

positive well. Stop reaction with 1N H₂SO₄ (blue color turns yellow). Read the yellow color at 450 nm.

If antibody is coupled to HRP- test the conjugate as above except that the antigen-coated plates should be used.

High background in ELISA is usually due to the use of **excessive** conjugate, improper conjugate diluents or blocking of coated antigens. The HRP-conjugate dilution will depend upon the strength of the antigen or antibody. If the antibody titer is weak then the conjugate dilution will be less. Typically, if HRP conjugate is used at 1:1K or less then background could become a problem. The HRP-conjugate can be purified to remove the unbound antigen or antibody to improve the working of the conjugate. Free HRP in conjugate is usually not a problem as it will not bind to the antigen or antibody and removed during washing.

Precautions

This kit contains lyophilized and preactivated HRP and it is stable for up to 1 year when kept at 4°C in lyophilized form. **Do not store HRP in solution or diluted forms. Preactivated HRP should be used immediately (within 30 min) upon reconstitution.**

Related Items Available from ADI

NEW 80025 Horseradish peroxidase (HRP) conjugation kit for antigen/antibodies (Micro; 3 x 200 ug HRP)

80012	High binding ELISA Strips plates (8 wellsx12 strips)	10/pk
80050	ELISA Plate Coating buffer concentrate (10X)	50 ml
80051	Phosphate Buffered Saline (PBS, pH 7.4) (20X)	100 ml
80062	ELISA Plate Blocking Buffer , (10X) milk-based	100 ml
80070	Antibody and Conjugate Diluents for ELISA (10X),	50 ml
80080	Wash buffer concentrate (20X) for ELISA	100 ml
80091	TMB substrate (1-component) for ELISA	500 ml
80100	Stop solution for TMB substrate (ELISA) (10X),	50 ml
80150	ELISA Kit for the detection of Mouse Antibodies	1 kit
80155	ELISA Kit for the detection of Rat Antibodies	1 kit
80160	ELISA Kit for the detection of Rabbit Antibodies	1 kit
80190	Western blot Kit for mouse or rabbit, human or goat antibodies using TMB color substrate	1 kit 0
80300	Protein (antigen/antibodies) Biotinylation Kit	1 kit

Instruction Manual No. M-80225

Horseradish Peroxidase (HRP) Conjugation Kit (Micro)

For Labeling Antigen And Antibodies

Cat. No. 80225

For In Vitro Research Use Only



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Introduction

HRP is widely used in ELISA/EIA as a detection molecule. HRP, a glycoprotein (40 kDa), is available in highly purified form, relative inexpensive, very stable, and provides several options (free amino, carboxyl, and carbohydrate groups) for covalent coupling to other antigens and antibodies. It has a very high turn over number and remains active after chemical modifications.

Conjugation of proteins (peptides, antigen or antibodies) requires activation of HRP and removal of activating agents. The activated HRP is then reacted with the protein. ADI has developed a unique method of HRP activation and keeping the activated HRP in lyophilized and activated state for extended period of time. The preactivated HRP (lyophilized) is then dissolved and simply mixed with other protein that have free amino group for an easy coupling. This kit therefore takes the time consuming and problematic steps out to provided a consistent and easy labeling of peptides and proteins. The labeling of proteins occurs via the activated amino groups of HRP to the amino groups of the coupling proteins.

Reagents Provided in the Kit

This kit is the same as #80220 that is supplied with 1 vial of 2 mg HRP. The micro kit allows coupling of three proteins or antibodies (~100 ug) and offers more convenience.

3 vial of Preactivated HRP (200 ug; lyophilized)	# 80226
Conjugation Buffer, 0.1 M NaHCO ₃ , pH 9.5 (10 X), 100 ml; (dilute 1:10 with water before use)	# 80222
Blocking Buffer; 500 ul;	# 80223
Conjugate Stabilizer; 2 ml.,	# 80224
Dialysis bag (Cut off size 10-12 kda)	# DB-10
If proteins of 10kda or less are being coupled then it is a good idea to use membranes with lower cut-off such as (3 kda). Wash the membrane thoroughly with 70% ethanol and then with distilled water.	

Reagents Required But not Provided

1. PBS, pH 7.4 for Dialyzing the conjugate (dissolve 0.26 g KH₂PO₄, 2.17 g Na₂HPO₄·7H₂O, and 8.71 g of NaCl in 1L H₂O).
2. Clamps for the dialyses membrane

HRP Conjugation Procedure

1. Dialyze the protein to be coupled in 1X conjugation buffer (0.1 M NaHCO₃, pH 9.5) extensively. The protein concentration should be 1-4 mg/ml. If the protein is in pure form or in saline or in H₂O, it is possible to adjust the pH by adding 10X conjugation buffer and omitting dialysis. If the protein is dry powder in water or PBS then it is possible to reconstitute it in directly in conjugation buffer.
2. Add 0.5-1 ml of dialyzed or pre-equilibrated protein (up to 1-2 mg) and add it to the vial containing preactivated HRP. Mix it at room temp. for 3 h or overnight at 40C.
3. Add Blocking Buffer (200 ul/ml of conjugate). Mix it for 1 h at RT.
4. Dialyzed the conjugate extensively against PBS at 40C. Note: It is possible to test the conjugate without dialyses as the conjugate is typically used at 1:1K-1:50K depending upon the antibody titer.
5. Add stabilizing buffer to the dialyzed conjugate (add 1 ml for each 500 ul of starting protein volume).
6. The conjugate can be kept at 40C for up to 6 months.

Notes: The functionality of the HRP-conjugate will depend upon the activity of its partner (antigen and antibody). Typically, 1 mg of antigen or antibody can be coupled to 2 mg HRP (1:2 Ag/Ab:HRP) as a starting point. It is possible to vary this ratio to 1:1 or 2:1 etc and test the resulting conjugate. This ratio will determine the working dilution of the conjugate, background, and also the sensitivity in a given technique. It is difficult to predict what ratio of the antigen/antibody and HRP will work the best.

Storage of the Conjugate

The stabilizer already has a bacteriostatic agent. **Do not add sodium azide (inhibits peroxidase)**. The conjugate should be kept at 40C for 6 months or it can be stored frozen in suitable aliquots. Avoid freezing and thawing. Glycerol (50%) can be added and conjugate kept at -200C with out freeing.

Recommended Usage of The Conjugate

ELISA testing of the Conjugate

ADI supplies both the single component TMB substrate for testing the conjugate (cat # 80091) and the general purpose ELISA kits that has all of the components for ELISA including 2-ab (cat # 80160 or others kits based upon the 1-ab used for conjugation).

If an antigen is coupled to HRP- the antigen-HRP conjugate can be tested using appropriate antibody coated plates (coat 1-5 ug/ml) and blocked. Incubate the conjugate (1:1K-100K) for 1-hr at room temp, wash, and then add TMB-substrate for 15-min. Blue color is obtained in