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Basic Methods for the Biochemical Lab

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4 Immunochemical Protocols

The protocols given in this chapter are selected examples of the universe of modern immunology. The demands of a biochemical lab gave the background of this selection; therefore, procedures which afford specialized knowledge, such as, for example, production of monoclonal antibodies, are omitted, and the reader is referred to the respective literature.

As with other biochemical methods, the immunochemical protocols are improved, but modifications are possible and sometimes desirable.

References

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4.1 Conjugation of Haptens (Peptides) to Carrier Proteins

Low-molar mass molecules, such as, for example, chemically synthesized peptides, are in most cases not able to induce antibodies in animals; therefore, these molecules have to be covalently coupled

(conjugated) to carrier molecules. Such macromolecular carriers are, for example, the hemolymph of the keyhole limpet *Megathura crenulata* (KLH), the hemolymph of the eatable snail *Helix pomatia*, the hemolymph of the horseshoe crab *Limulus polyphemus*, cationized serum albumin, hen egg ovalbumin, thyreoglobulin, or dextranes.

To get a directed coupling of a peptide via its N-terminal NH_2-group, the introduction of a monochloroacetylglycyl residue during peptide synthesis is recommended. Of course, coupling via NH_2-, SH-, or carboxyl groups of peptide side chains is also possible.

Production of antiserum with high titer and specifity is done by trial and error, especially because each immunized animal gives antisera with different characteristics; therefore, several groups of animals should be immunized with different antigen preparations.

Universal haptens, suitable especially in immunohistochemistry, dot blots, Western blots, or other kinds of immunoassays, are, for instance, biotin, fluorescein, or digoxigenin.

Some coupling reagents suitable for preparation of hapten-carrier conjugates are given in Table 4.1.

Table 4.1. Selected reagents for protein and peptide conjugation to carrier proteins

Coupling reagent	Peptide Binding to	Carrier protein
1-Cyclohexyl-3-(2-morpholin-4-yl-ethyl) carbodiimide	$-NH_2$, -COOH	-COOH, $-NH_2$
1-Ethyl-3-(3-dimethyl-aminopropyl) carbodiimide (EDC; EDAC)	$-NH_2$, -COOH	-COOH, $-NH_2$
2-Iminothiolane (Traut's Reagent)	$ClCH_2CO-NH-CH_2-CO-$	-SH
3-Maleimidobenzoic acid (MBS) N-hydroxysuccinimide ester	-SH, $-NH_2$	$-NH_2$, -SH
Succinimidyl 4-(N-male-imidomethyl)-cyclohexane-1-carboxylate (SMCC)	-SH, $-NH_2$	$-NH_2$, -SH
Aryldiazonium chloride	-SH, -Tyr	-SH, -Tyr
Bis-diazotated benzidine (BDB)	-Tyr	-Tyr
Glutardialdehyde	$-NH_2$	$-NH_2$
Sodium periodat	$-NH_2$	Saccharide side chain
N-Succinimidyl-3-(2-pyridyl-dithio) propionate (SPDP)	$-NH_2$	$-NH_2$
4-Azido-benzoic acid N-hydroxysuccinimide ester (HSAB)	(photoactivation)	$-NH_2$

Further coupling reagents are substances used in cross-linking proteins, which allow an estimation of interacting area between proteins or parts of a protein. Because of the chemical structure of these substances, spacers are introduced between hapten and carrier.

Cross-linking reagents may be divided into several types:

- Water soluble (contain mostly sulfonic acid residues) and water insoluble (penetrate mostly biological membranes)
- Homobifunctional (two identically reactive groups) and heterobifunctional (at least two chemically different reactive groups)
- Cleavable and not cleavable

Examples of cross-linking bifunctional agents are given in Table 4.2.

Table 4.2. Selected homo- and heterobifunctional cross-linking reagents

Reagent	Homo	Hetero	Water soluble	Cleavable	Distance (nm)
Glutaraldehyde	x		Yes	No	
Dimethyladipinimidate (DMA)	x		Yes	No	0.86
Disuccinimidylsuberate (DSS)	x		No	No	1.14
Bis[β-(4-azidosalicyl-amido)-ethyl]-disulfide (BASED)	x		No	Yes	3.47
Bis[2-(Sulfosuccinimido-oxycarbonyl-oxy)ethyl] sulfone (Sulfo-BSOCOES)	x		Yes	Yes	1.30
Disuccinimidyltartrate (DST)	x		Yes	Yes	0.64
Dimethyl-3,3′-dithiobispropionimidate hydrochloride (DTBP)	x		Yes	Yes	1.19
Succinimidyl 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (SMCC)		x	No	No	1.16
m-Maleimidobenzyl-N-hydroxysuccinimide ester (MBS)		x	No	No	0.99
N-Sucinimidyl-3-(2-pyridyldithio) propionate (SPDP)		x	No	Yes	0.68
4-Sucinimidyl-4-(p-maleinimidophenyl) butyrat (SMPT)		x	No	Yes	1.12

References

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4.1.1 Activation of Proteins with Traut's Reagent Yielding Proteins with Additional Free SH Groups

Solutions/Reagents

A 0.9 M NaCl, 0.1 M $NaHCO_3$, pH 8.0

B 0.9 M NaCl, 10 mM EDTA, disodium salt, 50 mM sodium phosphate, 0.02% NaN_3, pH 7.4

C PBS, 10 mM EDTA, disodium salt, 0.02% NaN_3

Mix 4 mg of KLH or a respective amount of a glycerol-containing solution of KLH (volume maximal 60 µl) with 190 µl Soln. A. Weigh 0.6 mg Traut's reagent (2-iminothiolane) into an Eppendorf tube and add the carrier protein solution. Shake at RT for 30 min.

Equilibrate a 10-ml Sephadex G-25 column with Soln. B and determine the void volume using dextran blue. Apply the reaction mixture and elute with Soln. B. Use the fractions containing the activated protein (in the case of KLH a light blue solution) immediately for conjugation (e.g., Protocol 4.1.3).

If BSA or ovalbumin is used, dissolve and elute the protein in Soln. C and monitor elution by UV reading.

Conjugate MCA-Gly peptides or maleimid-activated proteins to the iminothiolane-acivated carriers.

4.1.2 Conjugation of MCA-Gly Peptides to SH-Carrying Proteins

Weigh 4 mg of a monochloroacetylglycyl peptide (MCA-Gly peptide, peptide carrying a MCA-glycyl residue at the N-terminus) into an Eppendorf tube and add the activated KLH or other iminothiolane-activated carrier protein. Shake vigorously at RT for 3 h. Dialyze the reaction mixture twice at RT against PBS for 1 h each. Calculate protein concentration from 235-, 260-, and 280-nm readings (cf. Protocol 1.1.7).

Filter the conjugate through a 0.22-µm-tip filter and store in a refrigerator until immunization. For boostering, aliquot the sterile filtrate and freeze at −70 °C or mix with glycerol 1:1 and store at −20 °C.

Important: *Prepare conjugates with different carrier proteins for immunization and testing and use different coupling reagents, if possible.*

References

Lindner W, Robey FA (1987) Int J Peptid Res 30:794

4.1.3 Conjugation of Sulfhydryl Peptides Using 4-(N-Maleimidomethyl)-Cyclohexane-1-Carbonic Acid N-Hydroxysuccinimide Ester (SMCC)

Solutions/Reagents

A 50 mM HEPES, pH 7.4[1]
B 50 mM HEPES, 10 mM EDTA, pH 6.8
C 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2

Dissolve 4 mg of the carrier protein in 400 µl Soln. A. Dissolve about 0.5 mg SMCC (succinimidyl 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate, M_r 334.3)[2] in 50 µl DMF and add this solution to the carrier protein. Shake at RT for 1 h, centrifuge and desalt on a Sephadex G-25 column, equilibrated with Soln. B (the activated carrier appears in the void volume). Cool the receiving tube in an ice bath.

Dissolve 4 mg of a Cys-containing peptide in 400 µl Soln. B and mix with the solution of the activated carrier protein. Shake at RT for 1 h, aliquot and freeze at −70 °C.

Conjugation of antibodies

Dialyze an antibody solution against Soln. C and concentrate to 20–30 mg/ml. Add 3 mg of sulfo-SMCC (3-sulfosuccinimidyl-4-(N-maleimidomethyl)-cyclohexane-1-caboxylate, M_r 436.7) or of sulfo-GMBS (N-(γ-maleimidobutyryloxy)-3-sulfo-N-hydroxysuccinimide ester, M_r 382.3), dissolved in ddH_2O to 60 mg/ml, to 10 mg of antibody, rock at RT for 15 min and add further 3 mg of coupling reagent. Desalt on a Sephadex G-25 column, equilibrated with Soln. A, after total incubation time of 30 min and use the activated antibody (protein) for conjugation immediately (see above).

References

Hermanson G (1996) Bioconjugate techniques. Academic Press, San Diego. p 235, p 444

[1] If KLH is used, the buffer should contain 0.9 M NaCl and 0.25 mg SMCC/4 mg KLH are used.

[2] Instead of the DMF solution of the water-insoluble SMCC, the same amount of sulfo-SMCC in water my be used.

4.1.4 β-Galactosidase-Immunoglobulin Conjugate (Coupling via SH Groups)

β-Galactosidase from *Escherichia coli* contains sufficient SH groups for conjugation; therefore, only the antibody has to be activated by introducing maleimide groups which react with the SH groups of the enzyme. Conjugation of other proteins or haptens may be performed analogously.

