Supplementary Materials

Table S1. DoE table for experiment with BSA and ferritin.

Cross Flow (mL/min)	Channel Flow (mL/min)	Gradient exponential	Channel Thickness (mm)	Injection volume (µL)	Injection time (min)
2	0.25	0.88	0.35	20	10.0
2	0.44	0.50	0.19	20	1.0
1	1.00	0.88	0.19	80	3.3
1	1.00	0.63	0.19	20	3.3
4	0.25	0.50	0.35	80	10.0
5	0.25	0.88	0.19	50	1.0
4	0.81	0.88	0.19	50	3.3
5	0.44	0.88	0.5	50	3.3
2	0.25	0.88	0.19	80	7.8
4	0.25	0.50	0.35	80	1.0
4	0.81	0.50	0.35	20	7.9
1	1.00	0.75	0.35	20	10.0
5	1.00	0.50	0.5	50	3.3
1	1.00	0.63	0.5	50	1.0
1	0.25	1.00	0.35	80	1.0
2	0.81	0.63	0.35	80	3.3
4	0.44	1.00	0.19	80	3.3
4	0.25	1.00	0.5	80	10.0
2	0.44	0.63	0.35	50	7.8
5	1.00	0.88	0.19	20	10.0
5	0.44	0.88	0.35	50	7.8
5	0.81	0.63	0.35	20	3.3
2	0.81	0.63	0.35	80	3.3
5	0.63	0.63	0.5	80	1.0
4	0.81	0.75	0.5	20	1.0
4	0.25	0.75	0.5	20	10.0
5	0.25	0.63	0.5	20	1.0
2	0.63	0.50	0.5	20	3.3
4	0.81	1.00	0.5	20	7.8
1	0.25	0.50	0.35	20	5.5
2	0.63	0.50	0.19	20	10.0
4	1.00	0.63	0.5	20	10.0
1	0.44	1.00	0.35	80	10.0
5	1.00	1.00	0.35	80	1.0
1	0.25	0.50	0.5	80	3.3
1	0.44	1.00	0.19	20	3.3
1	0.63	0.50	0.5	50	10.0
5	0.25	0.63	0.19	20	5.5
4	1.00	0.88	0.35	80	10.0
4	1.00	0.50	0.19	50	1.0
1	1.00	1.00	0.5	80	10.0
1	1.00	1.00	0.35	20	1.0
3	0.89	1.00	0.5	80	1.0

Table S1. *Cont.*

Cross Flow (mL/min)	Channel Flow (mL/min)	Gradient exponential	Channel Thickness (mm)	Injection volume (µL)	Injection time (min)
1	0.81	0.50	0.35	80	10.0
5	0.25	1.00	0.35	20	3.3
4	1.00	0.63	0.35	50	10.0
1	0.44	0.50	0.19	80	5.5
5	1.00	0.63	0.19	80	7.8
5	0.25	0.50	0.5	80	5.5
3	0.63	0.75	0.5	80	5.5
5	1.00	1.00	0.19	20	1.0
2	0.81	1.00	0.19	50	7.8
5	0.81	0.63	0.5	80	10.0
1	0.25	1.00	0.5	20	3.3

Table S2. DoE table for experiment with human serum.

Pattern	Cross Flow (ml/min)	Channel Flow (mL/min)	Injection time (min)
+0−	4.5	0.65	3
+−0	4.5	0.45	5
0	3.75	0.65	5
+0+	4.5	0.65	7
0	3.75	0.65	5
0++	3.75	0.85	7
0−−	3.75	0.45	3
−0+	3	0.65	7
−+0	3	0.85	5
0	3.75	0.65	5
0	4.5	0.85	5
0+−	3.75	0.85	3
−0−	3	0.65	3
0−+	3.75	0.45	7
−−0	3	0.45	5

