SureLINK™

HRP Conjugation Manual

For Products:

Product	Catalog No.	Size
SureLINK HRP Conjugation Kit	84-00-01	6 x 0.1 mg rxn.
	84-00-02	6 x 1.0 mg rxn.
	84-00-03	2 x 0.1 mg rxn.
SureLINK Activated HRP	84-01-01	0.3 mg
	84-01-02	1.5 mg

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PRODUCT DESCRIPTION

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

BACKGROUND

Horseradish peroxidase (HRP) has been widely employed in enzyme immunoassays (EIA); namely ELISA, Western blotting and immunohistochemical staining techniques to detect and characterize analytes.[1,2,3]. HRP is a 44 kDa glycoprotein comprised of sugar moieties that can be modified to crosslink with antibodies or other proteins, without compromising the enzymatic activity. The sugar hydroxyl groups are activated with the sodium periodate ($NaPIO_4$)[4,5], to produce aldehydes which readily couple with primary amines present in all known proteins, via a Schiff base. Following the reduction of the Schiff base, the conjugates are stable for a long period of time.

The method has proven reliable, however, the need to purify activated HRP from excess $NaPIO_4$ renders the protocol inconvenient. Furthermore, once HRP is activated, conjugation must occur shortly after due to the significant drop in enzymatic activity within a few days after activation.

HRP's high substrate turnover rate combined with the commercial availability of several highly sensitive colorimetric and chemiluminescent substrates for ELISA and immunoblotting applications have made HRP an invaluable tool for antigen detection. In addition, the relative small size of HRP (44 kDa) is an advantage for assays that require the diffusion of enzyme conjugates through cell membranes; therefore, making HRP conjugates especially suitable for immunohistochemical staining techniques.

PRODUCT OVERVIEW

SureLINK™ HRP Conjugation Kits contain ready-to-use components to prepare HRP labeled antibody or protein within approximately 90 minutes. The resulting conjugates are stable for at least 6 months at 4°C. Kit reagents are sufficient to conjugate from 50 µg to 1 mg antibody samples per reaction, allowing users to prepare HRP conjugates using small amounts of precious monoclonal antibodies. The activated and lyophilized HRP enzymes supplied in the kits are enough to reproducibly prepare several HRP conjugates with comparable efficacies.

SureLINK™ HRP conjugates may be used in ELISA, blotting, and immunohistochmeistry techniques to detect and quantitate proteins by using colorimetric or chemiluminescent substrates. See Related Products on page 15 for a listing of HRP compatible substrates and assay reagents.

PRODUCT COMPONENTS

84-00-01, SureLINK HRP Conjugation Kit

Size: 6 x 0.1 mg rxn.

Kit Component	Part Number	Size	Quantity
SureLINK Activated HRP	84-01-01	0.3 mg	6
HRP Conjugation Buffer	84-02-01	12 mL	1
Reducing Reagent ($NaCNBH_3$)	84-03-01	0.1 mL	1
HRP Storage Buffer	84-04-01	12 mL	1

84-00-02, SureLINK HRP Conjugation Kit

Size: 6 x 1.0 mg rxn.

Kit Component	Part Number	Size	Quantity
SureLINK Activated HRP	84-01-02	1.5 mg	6
HRP Conjugation Buffer	84-02-01	12 mL	1
Reducing Reagent ($NaCNBH_3$)	84-03-01	0.1 mL	1
HRP Storage Buffer	84-04-01	12 mL	1

84-00-03, SureLINK HRP Conjugation Kit

Size: 2 x 0.1 mg rxn.

Kit Component	Part Number	Size	Quantity
SureLINK Activated HRP	84-01-01	0.3 mg	2
HRP Conjugation Buffer	84-02-01	12 mL	1
Reducing Reagent ($NaCNBH_3$)	84-03-01	0.1 mL	1
HRP Storage Buffer	84-04-01	12 mL	1

84-01-01, SureLINK Activated HRP

Size: 0.3 mg

Kit Component	Part Number	Size	Quantity
SureLINK Activated HRP	84-01-01	0.3 mg	1

84-01-02, SureLINK Activated HRP

Size: 1.5 mg

Kit Component	Part Number	Size	Quantity
SureLINK Activated HRP	84-01-02	1.5 mg	1

STORAGE AND STABILITY

- SureLINK™ HRP Conjugation Kits are shipped at 2-8˚C.
- Store all components at 2-8°C upon receipt.
- Kits are stable for at least one year.