Solutions/Reagents

A 0.1 M sodium phosphate, 0.15 M NaCl, 10 mM EDTA, pH 7.2
B 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2
C ***storage buffer tenfold***: 0.1 M sodium phosphate, 1 M NaCl, 10 mM magnesium acetate, 1% NaN_3 (w/v), 10 mg/ml BSA, pH 6.5

Dissolve the enzyme to a concentration of 1–2 mg/ml in Soln. A (if it is delivered in another buffer, dialyze against Soln. A). Mix 1 mg β-galactosidase in Soln. A with 0.25 mg SMCC activated antibody (concentration about 1 mg/ml; for activation see Protocol 4.1.3). Shake at RT for 2 h, dialyze or desalt on a Sephadex G-25 column against Soln. B and concentrate to about 2 mg/ml. Mix 9 vol. of the concentrated conjugate with 1 vol. Soln. C and store without further purification at 4 °C.

4.1.4.1 Enzyme Reaction of β-Galactosidase

Solutions/Reagents

A ***substrate buffer***: 3 mM p-nitrophenyl-D-galactoside (M_r 301.3), 10 mM magnesium acetate, 10 mM β-mercaptoethanol (M_r 78,13; ρ 1.114 g/ml) in TBS, pH 7.5
B ***stop solution***: 0.1 M EDTA in 2 N NaOH
TBS

In a well of a microtiter plate incubate 100 µl of an appropriate dilution of β-galactosidase conjugate at RT for 30 min. Wash thoroughly with TBS and start enzyme reaction by addition of 100 µl Soln. A per well. Stop after 5–20 min (the time period has to be the same for all wells) by addition of 100 µl Soln. B per well. Mix and read O.D. at 405 nm.

4.1.5 Carbodiimide Coupling of Peptides to Carrier Proteins with 1-Ethyl-3-(3-Dimethylaminopropyl)-Carbodiimide (EDAC, EDC)

Solutions/Reagents

A 0.1 M MES (4-morpholinoethanesulfonic acid) pH 4.7

Dissolve 4 mg of carrier protein (KLH, ovalbumin, BSA or the like) in about 400 µl of Soln. A[3]. Add a solution of 4 mg peptide in 400 µl Soln. A and mix well. The molar ratio should be at least 10 moles of peptide per mole carrier protein.

[3] If KLH is used, the buffer should contain 0.9 M NaCl.

Universal coupling protocol

Dissolve about 20 mg EDAC hydrochloride (M_r 191.7) in 200 µl Soln. A immediately before use and mix vigorously with the peptide-carrier solution. Shake at RT for 2 h.

If necessary, remove surplus reagents and/or make buffer exchange by gel filtration on Sephadex G-25 or dialysis. Concentrate the eluate and the dialysate, respectively, to about 1 ml.

As a variant, activate the peptide separately first and couple then to the carrier: Dissolve the peptide to 1 mg/ml in ddH_2O, add 10 mg EDAC hydrochloride per milligram of peptide, and adjust pH to 5.0. Incubate at RT for 5 min and correct the pH with diluted NaOH during this period. Then add the same volume of carrier protein solution. The amount of carrier protein should be in a ratio of 40 moles of COOH groups per mol peptide (ovalbumin: M_r 42.7 kD, 31 Asp, 48 Glu/Mole; BSA: M_r 67.7 kD, 54 Asp, 97 Glu/Mole). Shake at RT for 4 h and stop the reaction by addition of 1/10 volume of 1 M sodium acetate buffer, pH 4.2. Free the sample from surplus reagents by gel filtration or dialysis and concentrate to about 1 ml by ultrafiltration.

Amount and volume of the conjugate are sufficient for immunization of two rabbits (first immunization 200 µl each, first, second, and third boost, 100 µl each).

References

Hermanson G (1996) Bioconjugate techniques. Academic Press, San Diego. p 170

4.1.6 Conjugation of Horseradish Peroxidase (Glycoproteins) by Periodate Oxidation

Glycoproteins, such as horseradish peroxidase, are coupled selectively to other proteins or NH_2-groups bearing molecules via oligosaccharide side chain oxidation. The vicinal OH groups of oligosaccharide residues are oxidized by periodate to aldehyde groups, which react with amines to form imines (Schiff bases).

Solutions/Reagents

A 0.15 M sodium metaperiodate (32 mg/ml ddH_2O)
B 10 mM sodium acetate buffer, pH 4.5
C 0.2 M sodium carbonate buffer, pH 9.5
D 10 mM sodium carbonate buffer, pH 9.5
E 4 mg/ml $NaBH_4$ or $NaBH_3CN$ or 50 mM ascorbic acid, freshly prepared in ddH_2O
F 10 mg/ml BSA in PBS
PBS

Dissolve 2 mg purified enzyme ("Reinheitszahl" RZ $\approx$ 3; RZ = A_{403}/A_{274} at 0.5–1 mg/ml; for purification see Protocol 3.5.2.4) in 0.5 ml ddH_2O. Add 25 µl Soln. A to the stirred enzyme solution.

Important! *A color change has to occur. If no change is observed, discard the HRP.*

Continue stirring at RT for 20 min and dialyze twice against 100 vol. Soln. B each for 3 h. Transfer the dialysate into a fresh reaction tube, add quickly 10 µl Soln. C, vortex, and add immediately 5 mg antibody in Soln. D (about 10 mg/ml).

Incubate at RT on a shaker for 2 h, then add 1/10 vol. of Soln. E and mix again. Allow the reduction of SCHIFF bases at 4 °C for 2 h, and then dialyze against PBS overnight. Instead of dialysis, a gel filtration on a Sepahdex G-25 column, equilibrated with PBS, is possible. Concentrate the brownish dialysate and eluate, respectively, and add BSA to a final concentration or 10 mg/ml, mix with an equal volume of glycerol, and store at 20 °C.

Important! *Never bring HRP solutions in contact with sodium azide!*

Instead of dialysis or gel filtration, an affinity chromatography on Concanavalin A Sepharose (cf. Protocol 3.6.2.4) is recommended, because on the one hand, no conjugated antibody is removed, and on the other hand, the sugar used for elution stabilizes the enzyme-antibody conjugate in solution.

Take the enzymatic reaction as described in Protocols 2.5.4.1 and 4.13.1.

References

Nakana PK (1980) In: Nakamura RM, Dito W, Trucker ES III (eds.) Immunoassays. Liss, New York, p 157

Huson L, Hay F (1989) Practical Immunology, 3rd ed. Blackwell, Oxford, p 44

4.1.7 Conjugation of Peptides to Carrier Proteins Using Glutaraldehyde (Two-Step Procedure)

Solutions/Reagents

A PBS

B 50% glutaraldehyde (w/v) in pure water (stabilized solution)

Universal coupling protocol

Dissolve 5 mg of ovalbumin, BSA, or another suited carrier protein in 100 µl ddH_2O. The pH has to be 6, because aggregation occurs above pH 7. Add Soln. B to a final concentration of 2.5% glutaraldehyde and stir at RT for 30 min. Filtrate the reaction mixture on a PD-10 or Sephadex G-25 column, equilibrated with ddH_2O, and collect the activated protein in the void volume[4].

[4] A stock of glutaraldehyde-activated BSA with a concentration of 10 mg/ml may be stored frozen in aliquots. Activated BSA of high stability with about 20 moles aldehyde per mole BSA is made by a dialysis procedure given by ZEGERS et al. (1990) J Immunol Meth 130:195.

Dissolve the required amount of peptide (1 mole peptide per about 50 moles of lysine residues of the carrier, e.g., 60 µmol of peptide per 5 mg ovalbumin) in PBS and mix with the activated carrier. Stir at RT for 1 h and block with 10 mg/ml of solid $NaBH_4$ at RT for 20 min. Alternatively, reduce (block) the formed azomethines (SCHIFF bases) to secondary amines by addition of ascorbic acid (final concentration 5 mM).

Dialyze or desalt by gel filtration, aliquot, and freeze until immunization.

References

Kerr MA, Thorpe R (eds.) (1994) Immunochemistry Labfax. Academic Press, Oxford. p 66

4.1.8 Conjugation of HRP to Antibodies with Glutaraldehyde

Another possibility of conjugating HRP to proteins is the glutaraldehyde-mediated coupling. We prefer the oxidative coupling (see Protocol 4.1.6) because it does not influence the polypeptide backbone of the enzyme, but glutaraldehyde conjugation gives satisfying results, too.

Solutions/Reagents

A 25% glutaraldehyde (w/v) in ddH_2O
B 1 M sodium carbonate buffer, pH 9.5
C 0.2 M lysine in pure water
PBS

Dissolve 10 mg of purified HRP ("Reinheitszahl" RZ $\approx$ 3) in 0.2 ml PBS, then add 10 µl Soln. A. Incubate at RT overnight, dialyze against 25 ml PBS and fill up to 1 ml with PBS.

Mix 1 ml of the antibody solution (5 mg/ml PBS) with the activated HRP and with 0.1 ml Soln. B. Allow to react at 4 °C overnight, then add 0.1 ml Soln. C and incubate at RT for 2 h. Finally, dialyze against 25–30 ml PBS.