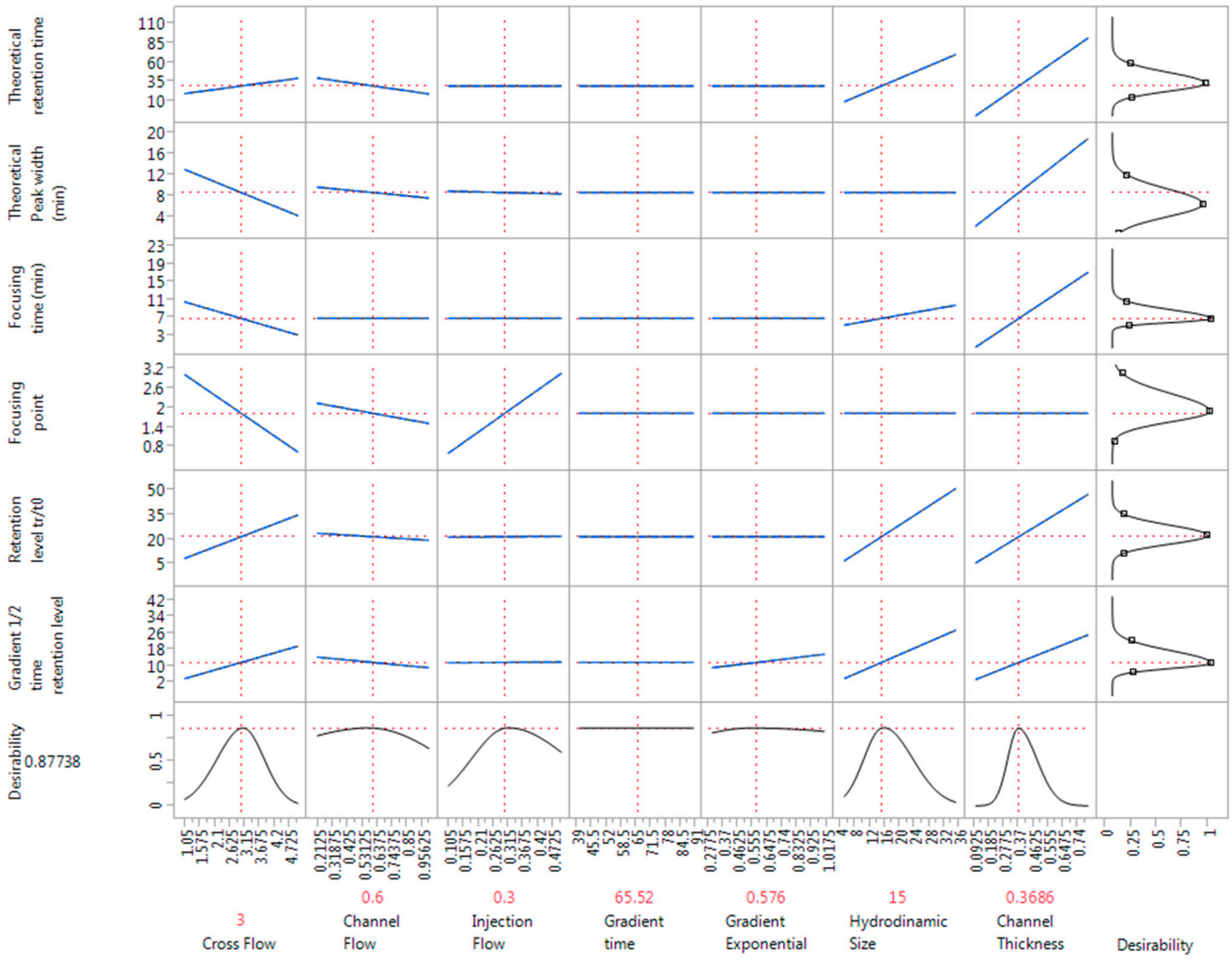

Figure S1. Prediction profiles based on theoretical outcomes *vs.* individual AF4 method parameters. Each prediction profile is a predicted response as one variable is changed while the others are held constant at selected values (vertical red lines). The desirably function of each outcome (far right column), was combined with the prediction profiles resulting in a combined desirability profile for each AF4 parameter (bottom row), which was used to determine the optimal value of each parameter.