BEFORE YOU BEGIN

SAFETY AND HANDLING

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

OTHER REQUIRED SUPPLIES AND EQUIPMENT

- Antibody or Protein to be labeled should be free of salts or contaminants (see Troubleshooting section for removing salts or other contaminants).
- 20 µl and 200 µl micropipettes
- Shaker
- Vortex
- Microcentrifuge

REAGENT PREPARATION

- Equilibrate buffers to room temperature prior to use.
- If precipitation occurs in HRP Conjugation Buffer at 4°C, dissolve by incubating in a 50-60°C water-bath and equilibrate to ambient temperature before use.
- All buffers are ready-to-use. Dilution or mixing prior to use is not required.

QUICK REFERENCE GUIDE

Re-suspend antibody/protein in HRP Conjugation Buffer

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Add antibody/protein to SureLINK™ Activated HRP

↓

Incubation with gentle agitation
1 hour at room temperature, or overnight at 4˚ C

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Add reducing agent and incubate with gentle agitation
15 minutes at room temperature

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Add HRP Storage Buffer (2X)
15 minutes at room temperature

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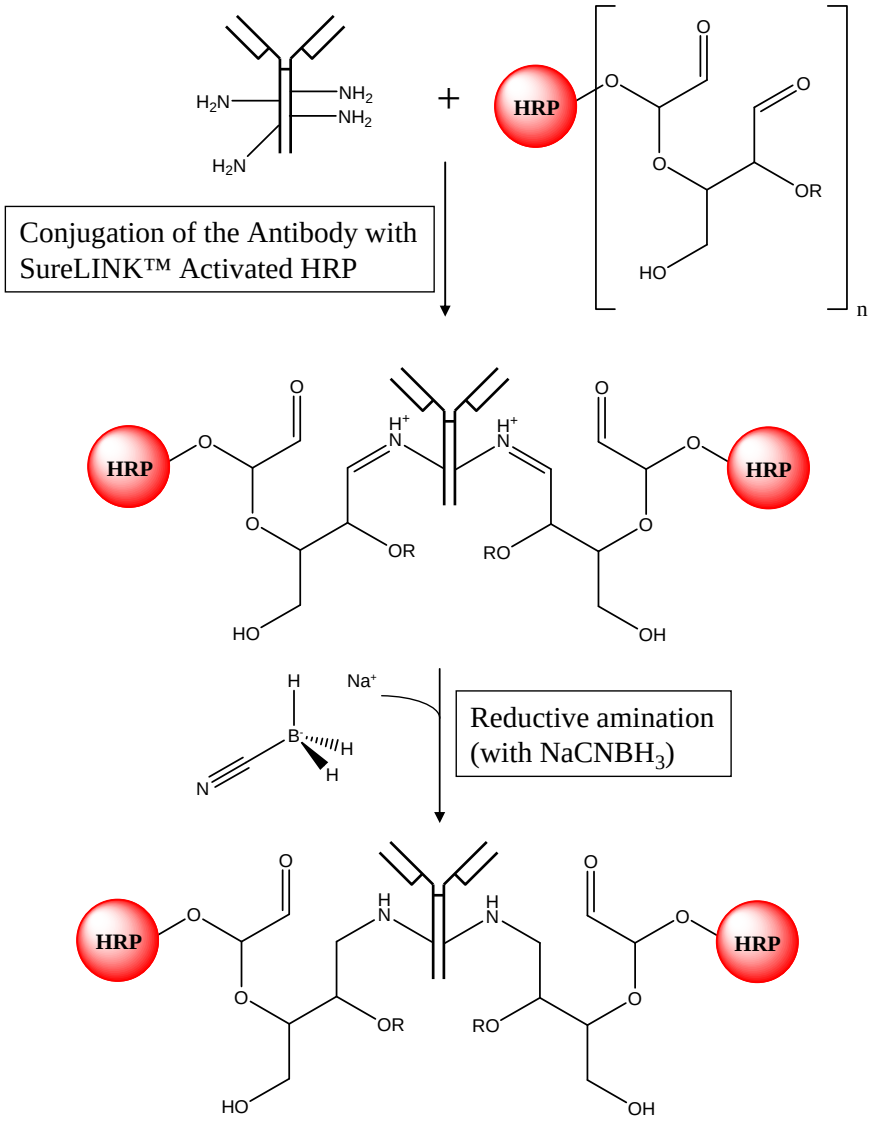
SureLINK™ HRP Conjugate is ready to use!

