Detergent Analysis in Protein Samples Using Mid-Infrared (MIR) Spectroscopy

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Quantitating relative levels of detergent present in protein preparations or samples derived from biological material, such as tissue or body fluids, is important because the presence of detergent may affect downstream analyses as well as protein structure/function. Especially because sample volumes, analysts' available time, and other resources may be limited, a method that consumes little sample and that is rapid and simple is needed for detergent analysis. It would also be preferable to have a method that is generally applicable across many aliphatic chain-containing molecules with many different physical properties. In this unit, methods are described for analyzing detergents and proteins in detergent-protein mixtures using mid-infrared (MIR) spectroscopy. A protocol is also included for efficient removal of unbound detergents from a protein sample accompanied by MIR-based monitoring of both detergent and protein content. This rapid monitoring of sample preparation during the workflow enables users to make timely decisions about sample preparation strategies that maximize both analyte purity and yield.

Keywords: mid-infrared (MIR) spectroscopy • infrared • detergent analysis • protein samples • detergent removal

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INTRODUCTION

This unit contains three protocols for MIR-based analysis of detergents and detergent-containing samples. Because detergents are ubiquitous in samples of biological interest, especially in protein preparations for structure/function studies, and because there are few practical methods that are capable of analyzing all detergents irrespective of chemical structure, these protocols are expected to be of interest to a wide audience, including protein biochemists, structural biologists, and researchers involved in biomarker discovery and development.

In Basic Protocol 1, we describe how to perform absolute quantitation of a specific detergent of interest by first calibrating the instrument with a detergent standard. Basic Protocol 2 describes how to use amide I absorption, separated by over a thousand wavenumbers from the aliphatic vibration signal used for detergent analysis, for protein quantitation in complex samples, many of which contain detergents. Basic Protocol 3 describes a novel, size-based method for removing detergent while maximizing protein recovery, facilitated by rapid monitoring of both detergent removal and protein recovery.

BASIC PROTOCOL 1

GENERAL MIR SPECTROSCOPY-BASED ANALYSIS OF DETERGENT-CONTAINING SAMPLES

This protocol uses a benchtop MIR spectrometer that enables non-specialists to analyze biological, aqueous samples that are spotted onto membrane cards (Strug et al., 2014). Quantitation using this system requires single-time instrument calibration with the detergent(s) of interest, permitting standardization across sample types and across processing runs. The presented protocol can be used in parallel with the majority of known detergent removal techniques.

The analysis described below can be performed using any Fourier transform infrared (FTIR) spectrometer capable of collecting spectra between 400 and 4000 cm^{-1}. However, using a traditional spectrometer (such as the ALPHA FTIR spectrometer from Bruker, the Nicolet FTIR spectrometer from Thermo Fisher Scientific, or the Spectrum Two infrared [IR] spectrometer from Perkin Elmer) requires a certain level of expertise for proper manipulation of spectra and data analysis. Literature on MIR-based quantitation using regular, non-specialized FTIR instrumentation recommends applying a multivariate approach, e.g., partial least-squares analysis (PLS; Janatsch et al., 1989; Dreissig et al., 2009; Sellick et al., 2010; Strug et al., 2014).

Materials

PBS
Milli-Q or other Type I Ultrapure water
Detergents and lysis buffers:
- 2% sodium deoxycholate in PBS (store up to 24 hr at room temperature)
- 4% sodium dodecyl sulfate (SDS) in PBS (store up to 6 months at room temperature)
- 2% NP-40 in PBS (store up to 1 year at 4°C)
- 2% Triton X-100 in PBS (store up to 1 year at 4°C)
- 2× radioimmunoprecipitation assay (RIPA) lysis buffer (see recipe; store up to 6 months at 4°C)

Assay-free sample cards (EMD Millipore, cat. no. DDAC00010-8P)
Direct Detect MIR-based spectrometer with most recent software version (EMD Millipore, cat. no. DDHW00010-00)
P2, P20 and P200 micropipettors (e.g., Rainin)

Generate calibration method

1. In eight 0.5-ml microcentrifuge tubes, prepare a dilution series of 2% sodium deoxycholate in PBS as described in Table 29.12.1. Mix by inversion or vortex gently and avoid foaming.

   The procedure outlined here describes calibration using sodium deoxycholate; however, the same procedure should be applied to preparing calibration methods for 4% SDS, 2% NP-40, 2% Triton X-100, 2× RIPA buffer and any other detergent/lysis buffer. Details regarding preparation of dilution series for SDS, NP-40, Triton X-110 and RIPA buffer are included in Table 29.12.1.

2. Using a calibrated P2 pipettor, carefully spot exactly 2 µl PBS onto position 1 of the assay-free sample card.

3. Using a calibrated P2 pipettor, carefully spot exactly 2 µl of each dilution series solution onto positions 2 through 4 of assay-free sample cards. Prepare one card per dilution point; prepare a total of eight cards, each containing a buffer blank and triplicate spots of calibration solutions.

