A New One-Media Monoclonal Antibody Purification Method Using Preparative Porous Ethylenediamine-N, N'-Tetra(methylphosphonic) Acid (EDTPA) Modified Porous Zirconia

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Abstract

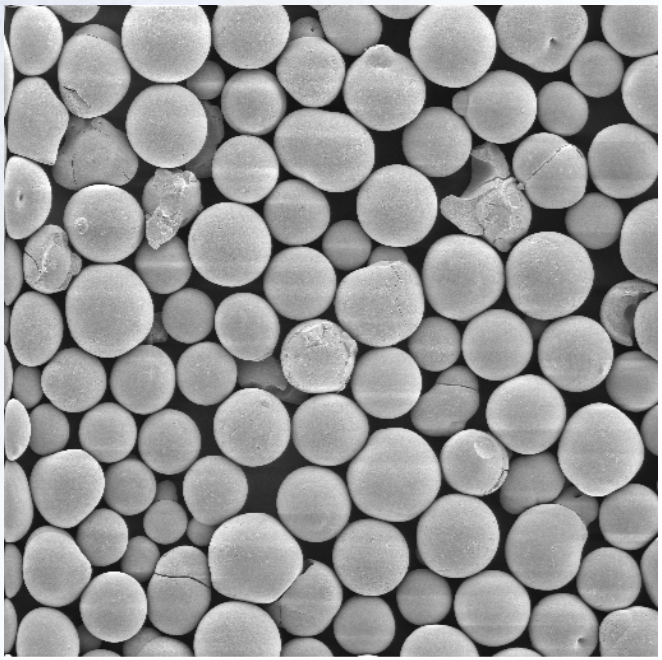
The research presented here describes the manufacture of a new class of *porous zirconia micro-spheres*, by spray drying, for large-scale preparative liquid chromatography of bio-molecules. Porous zirconia particles with an average diameter of 25 microns are coated with ethylenediamine-N, N'-tetra(methylphosphonic) acid (EDTPA) to produce a bio-compatible cation-exchange stationary phase for the purification of proteins. The coated zirconia particles can be packed into preparative liquid chromatographic columns and used for *rapid large-scale purification of monoclonal antibodies*. These mechanically stable zirconia columns can be run at very high mobile phase linear velocities compared to soft affinity gels functionalized with Protein A or Protein G. Thus *dramatic increases in purification throughput* are possible with the new zirconia phase. Most importantly, EDTPA modified zirconia (Rhinophase®-AB) can purify a wide range of IgG subclasses, as well as IgA and IgM, providing a *robust alternative to affinity chromatographic media*.

Outline

- Physical characteristics of 25 micron porous zirconia
- State-of-the-art Mab purification method
- Preparative Mab purification on 25 micron Rhinophase®-AB
- Direct Comparison of Mab purified with Rhinophase®-AB versus affinity gel Protein G media
- Binding Capacity of Different Subclasses of Mab on Rhinophase®-AB
- Binding Capacity of IgGs derived from different animal sources on Rhinophase®-AB
- Binding Capacity of IgG, IgA and IgM on Rhinophase®-AB
- IgY Purification from an Egg Yoke
- Conclusions

SEM and Nitrogen Porosimetry Data for Rhinophase®-AB

The spray dried particles are easily size classified using standard screens. The final material has large pores so that large bio-molecules can diffuse into the porous beads.

