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应用纪要

Evaluating the Ruggedness of the Alliance™ iS Bio HPLC System for SEC Separations

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Abstract

Biopharmaceutical companies are often progressing several monoclonal antibody (mAb) drug candidates to mitigate the risks associated with drug development. This strategic approach, aimed at increasing the likelihood of success, is accompanied by rugged technologies to improve efficiency and effectiveness of a labs operation to progress the most promising drug candidate forward. Specifically, in quality control (QC) environments, the implementation of robust methods is critical to ensure precision, accuracy, and reliability of results to make informed decisions throughout the manufacturing process. A key component of these robust methodologies is the selection of instrumentation and columns for the appropriate analytical method. For example, in size-exclusion chromatography (SEC), factors such as the type of stationary phase, column dimensions, instrument dispersion, and instrument ionic strength and pH tolerance should be considered. Recently, Waters has developed the Alliance iS Bio HPLC System for robust analysis of biopharmaceutical applications. This next-generation HPLC system, with biocompatible and exceedingly inert construction, contains MaxPeak™ High Performance Surfaces (HPS) Technology to reduce analysis variability. This application note will assess the robustness benefits associated with this surface technology for SEC separations.

In this study, the Alliance iS Bio HPLC System with an XBridge™ Premier Protein SEC Column (250 Å, 2.5 µm, 7.8 x 300 mm) was evaluated for method robustness and compared to a legacy stainless-steel system and column (250 Å, 5 µm, 7.8 mm x 300 mm). A widely adopted compendial SEC method outlined in the United States Pharmacopeia (USP) General Chapter <129> was adjusted by altering method parameters such as pH, injection volume, sample concentration, salt concentration, and salt type.[1] A comparative analysis was performed to evaluate the performance of each instrument configuration when method parameters were adjusted.

Benefits

- The Alliance iS Bio HPLC System coupled with an XBridge Premier Protein SEC Column showed improved tolerance to low ionic strength mobile phases when compared to a stainless-steel system and column

Introduction

Method robustness is defined as a methods capacity to remain unaffected by small variations in method parameters while providing reproducible results. This is typically evaluated in the method validation phase of the analytical development process to produce a reliable, efficient, and risk mitigating method. Throughout a validated methods lifecycle, variables such as different laboratories, analysts, instruments, and reagents can impact the accuracy of results. This is particularly important in the field of biotherapeutics, where the quality and safety of the drug product can be affected by the robustness of the method used during manufacturing. Therefore, it is crucial to establish a robust analytical method that can withstand these changes without compromising the integrity of results.

To provide QC labs with the tools to generate robust methods for biotherapeutics, Waters has recently launched the Alliance iS Bio HPLC System. This exceedingly inert system was constructed with MaxPeak HPS Technology to provide rugged and robust operation for biopharmaceutical applications. The system is complimented with MaxPeak Premier Columns and QuanRecovery™ Vials to reduce non-specific adsorption that is observed with biotherapeutics and stainless-steel metal surfaces. This universal platform for liquid chromatography, called MaxPeak Premier Solutions, is designed to reduce variability and would be a recommended system for QC labs concerned with reproducibility of HPLC

methods.

In this application note, the robustness of the MaxPeak Premier Solutions configuration was evaluated and compared directly to a stainless-steel HPLC system and column configuration. In a previous application note, the SEC method listed in USP General Chapter <129> was migrated and modernized to the Alliance iS Bio HPLC System.[2] Building on that work, this study will utilize the same compendial method to assess robustness on both systems by purposefully altering several method parameters listed in Figure 1. Evaluation metrics such as monomer, HMWS, and LMWS peak area %, peak tailing, half-height (HH) resolution, and peak width were compared between the systems to evaluate the advantages offered by MaxPeak HPS Technology.

Experimental

Potassium chloride (CAS 7447-40-7) was purchased from Sigma Aldrich.
Monobasic potassium phosphate (CAS 7778-77-0) was purchased from Acros Organics.
Dibasic potassium phosphate (CAS 7758-11-4) was purchased from J. T. Baker.
Ammonium acetate (CAS 631-61-8) was purchased from Sigma Aldrich.
Phosphate buffered saline, 10X was purchased from Gibco.
NISTmAb RM 8671 (humanized IgG1K monoclonal antibody) was purchased NIST SRM.