Purify the conjugate by affinity chromatography on Concanavalin A Sepharose (Protocol 3.6.2.4), add glycerol to 50% final concentration and store aliquots at −20 °C. The aliquots are stable for months.

References

Dent AH, Aslam M (1999) The preparation of protein-protein conjugates. In: Aslam M, Dent AH (eds.) Bioconjugation. Protein coupling techniques for the biomedical sciences. Macmillan Reference Ltd., London, p 216

Boorsma DM (1983) In: Cuello AC (ed.) Immunhistochemistry. IBRO Handbook Series: Methods in the neurosciences, vol. 3. Wiley, Chichester, p 87

4.1.9 Alkaline Phosphatase-Immunoglobulin Conjugate (Glutaraldehyde Protocol)

The advantage of the use of alkaline phosphatase (AP) lies in the high purity of commercially available enzyme. But it is difficult to get such highly specific activities as for HRP, because there is no simple procedure for separation of unbound antibodies from the conjugate.

A 1% glutaraldehyde (w/v) in ddH_2O
B 50 mM Tris·HCl, pH 8.0, 1 mM $MgCl_2$, 0.02% NaN_3(w/v) PBS

Mix 5 mg of affinity-purified immunoglobulin in 1 ml PBS with 10 mg AP[5] in 1 ml PBS. The molar ratio should be 1 mol IgG: 2 mol AP (M_r IgG 150 kD, M_r AP 140 kD). Dialyze the mixture twice at 4 °C against 100 ml PBS for 6–9 h each.

Add 0.15 ml Soln. A to the dialysate. Agitate at RT for 2 h and continue at 4 °C overnight. Dialyze three times against 100 ml PBS for 3 h each. Bring the dialysate up to 2 mg BSA and fill up to 10 ml with Soln. B. Aliquote and store at −20 °C.

A separation of conjugated AP from unbound enzyme in EIA is not necessary, because free AP is removed during the washing steps.

4.1.9.1 Enzymatic Reaction of Alkaline Phosphatase from Calf Intestine

This protocol gives soluble colored reaction products used for enzyme immunoassays in test tubes. A procedure yielding insoluble reaction products is given in Protocol 2.5.4.2.

Solutions/Reagents

A 2.7 mM p-nitrophenyl phosphate (pNP; M_r 371.15, disodium salt hexahydrate), 0.5 mM $MgCl_2$, 0.1 M diethanolamine, pH 9.5, in ddH_2O. Use highly pure colorless pNP
B 0.1 M EDTA in 2 N NaOH

Warm 0.5 ml of Soln. A up to 37 °C and add 5 µl conjugate and conjugate dilution in PBS, resp. Stop with 100 µl Soln. B after 5 to 10 min. If the assay is done in parallels, each probe has to be stopped after exactly the same time. Read the O.D. at 405 nm.

4.1.10 Labeling of Immunoglobulins with Fluorescent Dyes

Solutions/Reagents

A 0.2 M carbonate buffer, 0.5 M NaCl (2.12 g anhydrous Na_2CO_3, 2.92 g NaCl in 100 ml ddH_2O), adjust to pH 9.2 with HCl
PBS

[5] If AP is supplied as ammonium sulfate suspension, spin an aliquot containing 10 mg with 12 000 × g at 4 °C for 15 min. Discard the supernatant completely, add 1 ml PBS, followed by 1 ml of antibody solution in PBS.

Add 33 µl of fluorescein isothiocyanate (FITC; tetramethylrhodamine isothiocyanate, TRITC, or another fluorescent dye isothiocyanate derivative is used the same way), 50 mg/ml in DMF, to 1 ml of 5 mg/ml IgG in Soln. A. Shake at RT protected from light for 1 h. Remove surplus FITC and its hydrolysis products on a Sephadex G-25 column, equilibrated with PBS. The conjugate appears in the void volume and should be concentrated by ultrafiltration. Add sodium azide to a final concentration of 0.02% (w/v) and glycerol up to 10% (w/v) and store at 4 °C.

A selection of fluorescent dyes is given in Table 4.3.

Calculate the degree of substitution F/P (nmoles FITC per nmoles IgG) using the following equation:

$$F/P = \frac{A_{495} \cdot V \cdot 13.1515 \cdot 160}{P}$$

A_{495}, absorption of the sample at 495 nm and 1 cm path; V, total volume of the conjugate in milliliters; P, total amount of IgG in micrograms.

A good conjugate has an A_{495}/A_{280} quotient of 0.3 – 1.0.

The protein concentration P is determined by measuring the sample dissolved in PBS (reference: PBS) at 280 nm with consideration to the fluorochrome-specific absorption at 495 nm:

$$\text{Fluorescein:} \quad P = \frac{A_{280} \cdot (0.35 \cdot A_{495}) \cdot \text{dilution}}{1.38} \text{ (mg/ml)}$$

$$\text{Tetramethylrhodamine:} \quad P = \frac{A_{280} \cdot (0.56 \cdot A_{550}) \cdot \text{dilution}}{1.38} \text{ (mg/ml)}$$

The ratio F/P (moles fluorochrome per milligram of protein) is calculated by

$$\text{Fluorescein} \quad F/P = \frac{2.87 \cdot A_{495}}{A_{280} - (0.35 \cdot A_{495})}$$

$$\text{Tetramethylrhodamine} \quad F/P = \frac{4.47 \cdot A_{550}}{A_{280} - (0.56 \cdot E_{550})}$$

P: protein concentration in mg/ml; A_{280}, A_{495}, A_{550}: absorption at 280, 495 (fluorescein), and 550 nm (tetramethylrhodamine), respectively; 1-cm path, blank PBS; dilution: dilution of the sample (dilution is 101, if 10 µl of conjugate solution are added to 1000 µl).

References

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Hermanson GT (1996) Bioconjugate Techniques. Academic Press, San Diego, p 303

Hudson L, Hay FC (1989) Practical Immunology. 3rd ed. Blackwell Sci. Publ., Oxford, p 34

Table 4.3. Fluorescent dyes[6]

Fluorescent dye and derivative	Excitation (nm)	Emmission (nm)	Coupling to
2′,4′,5′,7′-Tetrabromosulfon fluorescein isothiocyanate	528	544	NH_2
2′,7′-Dichlorofluorescein isothiocyanate	510	532	NH_2
4′,5′-Dichloro-2′,7′-dimethoxy fluorescein	522	550	(DNA)
4′,6-(Diimidazoline-2-yl)-2-phenylindole (DIPI)	364	455	
Acridin Orange	460	650	(RNA)
Acridin Orange	500	526	(DNA)
Alexa 350 succinimidyl	346	442	NH_2
Alexa 430 succinimidyl	431	541	NH_2
Alexa 488 succinimidyl and maleimide	495	519	NH_2 and SH
Alexa 532 succinimidyl and maleimide	531	554	NH_2 and SH
Alexa 546 succinimidyl	556	573	NH_2
Alexa 568 succinimidyl	578	603	NH_2
Alexa 594 succinimidyl and maleimide	590	617	NH_2 and SH
BODIPY	500	506	NH_2
BODIPY FL	505	513	NH_2
BODIPY FL Br_2	533	548	NH_2
Cy2, NHS ester	489	506	NH_2
Cy3, NHS ester	550	570	NH_2
Cy3.5, NHS ester	581	596	NH_2
Cy5, NHS ester	649	70	NH_2
Cy5.5, NHS ester	675	694	NH_2
Cy7, NHS ester	743	767	NH_2
DY-495-X5-01, NHS ester	493	521	NH_2
DY-505-X5-02, amino derivative	505	530	COOH
DY-633-03, maleimide	637	657	SH
Eosin	535	542	NH_2
Erythrosin	530	555	NH_2
Ethidium bromide	518	605	(DNA)
Fluorescein isothiocyanate (FITC)	494	518	NH_2
FluorX	494	520	NH_2
Oregon Green 488	496	524	NH_2
Oregon Green 514	511	530	NH_2
R-Phycoerythrin	488	578	
PicoGreen	502	523	DNA
Propidium bromide	535	617	DNA
Rhodamin 6G	525	555	NH_2
Tetramethylrhodamine isothiocyanate (TRITC)	555	580	NH_2
Texas red	595	615	NH_2

4.1.11 Protein-Colloidal Gold Conjugates

Protein-labeled colloidal gold probes suit as well as for immunohistochemistry in electron microscopy as for detection of antigens or glycoproteins on blots. There are several protocols for enhancement of electron density and visible color resulting in higher sensitivity.

The size of the colloidal particles depends on reagents and their concentration during reduction of gold salts. Larger particles with size > 15 nm are obtained when citrate was the reduction agent, smaller particles result from tannic acid reduction. The unlabeled gold sol is stable in ddH_2O, but traces of salt let collapse the colloid. Covering with proteins increases stability, but especially colloids of small diameter tend to aggregate irreversibly during time; therefore, it is recommended to prepare sufficient amounts of colloidal gold and to store it unlabeled, and the protein-coated gold should be made as fresh as possible.

4.1.11.1 Preparation of Colloidal Gold Sol

Solutions/Reagents

A ddH_2O, filtered with 0.45 nm membrane filter and stored dust free and salt free in plastic bottles
B 1% gold(III)chloride (tetrachloroauric acid) (w/v) in Reagent A
C 1% trisodium citrate (w/v) in Reagent A
D aqua regia (chloronitrous acid): 1 vol. concentrated HCl+3 vol. concentrated HNO_3
E 1% tannic acid (w/v) in Reagent A; clear by centrifugation and store in a refrigerator for 1–2 days
F 1% K_2CO_3 (w/v) in Reagent A

To get gold colloids with reproducible size, the given volumes have to be observed exactly. Store the very hygroscopic gold(III)chloride in a desiccator on anhydrous calcium carbonate powder.

