Supplementary Material For:

Benchmarks

Improved Native Isolation of Endogenous Protein A-Tagged Protein Complexes

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S1: Peptide Synthesis and Purification

Novel peptides were synthesized by standard Fmoc solid-phase synthesis methods[1] using HATU as the coupling agent on a conventional automated synthesizer (Symphony, Protein Technologies) or alternatively using HBTU on a microwave peptide synthesizer (Liberty, CEM corp.). Incorporation of polyethylene glycol (PEG) spacers into the peptide sequence was accomplished using N-Fmoc-amido-(PEG)n-acid building blocks (Quanta Biodesign Inc.). Peptides were deprotected and cleaved from the resin with a TFA / triisopropyl silane / water / dioxa-1,8-octanethiol cleavage cocktail (25°C; 90 min). The cleaved peptides were precipitated in, and washed extensively with, t-butyl methyl ether. The dry crude peptides were cyclized (Cys-Cys disulfide) using Clear-OX polymer supported oxidant[2] (Peptide International). Typically the reduced peptides (40 mg) were dissolved in degassed 0.1 M ammonium acetate or bicarbonate / acetonitrile (1:1 v/v) at 5-6 mg/ml concentration. Each peptide solution was added to pre-swollen Clear-OX resin (200 mg; 0.2 mEq/g). The suspension was stirred for 2 hr at room temperature. Completion of the disulfide formation was confirmed by LC-ESI-MS analysis. The peptide was filtered and washed with a small volume of aqueous acetonitrile (50%). The solution was lyophilized and the cyclic peptide was purified by

preparative HPLC (Dionex U3000). The cyclic peptide fractions were reanalyzed by analytical LC-MS (Waters Acquity UPLC coupled to Thermo-LTQ mass spectrometer) and then lyophilized.

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Empower 2 software

Rockefeller University Proteomics Resource Center

SAMPLE INFORMATION

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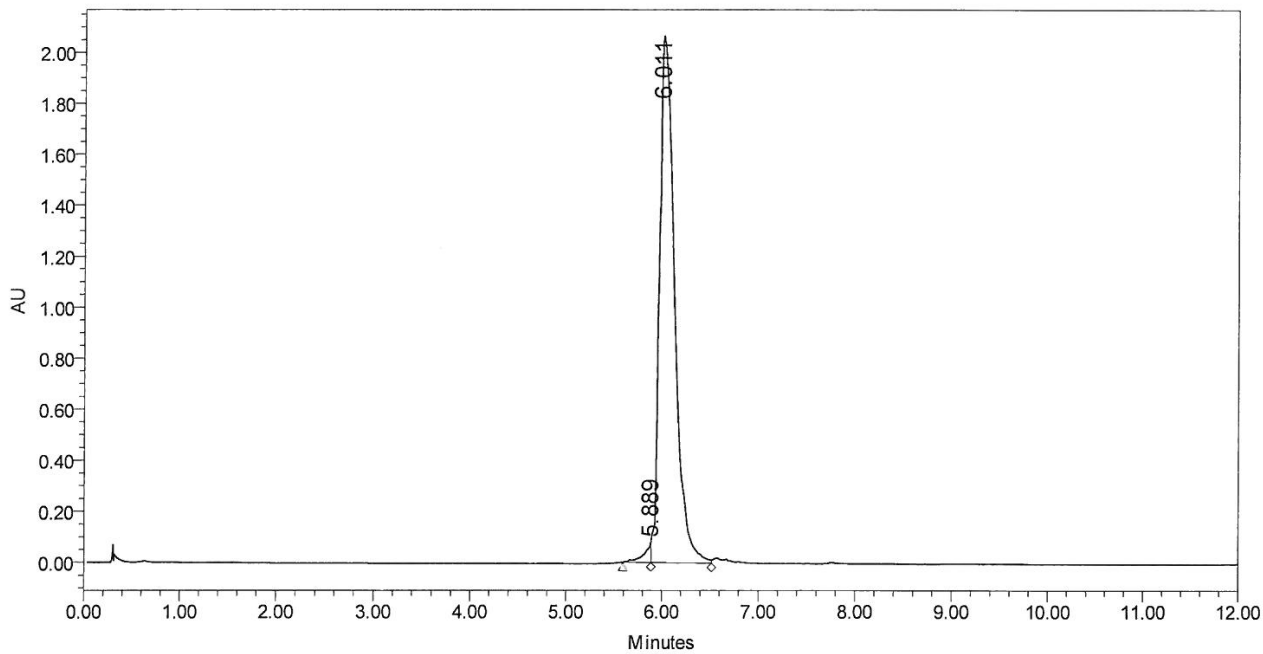

