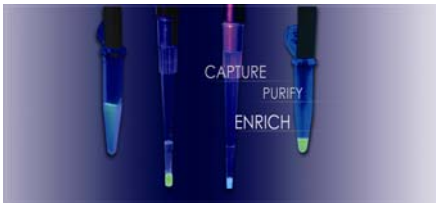


Application Note

PhyNexus
www.phynexus.com

Performance Characteristics and Assay Procedures for Protein A PhyTip® Columns

Introduction

The PhyNexus PhyTip® columns are innovative purification tools that radically simplify the capture, purification and enrichment of proteins from a variety of sources. Key to the success of these purification tools is the design of the mechanism to retain the affinity resin bed, with minimum dead volume and maximum capture potential. One group of PhyTip columns incorporates Protein A to specifically bind antibody (IgG) from different sources under certain optimal conditions, thus allowing nucleic acids and other contaminants to be removed. Following a rapid wash step, purified antibodies are easily eluted with commonly used low pH buffer. This technique allows for exceptionally high yields of IgG, depending on various conditions and provides for highly selective purification in the presence of very high concentrations of carrier proteins like BSA. PhyTip columns have extremely high binding capacity that can bind greater than 100 μg IgG, and can efficiently recover as little as 200 ng of IgG – representing a range of nearly three orders of magnitude. PhyTip columns are ideal for antibody screening from hybridoma supernatants or for high throughput extraction and purification of affinity tagged proteins.

Performance and Assay Procedures

IgG binds protein A with high affinity and specificity. The strength and selectivity of this interaction enables protein A to effectively purify IgGs from complex protein mixtures. To examine the performance of PhyTip columns with Protein A resin, the percent recovery of purified IgG (an anti-FITC-MAb) using 1000+ Protein A PhyTip columns was measured.

Materials and Methods

Sample: 10 μg mFITC-MAb (IgG_{2a} subclass) in 0.5 ml of

1. PBS containing 5 mg BSA, or
2. DMEM containing 10% FBS (Fetal Bovine Serum), or
3. SFM (Serum Free Medium) containing 5 mg BSA

Sample processing: All samples were processed on the semi-automated ME 1000 platform using the following protocols:

(1) Capture: Capture the specific protein by passing the sample over the resin bed with 2 in/out cycles (volume programmed for 0.6 ml @ 0.25 ml/min).

(2) Purify: Remove unbound proteins by washing the bound protein/affinity resin using 1 in/out cycle (volume programmed at 0.6 ml @ 0.5 ml/min) with 0.5 mL PBS (Wash Buffer I) followed by 1 in/out cycle (volume programmed at 0.6 ml @ 0.5 ml/min) with 0.5 mL of saline solution (Wash Buffer II).

(3) Enrich: Elute the IgG_{2a} with 4 in/out cycles (volume programmed at 0.1 - 0.15 ml @ 1 ml/min) with 15 μL of pH 3.0 elution buffer. Once eluted, 5 μL of neutralization buffer was added. Note that current elution buffer supplied by PhyNexus with Protein A and Protein G PhyTip columns has a pH of 2.5 and that the current neutralization buffer is at pH 9.0.

Quantitation procedure

(1) To prepare reduced $IgG_{2a,}$ 15 µl of final elution volume was reacted with 5 µl of freshly prepared TCEP (15 µl of 10 mg/ml TCEP in water for a final volume of 30 µl).

(2) 20 µl of reduced IgG_{2a} was injected into a non-porous polystyrene divinylbenzene reverse phase (C-18) column using an HP 1050 HPLC system. A gradient of 25% to 75% between solvent A (0.1% TFA in water) and solvent B (0.1% TFA in ACN) was used for 5 minutes. Detection: UV at 214 and 280 nm.

(3) Two major IgG_{2a} peaks eluted around 3.18 and 3.34 min. Areas under these two peaks were integrated from (3.13 – 3.5) min in each case and corresponding peak area was recorded at 214 nm.