Clean an Erlenmeyer flask and a Teflon-coated stirrer bar with Soln. D and wash thoroughly with Reagent A.

Pour 198 ml Reagent A into the flask, add 2.0 ml Soln. B and heat by strong stirring to boiling. Add quickly 2.0 ml Soln. C and reflux by strong stirring for 10 min. The color of the solution changes from pale yellow via gray to purple. The absorption maximum of the prepared colloid with a mean diameter of 20 nm is at 530 nm. Formation of aggregates is characterized by a shift to larger wavelength.

The cooled colloid is filled into and stored in a plastic flask cleaned with Soln. D.

[6] A lot of dye names are trademarks. For detailed information see, for example, Molecular Probes (www.probes.com), BD Biosciences (www.bdbiosciences.com/spectra/), Dynamics GmbH (www.dynomics.de), or GE Amersham Biosciences (www.amershambiosciences.com/life).

References

Frens G (1973) Nature (Phys Sci) 241:20

Gold colloid with smaller diameter is made by reduction of the tetrachloroauric acid with a citrate/tannic acid reaction mixture given in Table 4.4.

Mix 79 ml Reagent A with 1 ml Soln. B and heat to 60 °C while stirring. Prepare the reduction mixture according to Table 4.4 and heat to 60 °C, too. Add quickly the hot reaction mixture to the gold solution and continue heating and stirring for 15 min. When the color has changed to red, reflux for 10 min.

Table 4.4. Reduction mixtures for preparation of colloidal gold (5–10 nm)

Diameter (nm)	Solution A	Solution C	Solution E	Solution F
		(ml)		
≈ 10	16	4	0.09	0
≈ 6	16	4	0.4	0
≈ 4	16	4	2	2

References

Leunissen JLM, De Mey JR (1989) Preparation of gold probes. In: Verkleij AJ, Leunissen JLM (eds.) Immuno-gold labeling in cell biology. CRC Press, Boca Ratan, p 3

4.1.11.2 Adsorption of Protein to Colloidal Gold

Solutions/Reagents

A 5 mM buffer solution with a pH near to the pI of the respective protein
B 2 M KCl in pure water
C 5% Carbowax 20 M (polyethyleneglycol with M_r 20.000)[7]
D 0.05% Carbowax 20 M (w/v), 0.05% Tween 20 (w/v) in PBS
PBS

The adsorption of protein on colloidal gold occurs in a relative small pH range. Immunoglobulins are bound at pH 7.4, Protein A has an optimum at pH 6.5. For adjusting the pH, the gold colloid as well as the protein solution are dialyzed twice at RT against a 100-fold volume of Soln. A for 1 h each.

Determine the amount of protein necessary for saturation of the gold sol: Pipet 0.5 ml of the dialyzed colloidal gold into Eppendorf tubes and add 0.1 ml of a dilution series of the dialyzed protein to

[7] There are differences in polyethylene glycols of different manufacturers. If Carbowax 20 M is not available, check different lots of PEG 200 000.

the gold. Shake at RT for 15 min, then add 0.2 ml Soln. B to each tube. The color of the red gold sol switches to blue, if the amount of protein is not sufficient. Read at 650 nm and plot O.D. against amount of protein. The optimal amount is that dilution which gives just no color change.

Determination of optimal dilution

Dilute the protein solution to a concentration corresponding to the 1.5-fold of the optimal dilution. Pour 1 vol. of the diluted protein into a centrifuge tube and quickly add 5 vol. of dialyzed gold colloid. Vortex and incubate at RT for 15 min. Add 0.1 vol. Soln. C per 10 ml protein gold mixture and vortex again.

Spin with 11 000 × g at RT for 30 min and resuspend the pellet into a volume of Soln. D corresponding to the original volume of the gold sol. Spin again and carefully resuspend the pellet in 1/10 volume of Soln. D.

The red solution is stable at 4 °C for 2-3 weeks. If sediment occurs, spin with 500 × g for 5 min and discard the pellet.

For staining of Western blots use a 1:250 dilution in PBS.

References

Geoghegan WD, Ackerman GA (1977) J Histochem Cytochem 25:1187
Goodman SL, Hodges GM, Livingston DC (1980) Scanning electron microscopy 1980 II:133

4.2 Immunization of Laboratory Animals

Use 50 – 200 µg of antigen (if conjugates are used: of hapten) dependent on species and antigenicity per injection for immunization. If KLH conjugates are used, there is no need for adjuvants. In all other cases, mix the antigen solution with a commercially available adjuvant (the well-known FREUND's complete and incomplete adjurant should not be of first choice since it often induces severe necrosis). For the ratio of antigen solution to adjuvant, read the instructions for use given by the supplier. If the antigen is prepared by electrophoresis, mince the cutted gel band thoroughly in a small volume of PBS and immunize by intramuscular injection of the suspension into the hind legs nearby the lymph nodes or intradermal injection into the back. To treat animals with care, do not apply more than 150 µl of gel suspension per kilogram body weight. Never use suspensions or emulsions for intravenous injection! Especially when solutions are injected intravenously, filter these solutions through a 0.22-µm sterile filter.

Immunization using PAGE samples

As conjugates, complexes from biotinylated hapten and streptavidin may be used. In that case perform the detection with avidin-biotinylated hapten because avidin and streptavidin do not cross-react.

Mix 300 µl of antigen or hapten-carrier conjugate in PBS or TBS (without sodium azide or Thimerosal and without SDS) with 1 ml

of adjuvant based on mineral oil for a single injection dose of one rabbit (one-third of these volumes for injection of mice). Prepare an emulsion by homogenization with a syringe and a thin needle until a stable white cream is obtained. This water-in-oil emulsion is stable for several hours.

An immunization scheme for rabbits is given in Table 4.5. Do not forget to take some milliliters of blood for pre-immun serum immediately before or after the first immunization.

Table 4.5. Protocol of rabbit immunization

Day	Amount of antigen (µg/animal)	Collected blood (ml)	Remarks
0 (Preimmun serum)		5	
1 (First immunization)	50–200		
14 (First boost)	50–100	5	First test
28 (Second boost)	50–100	5	Second test
35 (Third boost)	50–100	5–50	Third test and/or first antiserum
43–44		25–100	Second antiserum

References

Cooper HM, Paterson Y (2000) Preparation of polyclonal antisera. Current protocols in molecular biology. Wiley, Unit 11.12

Green JA, Manson MM (1992) Production of polyclonal antisera. In: Manson MM (ed.) Immunochemical protocols. Methods in molecular biology, vol. 10. Humana Press, Totowa, N.J., p 1

Harlow E, Lane D (1988) Antibodies. A laboratory manual. Cold Spring Harbor Laboratory, p 92

Peters JH, Baumgarten H (eds.) (1992) Monoclonal antibodies. Springer, Berlin, p 39

4.3 Ammonium Sulfate Fractionation of Immunoglobulins

Stir freshly collected blood with a glass rod to bind fibrin and allow clotting first at RT for 1–2 h and then at 4 °C overnight. Pour the serum into a centrifuge tube and centrifuge at 100–300 × g for 10 min. If the serum is not used within the next day, aliquot the serum, freeze rapidly and store below −20 °C.

Solutions/Reagents

A 1 M Tris · HCl, pH 8.0

B saturated ammonium sulfate in ddH_2O (stir about 100 g $(NH_4)_2SO_4$ in 100 ml ddH_2O for several hours, then allow to

equilibrate at RT for at least 2 days; make sure to have a sediment of solid ammonium sulfate within the bottle; adjust pH 7.0 with ammonia)

C PBS

D 0.1 M $Na_2B_2O_7$, 0.02% (w/v) NaN_3 or Thimerosal, pH 8.4

E 10% (w/v) PEG 6000 in D

F 5% (w/v) PEG 6000 in D

Spin two 0.7-ml aliquots of (rabbit) serum at 4 °C and 15 000 rpm for 10 min. Join the supernatants and add 1/10 of volume of Soln. A. Determine volume. Add dropwise and slowly the same volume of Soln. B to give a 50% saturation. Shake at RT for 1 h and spin as described above. Carefully remove the supernatant, discard it, and resolve the precipitate with 1 ml ddH_2O. Determine the volume and add 40% of this volume of Soln. B. Allow to precipitate in the refrigerator overnight. Collect the precipitate by centrifugation, dissolve the pellet with ddH_2O, and dialyze against PBS or TBS. Whereas antibody solutions are stable at 4 °C for a longer period, aliquot the dialysate, determine immunoglobulin content by UV reading (equation d), Protocol 1.1.7, (absorption coefficients see Table 4.6), and store the aliquots at −70 °C.

For fractionation with low contamination by serum proteins of sera from other species the percentage of saturation given in Table 4.7 should be used.