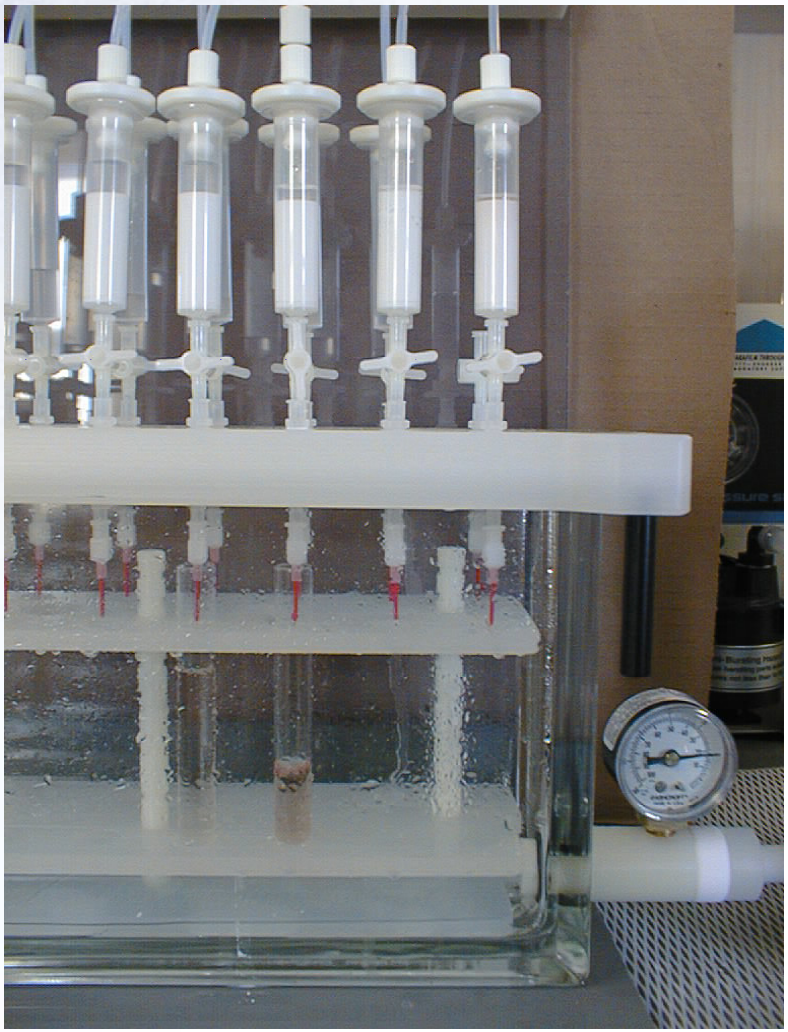


Sample	Surface Area (m^2/g)	Pore Volume (ml/g)	Average Pore Diameter (Å)