Stainless-Steel Configuration Benchmark Method Conditions

LC system:	Stainless-steel HPLC System
Detection:	λ = 280 nm
Column:	BioSuite™ Diol (OH) Column, 250 Å, 5 µm, 7.8 mm X 300 mm, (p/n: 186002165)
Vials:	LCGC Certified Clear Glass Screw Neck Total Recovery Vial, with Cap and Preslit

	PTFE/Silicone Septum (p/n: 186002630C)
Column temperature:	30 °C
Sample temperature:	8 °C
Injection volume:	20 µL
Flow rate:	0.5 mL/min
Mobile phase:	10.5 g dibasic potassium phosphate, 19.1 g monobasic potassium phosphate, and 18.6 g potassium chloride per liter of water, (0.20 M potassium phosphate and 0.25 M potassium chloride) pH 6.2
Run time:	30 minutes, isocratic
Chromatography software:	Empower™ 3, FR4

MaxPeak Premier Solutions Benchmark Method Conditions

LC system:	Alliance iS Bio HPLC System
Detection:	TUV, λ = 280 nm
Column:	XBridge Premier Protein SEC Column, 250 Å, 2.5 µm, 7.8 x 300 mm, (p/n: 186009962)
Vials:	QuanRecovery with MaxPeak HPS Vial and pre-slit PTFE silicone cap (p/n: 176004434)

Column temperature:	30 °C
Sample temperature:	8 °C
Injection volume:	20 µL
Flow rate:	0.5 mL/min
Mobile phase:	10.5 g dibasic potassium phosphate, 19.1 g monobasic potassium phosphate, and 18.6 g potassium chloride per liter of water, (0.20 M potassium phosphate and 0.25 M potassium chloride) pH 6.2
Run time:	30 minutes, isocratic
Chromatography software:	Empower 3.8.0

Results and Discussion

Injection Volume, Flow Rate, Sample Concentration, and pH

To evaluate the MaxPeak Premier Solutions and stainless-steel system configurations, the SEC method outlined in USP General Chapter <129> was used as the starting point. The stainless-steel HPLC system used a BioSuite Diol, 5-micron, 7.8 mm x 300 mm column, while the Alliance iS Bio HPLC System utilized an XBridge Premier Protein SEC, 2.5-micron, 7.8 mm x 300 mm Column. Each method parameter outlined in Figure 1 was evaluated individually using NISTmAb RM 8671, with a column temperature of 30 °C and wavelength of 280 nm. Initially, four method parameters were tested on both system configurations: injection volume (5, 10, and 20 µL), flow rate (0.4, 0.5, and 0.6 mL/min), NISTmAb concentration (10, 5, and 2.5 mg/mL), and mobile phase pH (5.6, 6.2, 6.8, 7.4, and 8.0). Figures 2 and 3 provide a comprehensive comparison of the performance of the two configurations under these varying

method conditions.

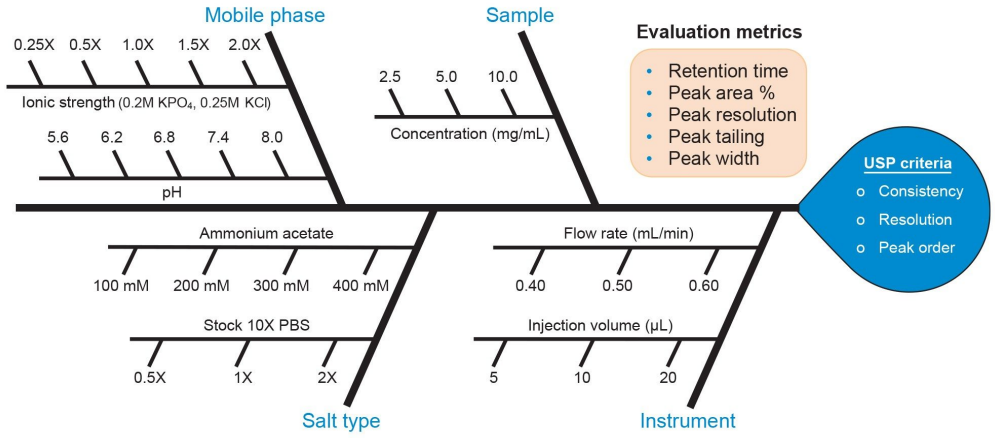


Figure 1. An Ishikawa diagram of the mobile phase, sample, salt type, and instrument parameters that were evaluated for ruggedness on the MaxPeak Premier Solutions and stainless-steel system and column configurations.

