EPIMAX™ AFFINITY PURIFICATION KIT USER MANUAL

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EPITOMICS AFFINITY PURIFICATION KIT USER MANUAL

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2.0 INTRODUCTION

Epitomics **EpiMAX™ Affinity Purification Kit** is a complete antigen-specific antibody purification kit. By utilizing Activated Serial-A coupling gel based technology, the kit enables purification of highly specific and ultra pure antibodies efficiently and quickly. This complete ready-to-use kit is provided with necessary reagents and activated matrix gel for purification of up to 5 antibodies

3.0 TECHNOLOGY

Activated Serial-A (ASA) coupling gel is a matrix gel support which contains a long spacer arm with a pre-activated reactive group. Once activated, this reactive group will form a highly stable covalent bond with free sulfhydryl or primary amine group. Due to this unique feature, ASA coupling gel allows a quick and simple immobilization of any SH- or NH_3- containing peptide which then can be used for simple affinity antibody purification (Fig 1).

Highly versatile and stable, ASA coupling gel can form stable covalent immobilization with any peptide regardless of its size, at a wide range of temperature (4°C -50°C), and at various buffer conditions (pH: 6-10, organic or inorganic solvent). Depending on the stability of the immobilized peptide, ASA coupling gel can be used multiple times with minimum loss of binding activity.

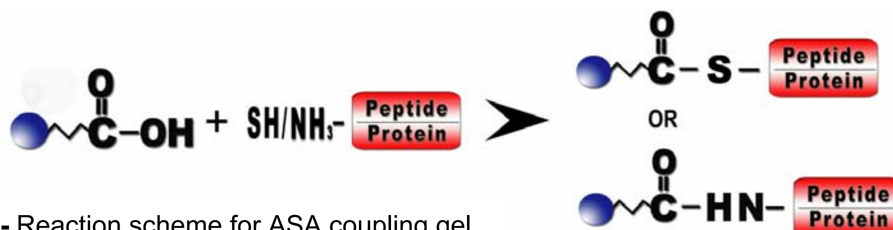

Figure 1 - Reaction scheme for ASA coupling gel

4.0 MATERIALS AND REAGENTS

Table 1 - Components/Reagents Provided

Reagent #	Reagent Name	Quantity	Storage Condition
Reagent 1	Activated Coupling Gel	1 bottle (5ml gel)	4-8 ^{o}C
Reagent 2	Antigen Coupling Buffer	1x 15ml	4-8 ^{o}C
Reagent 3	Reductant Sodium	5x 3mg/vial	4-8 ^{o}C
Reagent 4	Reductant Solution	1x 10ml	4-8 ^{o}C
Reagent 5	Peptide Dissolving Buffer	1x 1 ml	4-8 ^{o}C
Reagent 6	Antisera Sample Buffer	1x100ml	4-8 ^{o}C
Reagent 7	Sample Binding Buffer	1x250ml	4-8 ^{o}C
Reagent 8	Antibody Elution Buffer	1x100ml	4-8 ^{o}C
Reagent 9	Antibody Neutralization Buffer	15 ml	4-8 ^{o}C
	Chromatography Column	5	Room Temp
	Column Adaptor	1	Room Temp

Table 2 - Required Components/Reagents Not Provided

Reagent Name

- Deionized H_2O
- 20 - 50 ml disposable syringe
- 10 - 15 ml plastic tube
- Affinity column storage buffer:
  20 % ethanol (v/v), 80 % H_2O, 0.02 % sodium azide

5.0 STORAGE CONDITIONS

Activated Coupling Gel: 4-8 ^{o}C, sealed, 1 year;
Other components: 4-8 ^{o}C, sealed, 1 year.

