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A high-throughput differential filtration assay to screen and select detergents for membrane proteins

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Abstract

Structural studies on integral membrane proteins are routinely performed on protein–detergent complexes (PDCs) consisting of purified protein solubilized in a particular detergent. Of all the membrane protein crystal structures solved to date, a subset of only four detergents has been used in more than half of these structures. Unfortunately, many membrane proteins are not well behaved in these four detergents and/or fail to yield well-diffracting crystals. Identification of detergents that maintain the solubility and stability of a membrane protein is a critical step and can be a lengthy and “protein-expensive” process. We have developed an assay that characterizes the stability and size of membrane proteins exchanged into a panel of 94 commercially available and chemically diverse detergents. This differential filtration assay (DFA), using a set of filtered microplates, requires sub-milligram quantities of purified protein and small quantities of detergents and other reagents and is performed in its entirety in several hours.

Keywords

Membrane protein detergent stability; High-throughput assay; Precrystallization screening; Structural biology

The difficulties of working with membrane proteins are demonstrated by the fact that membrane protein structures represent less than 1% of the total number of protein structures in the Protein Data Bank despite integral membrane proteins encompassing 15–30% of most genomes [1–3]. Technical challenges in membrane protein structure determination include expression (to obtain suitable amounts of protein), purification (to obtain suitably stable and functional protein), and sample preparation (to obtain suitable two-dimensional crystals [for electron crystallography], three-dimensional crystals [for X-ray crystallography], or solutions [for nuclear magnetic resonance (NMR)² spectroscopy]). The assay technology developed and presented here focuses on the selection of appropriate detergents for use with a specific membrane protein, which is a critical aspect of both purification and sample preparation. In preparation for structural (and other) studies, membrane proteins are extracted from their native lipid bilayer environment, and this membrane bilayer is replaced by a membrane mimetic. The membrane mimetic solute is almost always a detergent at a

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concentration above its critical micelle concentration (CMC), where the detergent surrounds the hydrophobic membrane-facing portion of the membrane protein and forms the protein–detergent complex (PDC). PDCs are in equilibrium with detergent micelles and monomers in this solution. The chemical space of detergents is large, and the solution (and crystallization) properties of a membrane protein are intimately related to the properties of the detergent(s) comprising the PDC [4,5]. Also, the function of a membrane protein can be maintained at native or near-native levels or can be completely abrogated, depending on the detergent composition of the PDC.

Currently, according to the Membrane Proteins of Known Structure database (http://blanco.biomol.uci.edu/Membrane_Proteins_xtal.html), 252 unique integral membrane protein structures have been solved by X-ray crystallography. The Membrane Protein Data Bank (<http://www.mpdb.tcd.ie>) [6] lists 893 non-unique membrane protein X-ray crystal structures for which more than 50 different detergents have been used in their solubilization and/or crystallization. These detergents are not equally represented. For example, five detergents (*n*-dodecyl- β -D-maltopyranoside [DDM], *n*-decyl- β -D-maltopyranoside [DM], *n*-nonyl- β -D-glucopyranoside [NG], *n*-octyl- β -D-glucopyranoside [OG], and *n*-dodecyl-*N,N*-dimethylamine-*N*-oxide [LDAO]) have yielded the majority of α -helical membrane protein structures [7]. Although this speaks to the utility (and extensive use) of these five detergents, more than 40% of the structures solved to date required detergents other than those five. As such, a survey of membrane protein stability in “detergent space” is an important aspect of membrane protein structural biology (and biochemistry).

There are several methods to test detergent solubility of membrane proteins. These methods include size exclusion chromatography (SEC), dilution [5], and the ultracentrifugation dispersity sedimentation assay [8]. Inspection of the size exclusion chromatogram has been routinely used for both soluble and membrane proteins to assess the quality of a protein. The method of fluorescence detection size exclusion chromatography (FSEC) is a major advance in gel filtration chromatography of integral membrane proteins [9]. The unique optical signal of a fluorescently tagged recombinant protein enables that protein to be detected and characterized in a solubilized mixture prior to purification. Also, the use of fluorescence (vs. absorbance) detection increases the sensitivity by several orders of magnitude, requiring less solubilized (or purified) protein for the chromatography analysis. To evaluate detergent stability, SEC can be performed in either of two ways: (i) the column is equilibrated in the detergent to be tested and the protein is loaded onto the column (“detergent-specific mobile phase”) or (ii) the protein is exchanged into a new detergent and then injected onto a column equilibrated with a known “good” detergent for all chromatographic runs (“generic mobile phase”). The use of the generic mobile phase speeds up the SEC runs by eliminating the

²Abbreviations used: NMR, nuclear magnetic resonance; CMC, critical micelle concentration; PDC, protein–detergent complex; DDM, *n*-dodecyl- β -D-maltopyranoside; DM, *n*-decyl- β -D-maltopyranoside; NG, *n*-nonyl- β -D-glucopyranoside; OG, *n*-octyl- β -D-glucopyranoside; LDAO, *n*-dodecyl-*N,N*-dimethylamine-*N*-oxide; SEC, size exclusion chromatography; FSEC, fluorescence detection size exclusion chromatography; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; MWCO, molecular weight cutoff; DFA, differential filtration assay; DF, differential filtration; SBS, Society for Biomolecular Sciences; MW, molecular weight; PBS, phosphate-buffered saline; UV, ultraviolet; IMAC, immobilized metal ion affinity chromatography; RT, room temperature; CV, column volumes; PCR, polymerase chain reaction; TCA, trichloroacetic acid; EDTA, ethylenediaminetetraacetic acid; PES, polyethersulfone; GHP, GH hydrophobic polypropylene; TDM, *n*-tridecyl- β -D-maltopyranoside; B₂₂, second virial coefficient; FIAsH, fluorescein arsenical helix binder; GFP, green fluorescent protein; BCA, bicinchoninic acid.

column washing and equilibration steps for the next detergent. The generic mobile phase method rests on the assumption that if a protein sample has been exchanged into an incompatible detergent, then a compatible detergent in the mobile phase will not reverse the deleterious effects of that incompatible detergent [9]. Data from our laboratory suggest that this is not true for all cases, so we do not currently favor the generic mobile phase method. We note that the original FSEC publication [9] used a generic mobile phase; however, fluorescence detection is equally applicable to use of a detergent-specific mobile phase. For the dilution method, concentrated protein is diluted into a new test detergent and the $A_{320\text{nm}}/A_{280\text{nm}}$ ratio is recorded over time. Proteins typically possess little or no absorbance at 320 nm (and higher) wavelengths, so nonzero optical density in this region often arises from Rayleigh scattering of protein aggregates [10]. Thus, absorbance or light scattering at 320 nm has been used to assay aggregation of proteins, peptides, or viruses [11–13]. Because $A_{320\text{nm}}$ is indicative of protein aggregation, an increase in the $A_{320\text{nm}}/A_{280\text{nm}}$ ratio is diagnostic of the protein not being stable in the new detergent [5]. In the ultracentrifugation dispersity sedimentation assay, the protein is concentrated, diluted into the test detergent buffer with three concentration/dilution steps, and finally allowed to incubate overnight. At this point, a sample is taken while the rest of the protein is spun in the ultracentrifuge to pellet any aggregated protein. Another protein sample is taken after ultracentrifugation, and both the pre- and postultracentrifugation samples are run on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and compared. Any difference in band intensity between the two samples is indicative of aggregated protein being removed during the intermediate ultracentrifugation step and, thus, is related to detergent stability [8].