The method demonstrated robust performance on both system configurations when injection volume, flow rate, sample concentration, and mobile phase pH were adjusted from the conditions specified in the USP General Chapter <129>. As depicted in Figure 2, comparable peak area % results were obtained for the MaxPeak Premier Solutions (monomer: 96.58 ±0.14%, HMWS: 3.20 ±0.14%, and LMWS: 0.23 ±0.01%) when compared to the stainless-steel configuration for all four method parameters (monomer: 96.61 ±0.13%, HMWS: 3.19 ±0.13%, and LMWS: 0.21 ±0.02%). However, a significant improvement was observed with the MaxPeak Premier Solutions when evaluating the HH resolution between the monomer and adjacent HMWS peak, and the monomer peak width at 4.4% height as shown in Figure 3. The average HH resolution was 2.53 versus 1.88, and the average peak width at 4.4% was 0.79 versus 1.52 for the Alliance iS Bio HPLC System in comparison to the stainless-steel HPLC system for all four tested method parameters. These improvements can be attributed to both the reduction in particle size from the BioSuite 5-micron Column to the XBridge Premier Protein SEC 2.5-micron Column and the decreased system dispersion (20 µL, 4 σ) of the Alliance iS Bio HPLC System compared to the stainless-steel HPLC system (73 µL, 4 σ).

A notable limitation of the BioSuite Diol Column is its low maximum pressure of 1050 psi due to its

traditional silica particles. Although this column demonstrated robust performance between flow rates of 0.4–0.6 mL/min, the pressure at 0.6 mL/min reaches 780 psi, which is ~75% of the maximum pressure. In contrast, the XBridge Premier Protein SEC Column with its bridged ethylene hybrid particles and a maximum pressure of 6500 psi, is at ~30% of the maximum pressure at the same flow rate of 0.6 mL/min (1900 psi). As a result, the XBridge Premier Protein SEC Column is capable of operating at higher flow rates without approaching its maximum pressure limit, thereby allowing for greater flexibility in method development and optimization. While the capability to operate at higher flow rates is beneficial for applications requiring rapid analysis or high throughput, it should be noted that this can compromise the resolution of the separation.[3]

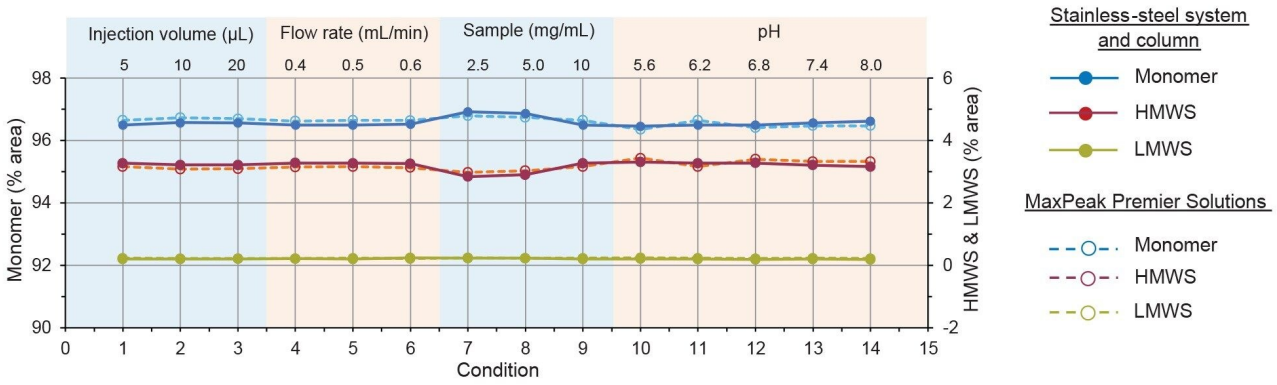


Figure 2. The peak % area for the monomer, HMWS, and LMWS are shown when injection volume, flow rate, sample concentration, and mobile phase pH method parameters are altered on both system configurations.

