

Tips for Antibody Production

Protocol for ELISA Titration of Anti-Serum

The ELISA test is performed to determine the interaction between the peptide and the antibody by calculating the titer of a specific polyclonal antibody serum.

Material and Buffers (stored at 4°C):

- Coating buffer: 0.05 M NaHCO₃/Na₂CO₃ buffer, pH 9.5
- EIA buffer: 0.10 M PBS with 0.1% BSA, pH 7.5
- Washing buffer: 0.01 M PBS-T, pH 7.0-7.2

ELISA Procedure:

- Coat 96-well plates with 100 µL peptide-conjugate (PP-BSA) per well at a concentration of 20 µg/mL in coating buffer.
- Incubate at 4°C overnight on orbital shaker.
- Remove unbound PP-BSA conjugate by washing with 200 µL washing buffer (2x) and pad dry on paper towel.
- Blocking: add 200 µL EIA buffer and incubate for 2 h at room temperature.
- Discard solution, wash with 200 µL washing buffer (3x), and pad dry on paper towel.
- Prepare serum dilution by diluting the serum with EIA buffer.
- Make 1:5 serial dilutions:

Sample preparation for pre-bleed serum (control) & test bleed:

A. 1:100 (10 µL A + 990 µL EIA)

Sample loading starting from B:

B. 1:1,000 (100 µL A + 900 µL EIA) (1K)
C. 1:5,000 (200 µL B + 800 µL EIA) (5K)
D. 1:25,000 (200 µL C + 800 µL EIA) (25K)
E. 1:125,000 (200 µL D + 800 µL EIA) (125K)
F. 1:625,000 (200 µL E + 800 µL EIA) (625K)

- Add 100 µL serum samples to wells, run in duplicate and incubate at room temperature for 2 h.
- Discard the serum dilution and wash wells thoroughly and rinse immediately with 200 µL washing buffer (3x).
- Dilute the secondary antibody of Goat anti-Rabbit HRP conjugate at proper dilution (e.g., 1:2,000) in EIA buffer.
- Add 100 µL secondary antibody per well and incubate at room temperature for 1 h.
- Discard the solution and wash wells thoroughly and rinse immediately with washing buffer (5x), followed by distilled water (2x).
- Add 100 µL K-Blue substrate and develop at room temperature for 10-30 min.
- Read plate at 630 nm (blue color) in a micro-well reader.
- Add 50 µL 1 N HCl to each well to stop the color development and read immediately at 450nm (yellow color).
- Determine the titer in serum; titer value is estimated to be the dilution where O.D. is about 0.1, except when pre-bleed O.D. is = 0.2 (when pre-bleed O.D. is = 0.2, the O.D. of pre-bleed is used to determine the titer).
- Prepare ELISA data for each anti-serum.

Affinity Purification Protocol

This method is used for affinity purification of anti-peptide antibodies from polyclonal serum.

Material and Buffers:

- 0.01 M Tris buffer with EDTA, pH 7.2
- PBS buffer, pH 7.2
- 0.1 mM Glycine buffer, pH 2.5

Peptide Coupling:

- Weigh out 10 mg peptide and 1.5 g thiosepharose 6B; adjust the amount of peptide and thiosepharose if necessary.
- Add ~10 mL purged HPLC grade H₂O to swell the resin for 10-15 min; centrifuge and discard the supernatant.
- Dissolve 10 mg peptide in minimal amount of 0.01 M Tris buffer.
- Couple the peptide and thiosepharose for 2 h at room temperature.

Extracting Antibodies:

- Pack the peptide-coupling column.
- Wash column with PBS buffer to eliminate excess peptides (O.D. = 0.08 at 280 nm)
- Dilute serum with Tris buffer (1:1) (use approximately 8-10 mL serum); add solution to the column at room temperature for 1 h and collect flow through.
- Wash the column with PBS buffer, and elute column with 25 mL Glycine solution and collect fractions.
- Read fractions at 280 nm; collect and dialyze appropriate fractions (highest O.D. against PBS).
- Determine the concentration of the antibody, aliquot, and store at -20°C for long-term storage.

Cell Lysate Preparation Protocol

Cell/tissue lysates for Western blots can be obtained from cultured cell lines or animal (mouse, rat, etc.) tissues.