Table 29.12.1 Preparation of Dilution Series for Various Detergents Required for Generating Calibration Methods

Final concentration (%)	Volume of stock (µl)	Concentration (%) of stock	Volume of PBS (µl)
Sodium deoxycholate			
0.031	6.25	0.5	93.75
0.063	12.5	0.5	87.5
0.125	6.25	2	93.75
0.250	12.5	2	87.5
0.500	25	2	75
0.750	37.5	2	62.5
1.000	50	2	50
2.000	100	2	0
NP-40			
0.031	6.25	0.5	93.75
0.063	12.5	0.5	87.5
0.125	6.25	2	93.75
0.250	12.5	2	87.5
0.500	25	2	75
0.750	37.5	2	62.5
1.000	50	2	50
2.000	100	2	0
Triton X-110			
0.031	6.25	0.5	93.75
0.063	12.5	0.5	87.5
0.125	6.25	2	93.75
0.250	12.5	2	87.5
0.500	25	2	75
0.750	37.5	2	62.5
1.000	50	2	50
2.000	100	2	0
SDS			
0.031	6.25	0.5	93.75
0.063	12.5	0.5	87.5
0.125	25	0.5	75
0.250	6.25	4	93.75
0.500	12.5	4	87.5
0.750	18.75	4	81.25
1.000	25	4	75
2.000	50	4	50
3.0000	75	4	25
4.0000	100	4	0

continued

Table 29.12.1 Preparation of Dilution Series for Various Detergents Required for Generating Calibration Methods, *continued*

Final concentration (×)	Volume of 2× RIPA (µl)	Concentration of stock (×)	Volume of PBS (µl)
RIPA			
0.016	3.125	0.5	96.875
0.031	6.25	0.5	93.75
0.063	12.5	0.5	87.5
0.125	6.25	2	93.75
0.250	12.5	2	87.5
0.500	25	2	75
0.750	37.5	2	62.5
1.000	50	2	50
2.000	100	2	0

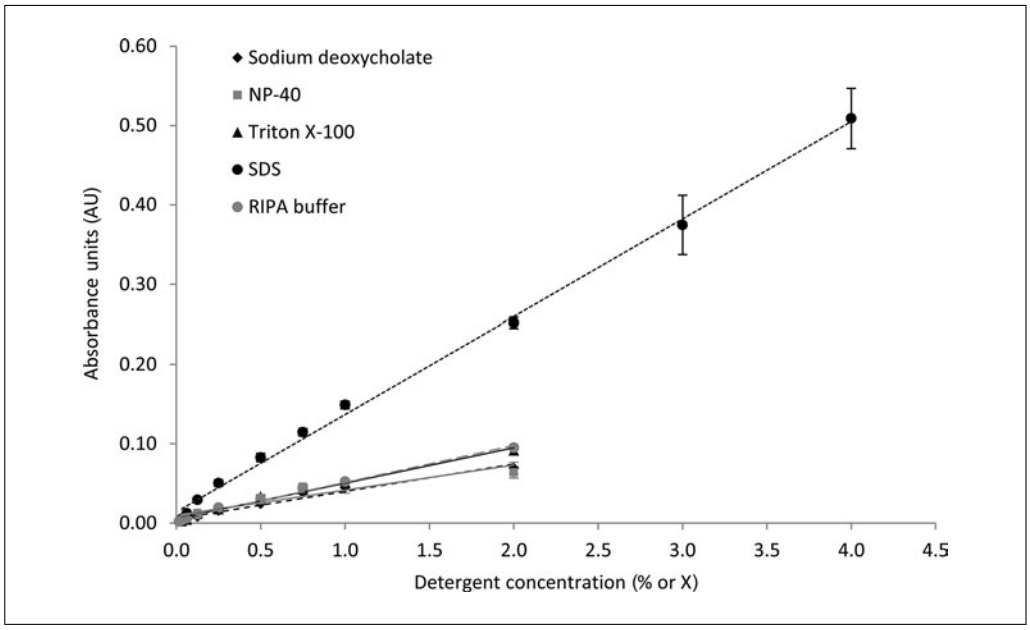

Figure 29.12.1 Calibration curves for structurally diverse detergents prepared using lipid standards and the Calibration Method in the instrument software. NP-40 (which is also part of RIPA buffer) and Triton X-100 have relatively low CMC, while SDS and sodium deoxycholate have a higher CMC. Because of the structural diversity of detergents, the calibration curve slopes vary greatly; therefore, it is important that the instrument be calibrated using the detergent of interest before attempting to determine the amount of that detergent in a sample of unknown composition.

4. Collect spectral data for all calibration cards, operating the spectrometer under "acquire lipid calibration spectra" mode, as described in the software user guide.

5. Use "Lipid calibration wizard" (embedded in the instrument software) to establish the linear quantitation range and to create a calibration method that will be used to quantify detergent concentration in further experiments.

   Eight dilution points usually result in a robust data set that permits establishment of a reliable linear quantitation range (Fig. 29.12.1). If needed, add more points following the trend shown in Table 29.12.1.

Attention should be paid to the precision of the measurement as determined by the percent coefficient of variation (%CV) among replicates. Significant variations in absorbance intensity among replicates usually indicate inaccurate pipetting that needs to be corrected before a reliable method can be developed.

Perform measurement and analyze data

6. Test accuracy of the newly prepared calibration method as follows: prepare fresh samples of sodium deoxycholate in PBS over a range of concentrations that cover at least one point at each of the edges of the dynamic concentration range (e.g., 0.05% and 1.8%; see Fig. 29.12.1) and at least one point in the middle of the range (e.g., 0.8%). Quantify all test samples using this new calibration method.

   Test concentrations of SDS, NP-40, Triton X-110 and RIPA buffer should follow the same trend.

   MIR-based detergent analysis uses the vibrational signal from aliphatic stretching. Because aliphatic content varies between specific detergents, preparation of a separate calibration method for each detergent (or detergent mixture) is required (Fig. 29.12.1). However, once prepared, the method can be stored, modified or improved with time and used for all future quantitation measurements.