These three methods all possess shortcomings. The biggest limitation is that the methods described above are not detergent exchanges but rather detergent dilutions (the exception is the single case where the protein is already in the same detergent as that present in the gel filtration mobile phase). This is a problem if the initial detergent is not diluted to a concentration below its CMC or, in the case of gel filtration, if the original detergent's micelles are not separated from the PDC or if a mixed detergent population exists. In these instances, the presence of the original detergent can “protect” a protein from a destabilizing detergent resulting in false positives. The original detergent's concentration is of great concern, especially when the method uses a protein ultrafiltration concentration step, because detergent micelles typically concentrate along with the protein even when a large molecular weight cutoff (MWCO) is used. Another limitation is that milligram amounts of protein and large amounts of expensive detergent reagents may be necessary, especially if there are a large number of conditions to be tested. Lastly, the time required to perform each method can be long, usually limiting the number of detergents surveyed, especially in the case of gel filtration where only one detergent can be tested at a time.

We have developed a microplate-based detergent screening assay named the differential filtration assay (DFA) (Fig. 1). Differential filtration (DF), the simple underlying principle, is that the variation of amounts of macromolecules in filtrates obtained by parallel passages of a macromolecular solution through several different MWCO filters provides information on the stability (aggregation) and size of a macromolecular solute as a function of buffer

composition. DF rapidly captures significant information that is typically obtained much more slowly by SEC. The combination of DF with a specific method for detection and quantitation of filtrates yields a DFA. For membrane proteins in PDCs, the detergent of the PDC is often the most significant buffer component; however, DF is equally well suited for examination of any solution conditions for any macromolecular solute. A standard buffer exchange technique, successfully implemented in screening detergents suitable for extracting proteins from their membrane environment [14–16], is used to exchange microgram quantities of purified membrane protein into each of the detergents of our 94-detergent panel (Table 1). This simple technique involves binding the protein to an affinity resin, extensively washing with the new detergent, and finally eluting from the column in the new detergent. In parallel, these eluents are each passed through high- and low-MWCO filter plates. In the initial development and validation of DFA presented here, the amounts of protein in the filtrates are measured by a rapid Western blot protocol. DFA is performed with 96-well SBS (Society for Biomolecular Sciences) format filter plates for further increasing speed and decreasing reagent and protein costs. Our assay addresses the limitations of the detergent screening methods described above and provides measures of both stability and rudimentary size information. In its current implementation, results can be obtained in approximately 2 h with only a few hundred micrograms of protein required for the assay. Furthermore, DFA can be completely automated if desired. To validate the assay, we present DFA data of two membrane proteins: AqpZ and KcsA. Both of these membrane proteins have been crystallized and their structures initially solved by other laboratories.

For the purposes of this article (and corresponding to a standard empirical definition), we define the stability of a membrane protein in a given detergent to be a quantity that is inversely proportional to the fraction of PDCs that form large particles or aggregates in that specific detergent. If all of the protein forms large particles or aggregates (which would be present, e.g., in the void volume of a suitable gel filtration column), then we would call that protein sample unstable. If none of the protein forms large particles or aggregates (such that the protein would be seen as one or more sizing peaks in the aforementioned gel filtration column), then we would call that protein sample stable. (Of course, stability, generally a time-dependent property, is not affected solely by detergent.)

Materials and methods

Detergent panel

All chemicals for the detergent panel were purchased from Anatrace, Avanti Polar Lipids, Bachem, and EMD Biosciences, as indicated in Table 1. Working stock solutions (2×) were made in ultrapure water, dispensed into 96-well plates, heat sealed with foil tape, and frozen at −20 °C until needed.

MWCO filtered microplate assessment

Stock solutions of each gel filtration molecular weight (MW) standard (Sigma or GE Healthcare) at 2–5 mg/ml were made in phosphate-buffered saline (PBS) buffer, and 30 µl of each stock solution was added to a 0.2 µm filter plate (product no. 5045, Pall) and 100 and 300 kDa MWCO filter plates (product nos. T-3180-14 and T-3180-21, ISC Bioexpress, or

product nos. CMR1411 and CMR1493-1, Seahorse Labware) and spun at 2000g for 2 min. We emphasize that these values of 100 and 300 kDa MWCO are the names given to these plates by the manufacturer. As will be shown, the actual MWCOs measured are different. Henceforth, we use the nomenclature "Low" and "High" for the 100 and 300 kDa plates, respectively. The filter plate flow-through and samples of the original stock solutions were transferred to an ultraviolet (UV) transparent 384-well plate, and $A_{280\text{nm}}$ was measured on a Molecular Devices SpectraMax 384 Plus spectrophotometer with PathCheck (i.e., 1 cm pathlength correction) active. The percentage difference between the stocks and eluate absorbance values was used to calculate the flow-through percentage for each standard through each filter type. The errors are the standard deviations obtained from measuring three separate samples in each filtered microplate.

Purification of AqpZ and KcsA

Both His6–AqpZ (Cys free) and His6–KcsA were overexpressed and purified from *Escherichia coli* by slight modification of published methods [17,18]. The AqpZ buffer was 20 mM Tris (pH 7.4), 500 mM NaCl, 10% glycerol, and 40 mM OG. The KcsA buffer was 20 mM Tris (pH 7.4), 150 mM KCl, and 1 mM DDM. Both purifications were carried out only to the first immobilized metal ion affinity chromatography (IMAC) step with Co-TALON (Clontech) resin and then desalted back into their respective buffers with PD10 columns (GE Healthcare). KcsA was further processed by digestion with chymotrypsin (Worthington Biochemical) for 4 h at room temperature (RT) with a 1:25 chymotrypsin/KcsA ratio by mass. The chymotrypsin was removed with immobilized benzamidine (GE Healthcare).

Protein binding, detergent exchange, and elution

AqpZ or KcsA (400 μg) was batch bound to 1.5 ml of Co-TALON resin (0.27 μg protein/ μl resin) along with additional buffer to a final volume of 7.5 ml, making a 20% slurry. Then 50 μl of the protein-bound resin slurry (10 μl resin) was added to the 0.2 μm filter microplate using a multichannel pipette with the ends of the tips cut to make a wider bore. The resin was pelleted in the microplate by spinning at 2000g for 2 min at 4 °C. For each protein, the detergent-containing wash solutions were prepared by a combination of the 2 $\times$ detergent stock panel with 2 $\times$ protein-specific buffer (i.e., the buffer in which the protein was purified).