Rhinophase®-AB	14	0.100	300

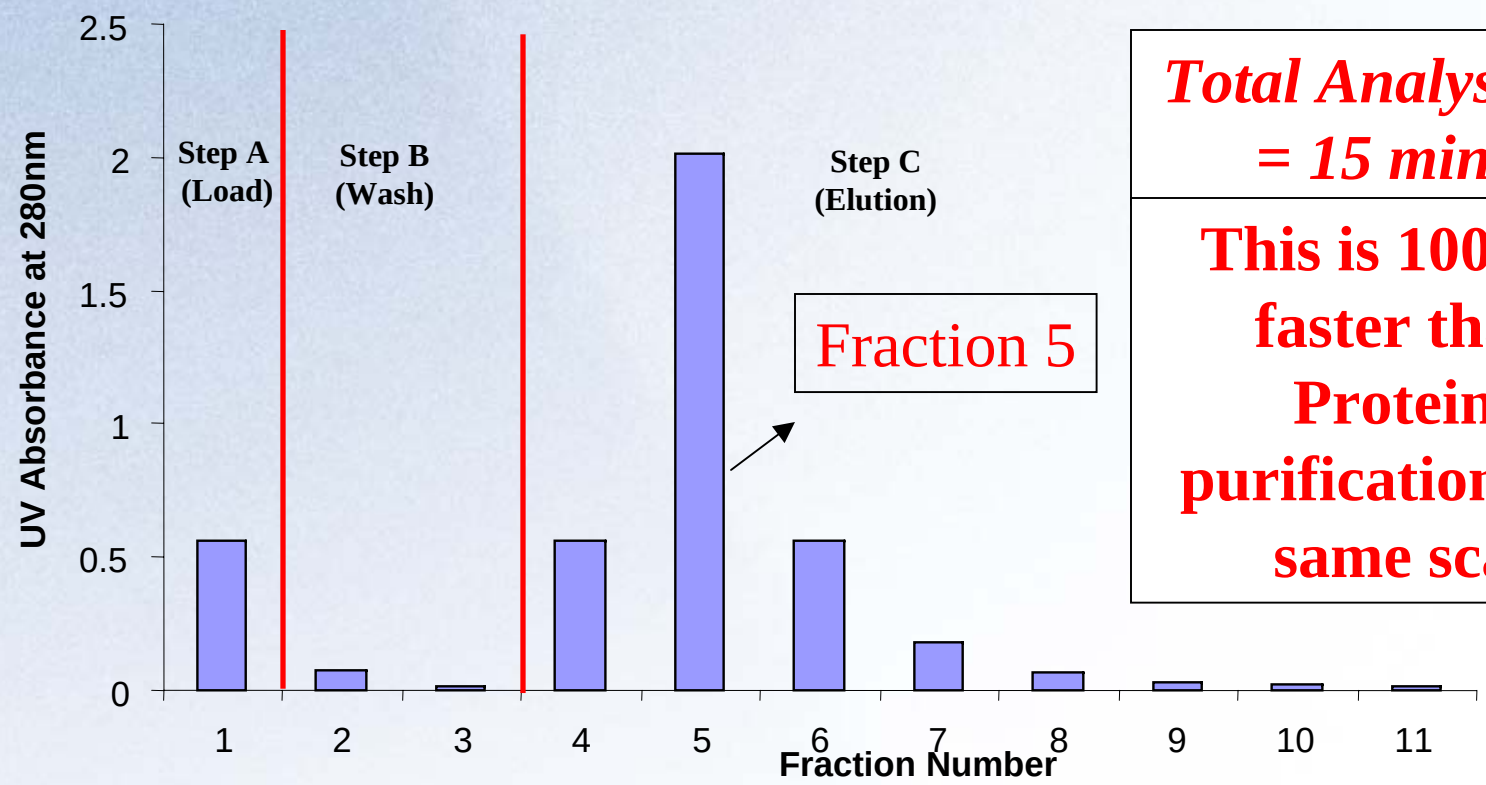
A New Ultrafast Preparative Purification Method Using Rhinophase®-AB

