TOSOH

BEGINNERS GUIDE FOR mAb TITER ANALYSIS

INTRODUCTION

In many stages of mAb development, cell culture samples must be screened for IgG titer. Antibody titer determination by Protein A affinity chromatography is much more robust, reliable and reproducible than enzyme-linked immunosorbent assays (ELISAs). Protein A affinity columns can be employed to determine the concentration of monoclonal antibody for the optimal time for harvest or to identify clones that express the most antibodies.

TSKgel® Protein A-5PW is a 20 µm, 4.6 mm ID × 3.5 cm L, column for high performance affinity chromatography. Made of PEEK hardware, this column has been designed for the rapid separation and robust quantification of a variety of antibodies. Monoclonal antibodies can be captured and accurately quantitated in less than 2 minutes per injection.

In this application note we want to focus on how to get you started when first using our TSKgel Protein A-5PW column for IgG-Titer analysis in your laboratory.

RAPID SEPARATION OF IgG FROM IMPURITIES

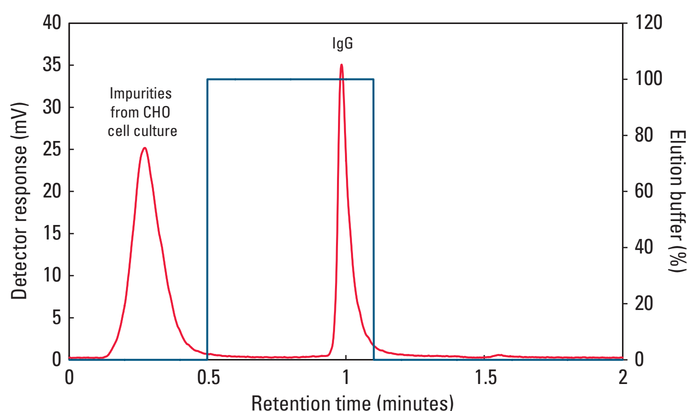


Figure 1

Column: TSKgel Protein A-5PW, 20 µm, 4.6 mm ID × 3.5 cm L
Binding buffer: 20 mmol/L sodium phosphate buffer, pH 7.4
Elution buffer: 20 mmol/L sodium phosphate buffer, pH 2.5
Stepwise gradient: 0 – 0.5 min: binding buffer
0.5 – 1.1 min: elution buffer
1.1 – 2.0 min: binding buffer
Flow rate: 2 mL/min; Detection: UV @ 280 nm
Sample: 20 µL CHO cell culture supernatant containing polyclonal IgG (0.5 mg/mL)

PROTEIN A AFFINITY CHROMATOGRAPHY

Protein A Chromatography relies on the specific and reversible binding of antibodies to an immobilized Protein A ligand. Protein A resins are the most frequently used affinity resins in biomanufacturing. Today it is the standard technique for capturing recombinant monoclonal antibodies due to its fast and easy method development, combined with high robustness and specificity.

Binding of monoclonal antibodies occurs under physiological pH values and in a wide range of conductivity. Therefore cell culture fluid can directly be applied to the column and only antibody binds to the column. Impurities, like host cell protein and DNA, do not bind to the column and can be washed out of the column with equilibration buffer. A pH shift to acidic pH (2-4) is used for the elution of the bound antibody, which will then elute in one single peak. Figure 1 shows a typical chromatogram of an analytical run on the TSKgel Protein A-5PW column.

CONNECTING THE COLUMN TO YOUR HPLC SYSTEM

The column end fittings are 10-32 female fittings as found on all TSKgel columns. Due to the PEEK hardware the fittings should always be tightened by hand. Tightening with a wrench is not necessary and can strip the threads of the column.

When connecting the column to your HPLC, first remove the plug on the inlet of the column and fill the inlet with mobile phase to avoid trapping air in the columnn. Set the flow rate of your system to 0.1 mL/min and connect the inlet tubing to the column. Wait until mobile phase is appearing at the outlet and connect the column to the detector inlet capillary of your HPLC system. Increase the flow-rate to 0.5 mL/min and check for leakage.

PREPARING THE COLUMN

1. Before using the column for the first time, the column should be equilibrated with 10 column volumes (CV) of equilibration buffer to remove the shipping solvent. The initial flow rate should not exceed 0.5 mL/min for the first 10 minutes. Afterwards the flow rate can be increased to the recommended flow rate of 2.0 mL/min
2. If the column has been stored for a long time cleaning of the column is recommended before use.

