Affinity purification

2011年12月12日
15:26

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Affinity Purification protocol with AminoLink Plus Immobilization Kit (adapted from Pierce)

last modified 06/30/08

- Preparation for antigen immobilization

1. Prepare pH 7.2 and pH10 buffer by dissolving BupH pack contents in 500mL ddH2O.
2. Dilute protein sample 4-fold in pH10 coupling buffer.
3. Prepare PBS (same as pH7.2 coupling buffer) containing 0.05% NaN3

- Coupling (all centrifuges are done at 1000 xg for 1min using a 15ml collection tube)

4. Suspend the AminoLink Plus Resin by end-over-end mixing. Sequentially remove the top cap and then the bottom tab to avoid drawing air into the column. Centrifuge the column to remove the storage buffer.
5. Add 2ml of pH10 buffer and centrifuge. Repeat this step once.
6. Replace the bottom cap. Add 2-3ml of the protein sample that was dissolved in pH10 buffer to the column. Save 0.1ml of the sample for subsequent determination of coupling efficiency.
7. Replace the top cap and mix column by rocking or end-over-end mixing at RT for 4 hours or overnight.
8. Remove top and bottom column caps, place column into a new tube and centrifuge to collect non-bound protein.
9. Save the flow-through.
10. Wash the column with 2ml of pH7.2 buffer and centrifuge. Repeat this step once.
11. Replace the bottom cap.
12. In a fume hood, add 2ml of pH7.2 buffer to 40ul of sodium cyanoborohydride solution, and add the resulting solution the column (50mM NaCNBH3).
13. Replace the top cap and mix column for 4 hours at RT or overnight at 4C.
14. Determine coupling efficiency by comparing the protein []s of the non-bound fraction to the starting sample.

- Block remaining active sites on column

15. Carefully remove the top cap. Some gas pressure may have formed.
16. Remove bottom column cap, place column into a new tube and centrifuge to remove buffer.
17. Wash the column with 2ml of Quenching buffer and centrifuge. Repeat this step once. Replace bottom cap.
18. In a fumehood, add 2ml of Quenching buffer to 40ul of NaCNBH3 solution (50mM NaCNBH3) and add the resulting solution to the column.
19. Replace the top cap and mix gently for 30 minutes by end-over-end rocking.

- Wash column

20. Carefully remove the top cap. Some gas pressure may have formed.
21. Remove bottom cap, place column into a new tube and centrifuge to remove quenching buffer.
22. Wash away reactants and uncoupled protein with 2ml of wash solution and centrifuge. Repeat this step four times. Monitor wash with Nanodrop for the presence of protein. Wash the column with more wash solution if needed. Protein coupled at high []s or at pH10 may require more extensive washing.

23. Equilibrate column for storage: add 2ml of storage buffer and centrifuge. Repeat this step two times.
24. Replace the bottom cap and add 2ml of storage buffer to the top of the resin bed.
25. Proceed to step 3 of the purification section or replace the top cap and store upright at 4C.

- Preparation for affinity purification

26. Make up PBS (or TBS or other buffers conducive to forming the intended affinity interaction; this is the binding/wash buffer). Degas buffers to avoid introducing bubbles into the resin bed that may impede flow (refer to Pierce tech tip #27).
27. Dissolve/exchange sample into PBS (If doing buffer exchange, refer to the section below).
28. Prepare 0.1-0.2M glycine-HCl at pH2.5-3.0 (Elution buffer)
29. Prepare 1ml of 1M sodium phosphate or 1M Tris-HCl at pH 8.5-9.0 (Neutralization buffer)

- (Optional) Buffer exchange (5ml columns)

30. Twist off the column's bottom closure and loosen cap. Place column in a collection tube.
31. Centrifuge column at 1000 xg for 2mins to remove storage solution.
32. Add 2.5ml PBS to the column and centrifuge at 1000xg for 2mins to remove buffer. Repeat this step twice.
33. Place column in a new collection tube, remove cap and slowly apply 2ml primary antibody to the center of the compact resin bed.
34. Centrifuge at 1000 xg for 2mins to collect the sample. Discard column after use.