ZirChrom®

Due to zirconia's very high mechanical strength, Mab purifications can be performed at high mobile phase linear velocities. A simple vacuum filtration apparatus can be use to achieve very high flow rates through a packed bed (90 mL/min). This approach is not possible using soft affinity gels such as Protein A and Protein G media.



Ultrafast Preparative Purification of IgG_1 Using Rhinophase®-AB



Total Analysis Time = 15 minutes

This is 100 times faster than a Protein G purification of the same scale!

Step A = 20 mM MES buffer, 4 mM EDTPA, 50 mM NaCl @ pH 4.0, Step B = 20 mM MES buffer, 4 mM EDTPA, 50 mM NaCl @ pH 4.0, Step C = 20 mM MES buffer, 4 mM EDTPA, 2.0 M NaCl @ pH 4.0. Flow Rate = 60 mL/min, Injection size = 31.6 mL serum-free cell culture supernatant diluted 4-times with loading buffer, (3.98 mg of Mab), Amount of Rhinophase®-AB in tube = 10 grams.

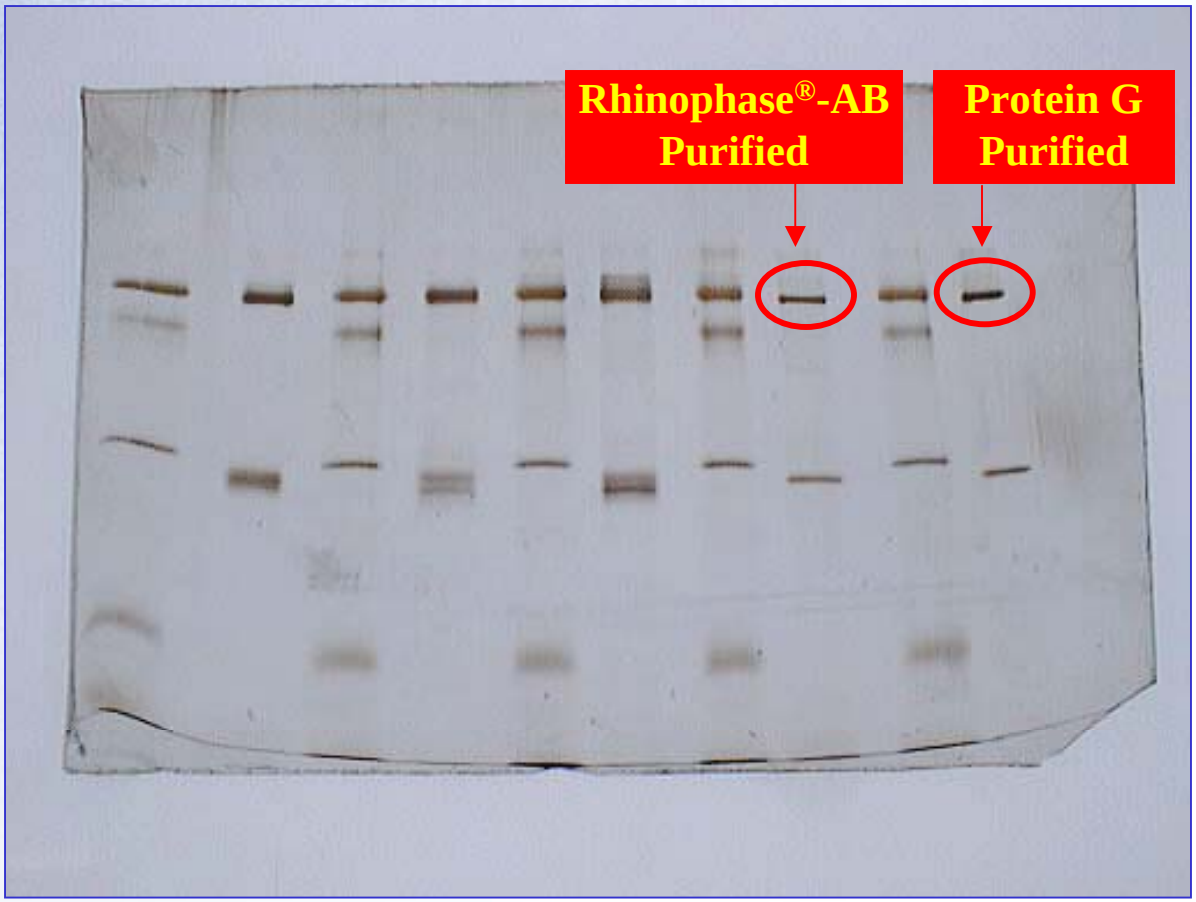
ELISA Plate Comparison of Protein G and the Ultrafast Purification on Rhinophase®-AB

	OD 280 nm		
Protein	Protein G	Rhinophase®-AB	
Offered	Purified IgG	Fraction 5	Fraction 5
15.6	0.0531	0.0502	94.5%
31.3	0.0946	0.0992	104.9%
62.5	0.1676	0.1892	112.9%
125	0.3176	0.3632	114.4%
250	0.5596	0.6362	113.7%
500	1.0166	1.1507	113.2%
1000	1.8151	1.8632	102.6%
		Average %	**108.0%**

An ELISA plate analysis using the same amount of Mab from Protein G and Rhinophase®-AB purifications showed an increased signal for the Rhinophase®-AB purified Mab.