(4) TCEP-treated IgG_{2a} standard under identical reaction condition was loaded into the column and used as an input or standard for recovery calculation.

Results of IgG_{2a} Recovery

Recovery in DMEM + 10% FBS = 77%

Recovery in SFM + 5 mg BSA = 64%.

It is known that carrier proteins like albumin induce an immune response and help in the production of good antibodies, so it is often present in high quantities when antibodies are being raised. For that reason, any purification and enrichment procedure must be capable of tolerating high concentrations of albumin. In addition, the results shown above seem to indicate that the presence of high background protein concentrations (such as BSA) may have a beneficial effect on recoveries.

Reproducibility of IgG2a recoveries in presence of carrier protein

In order to verify the role of carrier protein for maximum IgG2a recovery, and to demonstrate reproducibility, IgG2a was purified from PBS (and also PBS containing 5 mg BSA) from multiple samples (n=4). Results suggest increased recovery (at least 20% or more) of purified IgG2a in the presence of 500-fold excess of BSA concentration and that the procedure is also highly reproducible.

Recovery in PBS = 42.25% (SD = 4.66%)

Recovery in PBS + 5 mg BSA = 66.5% (SD = 7.5%)

Selectivity Performance and Assay Procedures

In order to characterize and verify the selectivity of the Protein A-IgG affinity interaction, 5 mg of BSA was added as a carrier protein to 15 µg IgG in a 0.5 ml sample volume (a 333-fold excess). The samples were processed using Protein A PhyTip columns.

Materials and Methods

Sample: 15 µg mFITC-MAb (IgG2a subclass) in 0.5 ml of PBS or PBS containing 5 mg BSA (10 mg/ml or 1% w/v BSA).

Sample processing: As described above in **Recovery Performance and Assay Procedures**.

IgG quantitation and purity analysis procedures:

(1) 15 µl of final elution volume was divided into two parts: 13 µl was reacted with freshly prepared 13 µl of 10 mg/ml TCEP (final volume = 26 µl and [TCEP] = 17.5 mM) at room temperature for ~16 hours while 2 µl was loaded onto gel in each experiment.

(2) 20 µl out of above 26 µl reduced IgG2a was injected into a non-porous polystyrene divinylbenzene reverse phase (C-18) column using an HP 1050 HPLC system. A gradient of 25% to 75% between solvent A (0.1% TFA in water) and solvent B (0.1% TFA in ACN) was used for 5 minutes. Detection: UV at 214 and 280 nm.

(3) Two major IgG2a peaks eluted around 3.17 and 3.3 min. The area under these two peaks was integrated from (3.13 – 3.5) min in each case and corresponding mAU was recorded at 214 nm.

(4) TCEP-treated IgG2a standards (injected amount 1.08, 2.16, 4.32, 6.48 and 8.64 µg of FITC-MAb) under identical reaction condition were loaded into the column and used as a standard curve for recovery calculation.

