KPL APPLICATION NOTE

Preparation of Conjugates with SureLINK™ HRP and Their Use in Immunoassays

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INTRODUCTION

Horseradish peroxidase (HRP)[1] is widely used in enzyme-based immunodetection techniques such as ELISA, Western blotting and immunohistochemistry to detect and characterize analytes. HRP is a glycoprotein with a molecular weight of approximately 40 kDa that has a high rate of substrate turnover[2]. The commercial availability of sensitive colorimetric and chemiluminescent substrates makes HRP an invaluable tool in biological research. The enzyme is naturally modified: it has eight carbohydrate side chains that can be oxidized with sodium periodate ($NaIO_4$), producing reactive aldehyde groups that readily couple with primary amines in proteins via a Schiff base linkage[3]. Following the reduction of the Schiff base bond, the conjugates are stable for extended periods of time. This modification has minimal effect on HRP activity. The performance of conjugates prepared with $NaIO_4$-activated HRP is superior to that of conjugates prepared by other common conjugation methods[4].

The periodate method is an efficient means to conjugate peroxidase and protein though it has several drawbacks.

- Reaction conditions including the concentration of $NaIO_4$, activation time, pH, and temperature must be optimized and controlled so as to minimize self-polymerization of HRP. The variability of the activated HRP preparations affects conjugation efficiency and reproducibility of the reaction.
- The stability of the activated HRP is highly dependent on the successful removal of the excess $NaIO_4$, subsequent to the activation reaction. Gel filtration or dialysis steps are required to remove the excess $NaIO_4$ and may result in lower yield of conjugate and loss of enzymatic activity.
- Finally, activated HRP preparations are not stable for longer than a few days, requiring the preparation of freshly activated HRP for each individual conjugation reaction.

KPL has overcome these limitations by providing SureLINK™ HRP in a ready-to-use, lyophilized form. No activation or purification is required. Conjugation of HRP to protein or antibodies occurs in 90 minutes elapsed time (with less than 20 minutes hands-on time). The activated HRP is packed in small, single-use vials and is stable for at least one year at 4°C. The SureLINK™ HRP Conjugation Kit contains all required reagents including Conjugation Buffer, Reducing Reagent and Storage Buffer. The conjugate preparations are stable for at least 6 months at 4°C with the use of the provided Storage Buffer. Each SureLINK™ HRP Kit contains sufficient reagents to conjugate as little as 50 µg of antibody per reaction, thus enabling users to prepare HRP-Ab conjugates with very small amounts of precious antibody samples.

Figure 1 demonstrates the labeling scheme for SureLINK™ HRP. In the first step, the activated HRP is added to the protein followed by additional of the Reducing Reagent, which stabilizes the covalent bonds between the protein and HRP.