Alternatively, immunoglobulins may be purified by affinity chromatography on Protein A, Protein G, Protein L or thiophilic

Table 4.6. Absorption coefficients of immunoglobulins of different species at pH 7.4

Species	Type of IgG	$A_{1cm}^{1mg/ml}$	λ (nm)
Chicken (yolk)	IgY	1.35	275
Goat	IgG	1.3	280
	IgM	1.3	280
Guinea pig	IgG1	1.357	278
Man	IgG	1.38	280
	IgM	1.45	280
Mouse	IgG	1.34	280
	Fab	1.4	278
	IgA	1.35	275
Rabbit	IgG	1.35	280
Rat	IgG	1.46	280
	IgM	1.25	280
Sheep	IgG	1.22	280

Data from Fasman G (ed.) (1992) Practical handbook of biochemistry and molecular biology. CRC Press, Boca Raton, Florida, p 265

Table 4.7. Ammonium sulfate fractionation of sera of different species

Species	Ammonium sulfate saturation (%)			Content
	1st Precipitation	2nd Precipitation[a]	3rd Precipitation[a]	of Ig (%)
Cat	35	35	30	71
Chicken	35	35	35	73
Goat	45	30	–	83
Guinea pig	40	40	35	74
Hamster	35	35	35	68
Horse	30	30	30	45
Man	50	45	45	
Mouse	40	40	35	75
Pig	35	35	35	72
Rabbit	35	35	35	91
Sheep	35	35	35	84

[a] The amount of ammonium sulfate resulting from the previous precipitation has to be taken into consideration

Data from Jones GL, Hebert GA, Cherry WB (1978) Fluorescent antibody techniques and bacterial applications. H.E.W. Publ., Atlanta

media. Note that loss of immunoglobulin subclasses may occur caused by different binding affinities to the Fc receptors Proteins A and G, and to the light chain receptor Protein L (cf. Table 4.8).

Antibody complexes are precipitated by relatively low concentrations of polyetyleneglycol 6000 (PEG 6000).

PEG precipitation

Dilute serum with Soln. D in a ratio of 1:25 and add the same volume of Soln. E to the dilution. Incubate at 4 °C overnight and spin with 20 000 × g for 20 min. Wash the pellet once with Soln. F and dissolve the precipitate after a further centrifugation in PBS.

References

Harlow E, Lane D (1988) Antibodies: a laboratory manual. Cold Spring Harbor Laboratory, New York, p 298

Digeon M, Laver M, Riza J, Bach JF (1977) J Immunol Methods 16:165

4.4 Removal of Unspecific Immunoreactivities

In Western blots and in immunofluorescence, antisera often give unspecific reactions, especially when rabbit sera are used. These unspecifities may be suppressed by pretreatment with liver powder.

Add about 50 mg of liver powder to 1 ml of antiserum and shake at RT for 15 min. Then spin at 10 000 × g. The supernatant is ready for preparing working dilutions.

Table 4.8. Binding affinities of immunoglobulin subclasses from different species to immobilized Protein A, Protein G, Protein L, and thiophilic chromatography supports

Species	Antibody class	Affinity to Protein A	Protein G	Protein L	Thiophilic medium
Chicken	IgY	–	–	–	++
Cow	IgG1	+	+++	–	
Cow	IgG2	+++	+++	–	
Cow	Total IgG	++	+++	–	++
Goat	IgG1	+	+++	–	
Goat	IgG2	+++	+++	–	
Goat	Total IgG	+	+++	–	++
Guinea pig	Total IgG	+++	++		
Horse	IgG(ab)	+	–		
Horse	IgG(c)	+	–		
Horse	IgG(T)	–	+++		
Horse	Total IgG	++	+++		++
Human	Fab	–	–	+++	
Human	IgA	+	–	+++	
Human	IgD	–	–	+++	
Human	IgE	+	–	+++	
Human	IgG1	+++	+++	+++	++
Human	IgG2	+++	+++	+++	++
Human	IgG3	+	+++	+++	++
Human	IgG4	+++	+++	+++	++
Human	IgM	+	–	+++	
Human	ScFv	–	–	+++	
Human	Total IgG	+++	+++		
Mouse	IgG1	+	++	+++	++
Mouse	IgG2a	+++	+++	+++	++
Mouse	IgG2b	+++	+++	+++	++
Mouse	IgG3	++	+++	+++	++
Mouse	IgM	–	–	+++	
Mouse	Total IgG	+++	+++	+++	++
Pig	Total IgG	+++	+	+++	++
Rabbit	Total IgG	+++	+++	+	++
Rat	IgG1	+	++		++
Rat	IgG2a	–	+++		++
Rat	IgG2b	–	+		
Rat	IgG2c	+++	+++		
Rat	Total IgG	+	++	+++	++
Sheep	IgG1	+	+++	–	
Sheep	IgG2	+++	+++	–	
Sheep	Total IgG	+	+++	–	++
–	Recombinant Ab	–	++	++	

– No binding, + weak binding, ++ medium binding, +++ strong binding

4.4.1 Preparation of Tissue Powder (Liver Powder)

Solutions/Reagents

A 10 mM phosphate buffer, pH 6.5
acetone
diethylether

Wash pig liver with water and remove as much blood as possible. Homogenize chopped pig liver in a blendor with the same volume of Soln. A and cool the homogenate to 0 °C. Pour slowly the homogenate to 10 vol. of acetone cooled to −15 °C. Allow to settle at −15 °C and filter the sediment on a BÜCHNER funnel. Wash the tissue twice with three volumes of cold acetone, followed by a wash with cold diethylether. Spread the dehydrated tissue on filter paper, grind up, allow the solvent to evaporate and store it in a desiccator containing anhydrous calcium chloride at 4 °C. The white liver powder remains stable for months.

References

Kleber H-P, Schlee D, Schöpp W (1997) Biochemisches Praktikum. 5th ed., G. Fischer, Jena, p 24

4.5 Preparation of Egg Yolk IgY Fraction

Solutions/Reagents

A TBS: 20 mM Tris·HCl, 0.5 M NaCl, pH 7.5
B 10% (w/v) dextransulfate in A
C 1 M $CaCl_2$
D 0.5 M EDTA, disodium salt, pH 7.5
E saturated ammonium sulfate solution (see Protocol 4.3)
solid ammonium sulfate

Separate yolks of chicken eggs from egg white and discard egg white. Wash the yolks carefully with water to remove adhering egg white. Suspend the yolks in 5 vol. Soln. A by vigorous stirring. Precipitate lipids and lipoproteins by addition of 6 ml Soln. B and 15 ml Soln. C per 100 ml yolk suspension. Stir at RT for 30–60 min and spin at 5000 × g for 10 min. Wash the pellet with a small volume of Soln. A (about 20 ml per yolk) and centrifuge again. Combine the supernatants and filter through a paper filter. Add Soln. D to the clear filtrate to a final concentration of 30 mM EDTA.

Add 24.3 g solid ammonium sulfate per 100 ml EDTA-filtrate (yields a 40% saturation) and stir at 4 °C for 30 min. Collect the precipitate by centrifugation. Wash the pellet once with 30% saturated ammonium sulfate (3 vol. Soln. E + 7 vol. ddH_2O), spin down again and dissolve the precipitated IgY in a small volume of Soln. A (about 1/10 of the original yolk volume). Dialyze against TBS and determine the IgY content spectrophotometrically.

References

Schwarzkopf C, Thiele B (1996) ALTEX 13 Suppl. 16:35
Staak C, Schwarzkopf C, Behn I, Hommel U, Hlinak A, Schade R, Erhard M (2001) In: Schade R, Behn I, Erhard M, Hlinak H, Staak C (eds.) Chicken egg yolk antibodies, production and application – IgY-technology. Springer, Berlin, p 65

4.6 Antibody Fragmentation

Since antibodies of different species and different subclasses are variably cleaved by pepsin and papain, a test run is recommended to check incubation time and cleavage conditions. Take samples of different incubation time, freeze rapidly, and monitor the fragmentation by SDS-PAGE at the end of the experiment. Apply the samples both with and without DDT.

4.6.1 F(ab')2 Fragments from IgG

Solutions/Reagents

A 100 mM sodium acetate buffer, pH 4.5
B IgG, 5 mg/ml in Soln. A
C 2 M Tris pH 8.8
D 10 mM Tris, 0.15 M NaCl, pH 7.6
pepsin

Test of cleavage conditions

Prepare a series of different concentrations of IgG (1–5 mg/ml) from Soln. A and aliquots of Soln. B. Add 5 µg pepsin per milligram IgG and incubate at 37 °C for 15–24 h. Stop proteolysis by addition of 1/10 volume Soln. C. Check by SDS PAGE for optimal IgG concentration and cleavage time (unreduced F(ab′)2: 110 kD; reduced F(ab′)2: doublet at 25 kD; unreduced Fc: 25 kD; reduced Fc: somewhat below reduced F(ab′)2).

Run the preparative cleavage with up to 10 mg IgG using the optimal conditions found in the previous experiment.

Separate the F(ab′)2 fragments by GPC on Sephadex G-100 from degradation products. The F(ab′)2 fragments elute in the void volume.

References

Harlow E, Lane D (1988) Antibodies: a laboratory manual. Cold Spring Harbor, p 630
Kerr MA, Thorpe R (eds.) (1994) Labfax immunochemistry. Academic Press, Oxoford, p 105

Solutions/Reagents

A 100 mM sodium citrate buffer, pH 3.7
B IgG, 2–10 mg/ml in Soln. A
C 1 mg/ml pepsin in Soln. A
D 2 M Tris base

Alternatively, add 20 µg pepsin (from Soln. C) per milligram IgG to Soln. B. Incubate rabbit IgG at 37 °C for 4 h, add 1/10 volume Soln. D and dialyze against TBS or PBS. Apply the dialysate on a Protein A column and collect the passage containing F(ab′)2 fragments.