   Step 6 will test the quality of the freshly prepared samples for the calibration method. Once validated in this way, the calibration method is ready to use for quantitation of sodium deoxycholate concentration in complex samples.

7. Using a calibrated P2 pipettor, carefully spot exactly 2 µl buffer solution (PBS; no detergent) onto position 1 of assay-free sample card.

8. Using a calibrated P2 pipettor, carefully spot three replicates of exactly 2 µl sample to be analyzed onto positions 2 through 4 of assay-free sample cards.

9. Quantify sodium deoxycholate concentration in the sample using the calibration method prepared in steps 1 through 5 and validated in step 6.

   Quantitation of SDS, NP-40, Triton X-110 and RIPA buffer concentrations in samples should be performed using the calibration method developed for each of the detergents/lysis buffer(s). Respective diluents or buffers (PBS or Milli-Q water in the described protocol) should be used as blanks.

MIR SPECTROSCOPY-BASED ANALYSIS OF PROTEIN CONTENT IN A COMPLEX MIXTURE CONTAINING BOTH PROTEIN AND DETERGENT

BASIC PROTOCOL 2

By virtue of its ability to detect the spectral absorbance bands for many structural entities, the MIR-based method is not limited to the analysis of detergent and lipid species. Several amide bands have been identified in MIR spectroscopy allowing for characterization and quantification of proteins. Among these, amide I (1600 to 1690 cm^{-1}) and amide II (1480 to 1575 cm^{-1}) are recognized as the most representative of all vibration modes (Miyazawa and Blout, 1960; Kong and Yu, 2007; Fig. 29.12.2). In Basic Protocol 2, amide I absorption, separated by over a thousand wavenumbers from the aliphatic vibration signal used for detergent analysis, is used for protein quantitation.

Quantitation of both species is performed using a single, fully automated measurement. Although the full MIR spectra of the analyzed sample and the appropriate buffer blank are collected, the buffer subtraction and quantitation are restricted to the regions of the spectra characteristic to detergents (3100 to 2600 cm^{-1}) and proteins (1850 to 1350 cm^{-1}; Fig. 29.12.3).

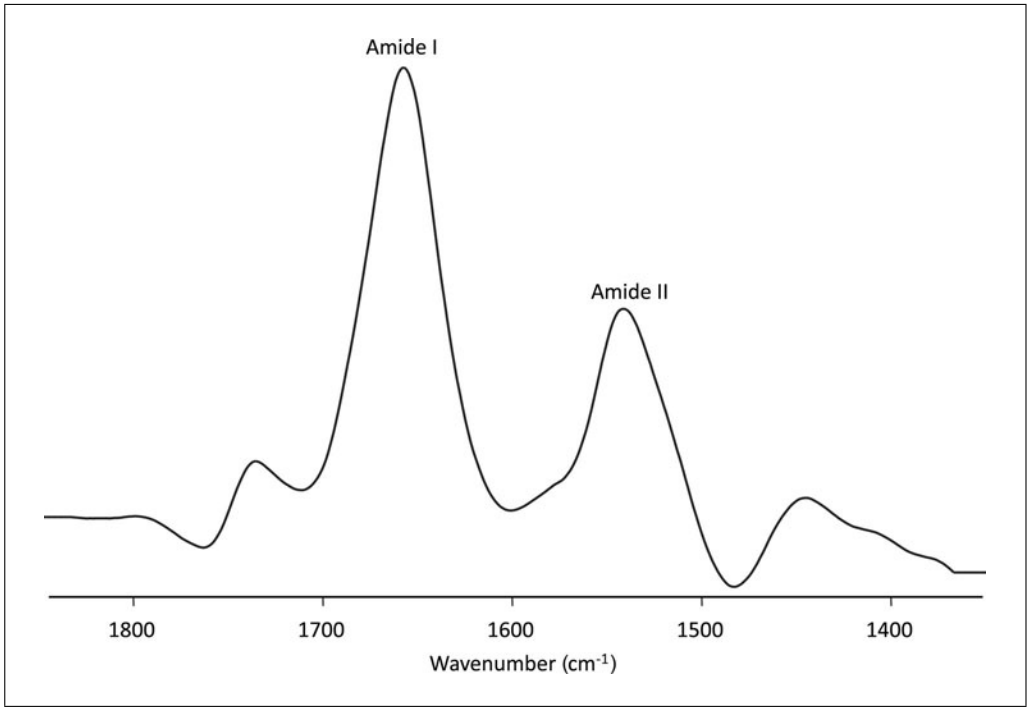

Figure 29.12.2 Example of the amide I and amide II regions of an IR spectrum of a protein sample, where the approximate ratio of the peak heights signifies that the spectrum may be used to generate reliable protein quantitation, and that sample component IR signals are not interfering with the analysis region of the spectrum.