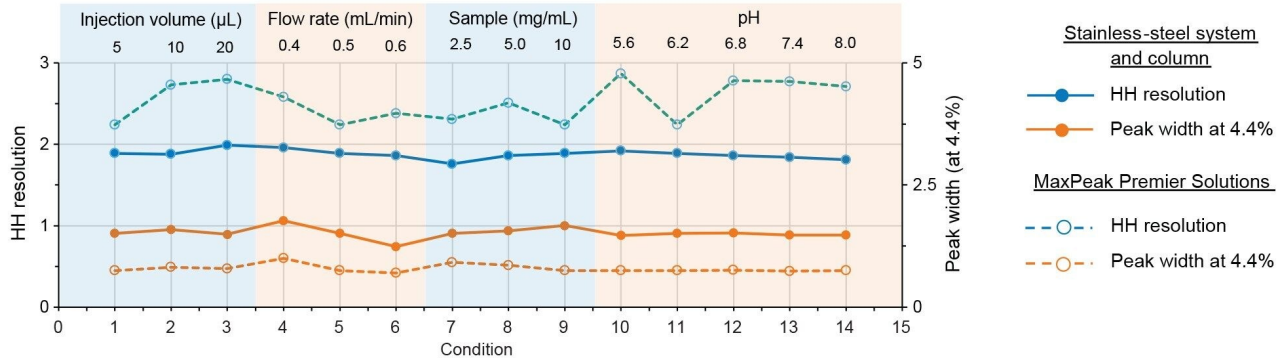
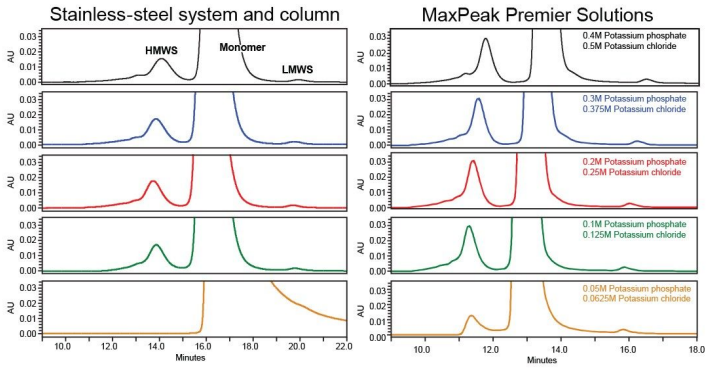


Figure 3. The half height (HH) resolution and monomer peak width at 4.4% peak height are shown when injection volume, flow rate, sample concentration, and mobile phase pH method parameters are altered on both system configurations.

Salt Concentration

Both system configurations demonstrated robust size variant separations for NISTmAb when parameters such as injection volume, flow rate, sample concentration, and mobile phase pH were adjusted. Salt concentration was chosen as the next parameter to assess method robustness on both systems. As outlined in USP General Chapter <129>, a 0.2 M potassium phosphate and 0.25 M potassium chloride buffer was used to evaluate the impact of salt concentration on robustness. This concentration is referred to as “1X” and several concentrations were evaluated that contained more or less salt, ranging from 0.25X–2X. The results of the salt concentration evaluation are shown in Figure 4. For the stainless-steel system and column, robust performance of the method was observed for 1X, 1.5X, and 2X salt concentrations. At a 0.5X salt concentration, the monomer and HMWS % area showed a 0.3% fluctuation while all evaluation metrics in the tabular data remained consistent. However, at a 0.25X salt concentration, the HMWS was not detected, and the monomer tailing increased significantly, resulting in the coelution of the LMWS with the monomer. The MaxPeak Premier Solutions configuration follows a similar trend to the stainless-steel system and column, albeit not to the same severity. Robust performance was observed for salt concentrations between 0.5X and 2X, however, at 0.25X salt concentration, the peak area % for the monomer, HMWS, and LMWS begin to fluctuate significantly. Although all size variants were still detected, unlike the stainless-steel system and column, these results suggest that the salt concentration in the mobile phase is a critical parameter for achieving robust and

reliable SEC separations.