CLEANING THE COLUMN

The TSKgel Protein A-5PW is a very robust column and lifetimes of over 2000 injections per column have been reported. With extended use of the column, monitor the backpressure and performance of the column with a control sample. Periodical cleaning will remove residual material from the resin and frits of the column and prolongs lifetime of the column.

Typical cleaning solutions include 0.1 M sodium hydroxide, 1 M acetic acid, 20 % ethanol and 1-2 M sodium chloride. To clean the column make multiple injections of 100 µL of the cleaning solvent using your normal analysis method. Usually, 2 to 5 injections of the cleaning solvent are sufficient to remove the majority of contaminants. Monitor the UV-Signal and as soon as no UV-Signal is detected the column is fully cleaned.

STORING THE COLUMN

1. Store column at 2 - 8 °C. Do not freeze the column!
2. Short-term storage (1-5 days): Wash column with 20 mmol/L sodium phosphate buffer, pH 7.4, for 20 CV at 0.5 mL/min. Cap both ends of column and store it in this buffer at the recommended storage temperature.
3. Long-term storage: Wash column with 20% ethanol at 0.5 mL/min for 20 CV and store column in this solvent at the recommended storage temperature

Do not use or store column above 20% organic solvent. More than 20% organic solvent will damage the Protein A ligand.

OPERATING CONDITIONS

Shipping Solvent: Aqueous solution containing 20% ethanol
Max. flow rate: 4.0 mL/min (standard operating flow rate: 2.0 mL/min)
Max. pressure: 2.0 MPa
pH range: 2.0 - 12.0
Bind and wash buffer: 20 mmol/L sodium phosphate buffer, pH 7.0 to 7.5

Note: 100-150 mmol/L NaCl can be added to the bind and wash buffer. However, high salt concentration can cause non-specific binding.

Elution buffer: Acidic buffers, pH 2.0 - 3.0
Optional acidic buffers:
- 12 mmol/L HCl (pH 2.0)
- 20 to 100 mmol/L sodium phosphate buffer, pH 2.5 - 3.5
- 20 to 100 mmol/L sodium citrate buffer, pH 2.5 - 3.5
- 20 to 100 mmol/L glycine, pH 2.5 - 3.5
- 2 - 5% acetic acid.
- 10 mmol/L sodium phosphate buffer, pH 2.5 + 10% ethanol (above 20% organic solvent should be avoided)

Note: To reduce high baseline, adjusting buffer pH with low (diluted) concentration of acid such as 100 mmol/L phosphoric acid is recommended.

Temperature: 4 – 30 °C

SAMPLE PREPARATION

To ensure efficient binding of the sample when injecting large quantities (> 500 µL), the sample should be diluted with equilibration buffer. For smaller quantities this is not necessary. To prevent fouling of the resin and blockage of the frits, buffer and samples should be filtered before use and injection with a 0.22 µm filter.

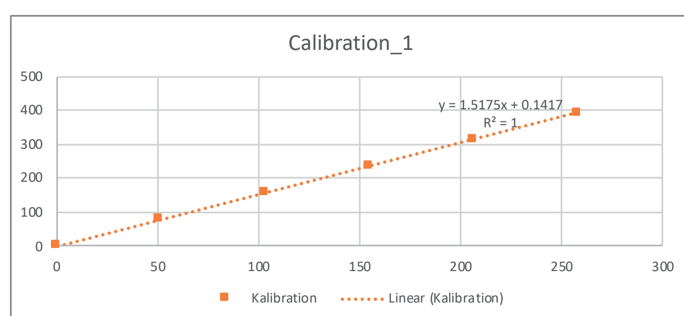
DETERMINING THE SAMPLE LOAD AND CREATING A CALIBRATION CURVE

For analytical purposes the maximum load is determined by the linearity of the calibration curve. When injecting too much sample, some of the sample might not bind to the column which results in partial breakthrough and flattening of the calibration curve.

To create a calibration curve a mAb-sample with known concentration is required. The calibration curve can be acquired with multiple approaches. Different sample volumes of the same sample can injected; identical sample volumes with samples of different (but known) concentration; different sample concentration and injection volumes. To create a baseline for all measurements, a blank of 10 µL mobile phase is injected. The calibration curve should be created by using at least duplicate injections and creating the average.