TIME TO COMPLETE REACTION
Approximately 90 minutes

SureLINK HRP CONJUGATION CHEMISTRY

SureLINK™ Activated HRP



Horseradish Peroxidase-Antibody Conjugate

CONJUGATION PROTOCOL

This protocol describes the experimental conditions for IgG conjugation. Other antibodies and proteins can also be labeled by varying the molar ratio of activated HRP to protein (see "Determining the required amount of SureLINK Activated HRP required for conjugation reaction" on page 9).

Note: Since activated HRP must be used within 1 hour after rehydration, the experimental schedule should be planned accordingly.

1) Rehydrate the lyophilized antibody sample (free of salts) with HRP Conjugation Buffer to a final concentration of 0.5-2.0 mg/mL.

A minimum volume of 100 µL antibody is required to perform the labeling reaction. See Troubleshooting guide, if antibody contains contaminants. If liquid antibodies are used, the protein concentration should be in the range of 0.5-2.0 mg/mL in buffer (KPL recommends two buffers: 0.1M carbonate/bicarbonate, pH 9.3; or borate buffer, pH 9.3-9.5).

2) Mix antibody solution gently with proper volume of SureLINK™ Activated HRP. Incubate for 1 hour at room temperature (20-25°C) or overnight at 4°C with gentle agitation.

See "**Determine the required amount of SureLINK Activated HRP for conjugation reaction**"on page 9 to calculate the volume of SureLINK™ Activated HRP. Activated HRP should be rehydrated with proper volume of HRP Conjugation Buffer or leave in powder form based on Table 2 recommended.

3) Add 10 µL of Reducing Agent ($NaCNBH_3$) to the reaction. Incubate for 15 minutes at room temperature.

4) Add equal reaction volume of HRP Storage Buffer (2X) to the reaction vial. Incubate for 15 minutes at room temperature. The conjugate is now ready for use.

See Page 11 example protocol for the calculation of final reaction volume. See page 12 for recommended starting concentrations for ELISA, Western blotting, and immunohistology applications.

Notes:

- *Final conjugate concentration is based on the antibody concentration and is calculated by dividing the total weight of antibody by final reaction volume.*
- *Conjugate is stable for at least 6 months at 4°C. For extended stability store conjugate at –20°C.*
- *The amount of the HRP in the conjugation reaction may be varied in order to optimize the efficiency of the conjugation reaction.*
- *The overall activity of the conjugate depends on the level of the antibody-antigen interaction and the enzymatic activity.*

Determine the required amount of SureLINK Activated HRP for conjugation reaction:

Part 1: Determine the HRP:Antibody (or protein) molar ratio (see Table 1)

Section 1: Labeling immunoglobulin:
For antibodies with a molecular weight of ~150 kDa, the optimal amount of HRP required to conjugate with an antibody sample varies as a function of the amount of the antibody in the reaction. A molar ratio of HRP:Antibody of 10:1 is recommended for antibody samples of 0.1 mg or less. A molar ratio of 5:1 is optimum for antibody samples greater than 0.1 mg. Reducing the HRP:Antibody ratio (for example from 5:1 to 1:1, using less HRP) favors the production of lower molecular weight conjugates which may possess enhanced abilities to penetrate cell membranes.

For higher molecular weight antibodies (>700 kDa), a molar ratio of HRP:Antibody of 25:1 is recommended.

Section 2:Labeling other proteins and peptides:
For lower molecular weight proteins/peptides (MW <5 kDa), the optimal HRP:Protein ratio may be much lower (i.e. 1:1 to 1:10). Further titration of the amount protein/peptide may be required to determine the optimal condition for the specific protein sample.

Table 1: Determination of HPR: Antibody (or Protein) Molar Ratio

MW of Antibody or Protein	Amount of Antibody or Protein	Molar Ratio of HRP: Antibody (or Protein)
< 5 kDa	0.05 – 1.0 mg	1:1 to 1:10
~150 kDa	0.1 mg or less	10:1
~150 kDa	> 0.1 mg	5:1
> 700 kDa	0.05 – 1.0 mg	25:1

Part 2: Determine the amount and volume of SureLINK Activated HRP for conjugation reaction

To determine the amount of SureLINK Activated HRP to use in Step 2 of the Conjugation Protocol, use the molar ratios described in Part 1. Table 2 provides examples of common antibody sample amounts and the volume of Activated HRP required.