Material and Buffer:

- Lysis buffer
 - 10 mM Tris
 - 5 mM EDTA
 - 50 mM NaCl
 - 50 mM NaF
 - 30 mM Na-Pyrophosphate
 - 200 μM Na-Orthovanadate
 - 1 mM Phenylmethylsulfonylfluoride (PMSF)
 - 1% Triton-X100
 - 5 μg/ml Aprotinin
 - 1 μg/ml Pepstatin-A
 - 2 μg/ml Leupeptin

* Note: Orthovanadate needs to be specially treated for lysis buffer preparation. Prepare 100 mM Na-Orthovanadate, adjust to pH 10.0; the solution is yellow in appearance. Boil the solution until it turns clear color, cool down to room temperature, readjust pH to 10.0, repeat cooling and boiling until colorless solution stays at pH 10.0. Store aliquots at -20°C.

Procedure:

- When the density of a cell line reaches 60-80% confluency, wash the cells with cold PBS (3x) and add 1 mL Lysis buffer per each 75-mL flask.
- Rinse tissue sample with cold PBS (3x) and add 3 mL Lysis buffer per 1 g wet tissue.
- Incubate in the Lysis buffer for 5 min at room temperature with gentle agitation and scrape the cells from the flasks.
- Sonicate with 5 second-pulses (4x) and cool on ice.
- Centrifuge homogenates at 2400xg for 20 min to eliminate cell debris.
- Add 1 mL 5x sample buffer per 4 mL supernatant.

5x Sample Buffer

		1x Final Conc
0.5 M Tris, pH 6.8	2.5 mL	25 mM
SDS	1 g	2%
1% Bromophenol Blue	0.33 mL	0.002%
Glycerol	5 mL	10%
2-Mercaptoethanol	2.5 mL	5%

- Aliquot and store at -80°C.

SDS-Polyacrylamide Gel Electrophoresis

Proteins can be separated on Polyacrylamide gels on the basis of size. Choose a polyacrylamide gel concentration that will allow a good separation of the band(s) of interest using the table below.

% Acrylamide	Best Resolution Range (kDa)
5	25-200
10	15-70
15	12-45

- Prepare a gel according to the recipe below. Mix the components in an Erlenmeyer flask in the fume hood with constant stirring under vacuum on for 15 min to remove air bubbles. Handle unpolymerized acrylamide powder with caution as it is a potent neurotoxin and its effects are cumulative.

	Resolving Gel						Stacking Gel						
	7.5%		10%		12%		15%		20%		4%		
# of Gel	1	2	1	2	1	2	1	2	1	2	1	2	4
H2O (mL)	2.5	5	2	4	1.75	3.5	1.25	2.5	0.25	0.5	3.1	6.2	12.4
0.5 M Tris-HCl(pH 6.8) -0.4% SDS (mL)	-	-	-	-	-	-	-	-	-	-	1.25	2.5	5
1.5 M Tris-Base(pH 8.8) -0.4% SDS (mL)	1.25	2.5	1.25	2.5	1.25	2.5	1.25	2.5	1.25	2.5	-	-	-
30% Acrylamide -0.8% Bis (mL)	1.25	2.5	1.75	3.5	2	4	2.5	5	3.5	7	0.65	1.3	2.6
10% Am Persulfate (μL)	30	50	30	50	30	50	30	50	30	50	30	50	100

TEMED (μL)	3	5	3	5	3	5	3	5	3	5	3	5	10
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- Pour running buffer into the anode and cathode chambers.

10x Running Buffer

		1x Final Conc
Glycine	144 g	192 mM
Tris-base	30 g	25 mM
SDS	10 g	0.1% (w/v)

q.s. to 1 L with distilled H₂O and dilute to 1x before use.

- Preparation of samples:
 For human cell extracts: add 100 μL of 4x sample buffer into 300 μL of cell extract (A₂₈₀=3.0).
 For bacterial cell extracts: dissolve the final pellet in equal amount of 2x sample buffer.
 For insect cells infected with baculovirus: use a lysate from 3000 cells per lane in 10-well comb with diluted sample buffer. Heat the samples at 95°C for 10 min before loading on the gel. Please refer to the formula for sample buffer.