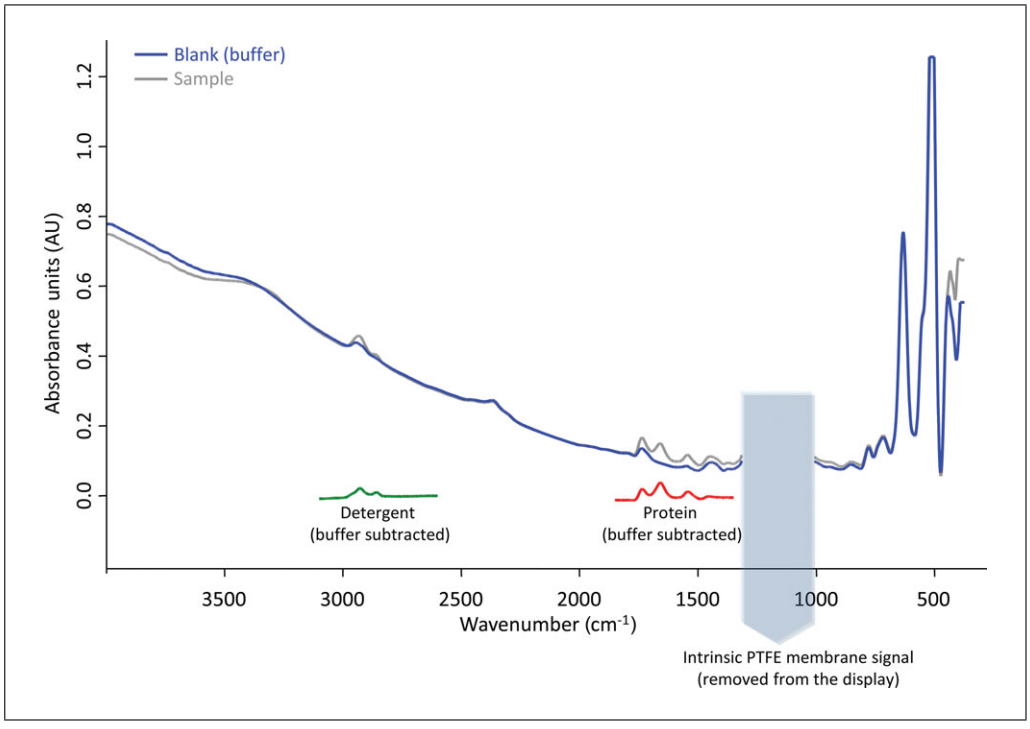

Figure 29.12.3 Analysis of protein and lipid content from a single sample measurement. Because the sample composition and ratio of individual components were unknown, phospholipid analysis was performed using the "Relative Absorbance" mode. For clarity, the intrinsic infrared signal from the membrane (1300-1100 cm^{-1}) was removed from the displayed spectrum.

Materials

PBS
Milli-Q or other Type I Ultrapure water
Detergents and lysis buffer:
- 2% sodium deoxycholate in PBS (store up to 24 hr at room temperature)
- 2% NP-40 in PBS (store up to 1 year at 4°C)
- 2× radioimmunoprecipitation assay (RIPA) lysis buffer (see recipe; store up to 6 months at 4°C)

Bovine serum albumin (BSA) SRM927d (National Institute of Standards and Technology [NIST] certified; prepare solutions fresh and use immediately):
- 10 mg/ml stock solution in PBS containing 0.5% sodium deoxycholate
- 10 mg/ml stock solution in PBS containing 1% NP-40
- 10 mg/ml stock solution in 1× RIPA buffer

Cytochrome C (EMD Millipore, cat. no. 250600; store solutions 24 hr at 4°C):
- 2 mg/ml in PBS containing 0.5% sodium deoxycholate
- 2 mg/ml in PBS containing 1% NP-40

ϒ-globulins from rabbit (Sigma-Aldrich, cat. no. G2018; store solutions 24 hr at 4°C):
- 2 mg/ml in PBS containing 0.5% sodium deoxycholate
- 2 mg/ml in PBS containing 1% NP-40

Rat liver lysate (homogenize frozen rat liver in 1× RIPA buffer containing phosphatase and protease inhibitor cocktails; spin down several times by centrifuge; collect clear lysate solution and store 24 hr at 4°C)

Assay-free sample cards (EMD Millipore, cat. no. DDAC00010-8P)
Direct Detect spectrometer (EMD Millipore, cat. no. DDHW00010-00)
P2, P20 and P200 micropipettors (e.g., Rainin)

Calibrate instrument

1. Prepare a calibration method using a detergent/buffer solution or a lysis buffer that most closely mimics the composition of the buffer that contains the sample (see Basic Protocol 1, steps 1 through 5 for method generation and step 6 for method validation) or use a previously generated method that is stored in the instrument's software.

2. Prepare protein calibration method:

   a. In ten 0.5-ml microcentrifuge tubes, prepare a dilution series of BSA in PBS containing 0.5% sodium deoxycholate. Mix by inversion or vortex gently. Avoid foaming.

      Details regarding preparation of BSA dilution series are included in Table 29.12.2. The dilution trend described in Table 29.12.2 should be followed while preparing the calibration series for BSA in 1% NP-40 and BSA in 1× RIPA buffer.

   b. Using a calibrated P2 pipettor, carefully spot exactly 2 µl 0.5% sodium deoxycholate in PBS (blank) onto position 1 of the assay-free sample card.
   c. Using a calibrated P2 pipettor carefully spot exactly 2 µl each BSA dilution series solution (prepared in step 2a) onto positions 2 through 4 of assay-free sample cards. Prepare one card per dilution point; prepare ten cards total containing buffer blanks and triplicate spots of each protein calibration solution.
   d. Collect spectral data for all calibration cards operating the spectrometer under "acquire protein calibration spectra" mode, as described in the software user guide; click on the 'Help' icon in the software to access the user guide.