Table 2:.Calculating Required Volume of SureLINK Activated HRP

Molar Ratio of HRP:IgG (Determined by Table 1)	IgG Sample Amount MW 160kDa	Required HRP MW 44kDa	Rehydration of Activated HRP with HRP Conjugation Buffer and Choices of Products	Required Volume of Activated HRP
10:1	0.05 mg (0.3 nmole)	0.15 mg (3 nmole)	Add **200 µL** of buffer to 1 vial of 0.3 mg HRP (84-01-01)	**100 µL**
10:1	0.10 mg (0.6 nmole)	0.3 mg (6 nmole)	Use 1 vial of 0.3 mg HRP (84-01-01) in powder form	Add IgG solution directly to HRP vial
5:1	0.25 mg (1.5 nmole)	0.38 mg (7.5 nmole)	Add **200 µL** of buffer to 1 vial of 1.5 mg HRP (84-01-02)	**50 µL**
5:1	0.50 mg (3.0 nmole)	0.75 mg (15 nmole)	Add **200 µL** of buffer to 1 vial of 1.5 mg HRP (84-01-02)	**100 µL**
5:1	0.75 mg (4.5 nmole)	1.13 mg (22.5 nmole)	Add **200 µL** of buffer to 1 vial of 1.5 mg HRP (84-01-02)	**150 µL**
5:1	1.00 mg (6.0 nmole)	1.5 mg (30 nmole)	Use 1 vial of 1.5 mg HRP (84-01-02) in powder form	Add IgG solution directly to HRP vial

Note: The calculations use molecular weight of IgG = 160 kDa and molecular weight of HRP = 44 kDa. Other antibody amounts and proteins with varying molecular weights can be used with this kit.

SAMPLE PROTOCOL

Example protocol for conjugating a lyophilized antibody (160 kDa, 0.5 mg) using the SureLINK HRP Conjugation Kit:

- The molar ratio of HRP:Antibody is determined to be 5:1 according to Table 1.

- Rehydrate the antibody sample (0.5 mg) using 400 µL of the HRP Conjugation Buffer. Antibody concentration is 1.25 mg/mL (within the concentration range of 0.5-2.0 mg/mL, with a volume of at least 100 µL).

- Rehydrate a 1.5 mg vial of the SureLINK Activated HRP (Cat # 84-01-02) with 200 µL HRP Conjugation Buffer yielding a HRP concentration of 7.5 mg/mL (See Table 2).

- Initiate the conjugation reaction by adding ***100 µL*** of the reconstituted Activated HRP to the entire ***400 uL*** antibody mix and incubate for 1 hour with gentle agitation at room temperature.

- Add ***10 µL*** of the Reducing Agent ($NaCNBH_3$) to the reaction mix and incubate for 15 minutes at room temperature.

- Add ***510 µL (100+400+10)*** of the HRP Storage Buffer (2X) to the conjugation reaction mix and incubate for 15 minutes at room temperature. Store the HRP conjugate at 4^{o}C. The final concentration of the HRP-labeled antibody is approximately 0.5 mg/mL based on the final concentration of antibody in the reaction mix (0.5mg/1020 µL).

RECOMMENDED USE OF CONJUGATES

SureLINK HRP labeled antibodies and proteins can be utilized 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 in your specific assay may vary and should be determined for each assay being developed.

Application	**HRP Conjugate Concentrations**
Direct ELISA	250 ng/mL to 2.5 µg/mL
Western Blotting	0.5 µg/mL to 50 µg/mL
Immunohistology	2.0 – 5.0 µg/mL

Most reagents required for performing ELISA, Western blotting, and Immunohistochemistry assays are available from KPL. A listing of products is described in the Related Products section.