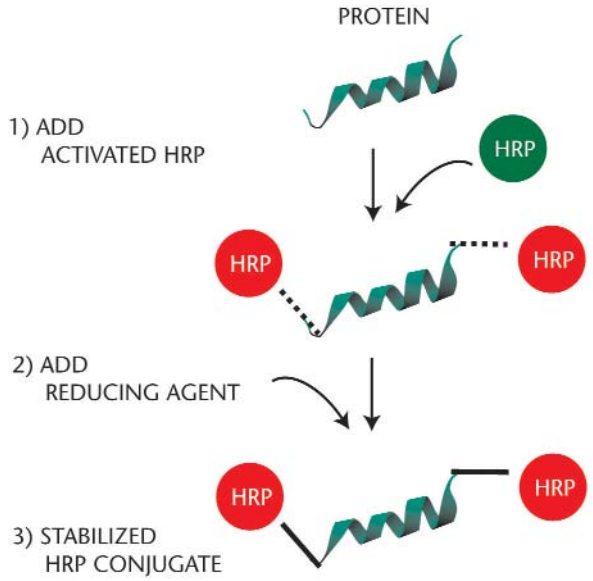

Figure 1: Labeling of Protein with SureLINK™ HRP

MATERIALS AND METHODS

The SureLINK™ HRP Conjugation Kit was obtained from KPL (Catalog #84-00-01). HRP substrates, including ABTS (1-C) (Catalog #50-62-00), TrueBlue (Catalog #71-00-67), DAB (Catalog #54-10-00), LumiGLO® (Catalog #54-61-02), and TMB Membrane substrate (Catalog #50-77-03) were also from KPL. Slides with formalin-fixed paraffin embedded human breast tissue (DakoCytomation) were deparaffinized with Xylene Substitute (Shandon, Catalog #9990507). SDS-PAGE protein gel and Western blotting reagents and supplies obtained from BioRad Laboratories were used according to the manufacturer's instructions.

Preparation of HRP conjugates: An HRP conjugate of Mouse Monoclonal Anti-goat IgG (OEM Systems, 5 mg/mL) was prepared using the SureLINK™ HRP kit as described in the product manual. Prior to conjugation, the monoclonal antibody was dialyzed against HRP Conjugation Buffer. A polyclonal antibody to mouse IgG was similarly prepared except that the lyophilized antibody was resuspended in HRP conjugation buffer and used directly. Mouse anti-human epithelial membrane antigen (EMA) monoclonal antibody (DakoCytomation) was conjugated to HRP.

Preparation of HRP-streptavidin conjugate: For preparation of HRP conjugated streptavidin (SA), lyophilized recombinant streptavidin (Roche Inc.) was rehydrated with the HRP Conjugation Buffer to 0.35 mg/mL. HRP-SA conjugate samples were prepared as described in product manual using a 4:1 or 2:1 molar ratio of HRP:SA. The HRP-SA conjugate used for the IHC assays was prepared as described above with minor modifications. The molar ratio of HRP:SA was maintained at 5:1 in the conjugation reaction, and the reaction was incubated for 4 hours at room temperature.

Determination of HRP conjugate activity in ELISA: For direct ELISA assays, microplate wells were coated in duplicate with 0.1 mL of antigen using two-fold serial dilutions of goat or mouse IgG starting at a dilution of 10 µg/mL. Plates were blocked with 1% BSA, washed with 2% sucrose, and stored desiccated at room temperature. HRP-labeled monoclonal anti-goat or HRP-labeled polyclonal anti-mouse IgG were added to each well at 5 µg/mL and 2.5 µg/mL, respectively. After 30 minutes of incubation at room temperature, each well was washed using 1X Wash Solution (KPL Catalog #50-63-00). The conjugate was detected using ABTS substrate at room temperature, and after the 15 minute incubation the optical density at 405 nm was measured using a 96-well plate reader.

For indirect ELISA assays, microplate wells were similarly coated using 1 µg of mouse IgG. Biotinylated goat anti-mouse IgG was added to each well in two-fold serial dilutions (starting at 2 µg/mL) and incubated for 30 minutes at room temperature. The wells were washed with 1X Wash Solution and incubated for 30 minutes with HRP-Streptavidin at 4:1 and 2:1 at 500 ng/mL. After washing, the detection reactions were initiated by addition of ABTS Substrate.

Stability testing of SureLINK™ HRP: Goat anti-mouse IgG conjugated to SureLINK™ HRP (as described above) was stored at 4°C for various lengths of time. As a control for each time point, a conjugate aliquot was stored at -20°C. Plates were coated with mouse IgG at 2.5 µg/mL. Conjugates (1µg/mL) were added to the plate and incubated for 30 minutes. After washing, the plates were incubated for 10 minutes with ABTS containing a range of concentrations of H_2O_2. Michaelis-Menton kinetic parameters Vmax and Km were calculated using Eadie-Hofstee plots and the efficiency of each conjugate was determined as Vmax/Km. Relative stability was defined as the ratio of the efficiency of conjugates prepared from the 4°C-stored HRP to the efficiency of the control conjugates.