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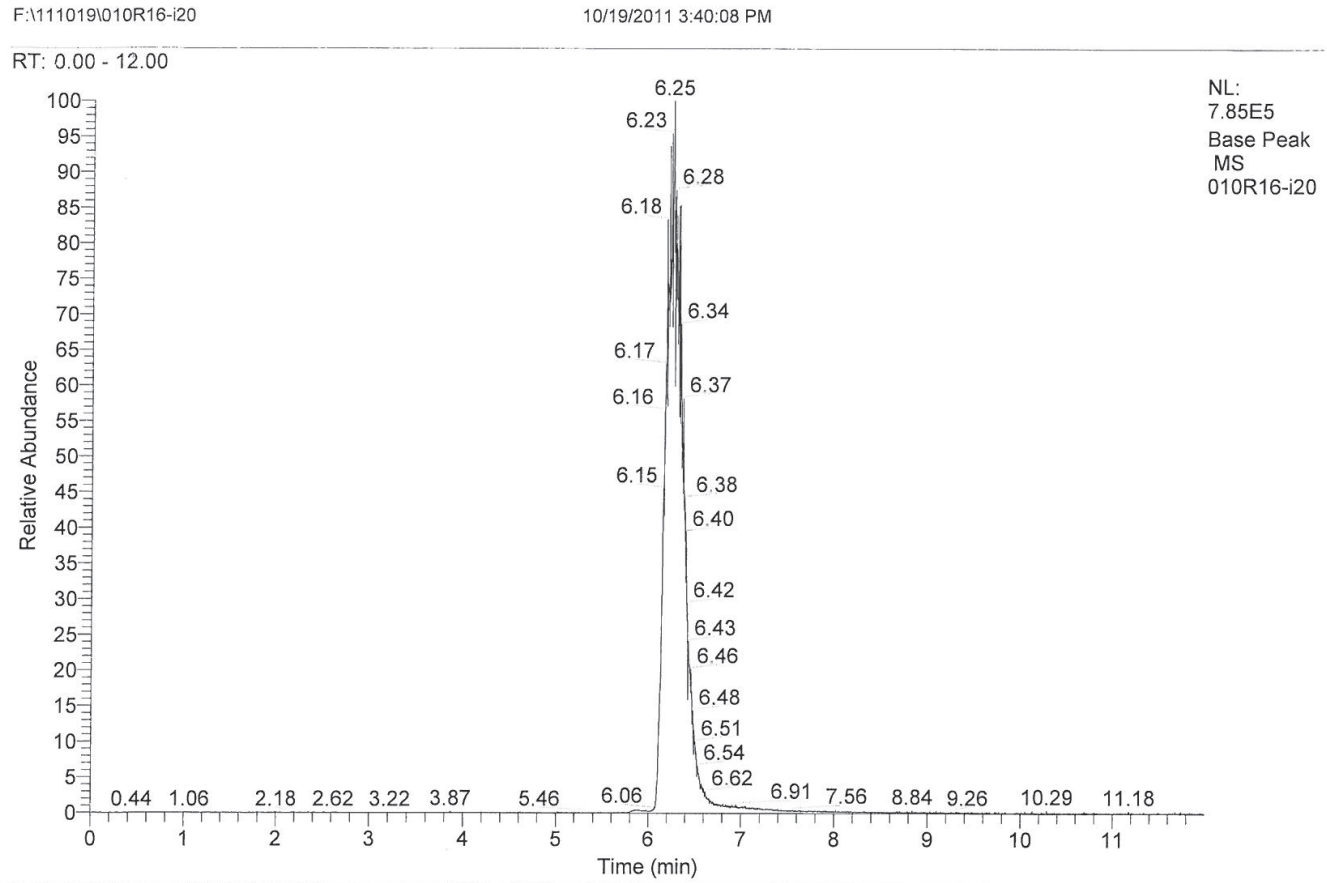
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6.15
6.40
6.42
6.43
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6.62
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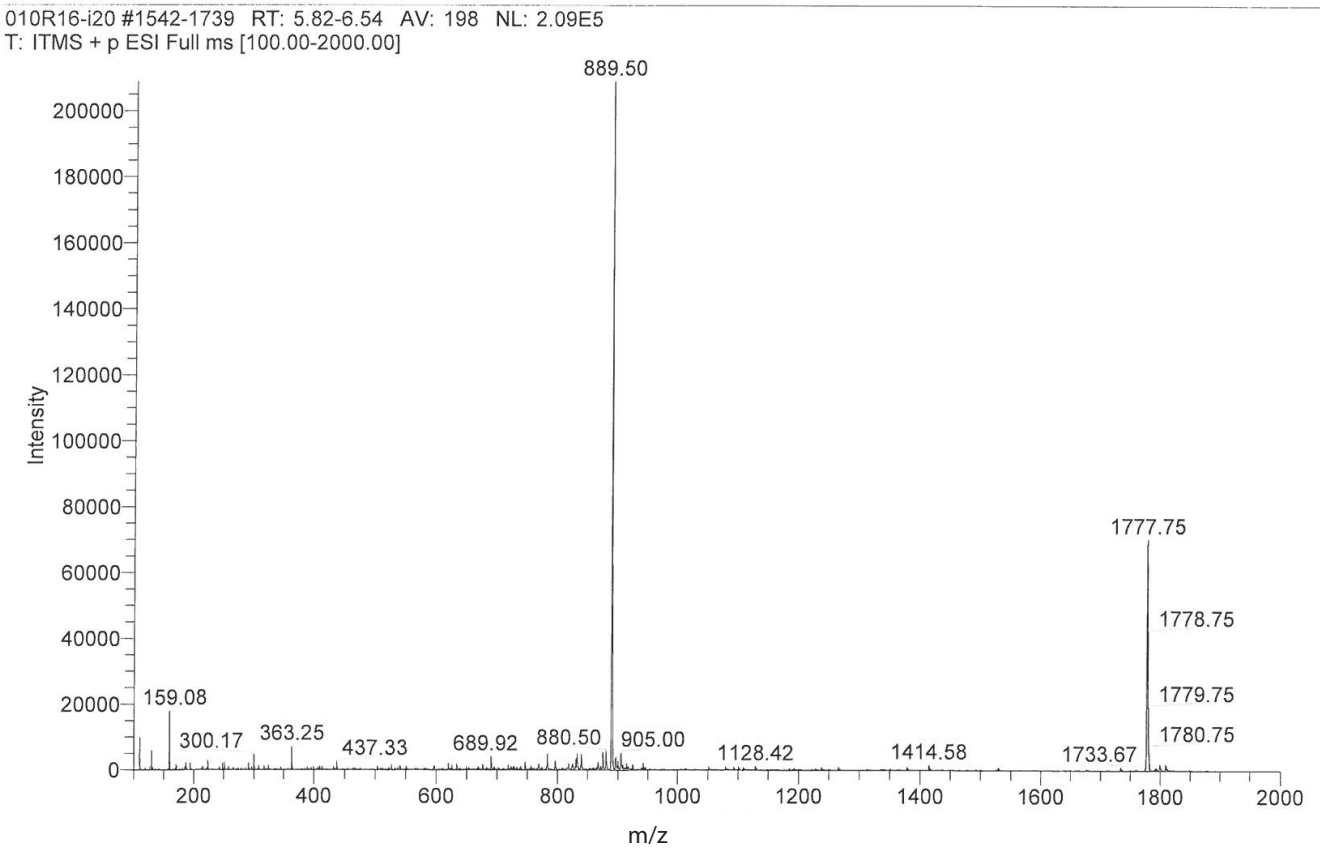
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S2: Peptide solubility and binding competition

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A: Initial attempts to resuspend lyophilized, PEG-modified FcIII peptide at final concentrations above 5 mM resulted in a large insoluble fraction and a final concentration in the hundreds-micromolar. This was observed in both HEPES buffered solution at pH 7.4 and Tris buffered solution at pH 8.0 - and was not resolved by extended incubation times - all indications are that an initial concentration of the peptide above 5mM has a deleterious effect on the final concentration. We found that resuspending lyophilized peptide at below 5 mM (*typically 3 mM*) in 40 mM Tris buffered solution at pH 8.0 with 100 mM NaCl, 0.01% (v/v) Tween 20, and 5% (v/v) ethanol provided for final concentrations of peptide solution at between 2 - 3 mM for PEG-modified FcIII peptide, depending on batch and precise buffer composition, and even extended the maximal solubility of Bio-Ox from the previously characterized 440 μM[1] to ~1.2 mM achieved in this study. We found that Tris at 40 mM was required to ensure a final pH of 8.0 for the resuspended peptides – important for maximizing solubility[1]. In all conditions tested, PEG-modified FcIII peptide exhibited at least double the solubility, greater retention of solubility at 4°C (table 1), and less precipitation on storage at -20°C (data not shown) as compared to Bio-Ox. See also file: FcIII_reagent_solubilities.xls.

