For life science research only.
Not for use in diagnostic procedures.

Peroxidase (POD), activated

Reagent for the labeling of water-soluble substances carrying primary amino groups with peroxidase from horse radish (HRP)
Lyophilisate from 0.5 ml

Cat. No. 11 428 861 001

8 mg (≙40 mg lyophilizate)
For 5 labeling reactions

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Store at +2 to +8°C

Application

The reagent can be used for labeling water-soluble substances with reactive and accessible primary amino groups (*e.g.*, peptides or proteins) with peroxidase for use in analytical methods. It is particularly suitable for the coupling of antibodies with peroxidase, as the resulting conjugate is used in immunochemical detection systems *e.g.*, ELISA, immunohistochemistry and immunoblotting procedures.

Product description

Reconstitution
On dissolving the lyophilisate in 0.5 ml redist. water, a peroxidase concentration of 16 mg/ml is obtained.

Stability
The lyophilisate is stable if stored dry at +2 to +8°C until the expiration date printed on the label. The reconstituted solution is stable for 3 months at +2 to +8°C. The solution can be aliquoted, shock-frozen at −60°C or below and then stored at −15 to −25°C; however, a loss of activity of 10 - 20% can be observed.

Capacity
The quantity of peroxidase is sufficient to conjugate ca. 6 mg immunoglobulin G. We recommend dividing the total quantity to be conjugated into 5 portions; each portion will then provide 0.5 - 1 ml of conjugate from ca. 1.2 mg IgG, a quantity that can be diluted 1:4,000 – 1:10,000 for ELISA applications.

Specification
Specific activity: ≥550 U/mg protein (+25°C, ABTS Substrate, H_2O_2; pH 5.0), Purity number ($A_{405\ nm}$-/$A_{275\ nm}$): 3.0 - 3.5. Isoenzyme distribution: > 90% homogeneous isoenzyme C.

Test procedure
The following procedure has been specially developed for the coupling of peroxidase to immunoglobulin G (IgG). It can however be equally successfully used for Ig Fab- and $F(ab')_2$-fragments from rabbit, mouse, sheep and goat (remarks). If other proteins are to be conjugated, we would recommend beginning with this procedure and checking the results with gel chromatography on HPLC, TSK 3000. If necessary, the procedure can then be adapted to individual requirements by altering the stochiometry and the concentration of reactants used for incubation. The test procedure describes the conjugation using 1/5 of the total quantity of reagent, sufficient to label 1.2 mg of IgG.

Reagents
Albumin, from bovine serum*
Glycine, analytical grade
HCl, 25%, analytical grade
KH_2PO_4, analytical grade
$K_2HPO_4 \times 3H_2O$, analytical grade
$NaBH_4$, analytical grade
NaCl, analytical grade
Na_2CO_3, analytical grade
$NaHCO_3$, analytical grade
NaOH, analytical grade
Triethanolamine, analytical grade
Kathon, CG**
Dialysis tube for 5 ml

Preparation of the solutions (+15 to +25°C).

1. 1 M Sodium carbonate/-hydrogencarbonate solution, pH 9.4

1 M Na_2CO_3: Dissolve 10.6 g Na_2CO_3 in 80 ml redist. water and make up to 100 ml.

1 M $NaHCO_3$: Dissolve 8.4 g $NaHCO_3$ in 80 ml redist. water and make up to 100 ml.

Adjust the pH of the $NaHCO_3$ solution to 9.4 by adding Na_2CO_3 solution.

2. 100 mM Sodium carbonate/-hydrogencarbonate solution, pH 9.8

Dilute 10 ml solution 1 to 100 ml with redist. water.

3. 200 mM Sodium borohydride solution.

NB: Prepare the solution immediately prior to use and keep cold on ice. Dissolve 8 mg $NaBH_4$ in 1 ml cold redist. water.

4. 2 M Triethanolamine solution, pH 8.0

Dilute 2.66 ml triethanolamine with 3 ml redist. water, adjust the pH to 8.0 with 25% HCl and make up to 10 ml with redist. water.