Determination of HRP conjugate activity in Western blotting: Goat or mouse IgG were separated by SDS-PAGE (Tris-HCl, 4-20% gradient) at 200V for 45 minutes. Proteins on the gels were transferred onto a 0.45 µm nitrocellulose membrane at 100 V for 33 minutes and blocked with 1X Detector Block solution (KPL) with 1% Detector Block Powder (KPL). For chemiluminescent detection, HRP conjugates of polyclonal goat anti-mouse and monoclonal mouse anti-goat IgG were diluted into Detector Block solution at final concentrations of 0.0125 µg/mL and 0.05 µg/mL, respectively. For colorimetric detection, the concentration of each conjugate was increased four-fold. Antigens were detected using LumiGLO® Chemiluminescent Substrate.

For detection with HRP-SA, the membrane was incubated for 1 hour with 0.05 µg/mL of biotinylated Goat anti-Mouse IgG in Detector Block solution. The membrane was washed 3 times, and incubated for 20 minutes with 0.05 ug/mL of HRP-SA in Detector Block Solution prepared using a 4:1 molar ratio of HRP:SA.

Preparation of slides for immunohistochemistry: Formalin-fixed, paraffin-embedded human breast tissue was deparaffinized using Xylene Substitute. The samples were rehydrated by soaking in different ethanol solutions for 10 minutes: starting from 100%, 80%, 40%, 20% and ending in deionized water. Slides were placed in 0.1M Tris-HCl, pH 7.6 for 10 minutes and endogenous peroxidase activity was blocked by incubating samples in KPL Blocking Solution (Catalogue #71-00-61) for 4 minutes and rinsing with distilled water. Slides were soaked in Tris-HCl for 5 minutes and blocked with 10% Normal Goat Serum (Catalog # 71-00-27) for 10 minutes. Slides were treated with mouse monoclonal anti-human EMA antibody (DakoCytomation) diluted in 1% Normal Goat Serum (4.8µg/mL) for 10 minutes and washed in Tris-HCl for 5 minutes. The primary antibody was omitted from the control slides.

Detection of epithelial membrane antigen E29 in human breast tissue using SureLINK™ HRP-SA conjugate: Breast tissue slides were incubated for 10 minutes with biotinylated goat anti-mouse IgG (KPL Catalog #71-00-29), washed in Tris-HCl for 5 minutes, incubated for 10 minutes with 2.5µg/mL of HRP-SA using a 5:1 molar ratio of HRP:SA, and washed in Tris-HCl for 5 minutes. The slides were incubated with TrueBlue Peroxidase Substrate for 10 minutes, followed by Contrast Red for counterstaining as described in the product

datasheets. Images were visualized using a compound light microscope. Similar procedures were used for DAB/Contrast Green staining.

RESULTS AND DISCUSSION

STABILITY OF SureLINK™ HRP CONJUGATES

SureLINK™ Activated HRP is stable for extended periods of time at refrigerated temperatures. The results depicted in Figure 2 demonstrate the stability of the lyophilized reagent. Conjugates prepared with SureLINK™ Activated HRP stored at 4°C for 12 months were >90% as active as conjugates prepared with fresh SureLINK™ Activated HRP.

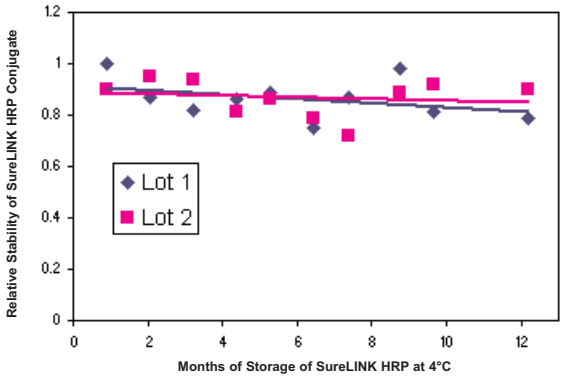

Figure 2: Stability of SureLINK™ Activated HRP. Two lots of the product were stored at 4°C for the indicated time intervals and then conjugated to antibody. Relative stability measurements were performed as described in Materials and Methods section.

