Chapter 32

A Spectrophotometric Turbidity Assay to Study Liquid-Liquid Phase Separation of UBQLN2 In Vitro

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Abstract

Liquid–liquid phase separation (LLPS) is hypothesized to be the underlying mechanism for how membraneless organelles or biomolecular condensates form inside both prokaryotic and eukaryotic cells. Protein LLPS is a biophysical process during which proteins demix from homogeneous solution to form protein-dense droplets with liquid-like properties. Disruptions to LLPS, such as changes to material properties of condensates or physicochemical parameters for LLPS onset, are implicated in neurodegenerative diseases and cancer. Therefore, it is essential to determine the physicochemical parameters that promote protein LLPS. Here, we present our UV-Vis spectrophotometric turbidity assay to characterize the temperature and concentration dependence of LLPS for UBQLN2, a protein that undergoes LLPS via homotypic interactions in vitro and forms stress-induced condensates in cells. Mutations in UBQLN2 cause amyotrophic lateral sclerosis (ALS) and disrupt UBQLN2 LLPS. We present a detailed expression and purification protocol for a C-terminal construct of UBQLN2 and how we use microscopy to image UBQLN2 LLPS. We use our UV-Vis assay to construct temperature–concentration phase diagrams for wild-type and mutant UBQLN2 constructs to determine the effects of domain deletions and/or mutations on UBQLN2 phase separation.

Key words Liquid-liquid phase separation, Turbidity, Microscopy, Spectrophotometric assay, LCST, UCST, Phase transitions, Phase diagrams, UBQLN2

1 Introduction

Liquid-liquid phase separation (LLPS) is a physical process that is hypothesized to underlie the formation of membraneless organelles or biomolecular condensates inside the cells, such as nucleoli, nuclear speckles, stress granules, processing bodies, and many more [1–4]. These condensates dynamically assemble and disassemble in response to stress and other cellular signals. Condensates are heterogeneous in composition, containing many biomolecules such as proteins, DNA, and RNA. Through selective enrichment and exclusion of these components, condensates modulate many

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cellular functions including transcription, translation, protein quality control, and transport mechanisms. Changes in expression levels and subcellular organization of macromolecules dynamically alter the conditions for condensate assembly and disassembly. Dysregulation of condensates is implicated in disease mechanisms of several neurological disorders and cancer [5–8]. Indeed, many disease-associated proteins including tau, TDP-43, and FUS all undergo LLPS in vitro and localize into condensates in cells [5, 9, 10].

In LLPS, biomolecules associate with each other to demix from a homogeneous solution into a phase-separated state with at least two liquid phases, specifically a biomolecule-poor (dilute) phase and a biomolecule-rich (dense) phase. The droplet phase exhibits liquid-like properties as these droplets coalesce and wet surfaces. The conditions to induce phase separation are determined by factors such as temperature, pH, salt concentration, and overall biomolecule concentration. In addition, recent studies have enumerated the molecular driving forces that promote LLPS of biomolecules. Common to all biomolecules that phase separate under physiological conditions is multivalency. Multivalency refers to a high number of sites that interact with other biomolecules. These "sites" can include multiple patches on folded domains and/or SLiMs (short linear motifs) in intrinsically disordered regions within macromolecules [4, 11–14]. Importantly, both homotypic (self) and heterotypic (e.g., protein-DNA, protein-protein) interactions promote phase separation among biological systems [15].

An important step to quantifying the phase separation behavior of a biomolecule is experimentally obtaining a phase diagram (*see* Fig. 1). Just as a phase diagram of water delineates the physiochemical parameters for solid, liquid, and gas phases, a phase diagram of a biomolecule delineates the conditions required for biomolecular phase separation into different liquid phases [16]. Although recent strategies enable the elucidation of phase diagrams inside the cells [17, 18], there is an extensive utility for obtaining temperature–concentration phase diagrams for purified proteins in vitro, where experimentalists have control over buffer conditions, temperature, and concentrations of biomolecules. Methods for obtaining phase diagrams include static and/or dynamic light scattering, centrifugation, microscopy, and temperature-ramp turbidity assays (discussed here) [19–21]. Temperature-ramp turbidity assays are used by several research groups to obtain the threshold or saturation concentration above which proteins undergo LLPS. At protein concentrations below the threshold, the protein solution exists in a single, homogenous phase.