RT	**A280**	**A320**	**net A280**	**dilution factor**	**[mM]**	**avg. [mM]**	
Bio-Ox	0.27	0.007	0.263	50	1.18	1.18	
1756.3 Da	0.272	0.002	0.27	50	1.21		
	0.26	0.005	0.255	50	1.15		
PEG(4)	0.628	0.006	0.622	50	2.80	2.73	
1776.3 Da	0.585	-0.001	0.586	50	2.63		
	0.621	0.004	0.617	50	2.77		
R-PEG(4)	0.534	0.007	0.527	50	2.37	2.36	
1932.88 Da	0.519	0.008	0.511	50	2.30		
	0.543	0.009	0.534	50	2.40		
4*C	**A280**	**A320**	**net A280**	**dilution factor**	**[mM]**	**avg. [mM]**	**% 4°C / RT**
Bio-Ox	0.209	0.006	0.203	50	0.91	0.86	72%
	0.192	0.005	0.187	50	0.84		
	0.186	0.005	0.181	50	0.81		
PEG(4)	0.568	0.005	0.563	50	2.53	2.55	93%
	0.547	0.004	0.543	50	2.44		
	0.598	0.004	0.594	50	2.67		
R-PEG(4)	0.508	0.007	0.501	50	2.25	2.22	94%
	0.515	0.007	0.508	50	2.28		
	0.484	0.009	0.475	50	2.13		

Table 1 - FcIII-based reagent solubilities (Figure 1B, main text): Three reagents were compared for solubility - the original Bio-Ox reagent[1], FcIII peptide containing an N-terminal 4-repeat polyethylene glycol moiety (PEG[4], this study), and FcIII peptide containing an N-terminal arginine and 4-repeat polyethylene glycol moiety (R-PEG[4], this study). The molar extinction coefficient at 280 nm wavelength for all three reagents (ε_{280} $M^{-1}cm^{-1}$) was estimated as 11125 $M^{-1}cm^{-1}$, based on the presence of two tryptophan residues and one cystine[2]. Three lyophilized aliquots of each reagent were resuspended at an intended final concentration of 5 mM (molecular mass shown below the reagent name) in the aforementioned Tris buffered solution at pH 8.0, also containing 1 mM EDTA. All samples were mixed using a vortex mixer, and centrifuged for 5 min at room temperature (RT) at full speed (14k RPM) in an Eppendorf 5417R microfuge. The supernatants were removed and readings of each reagent were made by UV spectrophotometry at 280 nm with simple background subtraction at 320 nm. The remainders of the samples were left overnight at 4°C, and the supernatants were assayed again after the samples were centrifuged, as previously. PEG[4] modified FcIII showed the greatest solubility at RT or 4°C and was carried forward as "PEGylOx." On the basis of these results, and in order maximize reagent, subsequent resuspensions of lyophilized peptide were carried out at 3 mM intended final concentration. Although similar solubility was observed for PEG[6] and PEG[8] (as for PEG[4]) modified FcIII peptides, both of these species exhibited an observable tendency to crash out of solution when held at below 4°C (data not shown) and were not carried forward.

B: To asses that the function of PEGylOx as a competitive elution reagent has not

been compromised by the PEG[4] moiety (as compared to Bio-Ox), its half maximal inhibitory concentration (IC_{50}) against wt *S. aureus* Protein A (SpA) was assessed and compared to that of Bio-Ox; shown in figure 1C, main text. See also file: IC50.xls. In this experiment, PEGylOx and Bio-Ox were compared against one another for ability to competitively displace SpA from SpA saturated IgG Dynabeads. Rabbit polyclonal IgG-conjugated Dynabeads (See http://www.ncdir.org/public-resources/protocols/) were incubated with SpA (Sigma-Aldrich #P7837; 165 µl of Dynabeads slurry and 165 µg SpA were incubated in 1 ml volume) in 20mM Tris pH 8.0, 100mM NaCl, 0.01% (v/v) Tween, 1mM EDTA, 5% v/v ethanol for 2 hr at 4°C with mixing. After SpA binding, the beads were washed two times with 1 ml of the binding solution without SpA and then resuspended in 1.65 ml of the same solution and thirty 50 µl aliquots were made (containing the equivalent of 5 µl of IgG beads slurry per 50 µl aliquot). Finally, solutions of Bio-Ox or PEGylOx were added to these aliquots, respectively, over a range of concentrations in 25 µl volume and incubated for 1 hr at RT. Bio-Ox was prepared at 10, 50, 100, 300, 500, and 900 µM; PEGylOx was prepared at the same and 2.2 mM as well; all displacement reactions were prepared in duplicate. A buffer only sample was also prepared in quadruplicate. The supernatants were collected to obtain the released fraction of SpA, and the beads were subsequently washed with 25 µl of 1X LDS loading buffer to remove the remaining bound fraction of SpA. 13 µl of each solution were loaded on SDS page gels, Coomassie stained, and the resulting images analyzed for the amount of SpA retained in each competitive displacement experiment (e.g. the fractional amount of SpA in the final LDS wash compared the total SpA in the reagent

elution plus the LDS wash – described in **S3**, below). Experimental replicates were averaged. The IC_{50} was calculated for both reagents based on the slope and y-intercept of the resulting lines plotted using a logarithmic fit. Both Bio-Ox and PEGylOx have substantially similar IC_{50} values, calculated at 159 and 166 µM respectively. We conclude that the affinity of PEGylOx for IgG, as compared to Bio-Ox, has not been diminished by the incorporation of the PEG[4] moiety and that the mechanism of competitive displacement of SpA (Fc binding) has been retained.