Next, 3CV (3 column volumes) of the detergent wash solution was added to each well, and the microplate was spun at 2000g for 2 min at 4 °C to remove the buffer. This washing was repeated until 20CV of detergent wash buffer had been added. The elution solutions were prepared from the 2 $\times$ detergent stock panel and 2 $\times$ elution buffer stocks (2 $\times$ wash buffer containing 1 M imidazole). The detergent-exchanged protein was then eluted from the resin in 6CV of elution solution by spinning the plate at 2000g for 2 min at 4 °C into a polymerase chain reaction (PCR) collection plate (Abgene). Then 25 μl of the eluted protein was added to the 300 kDa (High) MWCO filter microplate and 25 μl was added to the 100 kDa (Low) MWCO filter microplate, the plates were spun at 2000g for 2 min at 4 °C, and the eluate was collected into a PCR collection plate.

Western blot protocol

The Minifold I 96-well Dot Blot apparatus (Whatman) was used to blot the elution samples onto 0.2 μ m nitrocellulose (Whatman). Due to the spot-broadening effects of some detergents, the protein was precipitated in the blotting apparatus with trichloroacetic acid (TCA) prior to applying the vacuum. Here 150 μ l of 10% TCA was first added to each well of the assembled dot blot apparatus, followed by 10 μ l of the eluate from each MWCO filter plate. Vacuum was applied to filter the sample through the nitrocellulose, and each well was washed with 20 mM Tris (pH 7.4) and 500 mM NaCl three or four times. The membranes were quickly washed with ultrapure water and blocked for 10 min with Odyssey blocking buffer (LI-COR Biosciences). The membranes were probed for 10 min with an IRDye® 800CW-conjugated anti-6 $\times$ His tag antibody (Rockland Immunochemicals) diluted 1:10,000 in Western Breeze Primary Antibody Diluent (Invitrogen). The blots were washed with Western Breeze Antibody Wash (Invitrogen) four times for 2 min each and then with ultrapure water. The blots were scanned on an Odyssey Infrared Imaging System (LI-COR Biosciences) with the intensity adjusted to avoid saturation of the spots. Integrated spot intensities were measured with the Odyssey software, and the background for each spot was calculated from the median value of the baseline surrounding the spot. The background-corrected integrated spot intensities were then exported to a spreadsheet for normalization and graphing.

SEC runs

Detergent exchanges using larger amounts of protein (12 μ g protein/ μ l resin) for gel filtration were carried out in 0.22 μ m filter spin columns (Millipore) with a tabletop centrifuge using the same detergent exchange protocol as above except that the protein was eluted in only 3CV and 150 mM ethylenediaminetetraacetic acid (EDTA) was substituted for imidazole in the elution buffer to avoid imidazole fluorescence during SEC. Eluted protein (10 μ l) was loaded onto a calibrated Superdex™ 200 5/150 GL “short column” (GE Healthcare) at 0.4 ml/min. The column was equilibrated in the exchange detergent prior to sample injection. The intrinsic protein fluorescence (Ex_{280nm}/Em_{335nm}) was monitored using a Hitachi L-2485 fluorescence detector.

Results

Differential filtration

The use of multiple differing MWCO filters in parallel permits acquisition of rudimentary size information on PDCs. It is important to note that MWCO filters do not have a single sharp MWCO. Instead, a given filter will exclude approximately all particles above a certain size, will pass approximately all particles below a certain size, and will let through some fraction of particles between these two limits. For the purposes of DF, consider two MWCO filter plates: High and Low. The High plate excludes particles of large size and permits some or all of the remaining particles to pass through. The retentate of the High plate will consist solely of these large particles; nearly everything else will be in the filtrate. The Low plate has a lower size exclusion limit. Therefore, the retentate of the Low plate will include all of the retentate present in the High plate plus additional retentate arising from the lower cutoff.

Thus, in the absence of experimental error, the fraction of a sample in the filtrate of the Low plate will always be less than or equal to the fraction of that sample in the High plate filtrate.

During the conception of the detergent screening assay, SEC of the eluted samples was planned to be performed using either a generic mobile phase or detergent-specific mobile phase in conjunction with an in-line fluorescence detector to follow intrinsic protein fluorescence of the protein of interest. It became readily apparent that this would not work due to the fluorescence of a large population of the detergents in the panel themselves (data not shown). As an alternative to gel filtration for obtaining sizing information, or at the very least to remove any protein aggregate analogous to that present in the void volume of a gel filtration run, filtration through MWCO polyethersulfone (PES) filters was employed. Because MWCO PES filters are not absolute cutoffs but instead permit a range of particle sizes to pass through their membrane, SBS format MWCO filter plates from multiple manufacturers were evaluated. The size distribution range for each MWCO filter plate was measured using gel filtration MW standards. A sizing microplate that would disallow passage of blue dextran (the 2000 kDa void volume MW standard) and produce a suitable MW distribution for estimating size was sought. We emphasize that a single MWCO filtered microplate was not sufficient to satisfy both criteria given that the MW permeability range was too narrow for the Low plate and too broad for the High plate. To overcome this deficiency, Low and High MWCO microplates were paired together for the assay. The MW distributions of these two MWCO filtered microplates along with the 0.2 μ m GHP (GH hydrophobic polypropylene) filter plate are shown in Fig. 2. As expected, the 0.2 μ m plate allows passage of all the gel filtration standards, with equal and complete permeability of all standards except blue dextran (partially permeable). In contrast, neither the Low nor High plates allow blue dextran to flow-through and, thus, will prevent highly aggregated protein from passing through. With the use of both MWCO microplates, analysis of the High microplate filtrate reports primarily on stability and the ratio of the Low and High plate filtrates reports on size. Thus, analysis of the filtrates of several different MWCO filters captures significant data that are typically obtained by SEC. We call this technique differential filtration.

The detergent panel

The assay in its current form uses 96-well SBS format micro-plates. We use our 94-detergent panel (Table 1) plus negative and positive positions A1 and A2 of the microplates. The negative control contains detergent-free buffer that will cause the protein to precipitate on the resin, whereas the positive control is the current detergent-containing buffer. Even though it is highly probable that the current detergent used for a given protein is present in the panel, the working concentrations of the detergent may be different. The chemical name (or trademarked name), well location, abbreviation, working concentration, and CMC value for each detergent in the panel are shown in Table 1. All of the detergents were selected for their suitability for membrane protein studies based on several criteria: (i) commercial availability, (ii) moderate or high aqueous solubility, (iii) CMC values between 0.03 and 40 mM, and (iv) zwitterionic or nonionic headgroups. The detergent panel is first grouped into zwitterionic and nonionic sets and is further ordered by chemical class, then by chain length within each chemical class where applicable, and finally by CMC value. This permits the

facile recognition of patterns of stability. The detergent stock plate consists of 2× working concentration solutions dispensed and heat sealed into single-use microplates for ease in formulation of the wash and elution buffers. Each detergent's working concentration in the panel varies with respect to its CMC value: 2× (CMC > 10 mM), 2.5× (CMC 5–10 mM), 3× (CMC 1–5 mM), 10× (CMC 0.5–1 mM), 50× (CMC 0.1–0.5 mM), and 100× (CMC < 0.1 mM). The exception is *n*-tridecyl-β-D-maltopyranoside (TDM), where the working concentration is 50× due to solubility issues. The use of several CMC multiples is necessary due to low-CMC detergents being required at higher concentrations (in terms of their CMC) than high-CMC detergents [5].