Table 29.12.2 Preparation of Dilution Series for Protein Standard in Detergent-Containing Buffer[a]

BSA (mg/ml)	Volume of 10 mg/ml BSA stock in 0.5% sodium deoxycholate (μl)	Volume of 0.5% sodium deoxycholate in PBS (μl)
0.125	2.5	197.5
0.250	5	195.0
0.500	10	190.0
0.750	15	185.0
1.000	20	180.0
1.500	30	170.0
2.000	40	160.0
3.000	60	140.0
4.000	80	120.0
5.000	100	100.0

[a]Recommended for obtaining highest accuracy in protein quantitation.

e. Use "Protein calibration wizard" (embedded in the software) to create a calibration method that will be used to quantify protein concentration in complex samples containing sodium deoxycholate.

Ten dilution points usually result in a robust data set that permits establishment of a reliable linear quantitation range. If needed, add more points following the trend shown in Table 29.12.2.

Attention should be paid to amide I and amide II spectra; the shape of both signals and intensity ratio should be similar to the spectra seen in Figure 29.12.2.

Attention should be paid to the precision of the measurement as determined by the percent coefficient of variation (%CV) among replicates. Significant variations in absorbance intensity among replicates usually indicate inaccurate pipetting that needs to be corrected before a reliable method can be developed.

Perform measurement and analyze data

3. Test quality of the newly prepared calibration method as follows: prepare fresh samples of BSA in PBS containing 0.5% sodium deoxycholate over a range of concentrations that cover at least one point at each of the edges of the dynamic concentration range (e.g., 0.3 mg/ml and 4.5 mg/ml) and at least one point in the middle of the range (e.g., 1.8 mg/ml). Quantify the test samples using a freshly prepared calibration method.

MIR-based protein quantitation is based on amide I signal strength; hence BSA can be used to prepare a reliable and universal protein calibration. However, because the rate and degree to which samples dry on the membrane can depend on the buffer composition of the sample, a separate calibration method for each buffer is recommended for enhanced quantitation accuracy. Once generated, the method can be stored, modified or improved with time and used for all future measurements.

Step 3 will test the quality of the freshly prepared protein calibration method. Once validated in this way, the protein calibration method is ready to use for quantitation of complex and biologically derived samples.

4. Using a calibrated P2 pipettor, carefully spot exactly 2 μl PBS onto position 1 of assay-free sample card.

5. Using a calibrated P2 pipettor, carefully spot exactly 2 μl cytochrome C solution prepared in PBS containing 0.5% sodium deoxycholate onto positions 2 through 4 of assay-free sample cards.

6. Quantify protein and sodium deoxycholate concentrations using the calibration methods described in steps 1 and 2.

Rabbit ϒ-globulin solubilized in PBS containing 0.5% sodium deoxycholate should be analyzed following steps 4 through 6.

For high accuracy, protein quantitation from samples in buffers containing NP-40 and from RIPA (e.g., rat liver lysate described in Materials section above) should be performed using calibration methods developed (following step 2 and 3) for the corresponding buffers.

MIR SPECTROSCOPY-BASED MONITORING OF DETERGENT REMOVAL AND PROTEIN RECOVERY

BASIC PROTOCOL 3

During protein purification, the initial solubilization of a protein is usually achieved with an excess of detergent, resulting in a complex mixture containing two forms of detergent: free (unbound) detergent and protein-bound detergent. Removal of free detergent is relatively straightforward and sufficient for most downstream analyses; however, the disruption of protein-detergent interactions might result in protein precipitation. Therefore, it is desirable to measure both detergent and protein content at each step of a purification method.

This detergent removal protocol discusses a fast method for monitoring detergent removal efficiency while simultaneously monitoring protein recovery. This technique of detergent removal by continuous diafiltration, in which a constant sample volume is continually washed with the detergent-free buffer, may provide more efficient detergent removal and better protein recovery than the standard ultrafiltration removal techniques, which involve multiple dilution/concentration cycles, or dialysis.

This protocol includes instructions for removing unbound detergent while performing rapid MIR-based quantitation to monitor both detergent removal and protein recovery.

Materials

Sample to be analyzed (e.g., recombinant protein preparation solubilized in a sodium deoxycholate-containing buffer; store up to 24 hr at 4°C)
2% sodium deoxycholate in PBS (store up to 24 hr at room temperature)
PBS (store up to 1 year at room temperature)
Milli-Q or other Type I Ultrapure water
ϒ-globulins from rabbit (Sigma-Aldrich, cat. no. G2018; 2 mg/ml in PBS containing 0.5% sodium deoxycholate; store up to 24 hr at 4°C)
Bovine serum albumin (BSA) SRM927d (National Institute of Standards and Technology [NIST] certified; 10 mg/ml stock solution in PBS containing 0.5% sodium deoxycholate; prepare solutions fresh and use immediately)

Amicon Pro Purification System (EMD Millipore)
Assay-free sample cards (EMD Millipore, cat. no. DDAC00010-8P)
Direct Detect spectrometer (EMD Millipore, cat. no. DDHW00010-00)
P2, P20 and P200 micropipettors (e.g., Rainin)
Centrifuge with swinging bucket rotor (e.g., Beckman)
Microcentrifuge (e.g., Eppendorf)

Calibrate instrument

1. To prepare the spectrometer for detergent content monitoring, generate a lipid calibration method using the detergent present in the sample (sodium deoxycholate in this example), using the calibration method described in Basic Protocol 1.

