western Blot	KPL PhosphaGLO™ AP Substrate	100 mL	5430-0055 (55-60-04)
	KPl PhosphaGLO Reserve™ AP Substrate	100 mL	5430-0053 (55-60-02)
	KPl BCIP/NBT 1-Component AP Substrate	100 mL	5420-0038 (50-81-18)
Immunohistochemistry	KPl HistoMark™ RED AP Substrate	1000 slides	5510-0036 (55-69-00)
	KPL HistoMark™ BLUE AP Substrate	1000 slides	5510-0037 (55-70-00)
Support Reagents	KPL 20X Wash Solution	800 mL	5150-0008 (50-63-00)
	KPL 10X Coating Solution	50 mL	5150-0014 (50-84-00)
	KPL AP Stabilizer	200 mL	5290-0007 (55-15-00)
	KPL 10% BSA Diluent/ Blocking Solution	200 mL	5140-0006 (50-61-00)
	KPl Milk Diluent/Blocking Solution	200 mL	5140-0011 (50-82-01)
	KPL 5X Detector Block Solution	240 mL	5920-0004 (71-83-00)

REFERENCES

1. Kim, E.E., and Wyckoff, H.W. (1991) Reaction Mechanism of Alkaline Phosphatase Based on Crystal Structures. Two-Metal ion Catalysis. *J. Mol. Biol.* **218** (2), 449-464.

2. Manes, T., et. al (1998) Genetic Complexity, Structure, and Characterization of Highly Active Bovine Intestinal Alkaline Phosphatase. *J. Biol. Chem.* **273**, 23353-23360.

3. Engvall, E et al., (1971) Enzyme-Linked Immunosorbent Assay.II. Quantitative Assay of Protein Antigen, Immunoglobulin G, by Means of Enzyme-Labelled Antigen and Antibody-Coated Tubes. *Biochim. Biophys. Acta.* **251** (3), 427-434.

4. Ghosh, S.S. *et al*., (1989) Synthesis of 5'-Oligonucleotide Hydrazide Derivatives and their use in Preparation of Enzyme-Nucleic Acid Hybridization Probes. *Anal. Biochem.* **178**, 43-51.

5. Avrameas, S. (1969) Coupling of Enzyme to Proteins with Glutaraldehyde. *Immunochemistry* **6**, 43-52.

6. Teale, J. M., and Kearney, J.R.(1986) Clonotypic Analysis of the Fetal B Cell Repertoire: Evidence for an Early and Predominant Expression of Idiotypes Associated with the VH 36-60 Family *J. Mol. Cell. Immunol.* **2**, 283-292.

7. Migneault, I., et al., (2004) Glutaraldehyde: Behavior in Aqueous Solution, Reaction with Proteins, Application to Enzyme Crosslinking. *BioTechniques* **254**, 203.

8. Yoshitake *et al.*, (1979) Conjugation of Glucose Oxidase from *Aspergillus Niger* and Rabbit Antibodies using N-hydroxysuccinimide Ester of N-(4-carboxycyclohexylmethyl) Maleimide. *Eur. J. Biochem.* **101**, 395-399.

9. Schwartz, D. A. (2004) Hydrazine-Based and Carbonyl-Based Bifunctional Crosslinking Reagents, USPTO 6,800,728.

For Research Use Only

The products listed herein are for research use only and are not intended for use in human or clinical diagnosis.

Trademarks

KPL BluePhos, KPL HistoMark,KPL PhosphaGLO, and KPL SureLINK are trademarks of SeraCare Life Sciences. KPL SureLINK™ Kits and reagents are produced with components protected by U.S. Patents 6,800,728, 5,679,778, 5,420,285, 5,753,520 and 5,206,370.

Disclaimer

The recommendations of this bulletin are provided solely for the benefit of users who need practical guidance on immunoassay procedures. Because experimental conditions for the use of the suggested products are beyond the control of SeraCare, it is impossible for SeraCare to implicitly guarantee the performance of the mentioned products for any and all assay procedures. Users who need additional information or technical support should email Technical Services at kpltechserv@seracare.com for assistance.

SeraCare Life Sciences

508.244.6400 • 800.676.1881 Toll Free • 508.634.3334 Fax
www.seracare.com

L-1004176-01 August 2018