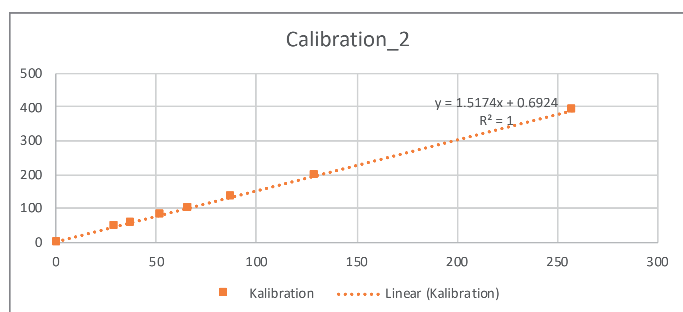
The X-axis of the calibration curve shows the average of the total area of the elution peak, the Y-axis shows the mass of antibody in µq.

1. Different sample volumes of the same sample



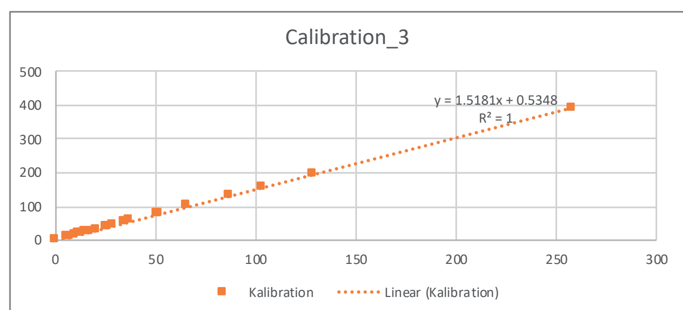
µL	c (mg/mL)	µg	Area (1)	Area (2)	Area (Avg)
50	7.83	391.25	258.26	257.37	257.81
40	7.83	313	206.1	206.22	206.16
30	7.83	234.75	154.6	154.73	154.66
20	7.83	156.5	103.09	102.73	102.91
10	7.83	78.25	51.31	51.08	51.2
10	0	0	0.18	0.18	0.18

2. Different sample concentrations and same sample volume



µL	c (mg/mL)	µg	Area (1)	Area (2)	Area (Avg)
50	7.83	391.25	258.26	257.37	257.81
50	3.94	197	129.05	128.87	128.96
50	2.67	133.5	87.31	87.27	87.29
50	2.03	101.5	65.98	65.95	65.97
50	1.59	79.5	52.04	52.04	52.04
50	1.15	57.5	37.3	37.27	37.29
50	0.9	45	29.04	29.03	29.03
50	0	0	0.46	0.44	0.5

3. Combined sample volumes and concentrations of Calibration 1 and 2



µL	c (mg/mL)	µg	Area (1)	Area (2)	Area (Avg)
50	7.83	391.25	258.26	257.37	257.81
50	3.94	197	129.05	128.87	128.96
50	2.67	133.5	87.31	87.27	87.29
50	2.03	101.5	65.98	65.95	65.97
50	1.59	79.5	52.04	52.04	52.04
50	1.15	57.5	37.3	37.27	37.29
50	0.9	45	29.04	29.03	29.03

By using the equation of the calibration curve, the total sample mass of any unknown mAb-sample within the calibration curve can be calculated. If the area of the unknown sample is above the range of the calibration curve, the easiest way is to inject the same sample with a reduced injection volume.

CALCULATION OF SAMPLE TITER FROM CALIBRATION CURVE

Equation 1: y = 1,5175 x + 0,1417
Y = Mass of mAb-sample
X = area of elution peak

	µL	c (mg/mL)	µg
CCF 1	50	?	?
CCF 2	50	?	?

Area (1)	Area (2)	Area (Avg)
129.86	129.92	129.89
47.76	47.81	47.78

To get the mass of CCF we use Equation 1:

Mass of mAb-sample = 1.52*129.89 + 0.14
= 197.25 µg

To get to the titer of CCF, we divide the result by the injection volume: 197.25 µg / 50 µL = 3.94 µg/µL

Equivalently, we can determine the titer of CCF 2:

Titer = (1.52 * 47.78 + 0.14)/50
= 1.45 µg/µL
= 1.45 g/L

CONCLUSION

TSKgel Protein A-5PW expands the line of TSKgel columns for antibody analysis with a high performance affinity chromatography column specifically designed for fast and accurate determination of monoclonal antibody concentration. The wide range loading capacity of the TSKgel Protein A-5PW column enables the accurate determination of mAb titers at various stages of mAb development: from initial screening in R&D to process control in upstream development. Its reproducibility of injection-after-injection allows the users to accurately monitor the titer of mAb with high confidence. The low level of protein A leaching also makes this column a good candidate for small scale purification of mAbs for initial characterization.