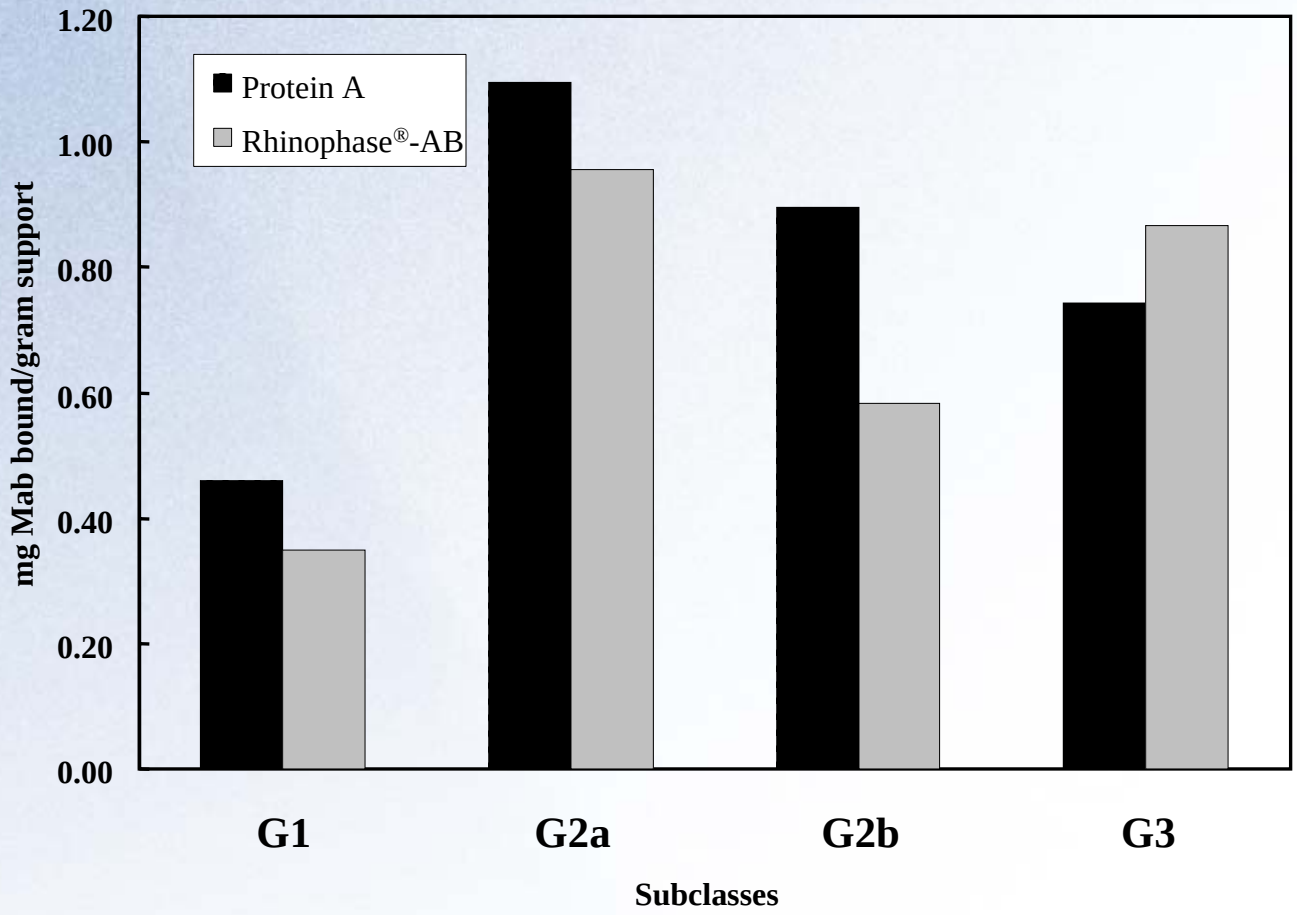
Mab Purity Comparison from Semi-Preparative Run

Purified Mab was equally pure using Protein G and Rhinophase®-AB.



Silver-stained, SDS-PAGE gel Comparing IgG_1 purified by Protein G (row 1 from right) and Rhinophase®-AB (row 3 from right). Electrophoresis was run under reducing conditions. Sample loading at 1 *u*g per lane. All other lanes are standards.

Relative Binding Strength of Different Subclasses of Mab



Rhinophase®-AB had high binding capacity for a variety of different Mab subclasses and is comparable to Protein A media.

Binding Capacity of Other Immunoproteins on Rhinophase®-AB

	Rhinophase®-AB
Sample	Capacity (mg antibody/ml particles)*
hIgG	28
hIgA	9
hIgM	2

*All values are reported as an average of 3-independent replicate experiments. The standard deviation is less than 5%. All capacities are reported as mg Ig bound per ml of beads.