Mobile phase ionic strength

System	Salt conc.	Main peak (monomer)					HMWS	LMWS
		RT	%Area	HH res.	Width at 4.4%	USP T	%Area	%Area
SS system and column	2X	16.30	96.69	1.64	1.59	1.45	3.11	0.20
	1.5X	16.07	96.65	1.77	1.55	1.49	3.14	0.20
	1X	15.96	96.65	1.89	1.51	1.49	3.16	0.20
	0.5X	16.01	96.95	1.88	1.55	1.54	2.84	0.20
	0.25X	16.33	100.00	N/A	3.02	3.24	N.D.	N.D.
MaxPeak Premier Solutions	2X	13.39	96.67	2.40	0.74	1.69	3.10	0.23
	1.5X	13.11	96.53	2.32	0.74	1.78	3.24	0.23
	1X	12.92	96.50	2.24	0.75	1.84	3.27	0.23
	0.5X	12.78	96.61	2.29	0.77	1.88	3.16	0.22
	0.25X	12.75	98.50	2.21	0.81	1.97	1.14	0.36

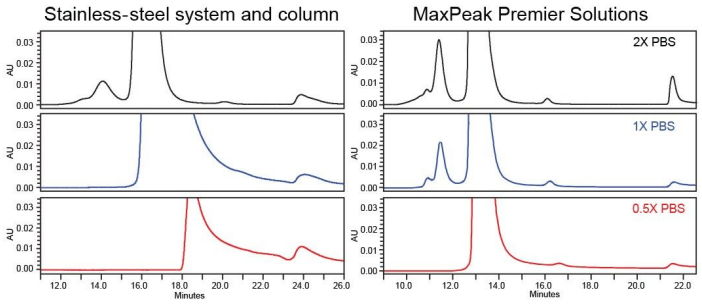
Figure 4. Chromatograms showing the impact of mobile phase ionic strength on both system configurations. Potassium phosphate and potassium chloride concentrations were altered and retention time, peak % area, half height resolution, peak width at 4.4%, and USP tailing were compared in the tabular data.

PBS and Ammonium Acetate

To further evaluate the importance of salt concentration on SEC separations, two other commonly used salts were examined on both system configurations, phosphate buffered saline (PBS) and ammonium acetate. PBS is widely used in SEC-UV separations due to its physiological pH and isotonicity. On the other hand, ammonium acetate is frequently used in SEC-MS analysis but has been utilized in SEC-UV analysis due to its bacteriostatic properties.[4] A 10X stock of PBS was diluted with water to create three concentrations to explore the effects of varying salt, 2X, 1X, and 0.5X at pH 7.4. For reference, 1X PBS is composed of 2.7 mM potassium chloride, 1.76 mM potassium phosphate monobasic, 137 mM sodium chloride, and 10.1 mM sodium phosphate dibasic. Similarly, ammonium acetate was prepared at a concentration of 400 mM and diluted with water to evaluate concentrations ranging from 100 to 400 mM at pH 7.0.

As depicted in Figure 5, even with a PBS concentration of 2X, the stainless-steel system and column configuration did not match the peak area % percentage observed with the potassium phosphate and potassium chloride mobile phase, as seen in Figure 4. When the PBS concentration was at 1X, the monomer exhibited similar peak tailing to the 0.25X salt concentration observed in Figure 4. More concerning, when the PBS concentration was halved (0.5X), the area counts of the monomer were

significantly reduced and recovery of NISTmAb was impacted. Conversely, at a 2X PBS concentration, the MaxPeak Premier Solutions configuration was able to closely match the results observed in Figure 4 with the potassium phosphate and potassium chloride mobile phase. Although there were peak area % changes observed at 1X and 0.5X PBS, the evaluation metrics for the monomer such as USP tailing, peak width, and area counts were not as severely impacted as they were for the stainless-steel system and column.