Depending on the nature of biomolecular interactions that promote phase separation, proteins may phase separate as temperature is increased (lower critical solution temperature or LCST phase transition) or phase separate as temperature is decreased (upper

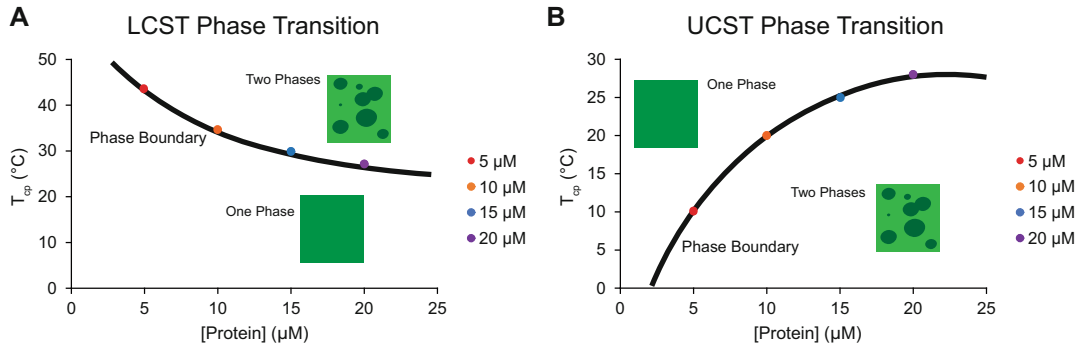

Fig. 1 Low-concentration arms of representative temperature–concentration phase diagrams for proteins undergoing (A) LCST (lower critical solution temperature) and (B) UCST (upper critical solution temperature) phase transitions. Cloud point temperatures (T_{cp}) are used to delineate the phase boundary (shown as a thick black line). Below the phase boundary for LCST phase transitions, the protein exists as a single-phase homogeneous solution. Above the phase boundary for LCST phase transitions, the protein demixes into a phase-separated solution consisting of a protein-dilute phase (outside droplets) in equilibrium with a protein-dense phase (inside droplets). The conditions for phase separation are reversed for proteins experiencing UCST phase transitions

critical solution temperature or UCST phase transition) (*see* Fig. 1). For example, systems whose phase separation is driven by hydrophobic interactions typically exhibit LCST phase behavior, such as PAB1 and UBQLN2 [22, 23]. Other systems whose phase separation is driven by polar and/or aromatic interactions often exhibit UCST phase transition behavior [24, 25]. Furthermore, a system may exhibit both LCST and UCST phase behavior, producing a closed-loop phase diagram or an hour-glass phase diagram [26, 27].

We have employed temperature–concentration phase diagrams to determine the molecular driving forces for UBQLN2, a protein involved in protein quality control mechanisms including proteasomal degradation and autophagy [28–30]. UBQLN2 is implicated in amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) [28, 31, 32]. UBQLN2 undergoes LLPS with both increasing salt concentration and temperature, similar to what would be expected for a biopolymer undergoing a LCST phase transition [23]. However, at least one UBQLN2 construct consisting of C-terminal residues 450–624 exhibits both LCST and UCST phase transitions between 16 and 60 °C, following the shape of a closed-loop phase diagram [27, 33]. We used temperature-ramp turbidity assays to rapidly screen the effects of amino acid substitutions on UBQLN2 phase separation [27, 33]. In this recent study, we generated a library of 95 single point mutants in UBQLN2 450–624 [27]. This minimal construct is easy to express and purify in bacteria, and we obtain milligram quantities that can be used for spectrophotometric turbidity assays. Using this mutant library, we determined that there were two classes of residues in UBQLN2,

specifically "stickers" and "spacers." Mutations at "sticker" positions substantially altered the shape and position of the LLPS phase boundary, whereas mutations in the "spacer" positions did not. Interestingly, hydrophobic amino acid substitutions at "stickers" modulated LLPS to the greatest extent, in line with expectations that UBQLN2 LLPS is driven in part by hydrophobic interactions. Furthermore, we also determined that certain disease-linked mutations of UBQLN2 promoted phase separation of UBQLN2 at lower temperatures and protein concentrations [33]. These same mutations also altered material properties of UBQLN2 condensates. Together, these experiments highlight the utility of elucidating temperature–concentration phase diagrams of proteins.