Visualization and quantification of elutions

Using a fast Western protocol, the elutions for each MWCO microplate are blotted to a nitrocellulose membrane, visualized using a conjugated primary antibody specific to the affinity tag used, and quantified in approximately 1 h. Dot blots of the elutions from the AqpZ and KcsA detergent exchanges are shown in Fig. 3. Quantification of dots on the blots measures the amount of protein eluted from each well. Because the same amount of protein was used in each well and an incompatible detergent will cause a membrane protein to precipitate on the affinity resin, the integrated dot intensity is directly related to the ability of a particular detergent to stabilize a membrane protein relative to the other detergents in the panel. As mentioned in the introductory paragraphs, the loss of membrane protein stability is assayed by the presence of, and amount of, irreversible aggregates (large particle formation) as a function of the detergent species that is present in the PDC. The Low and High dot blots are quantified and normalized to the highest intensity dot on each respective blot. The ratios of the normalized Low to High dot intensities are calculated to indicate the relative size of the PDC; this ratio is inversely proportional to the PDC size, with larger ratio values indicative of a smaller PDC size. The ratio is a better estimate of size than just the Low dot intensities alone. The Low dot intensities are related to both size and stability, whereas the Low/High ratio accounts for the removal of any aggregated protein retained by the High microplate (and thus also retained by the Low microplate). Figs. 4 and 5 demonstrate two representations of the quantified data. Fig. 4, the “audio equalizer representation,” presents the data to allow any stability and/or size patterns related to detergent type, chain length, or CMC to be easily elucidated because the detergent panel is organized in that manner. Fig. 5, the “size stability quad plot,” displays the data to allow quick assessment of PDC size related to stability without any regard for the detergent's physical or chemical properties. The data in both representations are binned into quartiles to help compensate for the inherent error in measuring only one data point. Interestingly, despite AqpZ being a larger tetramer (103 kDa), the KcsA tetramer (55 kDa) possesses a much more variable size dependence, as demonstrated by the broader distribution of relative PDC size seen in Fig. 5.

DF correlates with SEC

To further validate DF, larger scale detergent exchanges were performed on AqpZ and KcsA and samples were run over a calibrated SEC column. The retention times of the SEC runs were compared with the dot intensities obtained from the two MWCO filter plate elutions. Fig. 6 (top panel) shows three AqpZ gel filtration runs exchanged into TDM, LDAO, and OG along with the corresponding dot blots for those detergents. As predicted from the Low

elution dot blots, the apparent PDC size order of AqpZ is $OG < LDAO < TDM$, which is inversely proportional to their respective dot blot intensities. Fig. 6 (bottom panel) demonstrates an interesting phenomenon in which the same detergent at two different concentrations gives two different PDC sizes for KcsA. KcsA in the traditional working concentration of 1 mM DDM is predicted to have a larger PDC size than the same protein in 8.5 mM DDM by both SEC and DF. The reason for a higher concentration of detergent reducing the size of the PDC is currently under investigation.

Error analysis of Western blot detection method

In the implementation of DFA presented here, each detergent-unique PDC sample is measured a single time. To estimate the experimental uncertainty of the Western blot detection method, DFA (with the detergent panel) was performed with soluble His-tagged protein samples, at various protein concentrations, as well as with buffer- and water-only samples. We performed these experiments to estimate the standard deviations of the membrane protein DFA measurements. These samples covered a large range (more than an order of magnitude) of integrated background-corrected intensity (I). We observed a modest linear dependence of standard deviation with intensity: $\sigma = 0.142 \cdot I + 0.079$. Thus, on the normalized 0–1 scales used for the Low and High measurements, the errors range from 0.079 ($I = 0$) to 0.221 ($I = 1$). (Note that although the absolute error increases with signal size, the fractional error decreases.) The stability axis (High) of the size stability quad plot (Fig. 5) will range between these values. However, because the size axis (Low/High) of the size stability quad plot (Fig. 5) is a ratio, the errors are larger. These errors are listed for the AqpZ and KcsA data (Table 2). Also, we note that a small but systematic detergent effect was observed in these “error estimation” experiments; specifically, some detergents caused an apparent increase in the nonspecific binding of the fluorescent antibody to the nitrocellulose membrane relative to the majority of detergents in the panel. This effect is small compared with the intrinsic error of the individual measurements, so its correction was omitted.

Discussion

The detergent panel presented here, combined with DFA, provides a robust and rapid means to survey membrane protein stability in a large number of chemically diverse detergents as well as to obtain rudimentary PDC sizing information. Although the sizing information obtained from DF does not provide an absolute value for apparent MW, it does provide a coarse filter that allows the results to be binned and ranked for further analysis via traditional SEC as desired. The ability to quickly obtain this sizing and stability information is a significant benefit to this method, especially if the time that would be required to examine all 94 samples with SEC is considered. For the Superdex™ 200 5/150 GL column used in this work, each run is approximately 6 min in duration. The use of a generic mobile phase would require approximately 10 h, and the use of detergent-specific mobile phases would require an additional 44 h for column equilibration and pump washing. These time estimates assume no downtime and complete automation of the chromatography. Furthermore, many protein samples will aggregate or denature during this time, leading to ambiguous results.

On inspection of Fig. 4, various detergent stability and size trends are seen for AqpZ and KcsA. We present several examples of the types of observations that can be obtained from DFA. For AqpZ, there is increased stability with an increase in detergent chain length seen with dimethylamine-*N*-oxide detergents (A7–A9), whereas decreased stability is observed with increasing ethylene chain length in the C8En detergents (E2–E4). For KcsA, increasing detergent chain length for glucoside detergents (F5–F7) resulted in decreased stability, whereas just the addition of a hydroxyl group on Big CHAP (E12), forming Big CHAP deoxy (F1), increases protein stability.

As mentioned in Results, Fig. 6 shows an interesting phenomenon in which the smaller KcsA tetramer (55 kDa) has a broader size distribution than that of the larger AqpZ tetramer (103 kDa). This is most likely related to the amount of detergent KcsA binds relative to AqpZ to maintain its solubility (i.e., the more bound detergent, the larger the PDC size). The observation that a higher concentration of DDM resulted in smaller PDC size for KcsA (Fig. 6, bottom panel) suggests that more detergent is required to keep KcsA in a more compact state. Because the DDM monomer concentration should be relatively constant and approximately equal to the CMC at both 1 and 8.5 mM DDM concentrations [19], a higher number of DDM micelles appears to be required to form a smaller PDC. Experiments, planned to investigate this phenomenon, include determination of the amount of DDM bound to KcsA as a function of DDM concentration. The amount of detergent present in the PDC can be determined by static light scattering coupled with refractive index and UV detection [20–23]. In the context of the results observed with KcsA, we note that, contrary to the most simple expectations, detergent micelle size can change as a function of detergent concentration [24,25].