2. To prepare the spectrometer for monitoring protein content, generate a protein calibration method using dilutions of BSA in the same buffer and detergent that are present in the sample to be analyzed, for example, BSA solubilized in PBS with 0.5% sodium deoxycholate.

   Details of the protein calibration method are given in Basic Protocol 2.

Remove unbound detergent along with rapid monitoring of detergent removal and protein recovery

3. Clarify sample to be analyzed using either centrifugation (10,000 × *g*, 20 min at 4°C) or filtration to avoid clogging the detergent removal device.

   For example, samples between 10 and 50 ml can be clarified using a Steriflip 0.45-μm filter (EMD Millipore, cat. no. SE1M003M00), and samples <10 ml can be clarified using Millex 0.45-μm syringe filters (EMD Millipore, cat. no. SLHA033SB).

4. Use the Direct Detect spectrometer to estimate concentrations of detergent and protein in the starting material, using Basic Protocol 1 and Basic Protocol 2, respectively.

5. Add 50 μl pre-clarified sample to the Amicon Ultra 0.5-ml filter (included in the Amicon Pro system) and carefully attach to the Amicon Pro exchange device.

   Up to 75 to 80 μl of sample can be placed in the Amicon Ultra 0.5-ml filter and safely assembled with the Amicon Pro exchange device.

   Placing the sample in the Amicon Ultra 0.5-ml filter rather than in the Amicon Pro exchange device provides a stabilizing environment for the sample, given that the constant, gradual feed of fresh buffer prevents the sample from being rapidly diluted and concentrated from large volumes of potentially destabilizing, detergent-depleted buffer.

6. Add 1 ml PBS to the exchange device/Amicon Ultra 0.5-ml filter assembly. Fully assemble Amicon Pro device.

   The Amicon Pro device can safely accommodate up to 9 ml of buffer but will enable a 1000-fold buffer exchange, in the case of a 50-μl sample with only 1.5 ml fresh buffer, because of the way it meters in fresh solution during the centrifugation process.

7. Centrifuge at 4000 × *g*, 30 min in a swinging-bucket rotor.

8. Remove device from the centrifuge and disassemble Amicon Pro device.

9. Remove Amicon Ultra 0.5-ml filter and place an Amicon Ultra 0.5-ml collection tube over the top, and invert.

10. Spin in a microcentrifuge with fixed-angle rotor at 1000 × *g* for 2 min to recover sample.

11. Use Direct Detect spectrometer as described in Basic Protocol 1 and Basic Protocol 2 to estimate concentration of recovered protein and to confirm detergent displacement.

REAGENTS AND SOLUTIONS

Use Milli-Q-purified water or equivalent in all recipes and protocol steps. For common stock solutions, see APPENDIX 2E.

Radioimmunoprecipitation assay (RIPA) buffer, 2×

100 mM Tris·Cl, pH 7.4
300 mM NaCl
0.5% deoxycholic acid
2% NP-40
2 mM EDTA
Store up to 6 months at 4°C

COMMENTARY

Background Information

Detergents are widely used for purifying proteins, especially proteins with limited intrinsic solubility in aqueous solutions, such as therapeutically important amyloid-β plaques (Bucciantini et al., 2002), proteins with large hydrophobic areas on their surface (such as transcriptional activators) and membrane proteins. It has become clear that the proteins with high solubility represent the minority, not the majority. In fact, when 2078 randomly chosen genes from *Caenorhabditis elegans* were recombinantly expressed in *Escherichia coli*, only 11% were soluble without detergent (Finley et al., 2004). For the rest, isolation and purification strategies frequently involve the addition of detergents.

Excess free detergent molecules can interfere with many analyses and studies of protein structure-function relationships, requiring the removal of unbound detergent. Removing free detergent from protein preparations also prevents unwanted detergent micelles from forming during ultrafiltration-based concentration. Detergent removal methods include hydrophobic adsorption, size-exclusion chromatography, affinity chromatography, dialysis and centrifugal ultrafiltration (EMD Millipore Corporation, 2012). However, detergent and lipid molecules that are bound to the protein of interest can seldom be completely removed. Further, complete removal of detergent may adversely affect protein structure or function, for example, by causing protein precipitation or aggregation (Garavito and Ferguson-Miller, 2001).

Protein-detergent interaction, even when not detected using traditional methods, may greatly affect protein function (Tan and Ting, 2000; Garavito and Ferguson-Miller, 2001). For example, addition of the detergent, dodecylmaltoside, prevented the binding of a second calcium ion to a sarcoplasmic reticulum-associated ATPase, altering its enzymatic activity but had little effect on the protein's fluorescence spectrum (De Foresta et al., 1994). Given that this effect was not seen with other detergents, it is clear that in the course of developing a protein purification protocol, it is important to consider multiple detergents, their particular characteristics and their effects on both protein yield and protein activity. In cases where a specific amount of lipid or detergent must be present in order to allow a protein to assume its proper structure, activity or ability to form crystals, it is critical to accurately and precisely monitor the lipid/protein or detergent/protein ratio in samples (Privé, 2007).

Traditional methods used to analyze specific detergents within protein samples, in addition to involving multistep preparative procedures, have significant drawbacks: (1) radioisotopically labeled detergents have been used to quantitate detergent-protein binding, but this method is now rarely used because of the complexity of radiometric assays and associated waste disposal; (2) colorimetric assays, such as the phenol-sulfuric-acid-based detection of glucosides, maltosides and bile salt-based detergents (such as 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate [CHAPS]), or the methylene blue assay for SDS, are sensitive and accurate, but not universal and therefore impractical for analyzing multiple detergents simultaneously; (3) ashing procedures or enzyme-based assays can be used to quantify phospholipid-based detergents; these are also complex and, like colorimetric assays, are not universal for all detergents (Privé, 2007).