C: To determine the effect of the time of incubation on competitive displacement of SpA from IgG by PEGylOx, we incubated SpA saturated IgG Dynabeads with a saturated solution of PEGylOx for 5, 15, 30, or 60 min. The results are depicted in figure 1D, main text. As in **B** (above), rabbit polyclonal IgG-conjugated Dynabeads were incubated with SpA in 20mM Tris pH 8.0, 100mM NaCl, 0.01% (v/v) Tween, 1mM EDTA, 5% v/v ethanol. 66 µl of IgG beads slurry was combined with 66 µg SpA in 500 µl volume and incubated 1 hr at 4°C. The beads were washed twice and then resuspended in 660 µl and twelve 50 µl aliquots were dispensed (containing 5 µl Dynabeads slurry equivalent). To each aliquot, 10 µl of saturated PEGylOx solution (~2.5 mM) were added for the specified length of time, in triplicate. After the time period elapsed the supernatant was removed and combined with LDS loading buffer, and the beads were washed with 15 µl 1x LDS loading buffer (10 min at RT) to remove the remaining SpA. All samples were analyzed and quantified as in **B** (above), described in **S3** (below). The results indicate that ~70 - 75% of SpA can be competitively eluted from

IgG Dynabeads within 15 min, using a saturated solution of PEGylOx. See also file: POx_SpA_elution_time.xls.

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S3: Image quantitation and analysis

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All quantitative image analysis was carried out using the FujiFilm Multi Gauge v3.0 software and Microsoft Excel 2008. In all cases, bands of interest from gels were manually specified for intensity analysis within the Multi Gauge software. Equivalent regions of equal size were specified in blank regions of the gel for background subtraction. BSA standards were included for quantity calibration, typically at 100 and 400 ng bands in two separate lanes. All quantitative comparisons were carried out on normalized, calibrated measurements. An example image in processing (figure 1) and Multi Gauge readout (table 1) are provided below. Selected data from Multi Gauge generated files were transferred to Microsoft Excel for arithmetic, statistics and graphing. Two types of measurements were typically conducted: percent released and percent retained. “Percent released” assays express the intensity of a band measured in an elution fraction as a percentage of the total measured intensity (the sum of the intensities of both the eluted and retained fraction) - these were typically carried out to asses how much of a bait protein was obtained from the capture beads after treatment with elution peptide. “Percent retained” assays express the intensity of a band measured in a retained fraction as a percentage of the total measured intensity - these were typically carried out to asses how much wt SpA protein was retained on beads

after treatment with elution peptide. Retained fractions were obtained by washing with LDS loading buffer (2% lithium dodecylsulfate) which quantitatively interferes with the SpA / IgG interaction. All the Excel spreadsheets used to analyze Multi Gauge data presented here are included as supplemental data - these include a large degree of the raw data output from Multi Gauge (see file: Quant_BOx_POx_elution.xls). All original raw data are available in the original file formats on request.

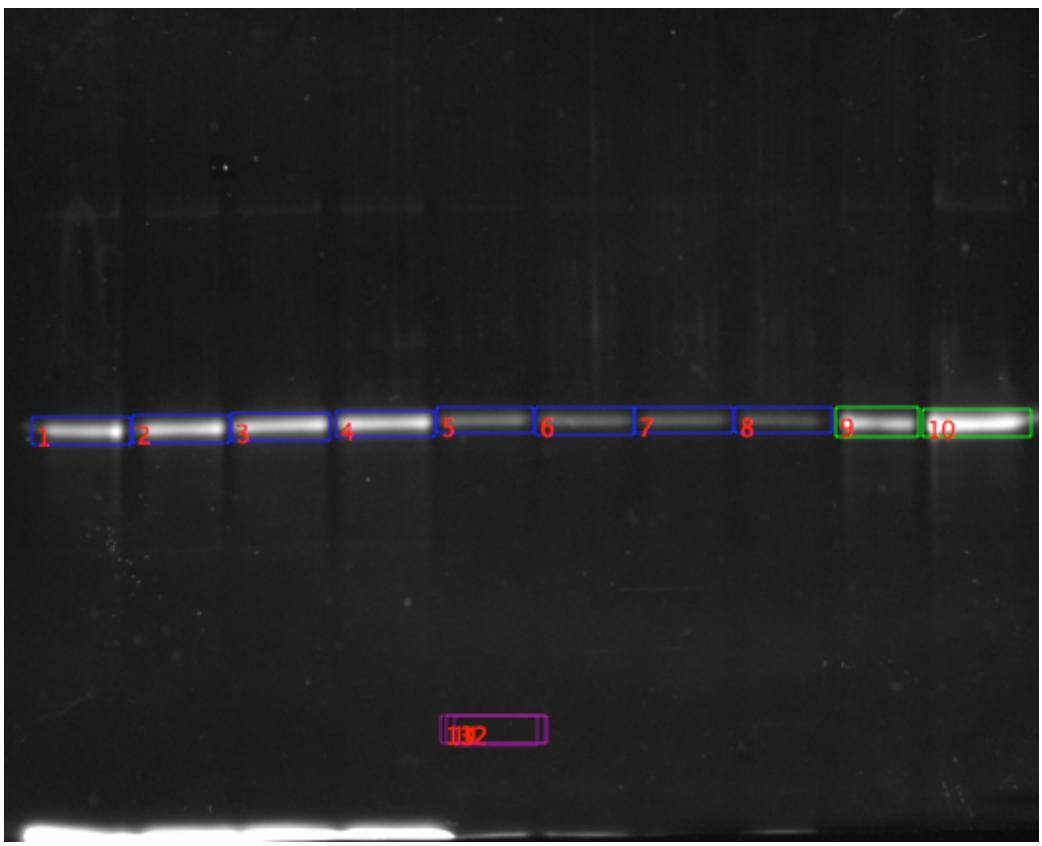

Figure 1 - Image processing and measurement in Multi Guage: This gel corresponds to the experiment carried out to measure the displacement of wt SpA from IgG Dynabeads with PEGylOx (detailed in **S2**, above). The blue boxes are drawn around the eluted SpA fractions (1 – 4, blue; PEGylOx can be seen at the bottom of the

gel) and the retained SpA fractions (5 – 8, blue). Boxes 9 and 10 (green) are BSA quantity standards. The remaining boxes (purple) are clustered in a “blank” region at the bottom of the gel and correspond in area to boxes drawn around samples and quantity standards to precisely subtract background from each measured signal.