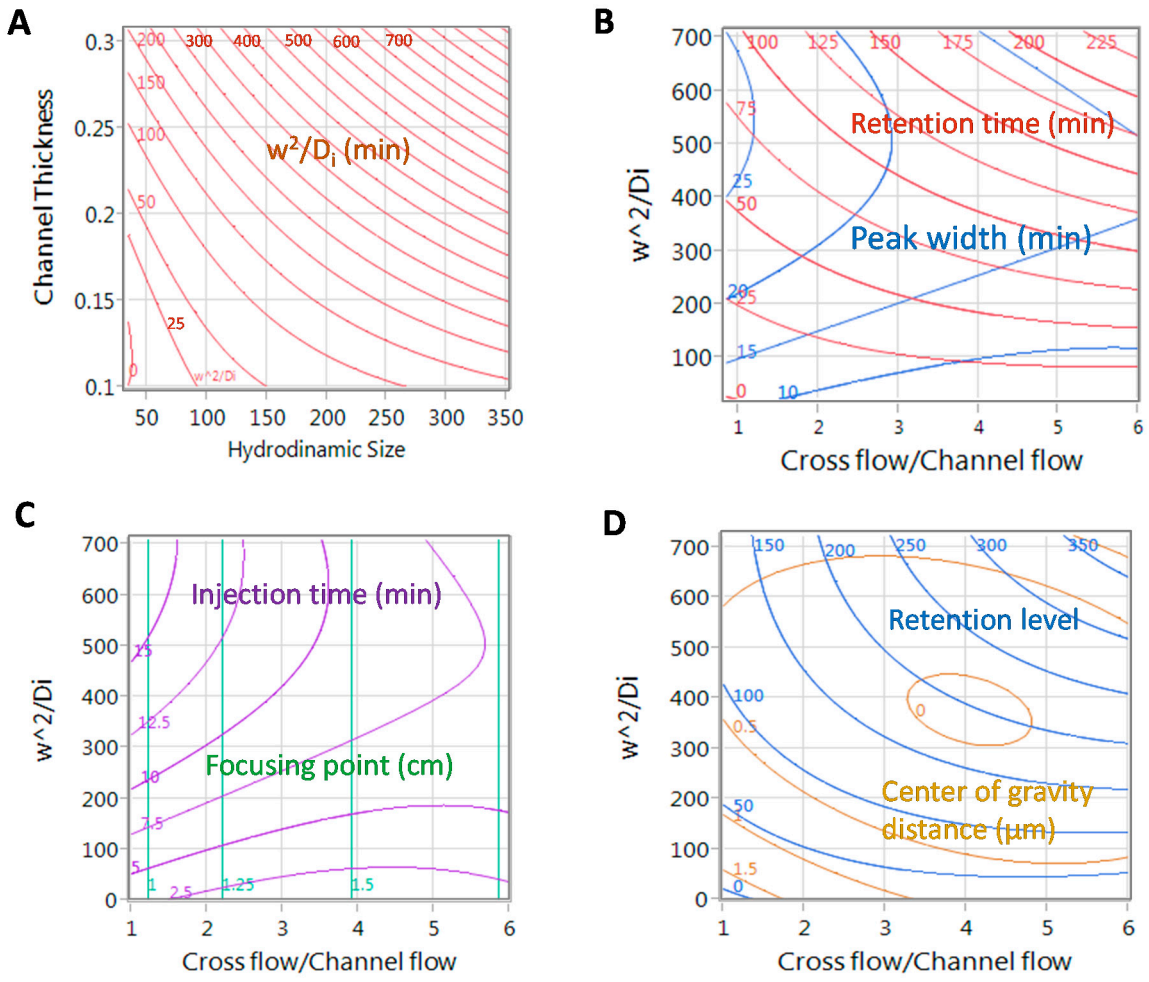

Figure S2. Theoretical contour profiles for 30–350 nm size range at F_{inj}/F_{cross} = 0.1with w = 0.1–0.3 mm, F_{cross} = 1–3 mL/min, F_{out} = 0.5–1 mL/min, First w^2/D_i, can be determined from Figure A based on hydrodynamic diameter and the channel thickness. Using the w^2/D_i values, the cross flow/channel flow ratio (F_{cross}/F_{out}) can be determined for a desired retention time (t_R) and corresponding peak width ($W_{b,t}$) from Figure B. With the selected F_{cross}/F_{out} ratio the injection time(t_{inj}) and the corresponding focusing point (z) is determined from Figure C. The corresponding retention level (R_L) and center of gravity distance from the membrane (l) can be determined from Figure D.