6.0 PUMP USAGE GUIDELINES

EpiMAX™ affinity purification kit is compatible with syringe pump usage and has been optimized for use with Epitomics Affinity Pump System (Cat# APS-D100EA, APS-D100NA). Protocol steps that can be used with the syringe pump are marked with Epitomics Affinity Pump Icon in this manual. For recommended flow rates for the syringe pump that can be used with the EpiMAX™ affinity purification kit (based on the syringe size), please refer to the following table:

Syringe Size	Minimum Flow Rate (ml/hr)	Maximum Flow Rate (ml/hr)
20 ml	0.1	399.9
30 ml	0.1	600.0
50 ml	0.1	1200.0

7.0 PROCEDURE (Please read through entire procedure before beginning)

7.1 Preparation of Affinity Column

7.1.1 Antigen Preparation:

For Peptide:

a. Dissolve 3-10 mgs of peptide in 100 ul of **Peptide Dissolving Buffer (Reagent 5)** until the peptide is completely dissolved.

b. Gently mix 1 ml of **Antigen Coupling Buffer (Reagent 2)** with 100 ul of dissolved peptide in a 15 ml centrifuge tube until the solution is clear.

Tips:

- Some peptides are hard to dissolve in H_2O depending on peptide's hydrophobicity. For these peptides, add additional 100-500 ul **Peptide Dissolving Buffer (Reagent 5)**. Extra peptide dissolving buffer will not affect conjugation reaction.

For Soluble Protein (powder):

a. Dissolve 3-10mgs of protein in 1 ml of **Antigen Coupling Buffer (Reagent 2)**.

For Soluble Protein (liquid):

a. For already dissolved protein, concentrate the sample to concentration to ≥1mg/ml.

b. Adjust pH to 8 (e.g. with 100 mM NaOH or Na_2CO_3) and mix directly with the coupling gel.

Tips:

- Coupling efficiency increases with decreasing protein's molecular weight (or higher molarity).

Please Note: Do not use any buffer that contains traces of primary amines (e.g. Cysteine, Tris, or Glycine). Prime amine groups in the buffer will interfere with antigen conjugation. We recommend complete buffer exchange before antigen coupling).

7.1.2 Gel preparation:

a. Re-suspend **Activated Coupling Gel (Reagent 1)** by gently shaking the bottle.

b. Remove the bottom cap on the column and transfer 1.1 ml of resuspended coupling gel. Attach the top adaptor to the column.

c. Allow the coupling gel to settle and residue buffer to flow through the column by gravity flow.

d. Attach a disposable syringe (20 ml) to the column nd apply 10 ml of ddH_2O, push plunger to allow deionized H_2O to pass through the gel completely.

e. Remove the top adaptor and attaché bottom cap; add 1 ml **Antigen Coupling Buffer (Reagent 2**) to the column. Use a pippet, gently re-suspend the gel and transfer gel suspension back to the 15 ml centrifuge tube containing 1 ml dissolved antigen solution from Antigen Preparation step.

f. Mix by gently swirling, then seal the tube and place the tube on a rotator and keep the gel suspended in the tube.

g. Allow conjugation for 2 hours at 37°C (for active protein conjugation, allow 4 hours at room temperature or overnight at 4°C)

   Tips:
   - During coupling reaction, color of reaction mix may change slightly in some cases. It is normal and color change will not affect purification efficiency.

7.1.3 Coupling Reagent Neutralization:

a. Add 1 ml of **Reductant Solution (Reagent 4)** to 3 mg of **Reductant Sodium (Reagent 3)**, and immediately add this mixture to the conjugated gel solution from previous step. Incubate for 2-6 hours at room temperature with gentle shaking.

   Tips:
   - It is normal to notice some formation of gas during this step. Allow the gas to escape by opening the top of the container.

b. Add 5 ml of deionized H_2O to the gel mixture. Gently re-suspend the gel for about 10 to 60 minutes in room temperature.