Channel	No	Group	Index	Name	AU	Area(mm2)	AU-BG	B	BG	Std	Ratio(%)		(A-B)/mm2	Calibrated
1	1	A	1		94026437.00	13.69	13023107.00			81003330.00		16.09	951018.02	182.62
1	2	A	2		95898689.00	13.69	14895359.00			81003330.00		18.40	1087740.03	208.87
1	3	A	3		97507435.00	13.69	16504105.00			81003330.00		20.39	1205219.40	231.43
1	4	A	4		97876200.00	13.69	16872870.00			81003330.00		20.84	1232148.63	236.60
1	5	A	5		87271272.00	13.69	6267942.00			81003330.00		7.74	457719.17	87.89
1	6	A	6		86558103.00	13.69	5554773.00			81003330.00		6.86	405639.70	77.89
1	7	A	7		85328077.00	13.69	4324747.00			81003330.00		5.34	315816.52	60.64
1	8	A	8		84505863.00	13.69	3502533.00			81003330.00		4.33	255773.99	49.11
1	11	A	9		81003330.00	13.69	0.00	B	81003330.00		-------	0.00	0.00	
1	9	B	1		88913850.00	12.94	12389784.00			76524066.00	S	100.00	957769.95	100.00
1	12	B	2		76524066.00	12.94	0.00	B	76524066.00		-------	0.00	0.00	
1	10	C	1		122140355.00	16.25	26021955.00			96118400.00	S	100.00	1601148.81	400.00
1	13	C	2		96118400.00	16.25	0.00	B	96118400.00		-------	0.00	0.00	

Table 1 - Multi Gauge output: A standard tabulated data output from Multi Gauge is shown, this one corresponding to the image above - a single replicate in the aforementioned experiment. The calibrated data in the far right column would be used for combining with other experimental replicates, statistics, and graphing. For each region of a given size specified to measure a signal [groups A (eluted or retained signal, boxes 1 - 8), B (100 ng BSA standard, box 9), and C (400 ng BSA standard, box 10)] an according, equal sized background region is specified for subtraction (No. 11, 12, & 13 in the table above).

S4: SpA-tag Supplement

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A: Both the Rout and Landick SpA-tags are based on the wt SpA sequence (sequence alignment shown below). The Rout tag SpA-tag[1-3] was used for C-terminal SpA-tagging of Nup53, Nup1p[3] and Psf2p[4]. The Landick SpA-tag[6] was introduced C-terminally in the *rpoC* gene as previously described[7,8]. Gray bars over the wt SpA sequence shown in the alignment (UniProt: P38507, SPA_STAAU) indicate the positions of consecutive IgG binding domains: E, D, A, B and C respectively[1,5]. As shown in the SpA-tag alignment below (fig. 1, also included as a Clustal file: SpA-tag alignment.aln), although there are some differences in the primary sequences, both the Rout and the Landick SpA-tags encompass a small C-terminal portion of domain E, all of domains D, A, & B, and a large N-terminal portion of domain C. Hence, these tags comprise 3 complete SpA IgG-binding domains, as well as flanking sequence of neighboring domains. In contrast the TAP-tag is based on two tandem repeats of the synthetic SpA-derived Z-domain[9-12]. These Z-domains are indicated as gray bars over the "ZZ from TAP-tag" sequence shown in the alignment. This is the SpA sequence that can be found in the large-scale TAP-tagging projects[12,13].

The expected primary protein sequence for Nup1p-SpA was determined by Sanger DNA sequencing (www.genewiz.com) of the PCR amplified[14] gene from genomic DNA

extracted[15] from the *NUP1::spa* strain. The *NUP1::spa* gene sequence was determined across the entire CDS and found to contain a point mutation, 580A>T in the *spa* sequence relative to that expected of the Rout lab SpA-tag, resulting in a premature stop codon and truncating 36 amino acids from the expected C-terminal of the SpA-tag (fig. 2, and included files: Nup1_CDS_contig.ace, SpA_seq.ace, Nup1-SpA.gbk, & Nup1-SpA vs SpA alignment.aln). This gene should produce a protein of ~135.3 kDa, based on primary sequence, whose *NUP1* CDS is identical to that annotated in GenBank M33632.1:1001..4231.