The preparation of F(ab′)2 fragments from monoclonal (mouse) IgG is similar, but check for optimal cleavage time and use a Protein-G column.

References

Kürzinger K (1993) Enzymatic and chemical modifications: antibody fragments. In: Masseyeff RF, Albert WH, Staines NA (eds.) Methods of immunological analysis. VCH, Weinheim, p 383

4.6.2 Fab′ Fragments (Rabbit)

Solutions/Reagents

A 550 mM Tris, 5 mM EDTA, pH 8.2
B IgG, 5 mg/ml in A
2-mercaptoethanol
iodoacetamide
PBS

Dialyze F(ab′)2 fragments against Soln. A. Add 2-mercaptoethanol to a final concentration of 0.2 M (15 µl/ml). Incubate at RT for 10 min, cool in an ice bath, and add iodoacetamide to a final concentration of 0.3 M (55.5 mg/ml). Incubate on ice for 1 h and dialyze against PBS or desalt on a Sephadex G-25 column, equilibrated with PBS (Fab′ fragments in the void volume).

4.6.3 Fab Fragments (Rabbit)

Solutions/Reagents

A 100 mM sodium acetate buffer, pH 5.5
B IgG, 5 mg/ml in A
C 1 M cysteine
D 20 mM EDTA
papain

Add 1/20 volume Soln. C, 1/20 volume Soln. D and 10 µg papain/mg IgG to Soln. B. Incubate at 37 °C for 8–12 h, and then add 13.8 mg per milliliter assay volume of iodoacetamide. Allow to react at RT for 30 min, then dialyze against PBS or separate on a Protein-A column (Fab fragments in the passage).

4.7 Heidelberger Curve (Precipitin Curve)

If two molecules having at least two binding sites for each other will interact, you will find a concentration range characterized by forming large aggregates. These aggregates are easy to precipitate.

If an antigen-antibody complex has to be precipitated by a secondary antibody (e.g., antigen bound by mAb precipitated by goat anti-(mouse-IgG) antibody), this range of equivalence must be known. For estimation of the range of equivalence the precipitin assay is used.

Solutions/Reagents

A 20% polyethyleneglycol 6000 (PEG 6000) (w/v) in ddH_2O
B 0.1 N NaOH, 0.1% SDS (w/v) in ddH_2O
PBS

Prepare a series of 1:1 or 1:2 dilutions of the first component, e.g., rabbit immunoglobulin or serum, in PBS. Pipet 0.5 ml of each dilution into a 2 ml tube. Add 0.5 ml of a dilution of the second component, e.g., goat anti-(rabbit IgG) antiserum 1:100 diluted in PBS, to each of the first dilutions. Vortex and incubate at 37 °C for 1 h or at 4 °C overnight. Enhance precipitation by addition of 0.25 ml of Soln. A.

Spin at 8000 × g for 20 min and wash the pellet twice with PBS. If unlabeled components were used, dissolve the pellet with 0.1 ml Soln. B and determine the protein content. If a radioactive labeled compound was involved, count for radioactivity.

Plot the amount of protein and radioactivity, respectively, against dilution. The maximum of the obtained HEIDELBERGER curve indicates the range of equivalence.

References

Heidelberger M, Kendall FE (1935) J Exp Med 62:697

4.8 OUCHTERLONY Double-Radial Immunodiffusion

4.8.1 Purification of Agar

If no agar of improved purity is present, the agar is purified by several dialyses. Suspend 4 g of agar in 100 ml ddH_2O; adjust pH to 7.0 with diluted hydrochloric acid or sodium hydroxide, and heat carefully in a water bath until a clear solution is obtained. Pour the solution onto a plate yielding a 5- to 10-mm-thick layer. After gelation, cut into about 1-cm^2 pieces and dialyze these pieces in a 100-fold volume of ddH_2O for a week. Change the water daily and add to the last portion 0.02% sodium azide. In a closed box, the agar remains stable at 4 °C for month.

4.8.2 Preparation of Slides

Solutions/Reagents

A 1.0 – 2.0% agar or agarose (w/v), 0.02% NaN_3 in barbital buffer, pH 8.4 (cf. Protocol 4.9)
B acetic acid/ethanol/water 1:5:4 (v/v/v)

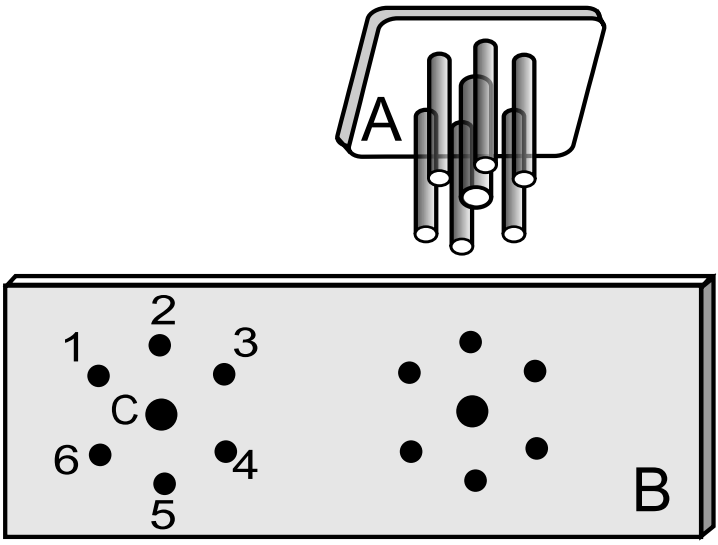

Fig. 4.1. Stamp (*A*) and slide (*B*) with two times of six sample wells (1–6) and larger central well (*C*)

C 0.05% Coomassie Brilliant Blue G250 (w/v) or 0.1% Amido Black 10 B in B
PBS

Melt Reagent A in a water bath or in a microwave oven. Dilute some milliliters of the molten solution with hot water to get 0.5% agar or agarose. Pour this solution onto a carefully defatted slide (3×7 cm) and dry in a stream of warm air. Store a stock of these pre-coated slides dustless; they are stable at RT for months.

Pipet 4 ml of the hot molten Soln. A onto the covered slides laying on a horizontally leveled table. After gelation, punch wells using a stamp as shown in Fig. 4.1. Suck out carefully residual material from the wells using a PASTEUR pipette connected to a pump.

4.8.3 Immunodiffusion

Depending on the question, fill the central well either with the antigen solution or with antiserum. Place the antigen(s) or antisera or antiserum dilutions into the peripheral wells. If the wells are not filled completely, adjust with PBS.

Place the slides horizontally in a humid chamber, e.g., Petri dishes containing wet filter paper, and store in a refrigerator at least for 24 h, better for 48–72 h.

4.8.4 Visualization of the Precipitin Lines

When diffusion is complete, strong precipitation lines are visible when the slide is viewed obliquely against a black background. Weak lines are visualized by staining.

Incubate the slide three times in PBS for 3 h each. Cover the slide with a sheet of filter paper and dry in air or with a hair dryer. Dampen the slide for a short period and remove the filter paper.

Place the slide into a tray, stain with Soln. C for 5–10 min, and discolor with Soln. B until the background is colorless.

4.9 Immunoprecipitation of Antigens

For analytical purposes, antigens are separated by immunoprecipitation from complex mixtures. So proteins are identified in cell lysates after genome expressions, but also receptors together with their ligands or protein complexes are separated by this method. Mostly, the precipitated immunocomplexes are dissociated and subsequently analyzed by SDS-PAGE. Because it is not possible to get large immune complexes in each case, since for example, antibodies are available directed against only one epitope, antigen-antibody complexes may be precipitated using secondary antibody or antibody receptor (Protein A, Protein G, Protein L) covalently immobilized on chromatographic support.

Solutions/Reagents

A ***dilution buffer***: 0.5% Triton X-100 (w/v), 1 mg/ml BSA in TBS

B ***lysis buffer***: 0.1 mM PMSF, 0.2 U/ml aprotinin in A
or: 1% Triton X-100 or Nonidet P-40 (NP40), 0.5% sodium deoxycholate (NaDOC), 0.1% SDS (w/v). 150 mM NaCl, 50 mM Tris, pH 7.5

C ***sample buffer*** (Laemmli system): 50 mM Tris · HCl, pH 6.8, 2% SDS (w/v), 10% glycerol (v/v)
precipitation aid: slurry of immobilized secondary antibody or Protein A or Protein G in PBS or TBS

If proteins were expressed within cells of cell cultures, the cells have to be disrupted as gently as possible. For instance, incubate 10^7 to 10^8 cells in 1 ml Soln. B at 0 °C for 30 min. Vortex and spin at 250 × g. Transfer the supernatant into a new tube and centrifuge at 13.000 × g for 10 min.

To reduce unspecific binding, mix a 200-µl aliquot of the clear supernatant with 2 µl pre-immune serum or unspecific antibody and a further 200 µl aliquot with 50 µl precipitation aid. Rock at 0 °C for 1 h and spin at 1000 × g. Transfer the supernatant into a fresh container and fill it up to 1000 µl with Soln. A. Add 0.5–5 µl of the specific antiserum and monoclonal antibody, respectively, and incubate on ice for 1 h. Prepare a second sample containing pre-immune serum instead of antiserum.