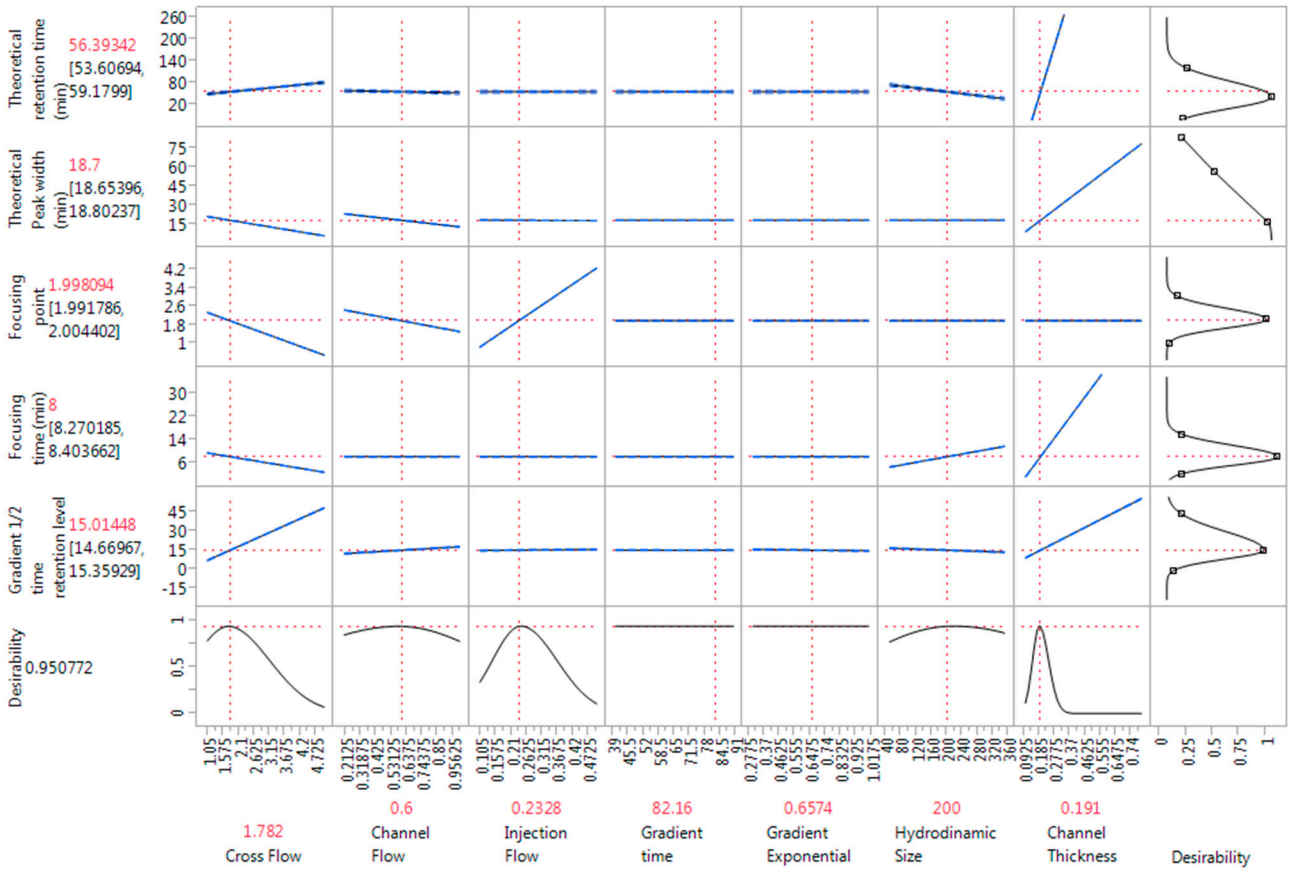

Figure S3. Theoretical desirability optimization of AF4 instrumental parameters based on full factorial DoE for 50–350 nm hydrodynamic diameter. Each prediction trace is the predicted response as one variable is changed while the others are held constant at the current values. The desirably function of each outcome (far right column), plate height, retention level, focusing point, and retention time at the half of the gradient time, was combined with the predicted correlation functions with instrumental parameters, cross flow, channel flow, injection flow, channel thickness, gradient time, and gradient exponential for 6 nm hydrodynamic diameter. The optimal range can be determined based on the combined desirability function for each parameter (bottom row).