Figure 1

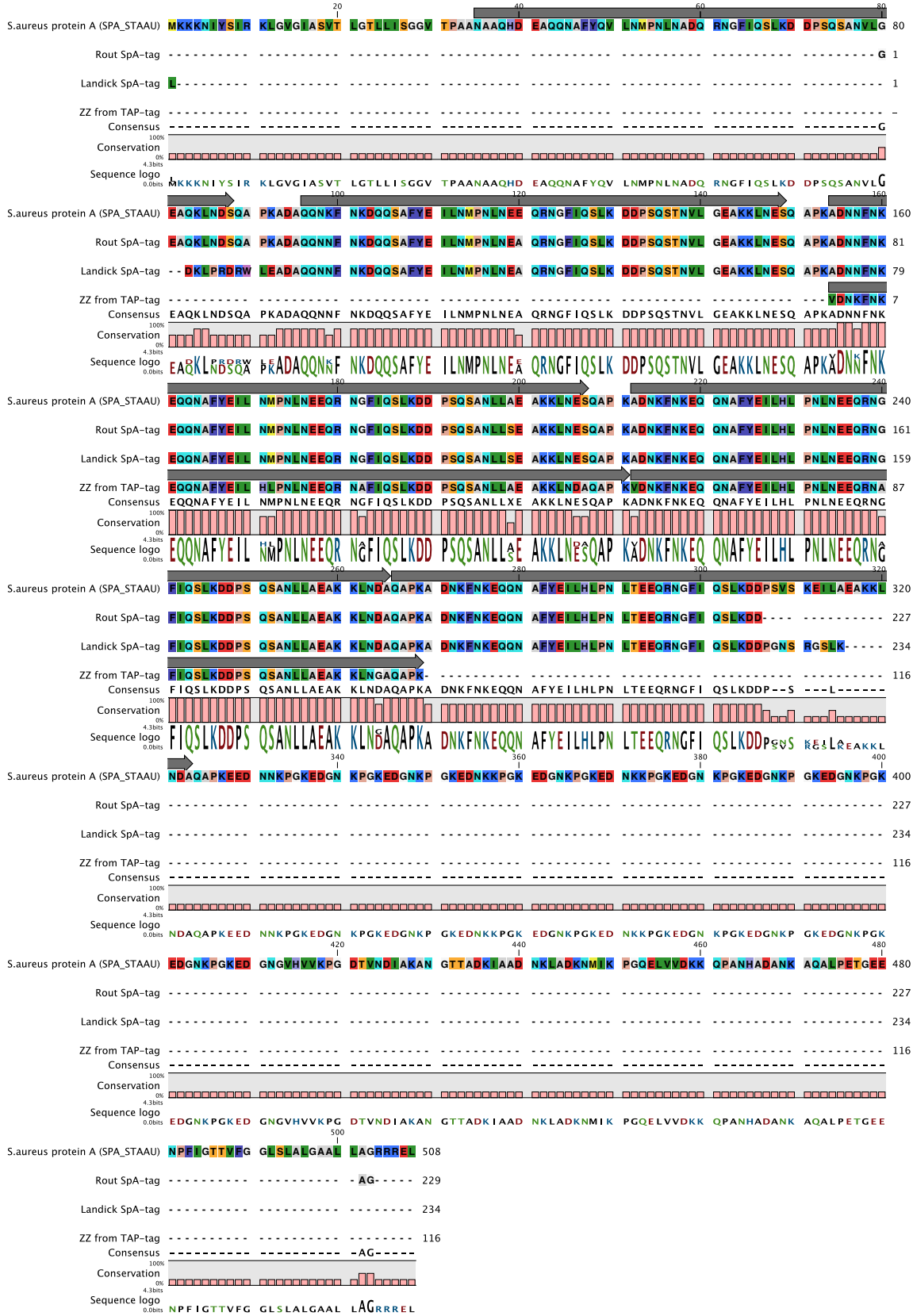
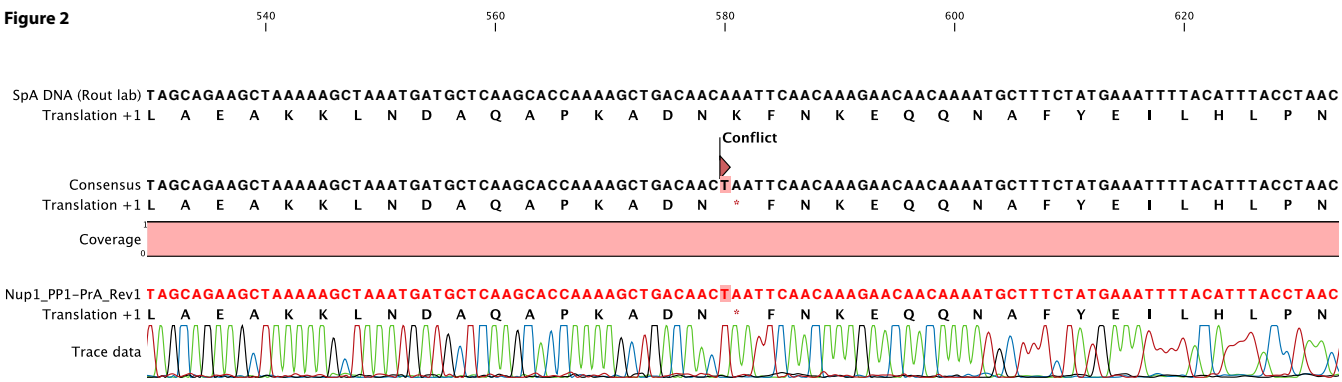

S.aureus protein A (SPA_STAAU)
Rout SpA-tag
Landick SpA-tag
ZZ from TAP-tag
Consensus
Conservation
Sequence logo

Figure 2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600
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Translation +1 L A E A K K L N D A Q A P K A D N K F N K E Q Q N A F Y E I L H L P N
Conflict
Consensus TAGCAGAAGCTAAAAAGCTAAATGATGCTCAAGCACCAAAAGCTGACAACTAATTCAACAAAGAACAACAAAATGCTTTCTATGAAATTTTACATTTACCTAAC
Translation +1 L A E A K K L N D A Q A P K A D N * F N K E Q Q N A F Y E I L H L P N
Coverage
1
0
Nup1_PP1-PrA_Rev1 TAGCAGAAGCTAAAAAGCTAAATGATGCTCAAGCACCAAAAGCTGACAACTAATTCAACAAAGAACAACAAAATGCTTTCTATGAAATTTTACATTTACCTAAC
Translation +1 L A E A K K L N D A Q A P K A D N * F N K E Q Q N A F Y E I L H L P N
Trace data

B: Nup53-SpA[3] (containing a multi-domain fragment of wt SpA) and Nup53-TAP[12] (synthetic, IgG-binding Z-domain; both discussed in **A**, above) were compared for total yield in affinity capture and efficiency of native elution using PEGylOx (fig. 3, below – procedure as described in fig. 2 of the main text and the online protocol).

