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High Performance Liquid Chromatography

Application News

Purification of Fluorescent Protein-Labeled Antibodies by Gel Filtration Chromatography

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User Benefits

- Both fluorescently labeled and unlabeled antibodies can be fractionated simultaneously under real-time monitoring using PDA and fluorescence detectors.
- With metal-free LC, stable data can be acquired even when mobile phases contain high salt concentrations.
- Low and medium pressure columns for protein purification can be applied to the LC system, helping reduce back pressure on the column.

■ Introduction

Fluorescently labeled antibodies are widely used in assays such as flow cytometry and immunofluorescence staining. Fluorescent substances for antibody labeling can be categorized into low-molecular-weight fluorescent dyes (e.g., FITC and Alexa Fluor) and fluorescent proteins (e.g., phycoerythrin (PE) and allophycocyanin (APC)).

Fluorescent proteins exhibit fluorescence intensities several tens of times greater than those of fluorescent dyes, thereby providing sufficient sensitivity for detecting antigens even at low abundance.

Unreacted fluorescent substances remaining in the antibody solution during the labeling process can cause high background levels. Therefore, purification of fluorescently labeled antibodies is expected to improve the signal-to-noise (S/N) ratio.

This article presents purification of antibodies labeled with the fluorescent protein R-phycoerythrin (R-PE) by gel filtration chromatography (GFC).

■ Overview of the Fluorescence Analysis and Purification System

The flow diagram of the system is shown in Fig. 1. Nexera™ lite inert is designed with enhanced resistance to salts and acids compared with conventional systems that use stainless steel for fluid-contact components. The fluorescence detector (RF-20Axs), photodiode array (PDA) detector (SPD-M40), and fraction collector (FRC-10A) are contained in the system, and a signal from the PDA detector is employed as a trigger for fraction collection.

The flow path of the PDA detector cell is modified with a larger inner diameter, which helps reduce back pressure on the column. This configuration enables the use of low and medium pressure columns packed with agarose gel, in addition to high-pressure columns typically used for HPLC. Multiple columns can be utilized because the system is equipped with two flow-changeover valves for column switching.

Furthermore, the system is suitable for small-volume fractionation owing to the use of microtubes and 96-well microplates.

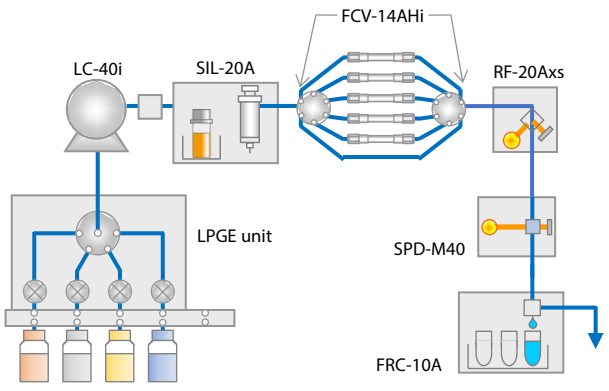


Fig. 1 Flow diagram

Labeling of Antibodies with R-PE

One hundred micrograms of mouse monoclonal IgG (mAb) was labeled by R-PE using a commercially available labeling kit (R-Phycoerythrin Labeling Kit-SH, DOJINDO Laboratories). The maleimide group in R-PE reacts with reduced disulfide bonds in the hinge region of IgG. If thiol groups are exposed on the IgG surface, one IgG molecule can be labeled by one to two or more R-PE molecules through the same mechanism. The resulting solution contains R-PE-labeled IgG, unlabeled IgG, and excess R-PE.

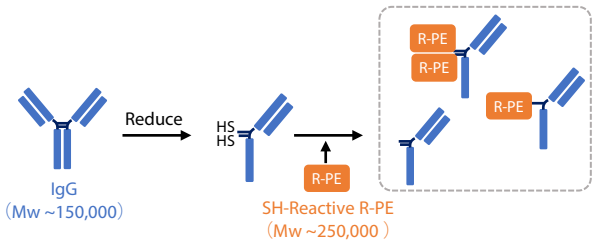


Fig. 2 Overview of R-PE Labeling of IgG

Purification of R-PE-Labeled IgG by Fluorescence Monitoring

Purification was carried out using a GFC column designed for protein purification (molecular weight range: 10,000–600,000) under the conditions listed in Table 1. The sample was prepared by ten-fold dilution of the R-PE-labeled IgG solution with the mobile phase.

A protein standard solution was analyzed under the same conditions to prepare a calibration curve for estimating the molecular weight of the target protein.