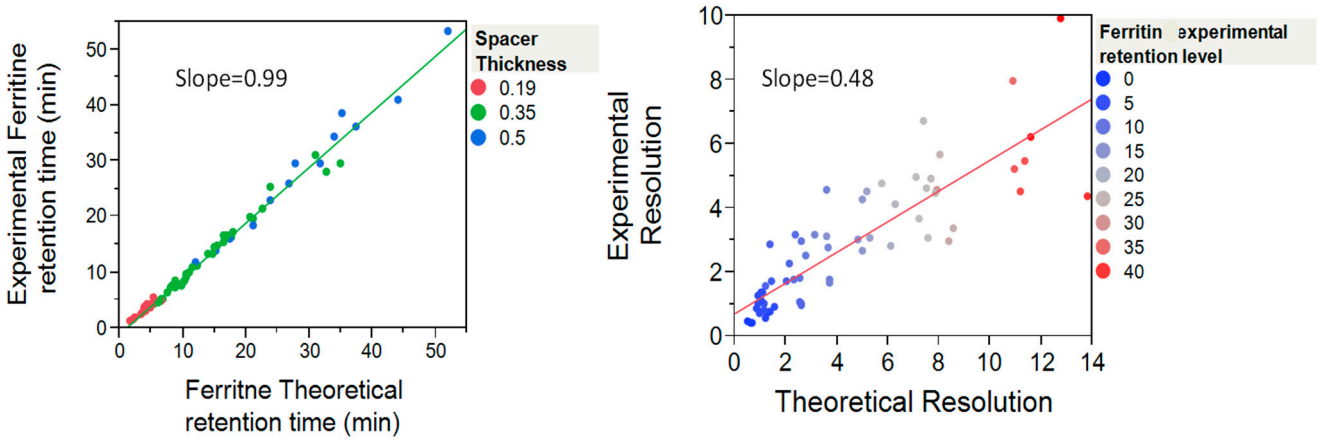

Figure S4. Correlation diagram of experimental versus theoretical retention time and resolution obtained by 54 run DoE experiment using BSA and ferritin standards.