USE OF SureLINK™ HRP CONJUGATE IN ELISA ASSAYS

SureLINK™ HRP conjugates can be used in a variety of ELISA assays. The use of these conjugates in a direct ELISA assay is illustrated in Figure 3. Mouse IgG is coated onto a plate and then probed with an antibody-enzyme conjugate. SureLINK™ HRP conjugates of monoclonal and polyclonal antibodies detected low nanogram amounts of IgG.

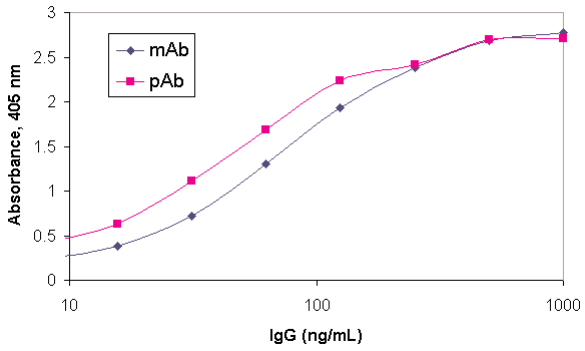

Figure 3: Detection of IgG (1-100 ng) in direct ELISA format with SureLINK™ HRP conjugates of monoclonal anti-goat IgG and polyclonal anti-mouse IgG antibodies.

SureLINK™ HRP conjugates can also be used in an indirect ELISA assay. In one example mouse IgG is coated onto a plate and then probed with a biotinylated antibody. After washing, the biotinylated antibody/antigen complex is detected with a streptavidin-HRP conjugate. (Figure 4)

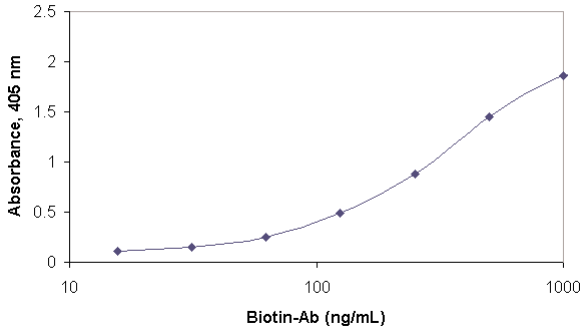

Figure 4: Detection of IgG (1 µg/well) in indirect ELISA format using biotinylated antibody (500 ng/ml), SureLINK™ HRP-streptavidin conjugate and ABTS Substrate.

USE OF SureLINK™ HRP CONJUGATES IN WESTERN BLOTS

The same three SureLINK™ HRP Conjugates were used to detect antigens on Western blot assays. Varying amounts of IgG antigen were separated on SDS-PAGE gels and transferred to nitrocellulose membranes. Antigens (panels A, B) were detected with the SureLINK™ antibody-HRP conjugate and LumiGLO Chemiluminescent substrate (Figure 5). With SureLINK™ HRP Conjugates, as little as 4 ng IgG was detected on Western blots.

Similarly, nitrocellulose membranes with varying amounts of IgG antigen were prepared and detected with an antibody-biotin conjugate followed by a streptavidin-HRP conjugate (panel C). As little as 2 ng IgG antigen could be detected.

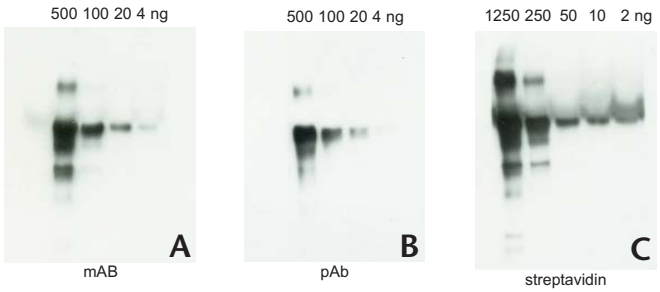

Figure 5. Western blots were prepared with various amounts of IgG and probed with the HRP conjugates with mAb, pAb and SA, respectively (panels A, B, C). LumiGLO substrate was used for chemiluminescent detection of HRP activity.