With the rising need for characterization of detergent-bound membrane proteins, more general methods have been developed for quantifying detergents. Absorbance at 275 and 280 nm can be used to estimate the concentration of Triton X agent (100 and 114) and NP-408; however, the limitation of this method is that it requires subtraction of protein

contribution to the signal, and this contribution is impossible to pre-estimate in complex biological samples (Antharavally et al., 2011). High-performance thin-layer chromatography has been used to analyze a mixture containing up to ten different detergents; however, the method requires that these detergents exhibit differential mobility in the liquid phase, and therefore it is not completely universal (Barret et al., 2013). Other general methods for detergent quantitation include the use of mass spectrometry (Antharavally et al., 2011) and refractive index (Strop and Brunger, 2005). Total organic carbon (TOC) analysis has also been used to monitor detergent content; however, TOC samples need to be free of protein in order to accurately estimate relative changes in detergent content (EMD Millipore Corporation, 2014). All of these require extremely complex analysis, costly instrumentation or both, and none of the methods described are fast and simple enough to perform on location (simultaneously with sample preparation), and are therefore impractical for guiding experimental design in real time.

Recently, FTIR spectroscopy has been used to monitor detergent concentration in protein samples (DaCosta and Baenziger, 2003; Strug et al., 2013). Because of their complex chemical structures, detergents absorb in many different regions of the MIR spectrum. However, comparing the spectra of structurally diverse detergents (digitonin, CHAPS, sodium deoxycholate, octylglucoside, SDS and cetyltrimethylammonium bromide [CTAB]) shows that there is significant overlap of the spectral bands resulting from symmetric stretching vibrations of aliphatic C-H bonds (2840 to 2870 cm^{-1}; Strug et al., 2013). This implies that IR-based analysis of detergents may be generally applied to many different detergents, unlike the traditional analysis methods described in the previous paragraph.

Another advantage of measuring signals in the 2840 to 2870 cm^{-1} region of detergent spectra is that these signals do not interfere with the band that arises from amide I vibrations (between 1600 and 1700 cm^{-1}) in proteins and peptides. Because of this spectral separation of the analysis regions used for detergent and protein quantitation, collection of a single FTIR spectrum permits simultaneous analysis of both detergent and protein. While some amide bond-containing detergents, like CHAPS, exhibit IR spectra that interfere with protein amide I band, using IR spectroscopy is, in general, an accurate method for simultaneous analysis of detergent and protein.

Critical Parameters

The protocols in this unit should be performed using an FTIR spectrometer capable of collecting spectra between 400 and 4000 cm^{-1}. Using a traditional spectrometer, however, requires a certain level of IR expertise for proper manipulation of spectra and data analysis. Literature on MIR-based quantitation using regular, non-specialized FTIR instrumentation recommends application of a complex chemometric data analysis. For investigators without access to this instrumentation or IR expertise, it may be necessary to use a benchtop MIR-based spectrometer, such as that described in the above protocols, to obtain reliable detergent analysis.

Because aliphatic content varies between specific detergents, preparation of a separate calibration method for each detergent (or detergent mixture) is required. However, once prepared, the method can be stored, modified or improved with time and used for all future quantitation measurements.

Pipetting accuracy is absolutely crucial. Significant variations in absorbance intensity among replicates usually indicates inaccurate pipetting that needs to be corrected before a reliable method can be developed. All users should undergo training to ensure that they can accurately dispense 2-µl volumes. Only P2 pipettors should be used, and they should be properly calibrated.

It is important to visually inspect the spectrum of a sample being analyzed to ensure that signals from sample components are not interfering with the signals of the analytes. For example, aliphatic chain-containing molecules in the sample, such as lipids, may interfere with detergent quantitation.

Troubleshooting

Problem: no signal or negative signal

The most common cause of missing or negative peaks is a simple misplacement of a sample in the blank position on the sample card. The bottom position on the card is normally reserved for the buffer blank, whose spectrum is subtracted from the sample spectra. It is also possible that the sample simply did not contain enough detergent or protein to detect (<0.1 mg/ml).

Problem: excessive variation in signal

The most common reason for variable results is inaccurate pipetting. Be careful not to