7.1.4 Column Packing:

a. Remove any seal on the column (bottom cap/top adaptor). Transfer re-suspended gel to the column. After allowing the gel to settle, seal the column with the top adaptor.

b. Connect the syringe to the column adaptor, Using a syringe, add 40 ml of deionized H_2O (for pump: the flow rate of 5-10 ml/minute) and wash the column thoroughly to remove any residues (e.g. unconjugated peptides)

c. Seal the column. The column can be stored in 20% ethanol at 4 ^oC.

7.2 Affinity Purification Protocol

7.2.1 Sample Preparation:

For Antisera Sample

a. Dilute the antiserum with equal volume **Antisera Sample Buffer (Reagent 6)**.
b. Mix for 1 minute then centrifuge the mixture for 10 minutes at ≥10,000 rpm.

c. Collect and save the supernatant (the sample).

For Supernatant Sample

a. Centrifuge the mixture for 10 minutes at ≥10,000 rpm.

b. Collect and save the supernatant (the sample), adjust the pH to 7-8

7.2.2 Column Equilibration:

a. Equilibrate the column with 10 volumes of **Sample Binding Buffer (Reagent 7)** with the flow rate of 5-10 ml/minute by manual syringe pressure or using an automated pump.

Tips:

- The bed volume of the conjugated affinity matrix gel is about 1 ml. For a purification of small amount of sample or low abundance antisera, 0.5 ml bed volume of affinity matrix gel should suffice.
- For column equilibration and sample loading, a 20 ml slip-tip disposable syringe can be used as a sample reservoir. The syringe can be directly attached to the top adaptor. For larger amount of sample, pump can be used during sample loading.
- Affinity matrix gel should NOT run dry. Always leave small volume (50-100ul) of buffer on top of the packed gel.

7.2.3 Sample loading:

a. Load diluted antiserum from Sample Preparation step to the column by gravity flow or pump pressure (flow rate 0.5-2 ml/minute).

Tips:
- Load the flow-through for second time to increase recovery rate.

b. Wash the column with 10 column volumes of **Sample Binding Buffer (Reagent 7)** by gravity flow or pump with the flow rate at 5-10 ml/minute.

7.2.4 Antibody Elution:

a. Add 100 ul of **Antibody Neutralization Buffer (Reagent 9)** to each fraction containers to neutralize the pH of the elutant.

b. Apply 10 ml of **Antibody Elution Buffer (Reagent 8)** to the column. Collect 1 ml per fraction manually or with a fraction collector. If using pump, adjust flow rate to 0.5-2 ml/minute.

Tips:
- To minimize antibody denaturation, the total elution time should not exceed 3-5 minutes. Mix elutent fractions rapidly with **Antibody Neutralization Buffer (Reagent 9)** upon elution.

c. Antibody concentration in each fraction can be determined by A280 reading.

Tips:
- Antibody Concentration = A280 reading/1.4 *(The protein concentration of IgG = ~1mg/ml at E(1%) = 14, 280 nm)*

d. Pool fractions of interest based on A280 reading, followed with total buffer exchange to desired buffer.

Tips:
- In most cases, the first 6 ml of fractions contains the majority of eluted antibody. However, if the sample is suspected to contain high amount of antibody, apply 10 ml of **Antibody Elution Buffer (Reagent 8)** or perform A280 reading to determine if further elution step is necessary.

7.3 Column Regeneration and Storage

a. Apply 5 volumes of Sample Binding Buffer (Reagent 7) to the column with a flow rate of 5-10 ml/minute.

b. Apply 2 ml of Column Storage Buffer (e.g. 20% ethanol or 20% ethanol +0.05% NaN_3) to the top of the column. Seal the column with Parafilm and store at 4-8 ^oC until re-use.

c. For the reuse of the same column – the maximum recommend usage is 5 times.

8.0 SAMPLE DATA

Table 1 - Representative example of antibodies purified using *Epi*MAX™ kit.