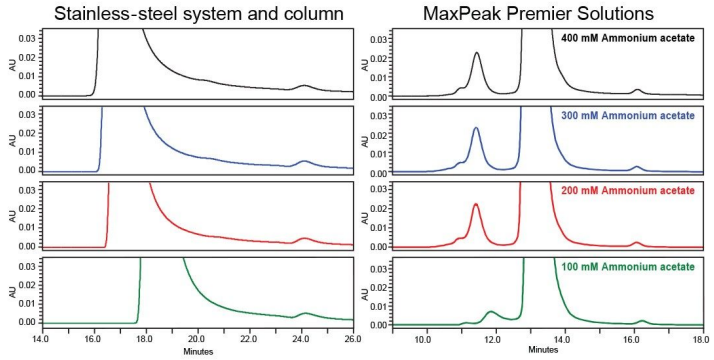
Stock 10X PBS

System	PBS conc.	Main peak (monomer)					HMWS	LMWS
		RT	%Area	HH res.	Width at 4.4%	USP T	%Area	%Area
SS system and column	2X	16.05	96.91	1.56	1.44	1.39	2.86	0.23
	1X	16.47	100.00	N/A	2.92	2.62	N.D.	N.D.
	0.5X	18.43	100.00	N/A	4.80	5.59	N.D.	N.D.
MaxPeak Premier Solutions	2X	12.89	96.47	2.73	0.74	1.78	3.29	0.24
	1X	12.92	97.76	2.77	0.74	1.80	2.00	0.24
	0.5X	13.07	99.38	N/A	0.79	1.82	N.D.	0.62

Figure 5. Chromatograms showing the impact of different PBS concentrations on both system configurations. PBS concentrations were evaluated between 0.5X–2X and retention time, peak % area, half height resolution, peak width at 4.4%, and USP tailing were compared in the tabular data.

Transitioning to Figure 6, the stainless-steel configuration and column were observed to be incompatible with ammonium acetate, likely caused by the lower ionic strength of ammonium acetate tested. Among the evaluated concentrations, neither the HMWS nor the LMWS were detected, and the monomer peak observed significant tailing. In stark contrast, the MaxPeak Premier Solutions configuration demonstrated robust performance for 200–400 mM ammonium acetate concentrations. The evaluation metrics were consistent across these salt concentrations and both the USP tailing and peak width at 4.4% were significantly reduced for the monomer when compared to the stainless-steel system and column. At a 100 mM ammonium acetate concentration, the HMWS was detected, but the recovery significantly decreased to 0.95%, compared to the ~2.3% at higher salt concentrations. This decrease in recovery was accompanied by a reduction in HH resolution but no significant changes were observed for USP tailing or peak width at 4.4%. Despite these results, the MaxPeak Premier Solutions configuration still outperformed the stainless-steel system and column, further highlighting the importance of selecting the appropriate system configuration and salt concentration for achieving

rugged SEC separations.



Ammonium acetate

System	AMA conc. (mM)	Main peak (monomer)					HMWS	LMWS
		RT	%Area	HH res.	Width at 4.4%	USP T	%Area	%Area
SS system and column	400	16.63	100.00	N/A	2.46	2.40	N.D.	N.D.
	300	16.73	100.00	N/A	2.38	2.52	N.D.	N.D.
	200	17.00	100.00	N/A	2.13	2.33	N.D.	N.D.
	100	18.23	100.00	N/A	2.28	2.33	N.D.	N.D.
MaxPeak Premier Solutions	400	12.98	97.48	2.96	0.71	1.69	2.16	0.36
	300	12.96	97.30	2.96	0.71	1.68	2.34	0.35
	200	12.94	97.32	2.98	0.71	1.67	2.33	0.35
	100	13.02	98.62	1.72	0.73	1.70	0.95	0.43

Figure 6. Chromatograms showing the impact of different ammonium acetate concentrations on both system configurations. Ammonium acetate concentrations were evaluated between 100–400 mM and retention time, peak % area, half height resolution, peak width at 4.4%, and USP tailing were compared in the tabular data.

Conclusion

While both system configurations exhibited robustness across various method parameters such as injection volume, flow rate, sample concentration, and mobile phase pH, the Alliance iS Bio HPLC System with an XBridge Premier Protein SEC Column outperformed the stainless-steel HPLC system and column by demonstrating a broader range of salt type and tolerance. In conclusion, the Alliance iS Bio HPLC System with an XBridge Premier Protein SEC Column proved to be a more rugged and reliable choice for SEC separations of mAbs. Its wider salt type and tolerance gives users more flexibility in method development and a greater degree of adaptability in the analysis of biotherapeutics, where new modalities are frequently introduced. For the future, as biopharmaceutical companies seek to improve efficiency in their QC operations, the Alliance iS Bio HPLC System is well-positioned to be at the forefront of biotherapeutic QC analysis.

References

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