- Affinity purification

35. Equilibrate the prepared affinity column to RT.
36. Remove top and bottom column caps. Centrifuge column to remove storage solution. Equilibrate column with 6ml of Binding/Wash buffer.
37. Add sample (<=2ml) in PBS to the column. Allow sample to enter the resin bed and replace bottom cap. Add 0.2ml of PBS. Replace top cap and incubate column at RT while rocking to allow binding to occur (e.g. 15-60 minutes). For samples > 2ml, add volumes in succession or process sample by batch method.
38. Remove top and bottom caps and centrifuge column. Without changing collection tubes, add 1ml of PBS and centrifuge again. Save the entire flow-through to evaluate binding efficiency and capacity.
39. To wash the resin, add 2ml of PBS and centrifuge. Repeat this step 2-4 times.
40. Elute the protein with 2ml of elution buffer collecting into a centrifuge tube containing 100ul of neutralization buffer and centrifuge. Save eluted, neutralized sample and repeat this step 2-3 times.
41. Test purity by SDS-PAGE and measure concentration by Nanodrop/protein assay. Proceed to Step 1 of the buffer exchange in antibody labeling protocol if desired.
42. To equilibrate the column (ASAP, refer to note 6), pass 4ml of PBS through the column.
43. Cap the column and add 4ml of PBS that contains a final concentration of 0.05% NaN3 for long-time storage. Cap the top and store column upright at 4C. Do not freeze the resin.

- Note:

44. To prepare the protein sample for affinity purification, dissolve 1-20mg protein or 1-2mg peptide in 2-3ml of coupling buffer (pH7.2 or pH10, see note 2 below). For proteins already in solution, dilute sample 4-fold in coupling buffer; alternatively, desalt/dialyze to buffer-exchange into coupling buffer.
45. Evidence suggests that higher pH coupling protocol can provide greater immobilization yields and ligand densities. If the protein is sensitive to the pH10 environment, refer to the alternative pH7.2 protocol (Pierce 44894).
46. For storage of excess coupling buffer, add 0.05% NaN3 and store at 4C.
47. Do not let resin to dry at any time.

48. If Pierce gentle elution buffer is used, use TBS instead of PBS for wash/binding buffer since phosphate is an interference.
49. Equilibrate the antigen column soon after use to prevent damage to the immobilized protein by the low pH elution buffer. Typically, an affinity column can be reused ~10 times, depending on the stability of the immobilized molecule.

    Buffer exchange with desalt column

    - 1000xg for 2mins with a 15ml collection tube.

50. C to remove storage buffer.
51. C 3x w/ buffer. (For 2mL columns use 1ml of buffer; for 5ml columns use 2.5ml of buffer)
52. Place column in a new collection tube, add sample and C to collect.

    Antigen immobilization

    - buffer volume = 2ml
    - 1000xg for 1min with a 15ml collection tube.

53. Dilute sample 4-fold/exchange sample into pH10 buffer.
54. C to remove storage buffer.
55. C 2x w/ pH10 buffer.
56. Add 2-3mL protein sample and rock for 4hrs at RT.
57. C to collect non-bound protein. Save the flow-through.
58. C 2x w/pH7.2 buffer.
59. Add {40ul NaCHBH3 in 2ml pH7.2 buffer} and rock for 4hrs at RT.
60. C to remove buffer.
61. C 2x w/quenching buffer.
62. Add {40ul NaCHBH3 in 2ml quenching buffer} and rock for 30mins at RT.
63. C to remove buffer.
64. C 5+x w/wash buffer.
65. C 3x w/storage buffer.
66. Add 2mL storage buffer.

    Affinity purification

    - buffer volume = 2ml
    - 1000xg for 1min with a 15ml collection tube.

67. Dilute/exchange sample into PBS.
68. C to remove storage buffer.
69. C 3x w/ PBS.
70. Add sample (<=2ml) in PBS to column. Add 0.2ml of PBS. Rock for 15-60mins at RT.
71. C to collect non-bound protein. W/o changing collection tube, add 1ml of PBS and centrifuge again. Save the flow-through.
72. C (2-4)x w/ PBS.
73. C (3-4)x w/ elution buffer each time collecting into a new centrifuge tube containing 100ul of neutralization buffer.
74. C 2x w/PBS.
75. Cap the column and add 4ml storage buffer.