

Phosphatase, alkaline (AP) activated

Orthophosphoric-monoester phosphohydrolase (alkaline optimum) EC 3.1.3.1

Reagent for the labeling of water-soluble substances carrying primary amino groups with alkaline phosphatase from calf intestine lyophilizate from 0.5 ml

Cat. No. 11 464 752 001

10 mg

Version November 2006

Store at +2 to +8°C

1. What this Product Does

Contents

40 mg lyophilizate (\cong 10 mg AP); sufficient for 5 labeling reactions

Storage and Stability

- If stored dry at +2 to +8°C, the lyophilizate is stable through the expiration date printed on the label.
- The reconstituted solution is stable for 5 days at +2 to +8°C. The solution can be aliquoted, shockfrozen at -60°C or below and then stored at -15 to -25°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 alkaline phosphatase for use in analytical methods.

It is particularly suitable for the coupling of antibodies with alkaline phosphatase, as the resulting conjugate is optimal for use in immunochemical detection systems *e.g.*, ELISA, immunohistochemistry and immunoblotting procedures.

The enzyme can be used without pre-activation for the labeling of Ig, Ig Fab, and Ig F(ab')₂ fragments from rabbit, mouse, sheep, and goat.

Enzyme Characteristics

Specific Activity	800 U/mg protein (25°C, pNPP, pH 9.8)
Purity	HPLC-TSK-3000-analysis: one peak >95%
Capacity	The quantity of the enzyme is sufficient to conjugate approx. 2 mg immunoglobulin G (IgG). We recommend dividing the total quantity to be conjugated into 5 portions; each portion will then provide 0.3-0.5 ml of conjugate from ca. 0.35 mg IgG, a quantity that can be diluted 1:4,000 - 1:10,000 for ELISA applications.
Reconstitution	On dissolving the lyophilizate in 0.5 ml double distilled water, an alkaline phosphatase concentration of 20 mg/ml is obtained.

Additional Reagents Required

- Albumin, from bovine serum*
- Glycine, analytical grade
- HCl, 25%, analytical grade
- KH₂PO₄, analytical grade
- K₂HPO₄ × 3 H₂O, analytical grade
- MgCl₂, analytical grade
- Sodium azide, analytical grade
- NaBH₄, analytical grade
- NaCl, analytical grade
- Na₂CO₃, analytical grade
- NaHCO₃, analytical grade

- NaOH, analytical grade
- Triethanolamine, analytical grade
- ZnCl₂, analytical grade
- Dialysis tube for 5 ml

2. How to Use this Product

2.1 Before You Begin

General Remark

The following procedure has been specially developed for the coupling of alkaline phosphatase to immunoglobulin G (IgG). It can however be equally successfully used for IgG, Fab- and F(ab')₂-fragments from rabbit, mouse, sheep and goat.

The test procedure describes the conjugation using 1/5 of the total quantity of reagent, sufficient to label 0.35 mg of IgG.

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 stoichiometry and the concentration of reactants used for incubation.

Influencing Parameters

Reaction ratio IgG to AP	The conjugation of IgG and Fab-fragments is optimized 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 AP that may be present.
Immunoglobulin	The procedure is optimized for the coupling of immunoglobulin G 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 15-25°C should be used.
Reaction temperature and time	The procedure is developed for a reaction temperature of +15 to +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.
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.

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 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. Figures 1 - 3 show the TSK-3000 profiles of the starting materials, immunoglobulin G from rabbit, AP 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.

Preparation of Additional Solutions Required

Prepare the following solutions at +15 to +25°C.

Solution	Composition/Preparation	Storage/Stability
1 1 M Sodium carbonate/hydrogencarbonate solution, pH 9.4	<ul style="list-style-type: none"> • 1 M Na₂CO₃: Dissolve 10.6 g Na₂CO₃ in 80 ml double dist. water and make up to 100 ml. • 1 M NaHCO₃: Dissolve 8.4 g 1 M NaHCO₃ in 80 ml double dist. water and make up to 100 ml. <p>ⓐ Adjust the pH of the NaHCO₃ solution to 9.4 by adding solution.</p>	Stable for 1 week at +2 to +8°C
2 100 mM Sodium carbonate/hydrogencarbonate solution, pH 9.8	Dilute 10 ml solution 1 to 100 ml with double dist. water.	
3 200 mM Sodium borohydride solution (0-4°C)	Dissolve 8 mg NaBH ₄ in 1 ml cold double dist. water.	Prepare immediately prior to use and keep cold on ice.
4 2 M Triethanolamine solution, pH 8.0	Dilute 2.66 ml triethanolamine with 3 ml double dist. water, adjust the pH to 8.0 with 25% HCl and make up to 10 ml with double dist. water.	Stable for 1 week at +2 to +8°C
5 1 M Glycine solution; pH 7.0	Dissolve 0.75 g glycine in approx. 6 ml double dist. water, adjust to the pH 7.0 with 0.1 M NaOH, and make up to 10 ml with double dist. water.	
6 Triethanolamine buffer, pH 7.6	50 mM Triethanolamine, 150 mM NaCl, 1 mM MgCl ₂ , 0.1 mM ZnCl ₂ , 10 mM glycine, 0.1% NaN ₃ (w/v), pH 7.6.	
7 Antibody solution	0.05 ml required for each labeling reaction. The IgG concentration of the solution to be used is c = 7 mg/ml (6-8 mg/ml). This value is critical for the coupling and hence should be checked photometrically for each test and adjusted if necessary: A280 nm, 1 cm, 1 mg/ml = 1.40. ⚠ Do not use preservatives, e.g., sodium azide, and detergents.	Prepare immediately prior to use.
8 Immunoglobulin, salt-free, lyophilized	Weigh 1.4 mg into a suitable vessel and dissolve in 0.2 ml solution 2. ⚠ Check the concentration and pH and correct if necessary.	