5. 1 M Glycine solution, pH 7.0

Dissolve 0.75 g glycine in ca. 6 ml redist. water, adjust to pH 7.0 with 0.1 M NaOH, and make up to 10 ml with redist. water.

6. PBS (phosphate-buffered saline); glycine; pH 7.4.

10 mM Potassium phosphate, 200 mM NaCl, 10 mM glycine, pH 7.5.

- Solution A (K_2HPO_4): Dissolve 4.56 g $K_2HPO_4 \times 3H_2O$, 23.4 g NaCl, and 1.5 g glycine in ca. 1,500 ml redist. water and make up to 2,000 ml with redist. water.
- Solution B (KH_2PO_4): Dissolve 2.72 g KH_2PO_4, 23.4 g NaCl, and 1.5 g glycine in 1,500 ml redist. water and make up to 2,000 ml with redist. water.
- PBS: Whilst controlling pH, add sufficient solution B to solution A until the pH is 7.4.

7. Antibody solution

0.3 ml required for each labeling reaction.
The IgG concentration of the solution to be used is c=4 mg/ml (3.8 - 42 mg/ml). This value is critical for the coupling and hence should be checked photometrically for every test and adjusted if necessary: A_{280nm}, 1cm, 1 mg/ml = 1.40.

Do not use preservatives *e.g.*, sodium azide and stabilizers e.g., albumin.

- **Immunoglobulin, salt-free, lyophilised:**
  Weigh 1.6 mg into a suitable vessel and dissolve in 0.4 ml solution. Check the concentration and pH and correct if necessary.
- **Immunoglobulin in buffer:**
  PBS buffer without additional proteins or preservatives: adjust the pH to 9.8 with solution 1 and if necessary dilute with solution 2 to obtain an IgG concentration of 4 mg/ml. Buffer with organic salts: Dialyse immunoglobulin into solution 2 and adjust the concentration to 4 mg/ml with solution 2.

Stability of the solutions

Solutions 1, 2, 4, 5 and 6 are stable for 1 week at +2 to +8°C. Solutions 3 and 7 should always be prepared immediately prior to use.

Procedure

1. Conjugation

Pipette exactly 0.3 ml antibody solution into a suitable 1-2 ml vessel and add 0.1 ml activated peroxidase. Mix well (reaction ratio: 1 M IgG: 5 M POD).
The MW of POD is 44,000 Da.

Incubate for 2 h at +15 to +25°C in a water bath or for 16 h overnight at +2 to +8°C.

2. Stopping the reaction

Add 40 µl solution 4 (triethanolamine solution) to the incubation solution, mix, pipette 50 µl solution 3 ($NaBH_4$) to the mixture, mix again and then incubate for 30 min at +2 to +8°C. Add another 25 µl of solution 4 and incubate again for 2 h at +2 to +8°C.

3. Stabilizing the conjugate

Pipette 10 µl of solution 5 into the incubation mixture and briefly mix.

4. Transfer of conjugate to storage buffer

Place the incubation solution in a dialysis tube (boiled water treated) and allow to dialyse extensively (*e.g.*, overnight) with 4 changes of 500 ml solution 6 (PBS, glycine).

5. Stabilizing the product for storage

Place the conjugate from the dialysis tube in a suitable vessel, add bovine serum albumin of 10 mg/ml and Kathon CG of 1 mg/ ml. Mix gently to dissolve.
The conjugate is stable for at least 2 months at +2 to +8°C. Should the conjugate have to be stored for a long period without loss af activity, it can be aliquoted, shock-frozen in liquid nitrogen and then stored at −60°C or below.
NB: Sodium azide should never be added as a preservative as it inhibits the peroxidase activity.
The reaction proceeds in such a way that practically no (<5%) immunoglobulin is left in the dialysate (see fig.3). The conjugate need therefore not be purified for normal immunoassay procedures. Residual amounts of POD do not normally interfere with such tests.