In this protocol, we describe how to express and purify UBQLN2 450–624, to construct the low-concentration arm of temperature–concentration phase diagrams for UBQLN2 using UV-Vis spectrophotometric assays, and to visualize UBQLN2 LLPS using bright-field microscopy. Critical issues, including salting-out purification techniques, preparation of protein samples for the turbidity assay and microscopy, preparation/cleaning of cuvettes, and spectrophotometric assay parameters, are discussed. Absorbance measurements at 600 nm are used as a proxy to monitor LLPS in the cuvette, while bright-field microscopy is used to visualize UBQLN2 droplet formation and droplet fusion. We present our methods for analyzing the temperature-ramp absorbance data using MATLAB scripts, but these can be adapted for other curve fitting software. By performing a series of temperature-based assays at different protein concentrations, a temperature–concentration phase diagram is constructed using cloud point temperatures (T_{cp}) of protein solutions. We define T_{cp} as the temperature at the midpoint (or inflection point) of the phase transition. This method has provided consistent results with UBQLN2 concentrations ranging from 5 μM to 300 μM. Our approach has enabled us to determine which domains and/or specific residues modulate phase separation of UBQLN2.

2 Materials

2.1 UBQLN2 450-624 W Expression and Purification

1. *UBQLN2 plasmid (Addgene 176852).*
2. *Chemically competent Rosetta2 (DE3) pLysS cells.*
3. *Ice and ice bucket.*
4. *PageRuler Unstained Protein Ladder 26614 for SDS–PAGE gel.*
5. *10-mL Fisherbrand Pipettes, Catalog No. 13–678-11E or equivalent pipette capable of dispensing up to 13.0-mL solution.*
6. *2.5-L Ultra Yield Flask, Part # 931136-B.*

7. *Sterilized LB Growth Medium:* 25 g LB powder, 1 L dH_2O in 2.5 L Ultra Yield Flask. Autoclave according to standard protocols.
8. Isopropyl β-D-1-thiogalactopyranoside *(IPTG)*.
9. *50-mM Tris pH 8, 1-mM EDTA buffer:* 6.057-g Tris and 0.372-g EDTA in 1-L dH_2O, pH adjusted to 8.0 (conc. HCl).
10. *Pierce Universal Nuclease or equivalent.*
11. *Lysis buffer:* pour 20-mL 50-mM Tris pH 8, 1-mM EDTA buffer into 50-mL conical tube, pipette 50 μL of 100-mM PMSF (in 100% ethanol, stored at −20 °C) and 50 μL of 1-M $MgCl_2$ into solution, and add 0.2-μL Universal Nuclease. Make immediately before use and store on ice until use.
12. *20-mM $NaPO_4$ buffer pH 6.8 (see* **Note 1***).*
13. *5-mL HiTrap Desalting Column (GE Healthcare).*
14. *Turner Model 340 Spectrophotometer or equivalent.*
15. *New Brunswick Scientific Excella E24 Incubator Shaker or equivalent.*
16. *Thermo Sorvall Legend XTR Refrigerated Centrifuge or equivalent.*

2.2 Protein Concentration Measurements at 280 nm

1. *NanoDrop ND-1000 Spectrophotometer or equivalent spectrophotometer* (e.g., *Thermo Scientific NanoDrop One) using 2-μL sample sizes.*

2.3 Turbidity Assay Experiments

1. *Agilent Cary 3500 UV-Vis Multicell (eight-cell) Peltier Spectrophotometer and Thermocycler or equivalent spectrophotometer with thermal cycling function (see* **Note 2***).*
2. *Microsoft Excel or preferred graphing software.*
3. *MATLAB or preferred curve fitting software.*

2.4 Turbidity Assay Preparations: Proteins, Solutions, and Buffers

1. *Ice and ice bucket (for preparation of protein samples).*
2. *Pipettes (P1000, P200, P20).*
3. *50-mL conical tubes (centrifugable).*
4. *15-mL conical tubes (centrifugable).*
5. *Concentrated UBQLN2 solution (or protein of choice):* Remove protein aliquots from the −80 °C freezer, and place them on ice to begin the thawing process.
6. *20-mM $NaPO_4$ buffer pH 6.8 (working buffer):* Same preparation as in Subheading 2.1.
7. *400-mM NaCl solution (2X Salt Solution, solution that induces phase separation of UBQLN2):* Transfer 30-mL 20-mM $NaPO_4$ buffer pH 6.8 to 50-mL conical tube. Add 0.9350-g NaCl to

solution. Dissolve NaCl. Transfer to a 50-mL graduated cylinder; fill to a 40 mL with buffer. Transfer back to a conical tube. Store at 4 °C.

2.5 Cuvettes

1. *Starna 28B-Q-10 Spectrosil Quartz Window Micro Cell, 10 mm path length, 0.7 mL.*
2. *Starna 28B-Q-10 Spectrosil Quartz Window Micro Cell Stopper.*
3. *Pressurized air.*
4. *Wash bottles* [3] *with ability to stream solutions.*
5. *Sparkleen or equivalent cleaning detergent.*
6. *Laboratory grade ethanol (100%).*
7. *Cuvette cleaning solution:* Transfer 800 mL of dH_2O into a beaker. Transfer 5 g of Sparkleen into the beaker and dissolve. Fill to 1 L with dH_2O. Transfer to a wash bottle.
8. *Ethanol:* 100% lab grade ethanol in a wash bottle.
9. *Deionized water (dH_2O):* Deionized water in a wash bottle.