The readout from the size stability quad plot (e.g., Fig. 5) may help to predict which detergents are best for crystallization because each quadrant represents different levels of stability and PDC size. Future experiments are planned to determine whether a “crystallization quadrant” exists. This would be somewhat analogous to the second virial coefficient (B_{22}) “crystallization slot,” where it was demonstrated that soluble proteins with B_{22} values within a specific narrow range had a propensity to form crystals [26]. Similar observations have been extended to membrane proteins [27–30]. Although detergents in the smallest, most stable quadrant would likely be useful for NMR, there are not enough data currently to suggest that this is necessarily the best criterion for membrane protein crystallography. Whether or not a crystallization quadrant exists, our assay and detergent panel will help with the critical “pipeline step” of choosing the proper detergents for any membrane protein of interest quickly and with minimal quantities of reagents.

The two test proteins used in this study, AqpZ and KcsA, both have had their structures determined [17,18,31]. What is the location in the size stability quad plot for the “crystallization” detergents of AqpZ and KcsA? AqpZ was crystallized in OG [18,32]. OG (F6 in Table 1) is located in the second-highest stability and second-smallest size quadrant (Fig. 5). For KcsA, Doyle and coworkers reported purification of KcsA in DM followed by detergent exchange via dialysis into LDAO [17]. DM (G10 in Table 1) is located in the second-highest stability and second-smallest size quadrant (Fig. 5). LDAO (A9 in Table 1) is located in the third-highest (second-lowest) stability and smallest size quadrant (Fig. 5).

Because the degree of exchange is not stated, we cannot unambiguously and accurately assign the crystallization detergent (or detergent mixture) of KcsA. However, it is interesting to note that crystal structures of KcsA/Fab complexes (see, e.g., Ref. [33]) have essentially all been in DM. Based on these scant examples, it is tempting to speculate that crystallization may be best pursued from the upper left “quarter” of the quad plot (i.e., those detergents that lay within the more stable and smaller halves of the distribution).

For the particular Western blot detection method used in the current study, it is critically important to state that the experimental errors (Table 2) decrease the precision of DFA. Thus, the above discussion of the DFA data is suggestive of various trends as well as being illustrative of the capabilities of DF for the rapid acquisition of stability and size data. However, even these rather noisy data are already of significant utility, especially for eliminating those detergents that most destabilize the protein. Along the stability (High) axis (Fig. 5), the data for both AqpZ and KcsA fall into two clusters of greater and lesser stability (values of High > 0.5 and < 0.5, respectively). All those in the less stable cluster could likely be omitted from further experiments. The larger errors along the size (Low/High) axis (Fig. 5) further degrade precision; however, SEC validation (Fig. 6) suggests overestimation of the errors for DFA determination of size. We prefer to be conservative about error analysis and note that these errors arise from the specific detection method used. Alternative detection methods (see below) will increase the precision of DFA.

The core technology of the DFA is the use of several different MWCO filters that yield DF of a macromolecular solute. Detection and quantification of the filtrates, required for application of DF to an assay, can be performed by a variety of methods. Factors that influence the choice of detection method include specific versus nonspecific detection, direct versus indirect detection, sensitivity, accuracy, precision, time required, and (of course) amount of protein required. Specific detection methods are those that will essentially detect only the expressed protein of interest. In this study, we performed a rapid Western blot protocol that used a fluorescent primary antibody to the polyhistidine affinity tag. Antibody-based methods are highly specific and indirect. A benefit of this high specificity is the ability to perform DFA on less highly purified samples. However, measurement of antibody binding to quantitate antigen, an indirect method, can introduce significant error, especially if the measurement is performed only once (illustrated by the analysis of error that we have presented here). Although error can be decreased by performing multiple measurements of each sample buffer condition, this increases the time, cost, and amount of protein required to perform the assay. A detection method that is specific and direct is measurement of the fluorescence of a small-molecule fluorophore (e.g., fluorescein arsenical helix binder [FIAsH] [34]) attached to a genetically encoded binding site of the recombinant protein or of a fluorescent protein (e.g., green fluorescent protein [GFP]) made as a fusion protein with the target. These are also direct detection methods because the protein itself is detected. Nonspecific direct detection methods are those that detect total amount of protein in the filtrate. These include Lowry [35] and bisinchoninic acid (BCA) [36] assays as well as nonspecific fluorescent labeling of proteins (e.g., by amine-reactive fluorophores [37]). A likely advantage of direct fluorescent methods, whether specific or nonspecific, is that the High and Low MWCO filtrates can go directly into fluorescence microplates for direct reading in a fluorescence plate reader.

With structural biology methodologies moving toward performing experiments on smaller scales with smaller amounts of material (especially important for difficult research problems with limited and/or expensive reagents), the development and use of high-throughput methodologies have increased. Here we have presented a true high-throughput membrane protein detergent screening assay that can be completed in approximately 2 h with microgram amounts of protein and microliter volumes of reagents. DFA helps to overcome the barrier of low protein yields that, unfortunately, are typical for membrane proteins, especially when using more complex and/or higher order expression systems (e.g., eukaryotic). The assay as presented here used 400 µg of protein to obtain stability and PDC sizing information on 94 different detergents from our panel. This amount of protein is not the absolute minimum amount required to perform the assay. DFA could be conducted with 10 to 50 µg of protein (or even less) if more sensitive detection of the Low and High MWCO elutions could be performed. Although we focused solely on the use of DFA for the parallel screening of multiple single detergents, DFA can readily be extended to the screening of detergent mixtures, additives, ionic strength, pH, and any other variable buffer components for both membrane and soluble proteins. Lastly, for proteins that possess native *in vivo* ligand-binding function, use of “physiological” ligand-binding affinity matrices in DFA can provide functional characterization as well as stability and size characterization.

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Fig.1. Flowchart of the detergent stability assay. The generic protocol for performing the assay is presented.

Fig.2.

Filter plate flow-through of molecular weight standards. The $A_{280\text{nm}}$ was measured for each stock solution, and the eluate from each plate was measured in triplicate. The percentage difference between the eluate and the stock is presented on the graph. The error bars represent the error propagated from the standard deviation of each triplicate measurement. No passage of blue dextran was observed in either the High or Low MWCO filter plates.

Fig.3.

Dot blots of eluted protein after exchange into the new detergent. Here 10 μ l of each of the elutions from the High and Low MWCO plates was spotted on nitrocellulose membrane and visualized by Western blot using an IRDye® 800CW-conjugated His tag antibody and a LI-COR Odyssey imaging system. The dot intensities were quantified with median background correction within the Odyssey software.