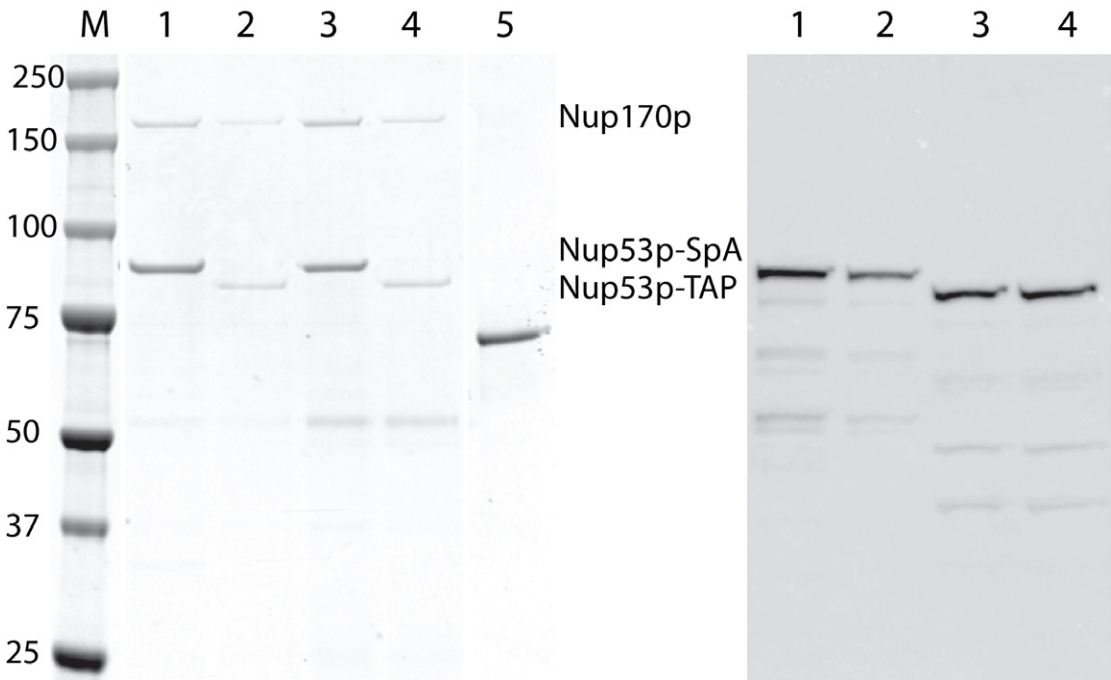

Figure 3 – Nup53-SpA vs Nup53-TAP: **(*LEFT*)** Colloidal Coomassie blue stained SDS-polyacrylamide gel showing Nup53p purified via either an SpA-tag containing wt sequences (SpA) or synthetic sequences (TAP). Lanes from left to right: (M) molecular mass marker, masses indicated are in kDa; (1) Nup53p-SpA eluted with PEGylOx; (2) Nup53p-TAP eluted with PEGylOx; (3) Nup53p-SpA retained fraction; (4) Nup53p-TAP retained fraction; (5) BSA standard, 200ng. **(RIGHT)** Western blot for SpA/TAP-tagged Nup53p using 5 µl of input cell extract and post-binding supernatant (0.5 g cell grindate combined with 2 ml extraction solution; batch binding 1hr). Lanes from left to right: (1)

Nup53p-SpA input; (2) Nup53p-SpA supernatant; (3) Nup53p-TAP input; (4) Nup53p-TAP supernatant. Transfer of proteins to the support membrane and HRP detection of SpA/TAP-tagged Nup53p carried out using standard procedures, PAP antibody complex (Sigma-Aldrich #P1291) at 1:5000 and Lumi-Light substrate (Roche #12015200001).

Comparing Nup53p-SpA eluted and retained fractions (lanes 1 vs. 3) to Nup53p-TAP eluted and retained fractions (lanes 2 vs. 4) in the gel shown at left, above, illustrates that PEGylOx releases the Nup53p/Nup170p dimer to roughly comparable efficacy in both cases. However, the yield in the Nup53p-SpA eluted fraction (lane 1) is significantly higher than the Nup53p-TAP eluted fraction (lane 2); and the combined yields (eluted plus retained fractions for each tagged protein [lanes 1+3 and 2+4], respectively) exhibit the same trend – indicating that more Nup53-SpA was captured, in total, from the cell extract than Nup53-TAP. This conclusion is further supported by the western blot shown above at right: Nup53p-SpA is noticeably depleted (lane 1 vs. 2), whereas Nup53-TAP is not (lane 3 vs. 4). These results are compatible with the SpA tag based on wt sequence being either of higher affinity through avidity effects or simply more accessible on account of its size and number of exposed IgG-binding repeats.

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S5: PEGylOx removal by spin column gel filtration

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PEGylOx was tested for depletion from solution by spin column gel filtration. The results are given in table 1, below. We compared Bio-Rad Bio-Gel P-6 (P6) with Bio-Rad Bio-Gel P-30 (P30) and Thermo Zeba 40k MWCO (Z40k) media. P30 and Z40k gave roughly equivalent results within the limits of our assay. We also tested Thermo Zeba 7k MWCO (Z7k), which gave results comparable to P6 (data not shown). All indications are that gel media with low molecular weight cutoff (e.g. 6 – 7 kDa in the case of P6 and Z7k) deplete PEGylOx only poorly, whereas those of higher molecular weight cutoff (e.g. 40 kDa for both P30 and Z40k) deplete peptide very well. Our assay was carried out by monitoring UV280 absorbance (A280) on a Beckman DU730 spectrophotometer using a 100 µl quartz cuvette. For theoretical and technical reasons, spectrophotometry used in this manner has a rather narrow linear dynamic range; typically assay readings should be held stringently (by dilution or concentration) to between ~0.2 – 0.7 AU for highest accuracy (~3-fold range). In practice a functional range of ~0.05 – 1.0 AU (~20-fold range) provides for useful estimations of protein abundance. Therefore our spectrophotometric assay can suffer from inaccuracy in the reading of both high concentration and highly depleted samples. To analyze pre- and post-column samples at the same dilution requires that the input sample be highly

concentrated, so as to be out of the linear dynamic range on the high side; but this works reasonably well for estimating e.g. PEGylOx depletion by P6, which leaves enough peptide in the eluted fraction to fall within functional detection range. Depletion with P30 and Z40k always reduced peptide levels to below those reasonable for accurate detection – and thus was outside of our ability to precisely assess by this assay. Our estimation based on the numbers we did obtain is indicative of better than 100-fold depletion of PEGylOx by P30 and Z40k media. Although UV is not the best assay, it provides a means for quick, rough checks on e.g. the reproducibility of spin column depletion and the behavior of PEGylOx with respect to depletion over columns equilibrated with different buffers (as buffer exchange of the protein complex of interest with simultaneous quantitative depletion of PEGylOx prior to downstream analysis is of great value). We have noticed (and this is reflected in table 1 for the two P6 readings) that there can be some column-to-column variability in the depletion results depending on e.g. how the resin bed settles during initial equilibration. Care must be taken to follow the manufacturer's specifications and settle the bed uniformly for best results. This may have been less apparent with P30 and Z40k because depletion, even with high % variability, was always below the accurate detection limit of our instrument. In order to convince ourselves that the depletion with was as good as it seemed by UV280, we analyzed a series of natively eluted experimental samples, including peptide depletion by spin column gel filtration, by SDS-PAGE and Coomassie staining (fig. 1, below).