IgY Purification Method

Sample Preparation

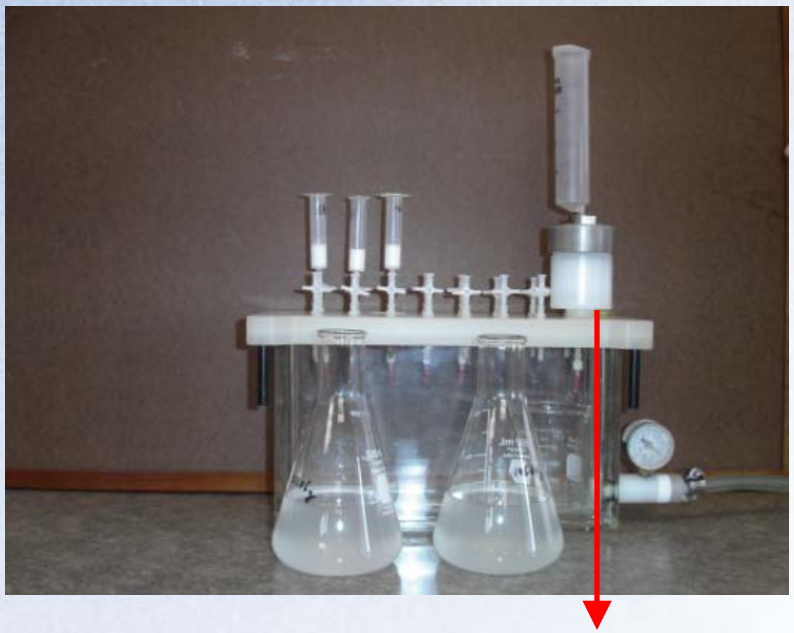
Separate the egg yolk from the egg wihte using the egg separator. Add the yolk into the beaker. Take 15.93 g of the yolk. Add 637.2 g buffer A (0.2 mM MES +0.04 mM EDTPA + 0.5 mM NaCl, pH 4.0 with NaOH pH adjustment). Mix it completely by shaking for 3 minutes. Centrifuge it for 15 min at 3750 rpm and filter supernatant with filter paper (Fisher Sci., Catalog No: 09-795G, 18.5 cm OD). The resulting solution is cloudy and can then be injected onto the SPE tube packed with the zirconia in loading buffer.

Elution Conditions

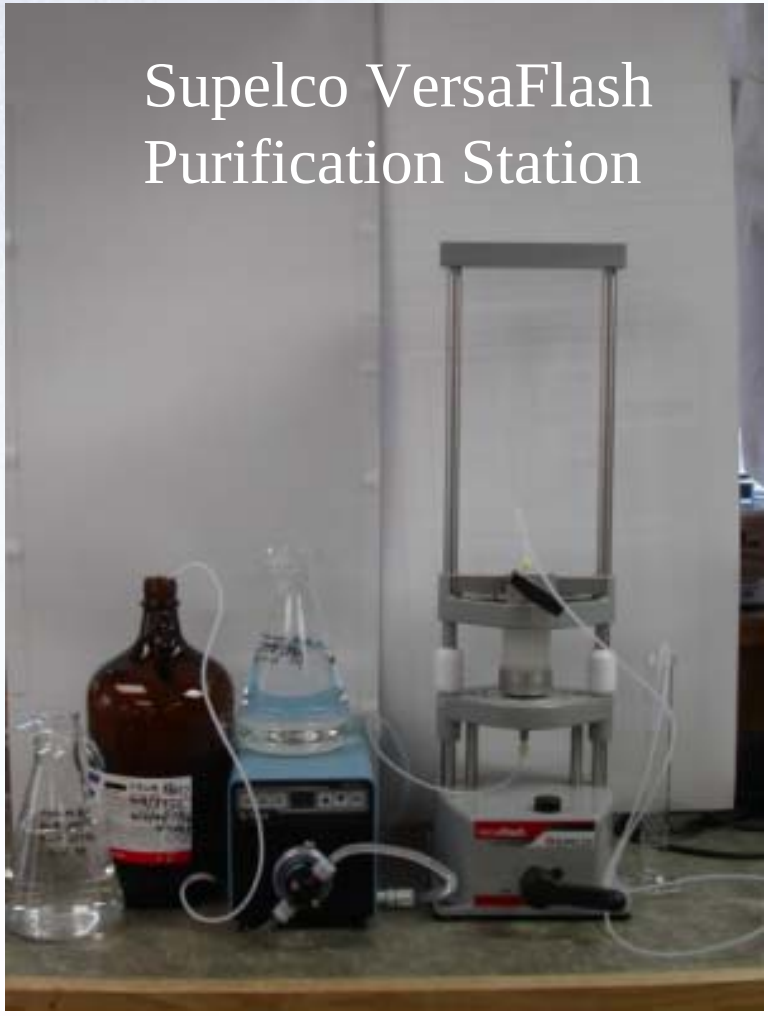
1. Loading Buffer: 0.2 mM MES +0.04 mM EDTPA + 0.5 mM NaCl, pH 4.0 with NaOH pH adjustment.
2. Matrix Protein Elution Buffer: 20 mM MES +4 mM EDTPA + 200 m M NaCl, pH 4.0 with NaOH pH adjustment.
3. IgY Elution Buffer: 20 mM MES +4 mM EDTPA + 400 m M NaCl, pH 4.0 with NaOH pH adjustment.
4. Wash Buffer: 20 mM MES +4 mM EDTPA + 1 .5 M NaCl, pH 4.0 with NaOH pH adjustment.