Fig.4.

Stability and relative size bar graphs. The normalized intensities from the High MWCO dot blots are plotted along with the ratio of Low/High normalized intensities for AqpZ and KcsA. The values are grouped into quartiles (indicated by the gridlines). The High intensity is directly proportional to stability, whereas Low/High is inversely proportional to the particle size. Nonreal ratio values (i.e., Low intensity > High intensity) are given in parentheses. These nonreal ratio values are all from High and Low intensities within the same quartile rank except those indicated by an asterisk (*).

Fig.5.

Quartile grid plot. The normalized intensities from the High MWCO dot blots are plotted on the horizontal axis, whereas the ratios of Low/High normalized intensities for AqpZ and KcsA are plotted on the vertical axis. The well numbers are shown next to each dot. Nonreal ratio values (i.e., Low intensity > High intensity) are located in the grayed out area of the plot. These nonreal ratio values are all from High and Low intensities within the same quartile rank except those indicated by an asterisk (*).

Fig.6.

Low MWCO elution intensity correlates to SEC retention time. Larger amounts of AqpZ and KcsA were detergent exchanged using spin columns, and then 10 μ l was injected onto a calibrated Superdex™ 200 5/150 GL gel filtration column equilibrated in the exchanged buffer. The retention times for MW gel filtration standards are shown on each chromatogram. The insets show the dot blot spots for each detergent.

Table 1

Detergent screen

Well	Abbreviation	Name	[Det] (mM)	Well	Abbreviation	Name	[Det] (mM)
A1		No detergent (–control)		E1	C7E5	<i>Pentaethylene glycol monoheptyl ether</i>	42 (21)
A2		Empty well for current detergent (+control)		E2	C8E4	<i>Tetraethylene glycol monooctyl ether</i>	20 (8)
A3	Z3-12	ZWITTERGENT® 3–12	8.4 (2.8)	E3	C8E5	<i>Pentaethylene glycol monooctyl ether</i>	17.75 (7.1)
A4	Z3-14	ZWITTERGENT® 3–14	10 (0.2)	E4	C8E6	<i>Hexaethylene glycol monooctyl ether</i>	25 (10)
A5	DMG	<i>n</i> -Decyl- <i>N,N</i> -dimethylglycine	38 (19)	E5	C10E5	<i>Pentaethylene glycol monodecyl ether</i>	8.1 (0.81)
A6	DOMG	<i>n</i> -Dodecyl- <i>N,N</i> -dimethylglycine	4.5 (1.5)	E6	C10E6	<i>Hexaethylene glycol monodecyl ether</i>	9 (0.9)
A7	DAO	<i>n</i> -Decyl- <i>N,N</i> -dimethylamine- <i>N</i> -oxide	21 (10.5)	E7	C10E9	<i>Polyoxyethylene(9)decyl ether</i>	3.9 (1.3)
A8	UDAO	<i>n</i> -Undecyl- <i>N,N</i> -dimethylamine- <i>N</i> -oxide	9.6 (3.2)	E8	C12E8	<i>Octaethylene glycol monododecyl ether</i>	9 (0.09)
A9	LDAO	<i>n</i> -Dodecyl- <i>N,N</i> -dimethylamine- <i>N</i> -oxide	3 (1)	E9	C12E9	<i>Polyoxyethylene(9)dodecyl ether</i>	5 (0.05)
A10	C-DDFOS	C-DODECAFOS™	44 (22)	E10	C12E10	<i>Polyoxyethylene(10)dodecyl ether</i>	10 (0.2)
A11	CF-4	CYCLOFOS™-4	28 (14)	E11	C13E8	<i>Polyoxyethylene(8)tridecyl ether</i>	10 (0.1)
A12	CF-5	CYCLOFOS™-5	13.5 (4.5)	E12	CHAP	Big CHAP	8.7 (2.9)
B1	CF-6	CYCLOFOS™-6	8.04 (2.68)	F1	CHAP-D	Big CHAP deoxy	4.2 (1.4)
B2	CF-7	CYCLOFOS™-7	6.2 (0.62)	F2	OHES	<i>Octyl-2-hydroxyethyl-sulfoxide</i>	48.4 (24.2)
B3	FC-10	FOS-CHOLINE®-10	22 (11)	F3	RDHPOS	<i>Rac-2,3-dihydroxypropyloctylsulfoxide</i>	48.4 (24.2)
B4	FC-11	FOS-CHOLINE®-11	5.55 (1.85)	F4	GX-100	<u>Genapol® X-100</u>	7.5 (0.15)
B5	FC-12	FOS-CHOLINE®-12	4.5 (1.5)	F5	HTG	<i>n</i> -Heptyl-β-D-thiogluconopyranoside	58 (29)
B6	FC-13	FOS-CHOLINE®-13	7.5 (0.75)	F6	OG	<i>n</i> -Octyl-β-D-glucopyranoside	36 (18)
B7	FC-14	FOS-CHOLINE®-14	6 (0.12)	F7	NG	<i>n</i> -Nonyl-β-D-glucopyranoside	16.25 (6.5)
B8	FC-I11	FOS-CHOLINE®-ISO-11	53.2 (26.6)	F8	CYGLU-3	CYGLU®-3	56 (28)
B9	FC-I11-6U	FOS-CHOLINE®-ISO-11-6U	51.6 (25.8)	F9	HECAMEG	HECAMEG	39 (19.5)
B10	FC-I9	FOS-CHOLINE®-ISO-9	64 (32)	F10	HEGA-9	Hega®-9	78 (39)
B11	FC-U10-11	FOS-CHOLINE®-UNSAT-11-10	15.5 (6.2)	F11	C-HEGA-10	C-Hega®-10	70 (35)
B12	DHPC	1,2-Diheptanoyl-sn-glycero-3-phosphocholine	4.2 (1.4)	F12	C-HEGA-11	C-Hega®-11	23 (11.5)
C1	LPC-10	LysoPC-10	20 (8)	G1	CYMAL-3	CYMAL®-3	60 (30)
C2	LPC-12	LysoPC-12	7 (0.7)	G2	CYMAL-4	CYMAL®-4	19 (7.6)
C3	FOSFEN-9	F0SFEN™-9	4.05 (1.35)	G3	CYMAL-5	CYMAL®-5	7.2 (2.4)
C4	CHAPS	CHAPS	20 (8)	G4	CYMAL-6	CYMAL®-6	5.6 (0.56)
C5	CHAPSO	CHAPSO	20 (8)	G5	CYMAL-7	CYMAL®-7	9.5 (0.19)
C6	DDMAU	<u><i>n</i>-Dodecyl-<i>N,N</i>-(dimethylammonio)undecanoate</u>	6.5 (0.13)	G6	DMHM	2,6-Dimethyl-4-heptyl-β-D-maltoside	55 (27.5)
C7	DDMAB	<u><i>n</i>-Dodecyl-<i>N,N</i>-(dimethylammonio)butyrate</u>	12.9 (4.3)	G7	OM	<i>n</i> -Octyl-β-D-maltopyranoside	39 (19.5)
C8	LAPAO	LAPAO	4.8 (1.6)	G8	NM	<i>n</i> -Nonyl-β-D-maltopyranoside	15 (6)
C9	TRIPAO	TRIPAO	13.5 (4.5)	G9	DαM	<i>n</i> -Decyl-α-D-maltopyranoside	4.8 (1.6)
C10	T-20	TWEEN® 20	5.9 (0.059)	G10	DM	<i>n</i> -Decyl-β-D-maltopyranoside	5.4 (1.8)
C11	BRIJ-35	BRIJ® 35	9.1 (0.091)	G11	UDαM	<i>n</i> -Undecyl-α-D-maltopyranoside	5.8 (0.58)
C12	TX-100	TRITON® X-100	11.5 (0.23)	G12	UDM	<i>n</i> -Undecyl-β-D-maltopyranoside	5.9 (0.59)
D1	TX-114	TRITON® X-114	10 (0.2)	H1	ωUDM	ω-Undecylenyl-β-D-maltopyranoside	3.6 (1.2)
D2	TX-305	TRITON® X-305	6.5 (0.65)	H2	DDαM	<i>n</i> -Dodecyl-α-D-maltopyranoside	7.5 (0.15)
D3	TX-405	TRITON® X-405	8.1 (0.81)	H3	DDM	<i>n</i> -Dodecyl-β-D-maltopyranoside	8.5 (0.17)