TROUBLESHOOTING GUIDE

Problem 1: Weak Level of Detection in Immunoassay

Causes and/or Observations	Possible Solutions
Inactive lyophilized HRP	Check expiration date and follow the storage condition of each component in the kit.
Low signal and/or high background levels	Titrate the amount of conjugate in the assay and optimize the signal to noise ratio.
Concentration of antibody in the conjugation reaction may have been overestimated, resulting in a smaller size conjugate. Underestimating the antibody concentration may result in significant level of unlabeled antibody in the final conjugate mix.	Centrifuge the sample prior to measuring the absorbance, and use triplicate measurements for concentration determination. Antibody samples must be purified from compounds that absorbance in the UV range.
Other proteins are present in the antibody sample—reducing the preparation of the desired HRP conjugate size.	Separate the protein sample on SDS-PAGE and detect protein bands using Commassie staining. Depending on the level of impurity, purification may be required.
Antibody or protein sample is in incompatible buffer for the conjugation reaction	The conjugation reaction is most efficient at basic pH buffer conditions. Exchange the sample buffer with 0.1M carbonate/bicarbonate pH 9.3 or 0.04M sodium borate pH 9.3-9.5. Other buffers such as 0.1M sodium phosphate buffer pH 7.4 may also be used to prepare lower molecular weight conjugates.
Sodium azide, an inhibitor of HRP activity, is present in the antibody preparation.	Dialyze the antibody sample against an appropriate buffer prior to conjugation.
Compounds with primary amine groups, ex. Tris, Glycine and NH_4OH, are present in the antibody sample, compromising efficiency of the conjugation reaction.	Dialyze the antibody sample against the appropriate buffer prior to conjugation.

Problem 2: High Background Levels in Immunoassay

Causes and/or Observations	Possible Solutions
Schiff Base linkages between the HRP and the antibody/protein are not reduced, increasing the probability of non-specific binding—high background.	Check the expiration date of the reducing agent. For basic pH reaction buffer condition, add $NaCNBH_3$ after the conjugation step. For neutral pH reaction buffer condition however, the $NaCNBH_3$ can be present throughout the duration of the reaction. Reducing agents such as $NaBH_4$, amine boranes, and ascorbic acids may also be used to stabilize the Schiff Base.[4,5] $NaBH_4$ must be prepared fresh before each use.
The conjugate size is very large.	Reduce the time of the conjugation reaction. Perform the conjugation reaction at lower temperature, with varying reaction times. Optimize the molar ratio of the HRP to antibody. Perform the conjugation reaction at a neutral pH, using buffers such as 0.1M sodium phosphate buffer, pH 7.4 in a 0.1M NaCl.

RELATED PRODUCTS

Product/Application Group	Product Name	Size	Catalog Number
Conjugation Kits & Reagents	SureLINK™ AP Conjugation Kit	3 x 0.1 mg	85-00-01
		3 x 0.5 mg	85-00-02
	SureLINK™ Modified AP	0.2 mg	85-01-01
		1.0 mg	85-01-02
	SureLINK Bioconjugation Kit	Kit	80-00-01
	SANH	10 mg (5 x 2 mg)	80-01-01
	SFB	20 mg (5 x 4 mg)	80-02-02
ELISA Products	ABTS Microwell Peroxidase Substrate	600 mL	50-62-00
	ABTS Peroxidase Stop Solution	200 mL	50-85-01
	SureBlue™ TMB Substrate	100 mL	52-00-01
	SureBlue Reserve™ TMB Substrate	100 mL	53-00-01
	TMB Stop Solution	400 mL	50-85-05
Western Blot Products	LumiGLO® Chemiluminescent Substrate	60 mL	54-61-02
	LumiGLO Reserve™ Chemiluminescent Substrate	600 cm2	54-71-01
	Detector Block (5X)	240 mL	71-83-00
Immunohistochemistry	Universal Block for Immunohistochemistry	100 mL	71-00-61
	HistoMark® BLACK	1000 slides	54-75-00
	HistoMark® ORANGE	1000 slides	54-74-00
	TrueBlue Peroxidase Substrate	10 mL	71-00-67
Support Reagents	Wash Solution Concentrate	800 mL	50-63-00
	Coating Solution Concentrate (10X)	50 mL	50-84-00
	Milk Dil./Blocking Solution Conc.	200 mL	50-82-01
	10% BSA Dil./Blocking Solution Conc.	200 mL	50-61-00
	HRPStabilizer	200 mL	54-15-01

Visit www.kpl.com for a complete listing of KPL products or contact KPL Technical Services at 800-638-3167, 301-948-7755 or techserv@kpl.com.

REFERENCES

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