Table 1 Conditions

System	:	Nexera lite inert
Column	:	Gel filtration chromatography column (300 mm × 10 mm I.D., 8.6 µm)
Mobile phase	:	0.1 mol/L phosphate (sodium) buffer pH 6.8 containing 0.2 mol/L NaCl
Flow rate	:	0.5 mL/min
Column temp.	:	Ambient temperature
Injection volume	:	100 µL
Detection	:	[Fluorescence] Ex = 564 nm, Em = 575 nm*1 (RF-20Axs) [PDA] 280 nm (SPD-M40)
Flow cell	:	[Fluorescence] Flow cell for inert LC [PDA] Inert flow cell for low pressure resistance column
Vial	:	Shim-vial™ H glass, 0.15 mL*2

*1 Gain: ×4, Sensitivity: Low *2 P/N: 227-34500-13

■ GFC Results of Protein Standard Solution

Fig. 3 shows the chromatogram of the protein standard solution, and Fig. 4 shows the calibration curve (molecular weight range: 13,700–440,000). LabSolutions™ GPC option software was used to generate the calibration curve.

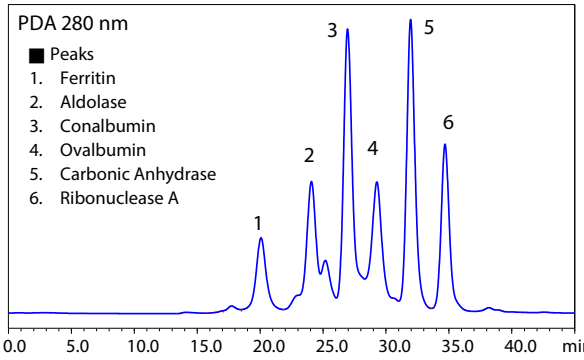
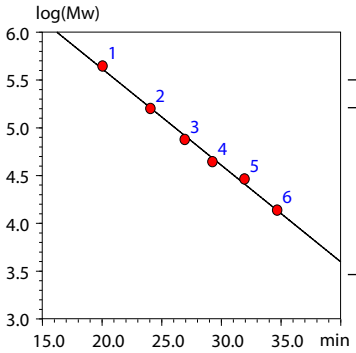


Fig. 3 Separation of the protein standard solution



#	Elution time	Mw
1	20.06	40,000
2	24.08	58,000
3	26.94	75,000
4	29.27	44,000
5	31.96	29,000
6	34.69	13,700

Fig. 4 Calibration curve

■ GFC Purification of R-PE-Labeled IgG

Fig. 5 shows the chromatogram of the sample solution. Peaks 1 and 2 were detected by both the fluorescence detector and the PDA, whereas Peak 3 was detected solely by the PDA.

Table 2 lists the elution times for each peak and the corresponding molecular weights calculated from the calibration curve.

Based on molecular weight information (approximately 150,000 for IgG and 240,000–290,000 for R-PE),

Peak 1 (highlighted in blue) is assigned to R-PE-labeled IgG,

Peak 2 corresponds to excess R-PE, and Peak 3 corresponds to unlabeled IgG.

Even at a maximum injected amount of approximately seven micrograms of IgG, the high-sensitivity fluorescence detector enabled sensitive detection and effective fractionation of trace-level IgG.

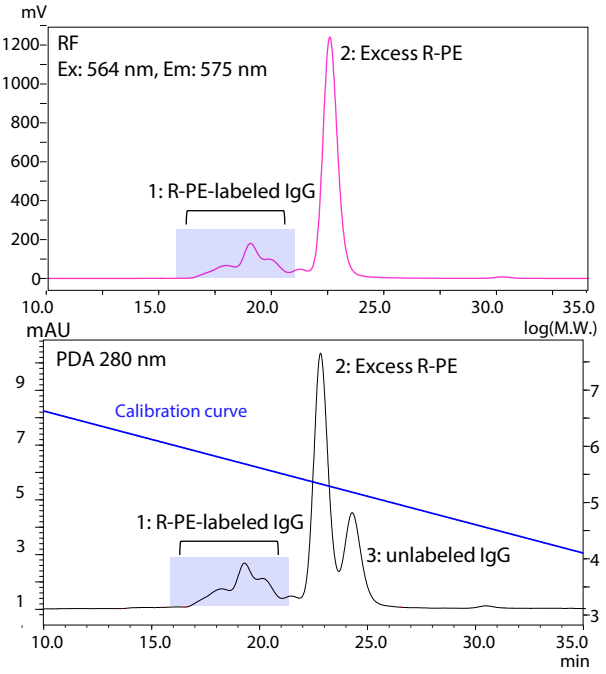


Fig. 5 Separation of the sample solution containing R-PE-labeled IgG

Table 2 Molecular Weights of Each Elution Peak

Peak #	Mw	
1	270,000-700,000	R-PE-labeled IgG
2	200,000	Excess R-PE
3	140,000	unlabeled IgG

■ Conclusion

This article presented purification of fluorescently labeled IgG using the corrosion-resistant LC system Nexera lite inert together with PDA and fluorescence detectors.

Use of a system that reduces back pressure on the column allows low and medium pressure agarose gel columns to be employed for purification of antibody-containing proteins.

The combination of PDA and fluorescence detectors enables simultaneous monitoring and reliable fractionation of both fluorescently labeled and unlabeled IgG.

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