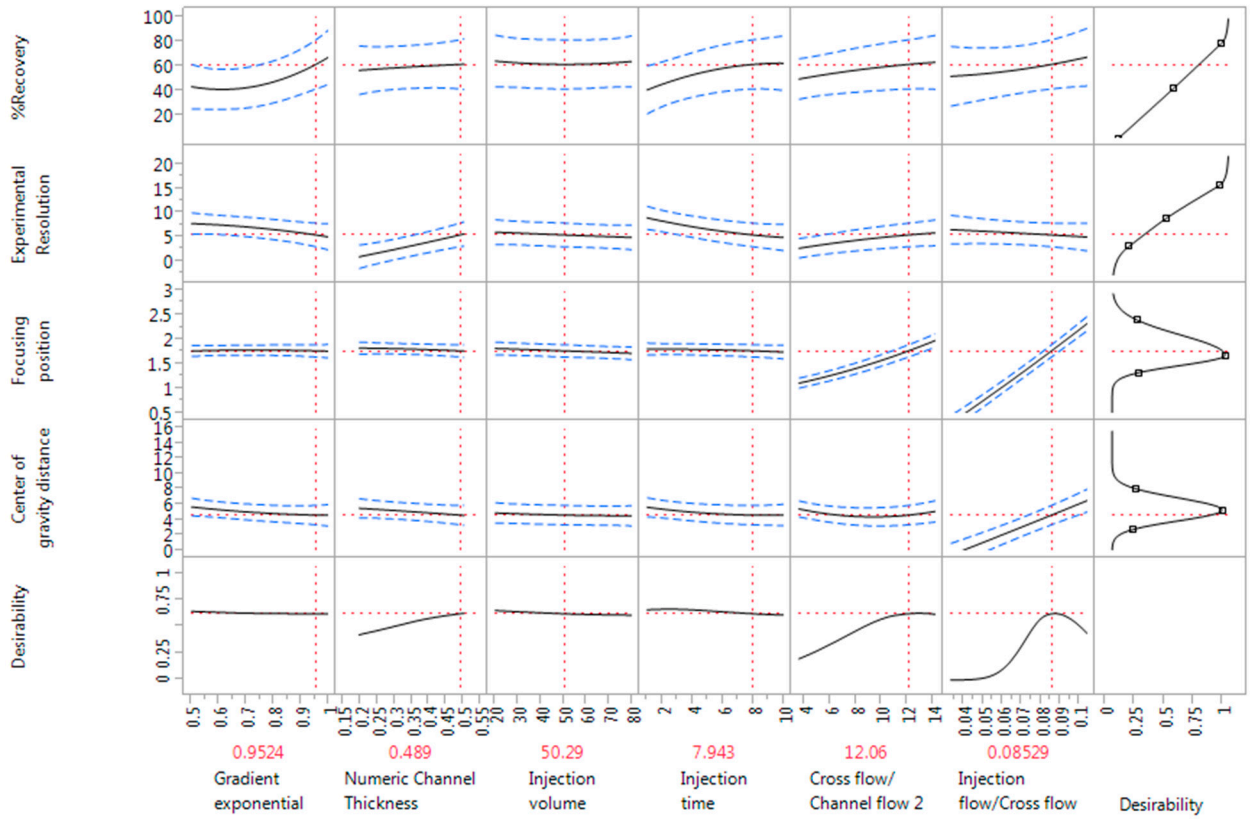

Figure S5. Prediction profiles of AF4 instrumental parameters based on fractional factorial DoE with protein standards. Each profile is a predicted response as one variable is changed while the others are held constant at selected values (vertical red lines). The desirably function of each outcome (far right column), was combined with the prediction profiles resulting in a combined desirability profile for each AF4 parameter (bottom row), which was used to determine the optimal value of each parameter.