Solution	Composition/Preparation	Storage/Stability
9 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 IgG concentration of 7 mg/ml. Buffer with organic salts: Dialyse immunoglobulin into solution 2 and adjust the concentration to 7 mg/ml with solution 2.	

2.2 Labeling Procedure

Protocol

To label antibody with activated AP follow the protocol below:

Conjugation

- Pipette exactly 50 µl antibody solution into a suitable 0.5 - 1 ml vessel and add 0.1 ml activated alkaline phosphatase.
- Mix well (reaction ratio: 1 M IgG: 6 M AP).
- Incubate for 2 h at 15 - 25°C in a water bath or for 16 h overnight at 2 - 8°C.

Stopping

- Add 20 µl solution 4 (triethanolamine solution) to the incubation solution, and mix.
- Pipette 40 µl solution 3 (NaBH₄) to the mixture, and mix again. Incubate for 30 min at 2-8°C.

- Add a further 5 µl of solution 4 and incubate again for 2 h at 2 - 8°C.

Stabilizing

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

Transfer

- 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 (triethanolamine buffer).

2.3 Stabilization for Storage

Place the conjugate from the dialysis tube in a suitable vessel, add bovine serum albumin of 10 mg/ml and sodium azide 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 of activity, it can be aliquoted, shock-frozen in liquid nitrogen and then stored at -60°C or below.

The reaction proceeds in such a way that the main IgG part is fixed in the conjugate (> 75%) and about 20% of the immunoglobulin is left in the dialysate (see fig. 3). The conjugate need therefore not be purified for normal immunoassay procedures. Should high demands be placed on sensitivity a purification by gel permeation chromatography (e.g., Sephacryl S-300) is recommended.

2.4 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) and the fractions tested for their suitability for the planned application.

2.5 Re-buffering of Antibody and Conjugate

The antibody can be re-buffered into solution 2 either using Sephadex G 25 or PD-columns or other suitable material. The conjugate can be re-buffered subsequent to step 3 by using column chromatography instead of the described dialysis (step 5).

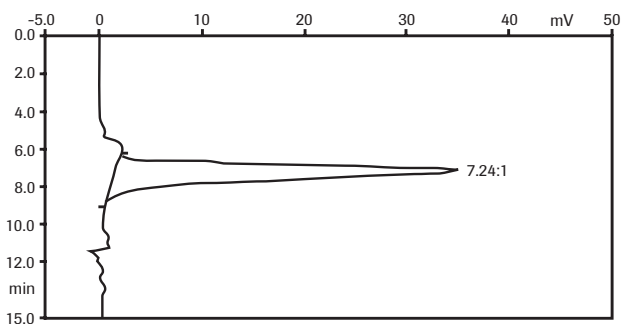


Fig. 1: HPLC, TSK-3000; IgG from rabbit

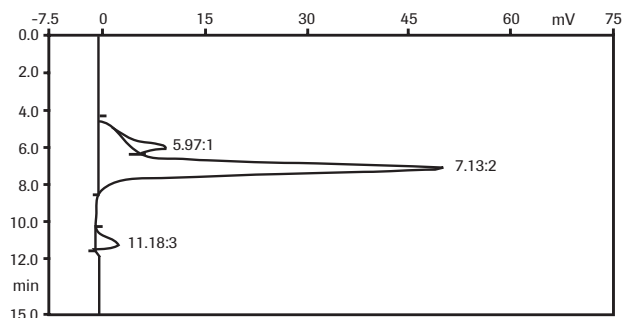


Fig. 2: HPLC, TSK-3000; alkaline phosphatase

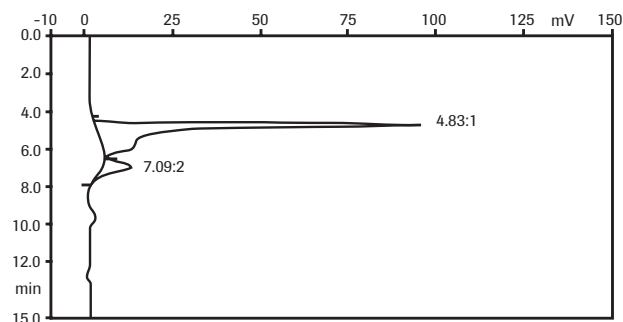


Fig. 3: HPLC, TSK-3000; Conjugate of IgG and alkaline phosphatase

3.3 Trademarks

Sephacryl, Sephadex, and PD-columns are trademarks of Pharmacia AB, Uppsala, Sweden.

3. Supplementary Information

3.1 Conventions

Symbols

In this Instruction Manual, the following symbols are used to highlight important information:

Symbol	Description
ⓘ	Information Note: Additional information about the current topic or procedure.
⚠	Important Note: Information critical to the success of the procedure or use of the product.

3.2 Ordering Information

Roche Applied Science offers a large selection of reagents and systems for life science research. For a complete overview of related products and manuals, please visit and bookmark our home page, www.roche-applied-science.com.

Product	Pack Size	Cat No.
Alkaline Phosphatase recombinant, highly active	10 mg	03 359 123 001
Alkaline Phosphatase (EIA grade, from calf intestine)	3 mg	10 567 744 001
	15 mg	10 567 752 001
Bovine Serum Albumin	1 g	10 238 031 001
	10 g	10 238 040 001

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