Sample #	Amount of Peptide Loaded, >75% purity (mg)	Affinity gel (ml)	Antisera (ml)	Antigen-specific Antibody Recovered (volume/amount)	Western Blotting Dilution Factor
Sample 1	6	1	15	26ml/7.592 mg	>1:5000
Sample 2	5	1	10	10 ml/4.36 mg	1:500
Sample 3	6.4	1	16	17.5ml/5.96mg	1:1000
Sample 4	6.5	1	15	17ml/5.78 mg	1:1000
Sample 5	5	1	15	13 ml/5.84 mg	1:5000
Sample 6	6.2	1	13	12.5ml/10.69 mg	>1:5000
Sample 7	7	1	15	13ml/5.84mg	>1:5000
Sample 8	1	0.3	1	1.5ml/ug	1:1000

Table 2 – Comparison between with *Epi*MAX™ to Vendor A's affinity purification kit (based on at least 3 purifications)

	EpiMAX™	Vendor A's Kit
Peptide/antigen Coupling Efficiency (%)	**59.3-93.6**	22.8-81.6
Total Purification Time	**4 hours**	7 hours
Total Protocol procedures/steps	**16**	34
Antibody Binding Capacity (mg/ml gel)	**5.34-15.7**	2.12-3.47
Target antibody Recovery rate	**6.3-20%**	1.1-18.9%
% Purity of eluent (on SDS-PAGE)	**≥ 95%**	≥ 95%
Folds improvement in purity	**≥ 200**	≥ 200

9.0 TROUBLESHOOTING

Problem	Cause	Solution
Low or no peptide coupling efficiency	• Peptide purity low • Peptide does not contain –SH or –NH_2 group; • -SH or –NH_2 group is not solvent accessible	• Purify peptide to >70% homogeneity; • Introduce –NH_2 through chemical modification; • Coupling antigen/peptide in organic solvent or under denaturing condition, i.e., 4 M urea
Peptide-coupling gel mixture becomes highly adhesive	• Too high coupling temperature	• Dilute gel mixture with 0.5-1 ml Coupling Buffer
Low antibody to antigen binding efficiency	• Impurities in Antisera are blocking binding sites	• Load column with less sample; • Dilute with antigen dilution buffer and centrifuge for 10-15 minutes @ 10, 000 rpm; • Perform ammonium sulfate fractionation and buffer exchange before affinity purification
Low flow rate	• Column clogged by insoluble impurities	• Centrifuge sample @ 7000 rpm for 10 minutes and load supernatant. • Apply chromatography pump.
Low activity of eluted antibody	• Partial denaturation of eluted antibody	• Elute antibody with higher flow rates • Mix with antibody neutralization solution immediately upon elution • Adjust pH of elution buffer to pH 3.5 to 5.0 with NaOH, and double the volume of the elution buffer used.
Prepared column loses affinity after several usage	• Accumulation of precipitates, denatured, and/or non-specific protein(s) within regenerated coupling gel. • Presence of hydrophobic compounds bound to regenerated coupling gel. • Damage to affinity gel/coupled peptides	• Remove precipitates, denatured, and/or non-specific protein(s) with 20 ml of 6M guanidine hydrochloride, followed by a wash step using 20 ml 1x PBS • Remove hydrophobic compound by washing with 20-30 ml of 70% ethanol or 1% Triton X-100, by a wash step using 20 ml 1x PBS • Generate new affinity coupling gel

For additional information and technical inquiries please visit Epitomics website or contact technical services at (877) 772-2622, support@epitomics.com

10.0 REFERENCES

1. Domen PL *et al.* Site-directed immobilization of proteins. *J Chromatogr.* 1990 Jun 27;510:293-302 (General affinity purification guideline reference)

11.0 RELATED PRODUCTS

Catalog #	Product Name	Size
3050-1	Goat anti-Rabbit IgG-HRP	200 ul
APS-D100NA	EpiMAX™ Affinity Pump System (North America Version)	N/A
APS-D100EA	EpiMAX™ Affinity Pump System (Asia/Europe Version)	N/A

12.0 CONTACT

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