Sample Buffer

	2x	3x	4x	5x
Tris-HCl (pH 6.8)	50 mM	75 mM	100 mM	125 mM
SDS	4%	6%	8%	10%
Bromophenol blue	0.004%	0.006%	0.008%	0.01%
Glycerol	20%	30%	40%	50%
2-Mercaptoethanol	10%	15%	20%	25%

- Load a lane of 10 μL of pre-stained molecular weight standards; 30 μL of sample/well or 300-500 μL/space.
- Run the gel according to the electrophoresis unit's manufacturer's instructions.
- Transfer proteins at 4°C from the SDS-PAGE gel to either a nitrocellulose or a PVDF membrane. (Note: PVDF membranes need to be activated with methanol before use. Smaller proteins require less transfer time than larger proteins.)

5x Transfer Buffer

		1x Final Conc
Tris-HCl	7.68 g	10 mM
Tris-Base	9.25 g	15 mM
Glycine	72.08 g	192 mM

SDS	2.5 g	0.05% (v/v)
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q.s. to 1 L with distilled H₂O and dilute to 1x before use.

Western Blot Protocol

In a Western blot, proteins can be separated on Polyacrylamide gels on the basis of size, transferred to a membrane, detected with antibodies, and visualized by the addition of an enzyme substrate.

- After the transfer is completed, membranes can either be air-dried or blocked immediately with 3-5% of either BSA or non-fat dry milk in TBST for at least 1 h or overnight. Stain gel with Coomassie Brilliant Blue R250 or Silver stain to check if transfer is complete. Any air-dried PVDF membranes need to be re-activated with methanol before use.

TBST Buffer

		Final Conc
Tris-Base	1.21 g	10 mM
NaCl	8.77 g	150 mM
Tween-20	0.5 mL	0.05% (v/v)

q.s. to 1 L with distilled H₂O.

- Apply primary antibody at 1-3 µg/mL or serum at 1:100-1:1000 dilution. Dilute in blocking solution (BSA or milk concentration can be decreased 2-5 fold) in TBST for 1-2 h at room temperature or at 4°C overnight.
- Wash membrane in TBST at room temperature (4x) for 5 min/wash.
- Apply a secondary antibody that is either alkaline phosphatase (AP) or horseradish peroxidase (HRP) conjugated against the host species of the primary antibody. For example, if the antibody was raised from a rabbit, then use either AP- or HRP-conjugated whole IgG (from goat, donkey, or mouse) of anti-rabbit. Incubate at 0.1-1.0 µg/mL in blocking solution for 1-2 h at room temperature with shaking.
- Wash membrane in TBST at room temperature (4x) for 5 min/wash.
- Develop blot using BCIP/NBT for AP-conjugated secondary antibodies or diaminobenzidine tetrahydrochloride (ABTS) for HRP-conjugated secondary antibodies or commercially available ECL kits.
- The probed membrane can be re-probed after stripping. Stripping buffer: 62.5 mM Tris-HCl (pH 6.8), 2% SDS, 0.1 M 2-Mercaptoethanol. Probe it using the above procedure.

Troubleshooting for Western Blot

High background	Increase concentration of BSA or milk
Low signal	Check the accessibility of the target protein Decrease the concentration of the blocking agent Increase concentration and/or length of primary antibody incubation period Increase concentration and/or length of secondary antibody incubation period Check the developing reagents
Fuzzy bands	Run gel using a lower voltage Presoak transfer membrane in the appropriate solution at the manufacturer's required length of time

Antibody Labeling

Immunoassay designs rely on the great specificity of antibodies and a suitable label which facilitates the generation of a quantitative signal. Antibody labeling techniques are involved in the interaction of reactive groups between enzyme/chemicals and proteins. It is very important to retain all the immunoreactivity of antibody and all the signal-generating capacity of the label. Here is a summary of protein/enzyme with broad estimation.