Add 50 µl of precipitation aid, 1:1 diluted with Soln. A, and rock at 0 °C for 1 h. Centrifuge at 500 × g for 1 min, discard the supernatant, and wash the pellet four times with 1 ml ice-cold Soln. A each.

Add 20–50 µl Soln. C to the pellet obtained after the last centrifugation. Heat to 95 °C for 5 min (if necessary, add 2-mercap-

toethanol or DTT to final concentration of 5% and 10 mM, respectively). Centrifuge and apply the supernatant directly onto the SDS-PAGE gel. The immunoprecipitated protein is identified by staining, Western blot, and/or autoradiography.

References

Åkerström B, Björck L (1989) J Biol Chem 264:19740
Harlow E, Lane D (1988) Antibodies: a laboratory manual. Cold Spring Harbor Laboratory, p 447

4.10 Immunoelectrophoresis

Electrophoresis in agarose or agar gels is, in contrast to SDS-PAGE, a nondenaturing method; therefore, the proteins move according to their net charge, which is at the given alkaline pH mostly negative, and are separated by electrophoretic mobility and not by molecular size. The electropherograms obtained after immunoelectrophoresis are not comparable to SDS-PAGE pictures.

Solutions/Reagents

A 15.4 g sodium diethylbarbiturate (barbital sodium, barbitone sodium, Veronal sodium), 2.76 g diethylbarbituric acid (barbital), 1 g NaN_3 dissolved in ddH_2O, pH adjusted to 8.4 and filled up to 1000 ml

B 1.0–1.5% agar (w/v) in 1:1 diluted Soln. A. Melt before use in a microwave oven or a boiling water bath

Pour 4 ml of molten Soln. B onto pre-coated slides (cf. Protocol 4.8.2). Punch wells according to Fig. 4.2 after gelation, and remove residues of agar from the wells.

Mix the probe 1:1 with Soln. A and add a trace of bromophenol blue. Fill the wells completely with sample.

Place the slide on the cooling plate of a horizontal electrophoresis apparatus, fill the tanks with 1:1 diluted Soln. B, and connect the ends of the slide to the buffer tanks by wetted filter paper wicks. Run the electrophoresis at 6 V/cm for about 45 min. Stop electrophoresis when bromophenol blue is about 4 mm from the the wicks.

Cut the antiserum slots "Ab" after finishing the electrophoresis and remove the agar within the slots. Pour antiserum into the slot(s) and store the slide in a humid chamber at 37 °C for 4–6 h or at 4 °C for 24–48 h. Visualize the precipitation lines as described in Protocol 4.8.4.

References

Hudson L, Hay FC (1989) Practical immunology, 3rd ed. Blackwell, London, p 236
Milford-Ward A (1977) In: Thompson RA (ed.) Techniques in clinical immunology. Blackwell, Oxford, p 1

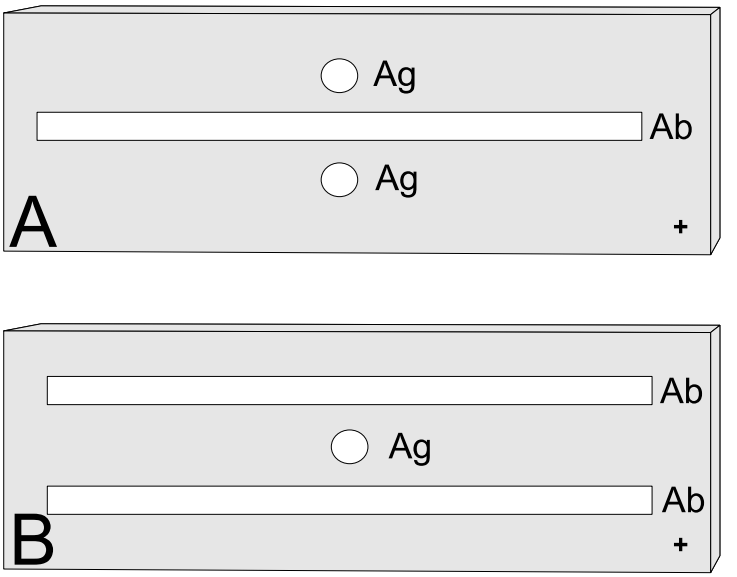

Fig. 4.2. Pattern of immunoelectrophoresis. **A** Two antigen (*Ag*) samples, one antiserum (*Ab*). **B** One antigen, two antisera. + Anode

4.11 Counterelectrophoresis

Solutions/Reagents

A barbital-acetate buffer, pH 8.2, I = 0, 1[8]
B 0.5% agarose (w/v) in ddH_2O
C 1.0% agarose with high endoosmosis (high EEO) (w/v) in 1:1 diluted Soln. A. Melt before use in a microwave oven or a boiling water bath

A synonym for "counterelectrophoresis" is "crossing over electrophoresis."

Pre-coat slides with Soln. B (see Protocol 4.8.2) and prepare the gel by pouring 0.2 ml/cm^2 molten Soln. C onto the slide placed on a leveled surface. Be sure to cover the whole slide with a uniform layer of agarose.

Punch at least two wells of 2- to 3-mm diameter using a PASTEUR pipet or a hypodermic needle. The wells are located in the middle of the slide and should have a distance of 5 mm (Fig. 4.3).

Place the slide into a horizontal electrophoresis apparatus, fill the tanks with Soln. A, and connect the small ends of the slide to the electrode tanks by moistened filter paper wicks. Fill the well directed towards the anode with antiserum dilution and pipet antigen solution containing traces of bromophenol blue in that well which is nearby the cathode.

[8] Ionic strength $I = \frac{1}{2} \cdot \sum_{n=1} (c_i \cdot z_i^2)$; c_i, concentration of ion i, z_i, charge of ion i

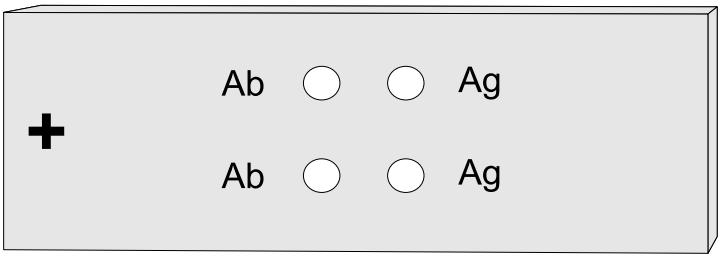

Fig. 4.3. Pattern of counterelectrophoresis. *Ag* antigen well, *Ab* antibody-containing well. + Anode

Run electrophoresis with 20–40 V/cm for about 45 min. It is recommended to cool the slide during electrophoresis.

When electrophoresis is finished, cover the gel with dry filter paper and several layers of tissue paper and press by a weight. Remove the paper after 15 min and agitate the slide in PBS for 2 × 30 min. Stain the gel as described in Protocol 4.8.4. The precipitin bands appear as fine blue lines.

References

Hudson L, Hay FC (1989) Practical immunology, 3rd ed. Blackwell, London, p 241

4.12 Dot-Blot Assay

Mostly plastics, such as nitrocellulose, polyvinylidene fluoride (PVDF), or polystyrene (used for microtiter plates), are used for immobilization of antigens. Because these materials interact with proteins by different mechanisms, sometimes it occurs that the binding material induces or removes epitopes necessary for binding of specific antibodies. So, if no reaction between antigen and antibody is observed, repeat the experiment by using another support (e.g., Nunc offers several types of polystyrene microtiter plates with different binding properties) or bind the antigen via a spacer (e.g., by biotinylation of antigen and immobilization of streptavidin on the support). A dubious positive result should be checked, if possible, by a competition experiment of incubation of the antibody-containing solution in the presence of dissolved (free) antigen.

Test of specifity by competition

Solutions/Reagents

A 0.1% gelatin (w/v) or 5% heat-inactivated calf serum or 0.1% serum albumin (w/v) or 0.2% non-fat milk powder (w/v) or 0.1% Tween 20 (w/v) in PBS

B secondary antibody-HRP conjugate dilution in PBS (dilution for instance 1:1000 to 1:50 000)

C 50 mg/ml 3,3′,4,4′-tetraaminodiphenylether or 3,3′-diaminobenzidine (DAB)or 4-chloro-1-naphthol in DMF or precipitating TMB according to Protocol 2.5.4.1. Stock solutions are stable at RT

Important! *If possible, do not use DAB because it is potentially carcinogenic*

D 0.4 ml C, 20 µl 30% H_2O_2 (alternatively: 6 ml 0.1% H_2O_2-urea adduct in ddH_2O; stable for several day at 4 °C), 0.1 ml 10% $CuSO_4$ (w/v), 0.05 ml 10% $NiSO_4$ or $NiCl_2$ (w/v) in 100 ml PBS. Prepare freshly before use
PBS

Indicate the dots with a pencil in a distance of 3 – 5 mm on a nitrocellulose sheet. Do not touch the nitrocellulose with unprotected fingers! Place the nitrocellulose onto a sheet of filter paper.

Apply 0.1 – 1 µl of antigen solution, diluted in PBS, on the marks. The diameter of the resulting dot should be not more than 3 mm. Dry at air for 10 min and block the nitrocellulose strip in Soln. A at RT for 15 min.