Well	Abbreviation	Name	[Det] (mM)	Well	Abbreviation	Name	[Det] (mM)
D4	NID-P40	[Octylphenoxy]polyethoxyethanol	15 (0.3)	H4	TDM	<i>n</i> -Tridecyl- β -D-maltopyranoside	1.5 (0.03)
D5	APO8	Dimethyloctylphosphine oxide	80 (40)	H5	OTM	<i>n</i> -Octyl- β -D-thiomaltopyranoside	21.25 (8.5)
D6	APO9	<u>Dimethylnonylphosphine oxide</u>	20 (10)	H6	NTM	<i>n</i> -Nonyl- β -D-thiomaltopyranoside	9.6 (3.2)
D7	APO10	Dimethyldecylphosphine oxide	13.98 (4.66)	H7	DTM	<i>n</i> -Decyl- β -D-thiomaltopyranoside	9 (0.9)
D8	APO11	<u>Dimethylundecylphosphine oxide</u>	3.6 (1.2)	H8	UDTM	<i>n</i> -Undecyl- β -D-thiomaltopyranoside	10.5 (0.21)
D9	APO12	<u>Dimethyldodecylphosphine oxide</u>	5.7 (0.57)	H9	DDTM	<i>n</i> -Dodecyl- β -D-thiomaltopyranoside	5 (0.05)
D10	C6E3	<i>Triethylene glycol monohexyl ether</i>	46 (23)	H10	S-8	<u>Sucrose8</u>	48.8 (24.4)
D11	C6E4	<i>Tetraethylene glycol monohexyl ether</i>	60 (30)	H11	S-10	<u>Sucrose10</u>	7.5 (2.5)
D12	C6E5	<i>Pentaethylene glycol monohexyl ether</i>	74 (37)	H12	S-12	Sucrose12	15 (0.3)

Note. The components in the detergent plate are shown along with their locations in the plate and concentrations used. The values in parentheses in the [Det] column are the CMC values for the detergents (as provided [with significant figures as shown] by the manufacturer. Detergents in **bold** were purchased from Avanti Polar Lipids, those in *italics* were purchased from Bachem, those underlined were purchased from EMD Biosciences, and all others were purchased from Anatrace. Detergents A3 through C9 are zwitterionic detergents, whereas detergents C10 through H12 are nonionic.

Table 2

Estimated errors.

	AqpZ	AqpZ	AqpZ	AqpZ	KcsA	KcsA	KcsA	KcsA
	H	$\sigma(H)$	L/H	$\sigma(L/H)$	H	$\sigma(H)$	L/H	$\sigma(L/H)$
A1	0.000	0.079			0.000	0.079	0.000	
A2	0.772	0.189	0.973	0.338	0.411	0.137	0.079	0.205
A3	0.697	0.178	0.764	0.295	0.988	0.219	0.593	0.210
A4	0.644	0.170	0.927	0.353	1.000	0.221	0.852	0.275
A5	0.431	0.140	0.695	0.362	0.311	0.123	0.609	0.417
A6	0.281	0.119	1.025	0.609	0.210	0.109	0.798	0.640
A7	0.393	0.135	0.705	0.386	0.248	0.114	0.938	0.624
A8	0.508	0.151	0.871	0.381	0.249	0.114	1.404	0.827
A9	0.693	0.177	0.454	0.213	0.383	0.133	1.065	0.515
A10	0.137	0.098	1.300	1.205	0.155	0.101	0.720	0.773
A11	0.150	0.100	0.938	0.911	0.182	0.105	0.393	0.539
A12	0.197	0.107	0.788	0.667	0.200	0.107	0.500	0.538
B1	0.130	0.097	0.724	0.894	0.157	0.101	0.650	0.728
B2	0.117	0.096	0.876	1.076	0.144	0.099	0.678	0.797
B3	0.160	0.102	0.933	0.862	0.226	0.111	0.801	0.608
B4	0.256	0.115	0.989	0.633	0.234	0.112	0.615	0.517
B5	0.242	0.113	1.043	0.681	0.208	0.109	0.621	0.569
B6	0.202	0.108	1.002	0.756	0.204	0.108	0.679	0.602
B7	0.312	0.123	0.947	0.538	0.145	0.100	0.866	0.893
B8	0.326	0.125	0.283	0.303	0.225	0.111	0.323	0.427
B9	0.291	0.120	0.634	0.447	0.260	0.116	0.873	0.579
B10	0.013	0.081	0.811	7.876	0.044	0.085	0.744	2.410
B11	0.134	0.098	1.554	1.400	0.167	0.103	0.916	0.824
B12	0.419	0.138	1.014	0.472	0.205	0.108	0.940	0.717
C1	0.627	0.168	0.808	0.324	0.505	0.151	0.787	0.357
C2	0.729	0.183	0.883	0.322	0.659	0.173	0.803	0.315
C3	0.343	0.128	1.270	0.626	0.234	0.112	0.912	0.639
C4	0.617	0.167	0.639	0.279	0.637	0.169	0.682	0.286
C5	0.537	0.155	0.765	0.338	0.503	0.150	0.793	0.359
C6	0.467	0.145	0.883	0.403	0.151	0.100	0.767	0.812
C7	0.258	0.116	0.788	0.547	0.231	0.112	0.763	0.581
C8	0.400	0.136	0.957	0.465	0.275	0.118	0.852	0.548
C9	0.559	0.158	0.842	0.353	0.633	0.169	1.017	0.382
C10	0.192	0.106	0.658	0.623	0.069	0.089	0.872	1.690
C11	0.158	0.101	0.742	0.769	0.094	0.092	0.664	1.136
C12	0.289	0.120	0.999	0.588	0.229	0.112	0.723	0.569
D1	0.154	0.101	1.979	1.519	0.261	0.116	0.949	0.608
D2	0.106	0.094	0.600	0.990	0.069	0.089	0.684	1.530