Note: All flow rates were approximately 16 mL/min

Fast Purification of IgY



Vacuum manifold for loading the VersaFlash Column



Supelco VersaFlash Cartridge packed with Rhinophase®-AB

Gel Electrophoresis Key

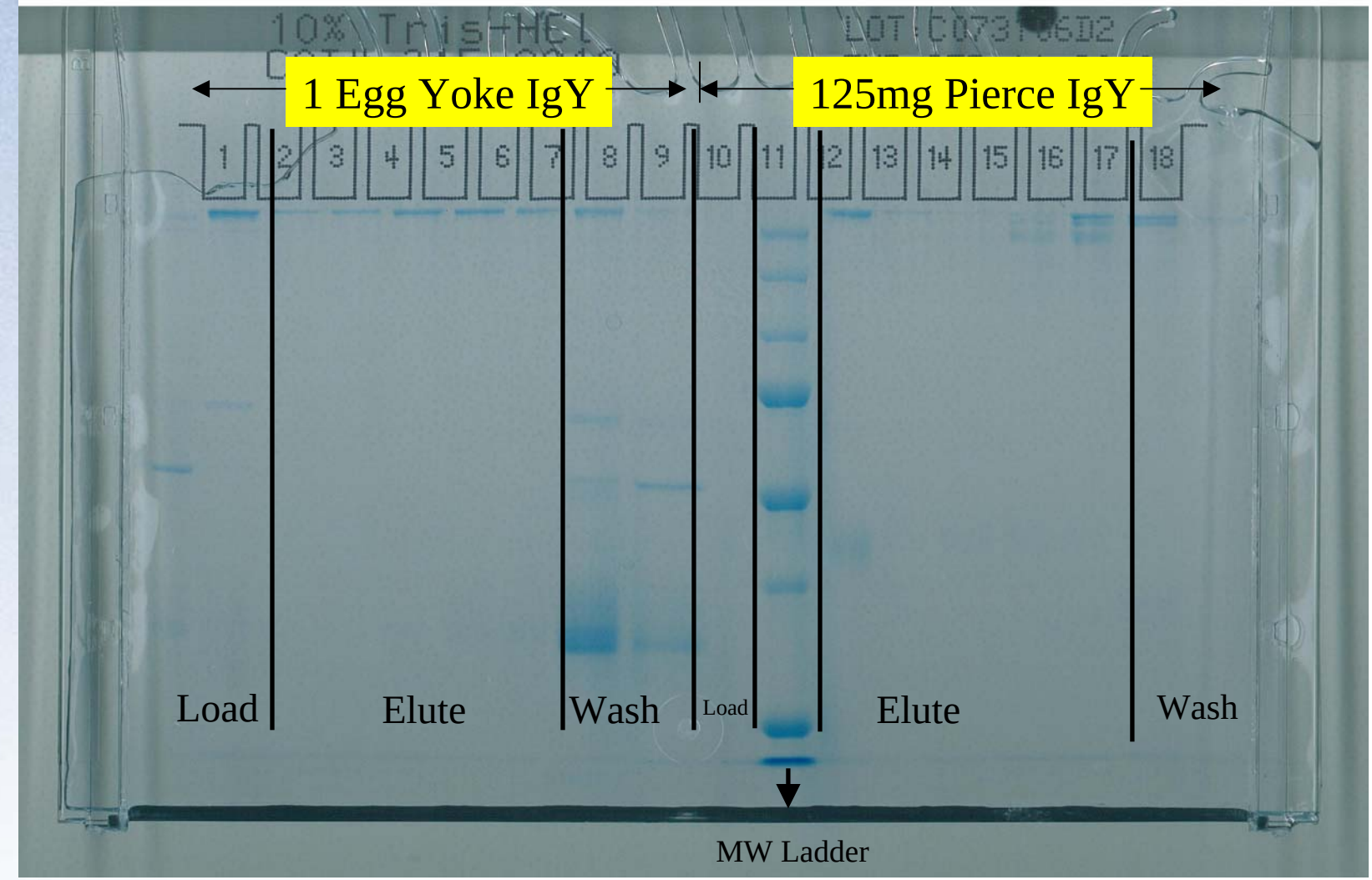
(elution conditions for following slide)