Incubate the wet strip, or parts of it, in antibody dilution in Soln. A at RT for 30 min. The incubation volume should be 0.5 – 1 ml per cm^2. This volume may be reduced if the incubation is done in a lockable tube on a roller desk. Wash the strip in a sufficient volume of PBS, at least three times for 5 min each.

Agitate the wet nitrocellulose in 0.5 ml/cm^2 of Soln. B at RT for 15 min and wash three times with PBS. Place the strip in a fresh container and incubate with Soln. D until the color appears. Stop the enzymatic reaction by discarding Soln. D and washing with water.

For a semiquantitative assay, cut each dot and put it into separate test tubes. Perform the enzymatic reaction with TMB substrate, stop with sulfuric acid and read O.D. at 450 nm. (This procedure is similar to an EIA on microtiter plates.)

4.13 Enzyme Immunosorbent Assay (EIA, ELISA)

The following protocols are not optimized procedures for EIA, but they are suitable for screening, e.g., for antibody titers of sera or mAb cell culture supernatants. A high-performance EIA has to be evaluated with respect to selection of type of microtiter plates, coating concentration, coating conditions, analyte dilution, sample buffer, washing buffer, incubation times and temperatures, conjugate dilution, and substrate composition.

The EIA described in this protocol is a so-called indirect EIA, because the antigen is immobilized onto the surface of the microtiter plate and a second species-specific antibody enzyme conjugate detects the bound antibody of the antiserum. As an example,

the enzyme label in the presented protocol is horseradish peroxidase (HRP), but when alkaline phosphatase or β-galactosidase or biotinylated secondary antibodies and streptavidin-enzyme conjugates are used, proceed analogously; only substrate solutions and stop solutions for AP and β-galactosidase have to be different.

4.13.1 Indirect EIA with HRP Conjugate

A 15 mM Na_2CO_3, 35 mM $NaHCO_3$, 0.02% NaN_3 (w/v), 0.001% phenol red, pH 9.6, in ddH_2O

B 0.05% Tween 20 (w/v) in PBS

C 0.05% Tween 20 (w/v), 0.1% serum albumin or gelatin (w/v), 0.001% phenol red in PBS

D 0.1 M citric acid (1.92 g anhydrous citric acid in 100 ml ddH_2O), adjusted to pH 5.0 with NaOH

E 0.40 mg/ml o-phenylenediamin (OPD), 0:5 µl/ml 30% H_2O_2 in D (prepare freshly)

E′ 0.55 mg/ml 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid), diammonium salt (ABTS), 0.5 µl/ml 30% H_2O_2 in D (prepare freshly each)

E″ 5 vol 0.1 M sodium acetate buffer, pH 5.0, 1 vol. of 1.2 mg/ml N,N,N′,N′-tetramethylbenzidine (TMB) in ethanol, and 4 vol. of 0.1% H_2O_2-urea adduct[9] (w/v) in ddH_2O are mixed immediately prior use

F 1 M sulfuric acid

F′ 0.1% NaN_3 in 0.1 M Tris, pH 7.4

PBS

Secondary antibody-enzyme conjugate, e.g., species-specific anti-(heavy chain)-IgG-HRP conjugate

Dissolve the antigen (pure protein or hapten-carrier conjugate) with a concentration between 0.1 and 50 µg/ml in Soln. A. Pipet 0.1 – 0.2 ml of the antigen solution into the wells of a 96-well microtiter plate (high binding capacity) and incubate on a shaker at RT for 1 h or at 4 °C in a humid chamber overnight.

Remove the antigen solution and wash three times with 200 µl Soln. B each. Beat out the last washing solution on tissue paper and block with 200 µl of Soln. C per well on a shaker at RT for 15 min. Remove the blocking solution and wash twice with PBS. Dry the plate at air and store it until use in a sealed plastic bag.

Pipet 100 µl of the antibody or antiserum dilution (for titer determination a serial dilution in PBS), blank (buffer without antiserum) and, if available, controls into the respective wells and incubate on a shaker at RT for 30 min. Remove the antibody dilution and wash three times with 250 µl of Soln. B each.

[9] H_2O_2-urea adduct (M_r 94,07) is used as a stable substance instead of H_2O_2 solution. The respective amounts of sodium percarbonate or sodium perborate are also suitable.

Pipet 100 µl of a suitable dilution of a secondary antibody-HRP conjugate into each well (the dilutions range from 1:500 to 1:100 000, dependent on the quality of the conjugate, and the amount of bound primary antibody; the dilution has to be checked empirically). Incubate on a shaker at RT for 30 min. Wash with Soln. B at least three times to remove traces of unbound conjugate.

Start enzyme reaction by addition of 100 µl Soln. E, Soln. E′, or Soln E″.

Important: *The time for enzymatic reaction has to be the same in each well; therefore, pipet substrate solution and stop solution exactly with the same rhythm. Enzyme reactions depend on time and temperature!*

Select the incubation time for an O.D. of the mostly colored well between 0.8 and 2.5. These values are reached usually between 5 and 15 min. Stop color development by addition of Soln. F and Soln F′, respectively. (If OPD or TMB are substrates, stop with Soln. F, in the case of ABTS use Soln. F′).

Read the O.D. at 492 nm (OPD), 450 nm (TMB), and 405 nm (ABTS), respectively, in a plate reader.

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4.13.2 Determination of Enzyme Activity by ELISA

Solutions/Reagents

A 15 mM Na_2CO_3, 35 mM $NaHCO_3$, 0.02% NaN_3 (w/v), 0.001% phenol red, pH 9.6, in ddH_2O
B 0.05% Tween 20 (w/v) in TBS
C 0.1% cold fish gelatin in Soln. B
D 5 vol. 0.1 M sodium acetate buffer, pH 5.0, 1 volume of 1.2 mg/ml N,N,N′,N′-tetramethylbenzidine (TMB) in ethanol and 4 vol. of 0.1% H_2O_2-urea adduct (w/v) in ddH_2O are mixed immediately prior use
E 1 M sulfuric acid
TBS

Dilute anti-(mouse-IgG)-IgG (in the case of monoclonal antibodies; for testing conjugates prepared from antibodies of other origin, use the respective anti-species specific antibody) to 5 µg/ml in Soln. A. Coat the wells of a microtest plate with 100 µl/well of this dilution and incubate at 4 °C overnight. Remove antibody solution and wash once with TBS. Add 150 µl Soln. C per well and incubate at

RT for 30 min. Remove blocking solution, rinse once with Soln. B and add 100 µl/well of a dilution series (e.g., 1:500, 1:1500, 1:4500, 1:13 500, 1:40 500, 1:121 500, 1:364 500 in TBS) of antibody-enzyme conjugate to the wells. Shake at RT for 30 min, remove conjugate solution, knock out the plate on paper tissue, and rinse three times with Soln. B.

Perform the enzymatic reaction with Soln. D for 10–30 min (each well has to react for the same time and at the same temperature) as described above, stop with Soln. E and read O.D. at 450 nm. Plot O.D. against conjugate dilution. The working dilution of the conjugate should give an O.D. of 1.5–2.5.

4.13.3 Isotype Determination by EIA (AP Conjugate)

This protocol describes the use of an alkaline phosphatase conjugate; of course, a HRP conjugate works well, too.

Solutions/Reagents

A 15 mM Na_2CO_3, 35 mM $NaHCO_3$, 0.02% NaN_3 (w/v), 0.001% phenol red, pH 9.6, in ddH_2O

B 0.05% Tween 20 (w/v), 0.02% NaN_3 (w/v) in PBS

C 0.1% serum albumin or gelatin (w/v), in Soln. B

D 1 mg/ml p-nitrophenyl phosphate (pNP; M_r 371.15, disodium salt hexahydrate), 0.5 mM $MgCl_2$, 0.1 M diethanolamine, pH 9.5, in ddH_2O. Use highly pure colorless pNP and prepare substrate solution freshly

E 1 N NaOH

PBS

antibody recognizing all subclasses of heavy chains of an antibody class of a species, e.g., anti-(mouse-γ-chain)-IgG (goat) secondary isotype-specific antibody-AP conjugate, e.g., anti-(mouse-IgG1)-IgG (goat) AP conjugate

Dilute the class-specific capture antibody to 2 µg/ml in Soln. A. Apply 100 µl/well of the coating solution to a microtiter plate and incubate at 4 °C overnight. Remove the liquid from the plate and wash once with Soln. B. Block the wells with 150 µl Soln. C at RT for 2 h. Knock out the blocking solution and wash once with Soln. B. Store the plate in a sealed bag at 4 °C.

Dilute antiserum or hybridoma supernatant in a geometric series with Soln. B. starting at 1:100 and 1:10, respectively. Pipet 100 µl of each dilution in duplicates into the wells and also for positive as well as for negative control of an appropriate dilution of a characterized immunoglubulin. Incubate at RT on a shaker for 1 h. Wash three times with at least 200 µl/well of Soln. B. Add 100 µl of AP conjugate dilution in Soln. B (if the conjugate is not tested and no recommendations are given by the supplier, start with a 1:10 000 dilution). Incubate at RT for 30 min, knock out the conjugate solution, and wash three times with Soln. B. Start enzymatic reaction by addition of 100 µl/well of Soln. D. Stop after 10.0 min at RT with 100 µl/well of Soln. E. Read absorption at 405 nm in a plate reader.