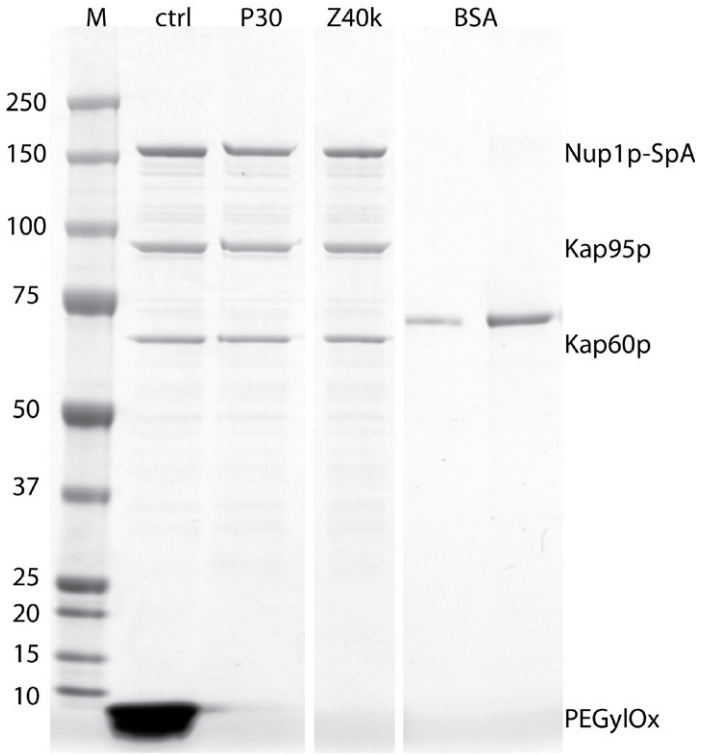
Table 1.

PEGylOx	net A280	dil. Factor	tot. A280	avg.	Δ
Pre	1.341	20	26.82	26.45	n/a
	1.304	20	26.08		
Post P6	0.32	20	6.4	6.33	4.18
	0.313	20	6.26		
Pre	1.447	20	28.94	28.99	n/a
	1.452	20	29.04		
Post P6	0.051	20	1.02	0.99	29.28
	0.048	20	0.96		
Post P30	0.007	20	0.14	0.15	193.27
	0.008	20	0.16		
Post Z40k	0.013	20	0.26	0.27	107.37
	0.014	20	0.28		
Pre	0.549	50	27.45	27.075	n/a
	0.534	50	26.7		
Post P30	0.007	10	0.071	0.0855	316.67
	0.010	10	0.1		
Post Z40k	0.007	20	0.14	0.12	225.63
	0.005	20	0.1		
Pre	2.452	20	49.04	49.3	n/a
	2.478	20	49.56		
Post P30	0.02	20	0.4	0.37	133.24
	0.017	20	0.34		
Post Z40k	0.033	20	0.66	0.68	72.5
	0.035	20	0.7		

	avg. Δ
P6	17
P30	214
Z40k	135

Table 1 – PEGylOx depletion by spin column gel filtration: A legend is present below the table. **(LEFT)** Four independent experimental series are presented, demarked by boxes containing all samples for that series. Each reading within each series was made in duplicate from a single experimental source sample; replicate readings are grouped with the sample type (Pre, Post P6, Post P30, and Post Z40k) and means were taken for the total A280 of each replicate series after dilution. Pre-column samples (i.e. saturated PEGylOx stock solutions) were analyzed directly at the given dilution in 40 mM Tris buffered solution at pH 8.0 with 100 mM NaCl and 0.01% (v/v) Tween 20. For samples applied to P6 and P30 media, 26 µl of stock were first applied to the spin column, pre-equilibrated with the Tris buffered solution mentioned, and then subsequently read at the given dilution. For samples applied to the Z40k medium, 13 µl of stock were first applied to the pre-equilibrated spin column, and then subsequently read at the given dilution. The fold-depletion for each column type is given in the table. **(RIGHT)** Like samples are averaged together to give a rough fold-depletion observed for each column-type.

Legend **– PEGylOx**: Pre (A280 of PEGylOx stock solution), Post (A280 of spin column flow-through), P6, P30, Z40k (different media, as in text); **net A280**: A280 background subtracted for A320 at the given dilution; **dil. Factor**: dilution of sample into buffer before reading out; **tot. A280**: calculated A280 compensated for dilution; **avg.**: mean of technical replicates; **Δ**: fold-depletion in A280 relative to the Pre column sample; **avg. Δ**: mean of fold-depletion for all like experimental replicates.



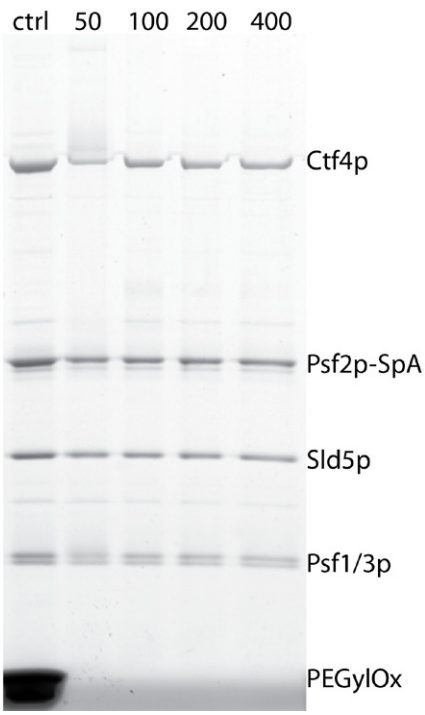


Figure 1 – PEGylOx depletion by spin column gel filtration with simultaneous buffer exchange: **(TOP)** Peptide removal trials from Nup1p-SpA affinity purifications utilizing 0.5 g of grindate eluted in 10 µl saturated PEGylOx solution, then either loaded directly on the gel (control - ctrl) or gel filtered through a Z40k or through a P30 (an additional 10 µl of buffer added to the sample to accommodate the loading requrements of P30 micro bio-spin columns). In both cases the columns were pre-equilibrated with 40 mM Tris buffered solution at pH 8.0 with 100 mM NaCl and 0.01% (v/v) Tween 20. BSA is present in the right most lanes at 50 ng and 200 ng. **(BOTTOM)** Peptide removal trials from Psf2p-SpA affinity purifications utilizing 1 g of grindate eluted in 10 µl saturated PEGylOx solution, then either loaded directly on the gel (ctrl) or gel filtered through a

Z40k. Each Z40k was equilibrated with a different concentration of ammonium acetate solution from 50 – 400 mM (as indicated). In both TOP and BOTTOM PEGylOx was quantitatively removed to below the limit of detection by our colloidal Coomassie blue stain (~1 ng)[1] – commensurate with a drastic reduction in PEGylOx, as previously determined by UV280 spectrophotmetry.

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