Protein	Source	MW(kDa)	Number of-NH ₂	Number of-SH	Number of-COOH
HRP	Horseradish	44	6	none	28
AP	E. Coli	94	56	none	98
AP	Bovine	125	42	none	106
β-Galactosidase	E. Coli	465	80	64	508
BSA	Bovine	68	59	35	98

Antibodies can be labeled by covalent coupling to enzymes, biotin, fluorochromes, etc. The choice of the label depends on the technique or immunoassay methods in different applications. Horseradish peroxidase (HRP), alkaline phosphatase (AP), β-galactosidase are also used for labeling antibodies.

Biotinylation of Purified Antibody Protocol

Biotinylation of primary antibodies is the most commonly used method. This reaction does not inactivate the antibody and comprise simple techniques. Biotinylated primary antibodies can be detected using the biotin-binding protein streptavidin.

Materials:

- 1 M NH₄Cl
- N-hydroxysuccinimide biotin
- Dimethyl sulfoxide (DMSO)
- 0.1 M NaBH₄, pH.8.8
- Primary antibody

Procedure:

- Dialyze antibody against 0.1 M NaHCO₃ at 4°C overnight.
- Prepare a solution of N-hydroxysuccinimide biotin at 10 mg/mL in DMSO.
- Mix the antibody solution with biotin ester at a ratio of 25-250 µg of ester per mg of antibody; stir the mixture for 4 h at room temperature
- Add 20 µL 1 M NH₄Cl per 250 µg ester; incubate for 10 min at room temperature
- Dialyze the mixture against PBS, pH 7.4.
- Adjust the pH of the antibody solution and add stabilizer for long-term storage.

Labeling Antibodies with Horseradish Peroxidase (HRP) Protocol

Materials

- Horseradish Peroxidase (HRP) 0.1 M Sodium Periodate
- 0.1 M NaHCO₃, pH 9.5
- NaBH₄ solution (4 mg/mL)

Procedure

- Antibody preparation: Dialyze purified antibody against 0.1 M NaHCO₃, pH 9.5 at 4°C overnight.
- HRP preparation: Dissolve 10 mg HRP in 2 mL HPLC-grade DI water. Add sodium periodate to final concentration of 5 mM and incubate for 20 min at room temperature.
- Combine HRP with antibody and incubate for 3 h at room temperature.
- Add 100 µL NaBH₄ solution (4 mg/mL) and incubate for 30 min at room temperature.
- Dialyze the mixture against PBS, pH 7.4 at 4°C overnight.
- Add conjugate stabilizer and aliquot for long-term storage.

Labeling Antibodies with Alkaline Phosphatase (AP) Protocol

Materials

- Alkaline Phosphatase (AP) 0.1 M SPBS
- 25% Glutaraldehyde
- 1 M Ethanolamine

Procedure

- Mix 10 mg antibody with 5 mg AP in a final volume of 1 mL.
- Dialyze the mixture against four changes of sodium phosphate buffer at 4°C overnight.
- Place the mixture in a small container, add small stir bar.
- Add 50 µL 1% EM grade glutaraldehyde; stir for 5 min, then incubate for 3 h at room temperature.
- Add 0.1 mL of 1 M ethanolamine, pH 7.0 and incubate for 2 h.
- Dialyze overnight at 4°C against 3 changes of PBS.
- Spin the mixture at 40,000g for 20 min and store the supernatant at 4°C in the presence of conjugate stabilizer.

Labeling Antibodies with Fluorochromes Protocol

Materials

- Fluorescein Isothiocyanate (FITC) Dimethyl sulfoxide (DMSO)
- Purified antibody
- 0.1 M Sodium Carbonate, pH 9.5
- G-25 column

Procedure

- Antibody preparation: Dialyze purified antibody against 0.1 M sodium carbonate, pH 9.5 at 4°C overnight.

- FITC preparation: Dissolve FITC in DMSO at 10 mg/mL.
- Mix FITC and antibody at the ratio of 1:15 for monoclonal antibody and 1:17.5 for polyclonal antibody; incubate at room temperature for 1 h.
- Separate the unbound dye from the conjugate by gel filtration at the volume ratio of gel:conjugate = 5-10:1; collect the first color peak.
- Measure A495 and A280 to get the ratio (ideal ratio is ~ 0.9-1.1).
- Store the labeled antibody in a conjugate stabilizer, aliquot and store them at -20°C for long-term storage.

References

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