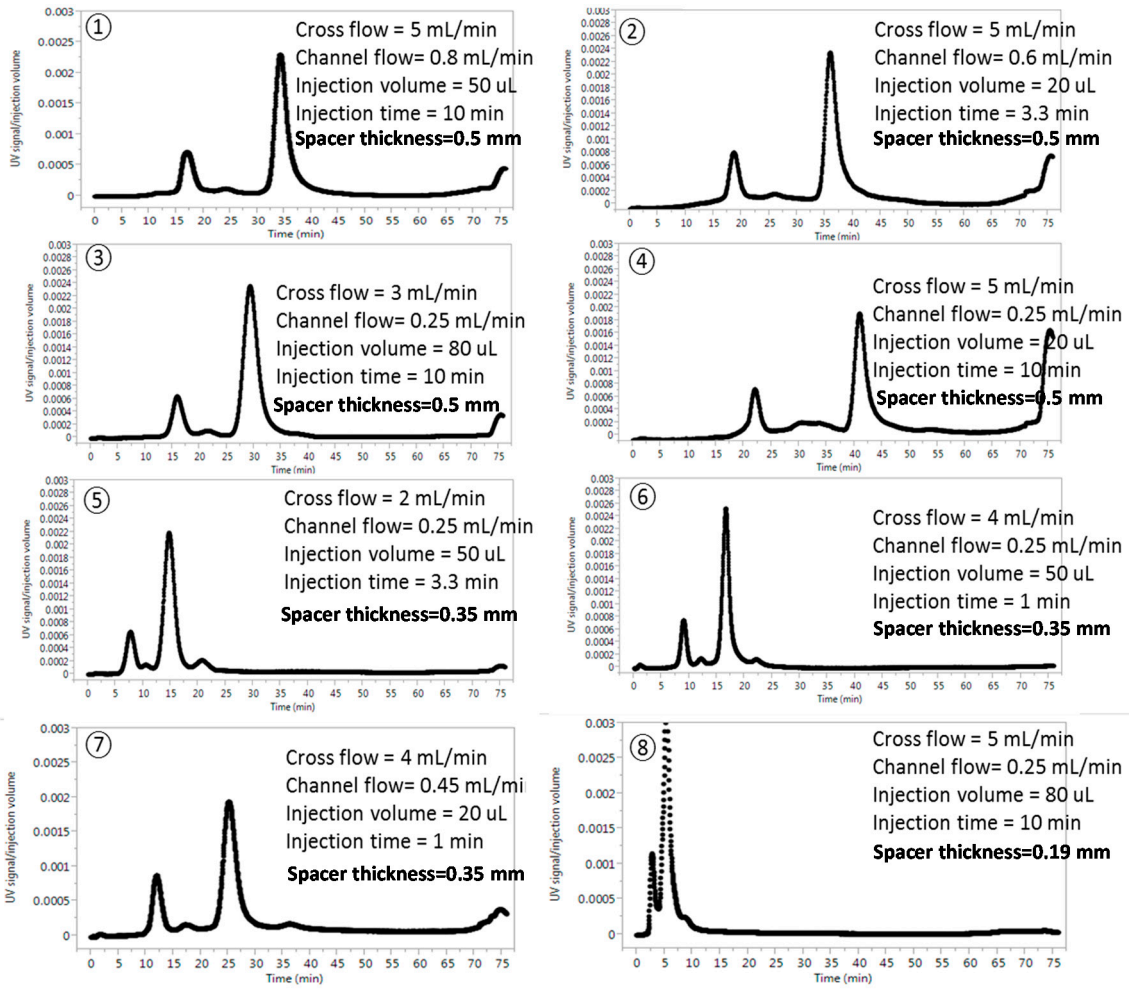

Figure S6. Representative fractograms from the DoE experiment with a mixture of bovine serum albumin and ferritin. High flow field (F_{cross}/F_{out} > 10), leads to good resolution but low channel recovery; along with increased UV absorption during purge (fractogram 4). High flow field and 0.19 mm spacer thickness (w=) resulted in both pure resolution and recovery (fractogram 8). Best channel recoveries and resolution were obtained at moderate flow field, F_{cross}/Fout ~ 7 (fractograms 1 and 5) and 0.35 and 0.5 mm spacer thickness.

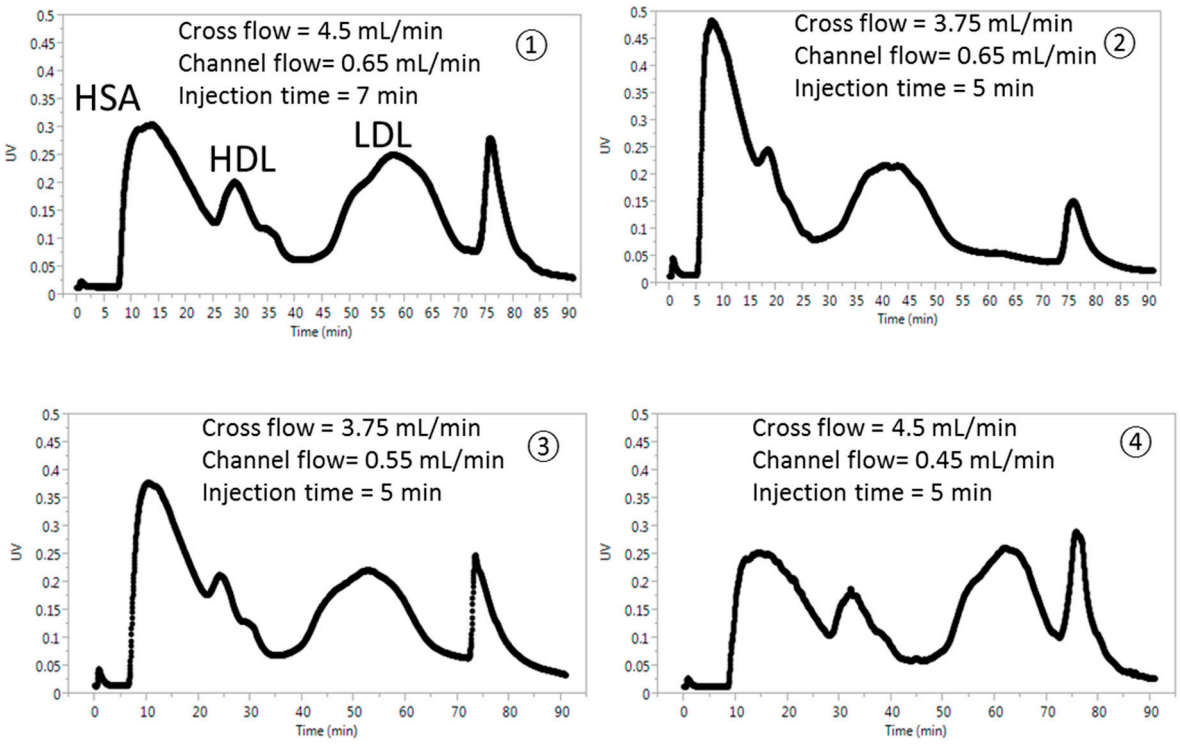

Figure S7. Representative fractograms from the DoE experiment with human serum. Most prominent components are human serum albumin (HSA), high density liporpteins (HDL) and low density liporpteins (LDL).