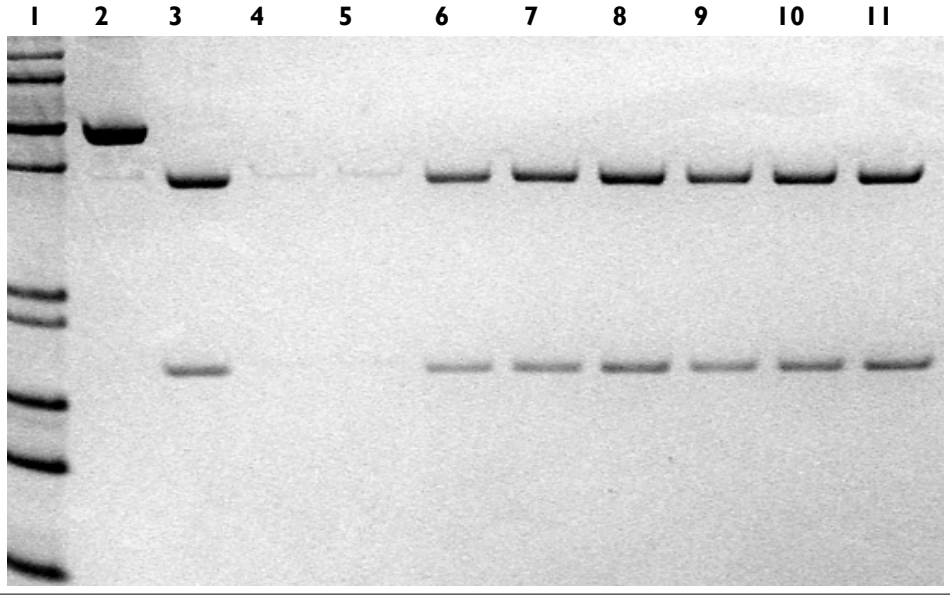
Selectivity of Protein A PhyTip columns

Data shown below from these experiments indicates that IgG purification using the Protein A PhyTip column is highly selective. A 333-fold excess of BSA can quantitatively be removed by using Protein A PhyTip columns in a very fast process. Similarly, as described previously, the same IgG_{2a} can be selectively purified from serum free hybridoma medium containing BSA or FBS (purity data not shown here).

Recoveries from selectivity assay (determined by HPLC method)

Procedure	Recovery
15 µg IgG_{2a} /0.5 ml PBS (2 cycles of loading)	**43%**
15 µg IgG_{2a} /0.5 ml PBS+5 mg BSA (2 cycles of loading)	**56%**
15 µg IgG_{2a} /0.5 ml PBS+5 mg BSA (5 cycles of loading)	**62 %**

Nu-PAGE 4-12% Bis-Tris gel with MES running buffer



Lane 1: marker, **2**: 2 µg BSA, **3**: 2 µg IgG_{2a}, **4, 5**: Protein A resin only, respectively; **6, 7, 8**: 2 µl each of Protein A purified IgG_{2a} from PBS, PBS containing 5 mg BSA (2 and 5 cycles capture), respectively; **9, 10, 11**: 2 µl each of Protein A purified IgG_{2a} from PBS, PBS containing 5 mg BSA (2 and 5 cycles capture), respectively.

Binding Capacity Performance and Assay Procedures

Protein A PhyTip columns bind appropriate IgG isotypes with very high capacity (see product insert for isotype binding characteristics). In order to characterize and determine the IgG capacity of a 10 µl bed volume of Protein A resin in a PhyTip column, different masses of IgG were taken in 0.5 ml of PBS and processed; breakthrough samples were removed for analysis to determine the amount of unbound IgG material, and the difference between the amount in the breakthrough and the amount loaded was taken to be the amount bound to the resin.

Materials and Methods

Samples: 10, 50, 100, 150 and 200 µg anti-FITC-MAb (IgG_{2a} subclass) in 0.5 ml of PBS.

Sample processing : Sample loading procedure is described in **Recovery Performance and Assay Procedures**. Loading cycle = 5 in/out cycles, so as to ensure maximum binding capacity.

IgG quantitation in breakthrough: The HPLC method described earlier was used for quantitation of the IgG still present in the breakthrough volume. From this procedure it was determined that 60 ng of IgG is the limit of detection when taken from a 15 mL sample prior to TCEP reduction. This corresponds to 2 mg of IgG being present in the 0.5 mL breakthrough volume.

Capacity and Recovery Assay Table

This data below shows that the IgG capacity is well over 100 mg for the PhyTip columns, and that over 80% of the IgG is taken up when this amount of material is introduced to the resin.

Initial IgG(µg)	Breakthough Mass(µg)	IgG Bound(µg)	% Bound
10	<2	>8	>80
50	4	46	92
100	16.5	83.5	83.5
150	47.65	102.35	68.2
200	71	129	64.5

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