		AqpZ	AqpZ	AqpZ	AqpZ	KcsA	KcsA	KcsA	KcsA
		H	$\sigma(H)$	L/H	$\sigma(L/H)$	H	$\sigma(H)$	L/H	$\sigma(L/H)$
D3		0.096	0.093	0.853	1.252	0.064	0.088	0.709	1.651
D4		0.106	0.094	0.984	1.246	0.153	0.101	0.811	0.826
D5		0.316	0.124	0.842	0.495	0.364	0.131	0.800	0.438
D6		0.378	0.133	0.918	0.468	0.449	0.143	0.746	0.368
D7		0.428	0.140	0.876	0.421	0.449	0.143	0.945	0.432
D8		0.234	0.112	0.345	0.421	0.562	0.159	0.488	0.251
D9		0.408	0.137	0.149	0.220	0.585	0.162	0.275	0.190
D10		0.006	0.080	1.243	21.267	0.041	0.085	0.344	2.100
D11		0.009	0.080	1.457	16.568	0.050	0.086	0.842	2.243
D12		0.012	0.081	1.092	10.055	0.067	0.088	0.763	1.646
E1		0.355	0.129	1.349	0.643	0.250	0.115	0.814	0.570
E2		0.482	0.147	1.240	0.509	0.275	0.118	1.831	0.957
E3		0.410	0.137	1.403	0.612	0.326	0.125	0.953	0.527
E4		0.281	0.119	1.031	0.611	0.249	0.114	1.008	0.653
E5		0.238	0.113	1.257	0.785	0.249	0.114	0.928	0.620
E6		0.214	0.109	1.230	0.833	0.266	0.117	0.999	0.621
E7		0.452	0.143	0.916	0.421	0.269	0.117	1.112	0.662
E8		0.191	0.106	0.788	0.685	0.253	0.115	0.788	0.554
E9		0.260	0.116	0.625	0.481	0.221	0.110	0.772	0.605
E10		0.185	0.105	0.656	0.640	0.200	0.107	0.900	0.711
E11		0.116	0.096	0.870	1.073	0.142	0.099	1.014	0.999
E12		0.664	0.173	0.457	0.219	0.384	0.134	0.319	0.274
F1		0.662	0.173	0.778	0.307	0.687	0.176	0.783	0.303
F2		0.582	0.162	1.003	0.394	0.277	0.118	0.737	0.501
F3		0.583	0.162	0.974	0.385	0.548	0.157	0.935	0.385
F4		0.145	0.100	0.785	0.849	0.188	0.106	0.873	0.732
F5		0.671	0.174	0.763	0.300	0.644	0.170	0.632	0.270
F6		0.661	0.173	0.724	0.292	0.317	0.124	0.262	0.304
F7		0.637	0.169	0.557	0.251	0.162	0.102	0.311	0.566
F8		0.481	0.147	0.599	0.309	0.129	0.097	0.821	0.958
F9		0.675	0.175	0.660	0.271	0.838	0.198	1.194	0.386
F10		0.542	0.156	0.572	0.280	0.459	0.144	1.221	0.516
F11		0.843	0.199	0.604	0.229	0.893	0.206	1.225	0.385
F12		0.857	0.201	0.712	0.255	0.893	0.206	0.922	0.305
G1		0.588	0.162	0.839	0.343	0.819	0.195	1.033	0.346
G2		0.626	0.168	0.835	0.332	0.557	0.158	0.864	0.361
G3		0.816	0.195	0.785	0.280	0.856	0.201	0.620	0.232
G4		1.000	0.221	0.683	0.232	0.765	0.188	0.546	0.225
G5		0.919	0.209	0.826	0.277	0.719	0.181	0.542	0.232
G6		0.523	0.153	0.749	0.338	0.224	0.111	0.081	0.367
G7		0.635	0.169	0.788	0.316	0.854	0.200	0.740	0.263

	AqpZ	AqpZ	AqpZ	AqpZ	KcsA	KcsA	KcsA	KcsA
	H	$\sigma(H)$	L/H	$\sigma(L/H)$	H	$\sigma(H)$	L/H	$\sigma(L/H)$
G8	0.616	0.166	0.854	0.340	0.789	0.191	0.616	0.240
G9	0.503	0.150	0.550	0.287	0.576	0.161	0.453	0.238
G10	0.556	0.158	0.793	0.340	0.713	0.180	0.566	0.239
G11	0.712	0.180	0.757	0.291	0.826	0.196	0.641	0.241
G12	0.790	0.191	0.971	0.334	0.902	0.207	0.784	0.268
H1	0.448	0.143	1.124	0.491	0.938	0.212	0.543	0.203
H2	0.709	0.180	0.979	0.352	0.830	0.197	0.631	0.238
H3	0.682	0.176	1.013	0.368	0.866	0.202	0.811	0.280
H4	0.399	0.136	0.173	0.230	0.775	0.189	0.106	0.120
H5	0.647	0.171	0.865	0.335	0.774	0.189	0.750	0.278
H6	0.721	0.181	0.949	0.342	0.720	0.181	0.542	0.231
H7	0.642	0.170	1.084	0.399	0.677	0.175	0.531	0.236
H8	0.659	0.173	1.013	0.374	0.670	0.174	0.649	0.269
H9	0.782	0.190	0.892	0.314	0.774	0.189	0.478	0.206
H10	0.606	0.165	0.811	0.330	0.728	0.182	0.636	0.255
H11	0.736	0.184	1.105	0.382	0.826	0.196	0.900	0.309
H12	0.770	0.188	0.997	0.345	0.757	0.186	0.756	0.282

Note. As described in Results (“Error analysis of Western blot detection method” subsection), the standard deviation of a single measurement is given by $\sigma = 0.142 \cdot I + 0.079$, where I is the integrated background-corrected intensity (normalized to a scale of 0–1). This expression for σ , yielding the error $\sigma(H)$ or $\sigma(L)$ of a single High (H) or Low (L) measurement, respectively, can be calculated for the values of stability (High) on the size stability quad plots (Fig. 5). The size value is given as the ratio of Low/High (L/H); therefore, error arises from both the L and H measurements. The error $\sigma(L/H)$ is given by $(L/H) \cdot \text{SQRT} \{ [\sigma(L)]^2 + [\sigma(H)]^2 \} = (L/H) \cdot \text{SQRT} \{ [(0.142 \cdot L + 0.079)^2 / L^2] + [(0.142 \cdot H + 0.079)^2 / H^2] \}$.

The values of H and L/H , along with their associated errors $\sigma(H)$ and $\sigma(L/H)$, respectively, are tabulated for AqpZ and KcsA.