IgY from Zirconia Purification

IgY Standard From Kit Purification

			mL
1	2006072501	Sample	150
2	2006072511	20 mM MES+4 mM EDTPA+ 200 mM NaCl, pH 4	611
3	2006072512	20 mM MES+4 mM EDTPA+ 200 mM NaCl, pH 4	661
4	2006072521	20 mM MES+4 mM EDTPA+ 400 mM NaCl, pH 4	1036
5	2006072522	20 mM MES+4 mM EDTPA+ 400 mM NaCl, pH 4	1086
6	2006072523	20 mM MES+4 mM EDTPA+ 400 mM NaCl, pH 4	1136
7	2006072524	20 mM MES+4 mM EDTPA+ 1500 mM NaCl, pH 4	1186
8	2006072541	20 mM MES+4 mM EDTPA+ 1500 mM NaCl, pH 4	1717
9	2006072542	20 mM MES+4 mM EDTPA+ 1500 mM NaCl, pH 4	1767
10	2006071701	Sample	50
11	Protein Standard MW Ladder		
12	2006071711	20 mM MES+4 mM EDTPA+ 200 mM NaCl, pH 4	130
13	2006071712	20 mM MES+4 mM EDTPA+ 200 mM NaCl, pH 4	180
14	2006071721	20 mM MES+4 mM EDTPA+ 400 mM NaCl, pH 4	455
15	2006071722	20 mM MES+4 mM EDTPA+ 400 mM NaCl, pH 4	505
16	2006071723	20 mM MES+4 mM EDTPA+ 400 mM NaCl, pH 4	555
17	2006071741	20 mM MES+4 mM EDTPA+ 1500 mM NaCl, pH 4	1130
18	2006071742	20 mM MES+4 mM EDTPA+ 1500 mM NaCl, pH 4	1180

Gel Electrophoresis of Purified IgY from a Commercial Kit and on Rinophase® AB



Conclusions

- Rhinophase®-AB provides a widely applicable alternative to currently used Protein A and Protein G antibody purification media.
- Typical yields of Mab purifications greater than 95%, with purity levels equal to or greater than affinity gel-type media.
- Due to Rhinophase®-AB's excellent mechanical stability, purifications can be performed 100-fold faster with equivalent results.
- Rhinophase®-AB is chemically durable over the entire pH range, which allows for cleaning and depyrogenation (data not shown).
- ELISA plates produced with Rhinophase®-AB purified Mab showed greater signal than those produced with Protein G purified Mab.
- Rhinophase®-AB has affinity for a wide range of immunoprotein classes and subclasses including monoclonal and polyclonal IgG, IgA, and IgM.

Conclusions Continued

- IgY purity in elution fractions looks very high on Rhinophase®-AB.
- Some IgY was observed in the fall-through for the Rhinophase®-AB, but this may be due to channeling.
- Matrix proteins are eluted at high ionic strength (>400 mM NaCl) for both samples.
- Rhinophase®-AB is able to fractionate Commercial Kit IgYs at different ionic strengths.
- 125 mg of Commercial Kit purified IgY was run versus the IgY from an egg yoke. Similar *overall recovery was observed based on gel electrophoresis spot intensity*.
- Fast, Easy Scale-up of IgY purification from egg yokes is feasible using the Supelco VersaFlash Purification Station and Rhinophase®-AB.
- Acknowledgement: National Institutes of Health Grant # 5 R44 GM58354-03.