USE OF SureLINK™ HRP CONJUGATES IN IMMUNOHISTOCHEMISTRY

SureLINK™ HRP conjugates can also be used in immunohistochemistry (IHC) applications. Deparaffinized human breast tissues on slides were treated with a monoclonal antibody to human epithelial membrane antigen (EMA). The slides were treated sequentially with biotinylated anti-IgG and a streptavidin-HRP conjugate. Figure 6 demonstrates detection with DAB/Contrast Green (A and C) or TrueBlue/Contrast Red (B and D). With DAB/Contrast Green, the EMA protein was stained an orange-brown whereas nuclei were counterstained with a blue-green color. With TrueBlue/Contrast Red, EMA is stained dark purple and the nuclei are stained pink. Primary antibody was omitted for the control slides (C and D) and only the counterstain is visible in these slides.

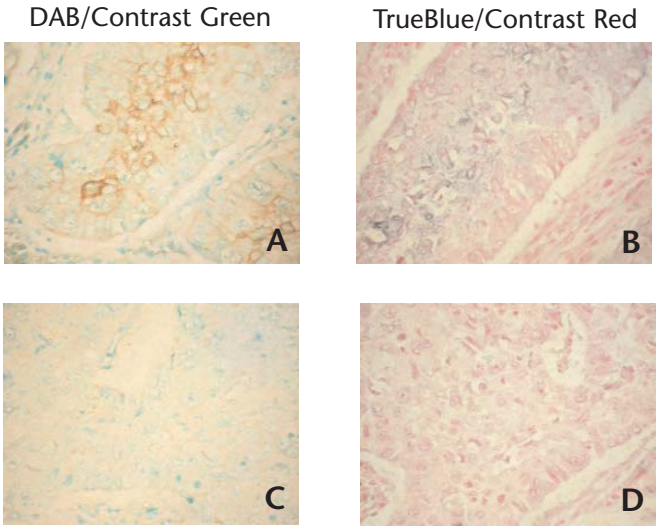

Figure 6: Detection of human EMA protein in human breast tissue. 2.5ug/ml of SureLINK™ HRP-SA conjugate was added to each slide. Panels A & B: anti-EMA mAb. Panels C & D: anti-EMA primary mAb was omitted.

CONCLUSION

HRP conjugates prepared using SureLINK™ Activated HRP are useful in a range of immunoassays including ELISA, Western blot and immunohistochemistry. These conjugates may also be used in other assay formats where HRP is a suitable reporter. SureLINK™ HRP Conjugation kits contain all the required reagents for quick and reliable labeling of antibodies or other proteins with HRP. These kits save time and minimize the need for optimizing the HRP activation procedure. The activated HRP is stored in a stable lyophilized form, thus allowing for reproducible conjugate preparations.

ML321-03

REFERENCES

[1]Abbreviations-HRP, horseradish peroxidase; SA, streptavidin; Ab, antibody; mAb, monoclonal antibody; pAb, polyclonal antibody; ELISA, Enzyme-linked immunosorbent assay; IHC, immunohistochemistry.

[2]KPL's Technical Guide for ELISA -- http://www.kpl.com/docs/techdocs/chapters%201%20-%204.pdf

[3]Nakane, P.K. and Kawaoi, A. (1974) Peroxidase-labeled antibody: a new method of conjugation. *J. Histochem Cytochem*. **22**: 1084-91.

[4]Tsang, V.C.W., Greene, R.M., and Pilcher, J.B. (1995) Optimization of the covalent conjugating procedure (NaIO4) of horseradish peroxidase to antibodies for use in enzyme-linked immunosorbent assay. *J. Immunoassay* **16(4)**, 395-418.

Product Information

Description	Size	Catalog No.
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 reaction	84-00-02
SureLINK HRP Conjugation Kit	6 x 0.1 mg reaction	84-00-01
SureLINK HRP Conjugation Kit	2 x 0.1 mg reaction	84-00-03
SureLINK Activated HRP	1.5 mg	84-01-02
SureLINK Activated HRP	0.3 mg	84-01-01

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