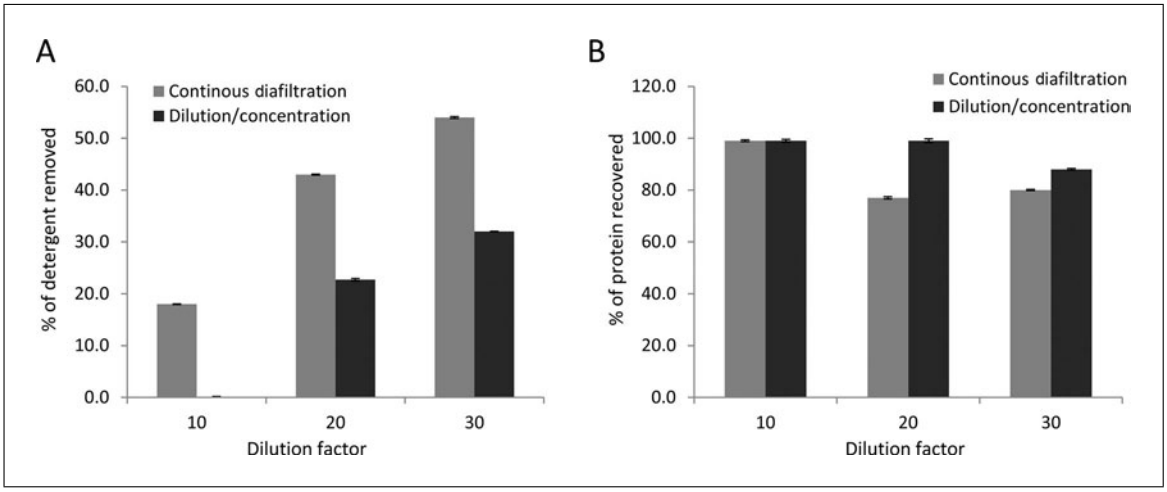

Figure 29.12.4 Comparison of detergent "stripping" (red bars) to dilute/concentrate approach (blue bars). Both experiments were performed using Amicon Pro devices. For "stripping", 50 μl sample was placed in the Amicon Ultra 0.5-ml filter and washed with 0.5, 1.0 and 1.5 ml buffer. In the case of "diluting," 50 μl sample was diluted with 0.5, 1.0 and 1.5 ml buffer and placed into an Amicon Pro exchange device/Amicon Ultra 0.5-ml filter assembly and concentrated (to a final volume of 50 μl) via centrifugation. (**A**) Efficiency of each method in detergent removal. (**B**) Effectiveness of each method in protein recovery.

transfer extra droplet volumes from the sample and ensure all of the liquid in the tip is dispensed. With a little practice, the variability can be <5%. Placing the pipette tip firmly against the membrane to avoid any losses caused by liquid shifting from inside to the side wall of the tip is also recommended.

Problem: protein amide I signal is inflated by detergent IR signal from amide bonds present in the detergent structure

Amide bond-containing detergents, such as CHAPS, can interfere with MIR-based protein quantitation if these detergents are present at high enough concentrations. We recommend choosing a non-amide bond-containing alternative if the sample is to be qualified using MIR-based quantitation. For example, CHAPS, a mild detergent used when maintaining protein structure and activity is of particular concern, can be substituted with octylglucoside, Triton X-100, sodium deoxycholate (see Internet Resources, Applichem, 2008). Since the detergent is added to the sample at a known concentration, its potential interference can be assessed prior to protein analysis. While verifying a new detergent calibration method check for absorbance in the amide I region. Pre-loaded calibration methods can be used to test the amide I absorption strength of amide bond-containing detergents. If the signal in the amide I region is strong enough to be detected using a pre-loaded calibration method, determine if the signal coming from the detergent can be subtracted, thereby providing a reliable protein concentration estimation. To do this, prepare two spots on one assay-free card using a buffer that contains detergent with amide bond(s). Use one spot as a blank and the other as a sample in the protein quantitation measurement that uses one of the pre-loaded calibration methods. In the majority of cases, the buffer subtraction step will compensate for the amide I signal coming from the detergent.

Problem: sample fails to dry quickly

Drying rate is highly dependent upon the buffer additives. Some additives, like glycerol or sucrose, will not dry. Some salts are also very hygroscopic and are very difficult to dry.

Anticipated Results

Figure 29.12.3 shows a typical IR spectrum of a complex sample containing both protein and detergent. The region of the spectrum in which the membrane absorbs has been removed for clarity. Note that the amide I peak corresponding to the protein and the signal corresponding to the aliphatic groups of the detergent are sufficiently separated to enable analysis of both types of molecules in a single measurement.

Figure 29.12.4 shows the results of detergent removal from a 50-μl protein sample while monitoring percent detergent removed (Fig. 29.12.4A) as well as percent protein recovered (Fig. 29.12.4B). These results were obtained using two different methods of detergent removal. The red bars reflect the results of removing detergent using continuous diafiltration using ten times, twenty times, and thirty times the volumes of detergent-free buffer (see

Basic Protocol 3) while the blue bars reflect the results of removing detergent by first diluting the sample by ten times, twenty times, or thirty times, followed by concentration by ultrafiltration.

These results, which provide information on choosing the optimal method for detergent removal, illustrate the utility of analyzing protein and detergent content using a single measurement and a small aliquot of the protein preparation. Specifically, continuous diafiltration (unlike rounds of dilution and concentration) maintains an unbound detergent concentration below the critical micelle concentration (CMC); as a result, fewer micelles form, and detergent removal through the ultrafiltration membrane is therefore more complete. Continuous diafiltration also prevents proteins from rapid concentration in a detergent-free buffer, a process which may lead to precipitation, unfolding and loss of yield. By measuring detergent and protein recovery at each step of the protein preparation process, it was possible to determine a protocol that provided an acceptable balance between detergent removal and protein recovery.

Time Considerations

Calibration method development and validation, if performed by a user without prior experience in these methods, can require up to 2 hr, depending on the level of the user's comfort with pipetting, software navigation and general laboratory procedures. For a user skilled in the art, ~30 min are required for these steps.

If using a pre-generated calibration method, one assay-free card can be spotted, dried and analyzed in 5 min. Re-analyzing a previously spotted card can take as little as 2.5 min, depending on whether the card is re-dried and if it has been properly stored.

For Basic Protocol 3, detergent removal by continuous diafiltration takes approximately 30 to 40 min.

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Internet Resources

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