Remarks

Purification/fractionation of the conjugate

If the conjugate is to be used for special applications like *e.g.*, highly sensitive ELISA procedures or measurement in problematic matrices, it can be further purified subsequent to dialysis and prior to stabilization with bovine serum albumin by gel permeation chromatography (*e.g.*, Sephacryl S 300, Pharmacia) and the fractions tested for their suitability for the planned application.

Re-buffering of antibody and conjugate

The antibody can be re-buffered into solution 2 either using Sephadex G 25 or PD-columns (Pharmacia) or other suitable material. The conjugate can be re-buffered subsequent to step 3 by using column chromatography instead of the described dialysis (step 4).

Reaction ratio IgG to POD

The conjugation of IgG and fab-fragments is optimised for the above reaction ratio. Other stoichiometric proportions can be considered for special applications, but it must be stressed that the protein concentrations indicated in the test procedure should be held constant and that a molecular sieve fractionation should be carried out in order to separate out residual amounts of IgG or POD that may be present.

Immunoglobulin

The procedure is optimised for the coupling of immunoglobulin from rabbit; it can, however, be equally conjugated with IgG from sheep and goat. If Fab- or $F(ab')_2$-fragments of these species or immunoglobulin G from mouse are to be used, a reaction time of 3 h at +15 to +25°C should be used.

Reaction temperature and duration

The procedure is developed for a reaction temperature of +25°C but is relatively tolerant to the time of the reaction. At +15 to +25°C, the reaction time should be 2 h but this can be extended to 3 h without influencing the results.
Alternatively, the reaction can be carried out at +2 to +8°C; the reaction time, however, then has to be 18 h, but can also be extended to 24 h.

Influence of pH

The pH should never be allowed to fall below 9.8.
To ensure best reproducible results, it should also be kept constant. The maximum allowable pH is 10.8.

Influence of NaCl and potassium phosphate concentration

(Coupling in PBS with subsequent pH adjustment)
NaCl concentrations of 50 - 400 mM and simultaneous phosphate concentrations of 10-20 mM have a marginal effect only on the reaction. If the potassium phosphate concentration used is in the range of 30 - 100 mM, the NaCl concentration should not exceed 150 mM.

HPLC chromatography

Should high demands be placed on reproducibility, the reaction should be carried out under HPLC TSK 3000 control. Figs 1 - 3 show the TSK 3000 profiles of the starting materials, immunoglobulin G from rabbit, POD and the final product. It can be seen that the IgG is completely bound in the conjugate, corresponding to the resolution of the TSK 3000 column.

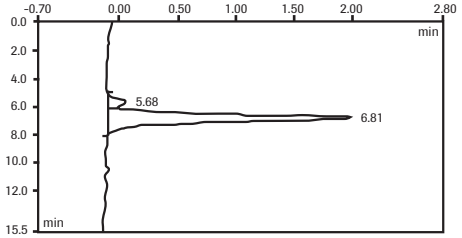

Fig. 1: HPLC. TSK-3000; IgG from rabbit

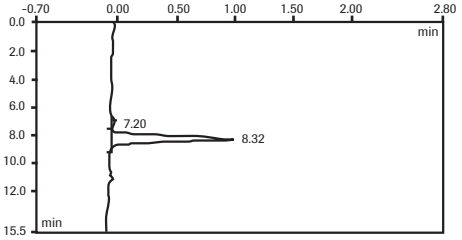

Fig. 2: HPLC, TSK-3000; Peroxidase

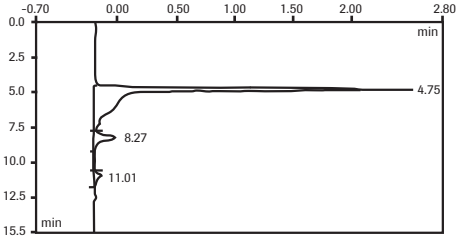

Fig. 3: HPLC TSK-3000; Conjugate of IgG and POD

* available from Roche Diagnostics

Changes to previous version

- Editorial changes.

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Roche Diagnostics GmbH
Sandhofer Strasse 116
68305 Mannheim
Germany