2.6 Microscopy

1. *Concentrated UBQLN2 solution (or protein of choice):* Remove protein aliquots from −80 °C storage, and place them on ice to begin the thawing process.
8. *20-mM $NaPO_4$ buffer, pH 6.8:* Same preparation as in Subheading 2.1.
2. *Eisco Cavity Slides Catalog # S99368 or equivalent.*
3. *MatTek Uncoated Glass Coverslips 22 × 22 mm or equivalent.*
4. *Tweezers.*
5. *5% bovine serum albumin (BSA):* Dissolve 0.5-g BSA into 10-mL 20-mM $NaPO_4$ pH 6.8 buffer in a 50-mL conical tube. Store at 4 °C.
6. *Table-top incubator capable of 37 °C.*
7. *ONI Nanoimager (Oxford Nanoimaging Ltd.) or inverted microscope with at least a 20x objective; ours is equipped with an Olympus 100 × /1.4 NA objective and Hamamatsu sCMOS ORCA flash 4.0 V3 camera.*
8. *Fiji imaging software.*

3 Methods

Below, we describe our expression and purification protocols for UBQLN2, our temperature-ramp turbidity assay protocol for UBQLN2, and how we image UBQLN2 LLPS via microscopy. We provide specifics on important points regarding purification of UBQLN2 without affinity tags, sample preparation,

spectrophotometer setup, as well as data analysis to extract meaningful "cloud point" temperatures in construction of temperature–concentration phase diagrams. We include notes for steps that can be adapted for other proteins that phase separate with decreasing temperature (UCST phase transitions).

3.1 Expression and Purification of UBQLN2 450-624 W

This section explains the steps we use to express and purify UBQLN2 construct 450-624 W (*see* Fig. 2a). UBQLN2 450-624 W includes residues 450–624 and an additional C-terminal tryptophan for determining protein concentration via A_{280} measurements. We use its phase separation property to salt out UBQLN2 from bacterial lysate. This method yields milligram quantities of UBQLN2 450-624 W from 1 L of bacterial culture.

1. Transform UBQLN2 450-624 W in pET-24b(+) plasmid into Rosetta2 (DE3) pLysS cells using standard protocols. Select for plasmid-containing cells using LB plate containing 50 $\frac{\mu g}{mL}$ Kanamycin (Kan) and 35 $\frac{\mu g}{mL}$ Chloramphenicol (Chl). (Or streak UBQLN2 450-624 W in pET-24b(+) plasmid in Rosetta2 (DE3) pLysS cells from previously prepared glycerol stock onto a Kan/Chl plate.) Grow overnight at 37 °C.
2. Inoculate a 25-mL "starter" culture with a colony from overnight plate (autoclaved 225 mL Erlenmeyer containing 25-mL LB; add antibiotics to the final concentrations of 50 $\frac{\mu g}{mL}$ Kan and 35 $\frac{\mu g}{mL}$ Chl prior to inoculation). Allow the starter to grow at 37 °C in shaking incubator until turbid (usually 4 to 4.5 h).
3. Transfer 12.5-mL starter to 1 L "culture flask" (autoclaved 2.5-L Ultra Yield Flask containing 1-L LB solution prepared using tap water; add antibiotics as noted in step 3). Allow bacteria to grow in the culture flask at 37 °C in a shaking incubator until A_{600} of 0.6 to 0.8 is reached (approximately 4–5 h).
4. Induce expression with 500-μL 1-M IPTG in a shaking incubator at 37 °C overnight (12–16 h). To prepare 1-M IPTG, dissolve 11.915-g IPTG into 35-mL dH_2O, and bring up to a total of 50 mL with dH_2O. Mix well, aliquot into microfuge tubes, and store at −20 °C.
5. Pellet cells by centrifuging 1-L culture at 4000 × G for 15 min at 4 °C. Remove supernatant and properly dispose (e.g., bleach). Place a centrifuge bottle with a cell pellet in −80 °C freezer for ≥1 h.
6. Begin to thaw centrifuge bottle on bench. Before pellet is completely thawed, add 20-mL lysis buffer and mix with disposable pipette until the mixture is not chunky or viscous.
7. Transfer mixed lysis solution into centrifuge tubes. Centrifuge at 20,000 × G for 20 min at 4 °C.

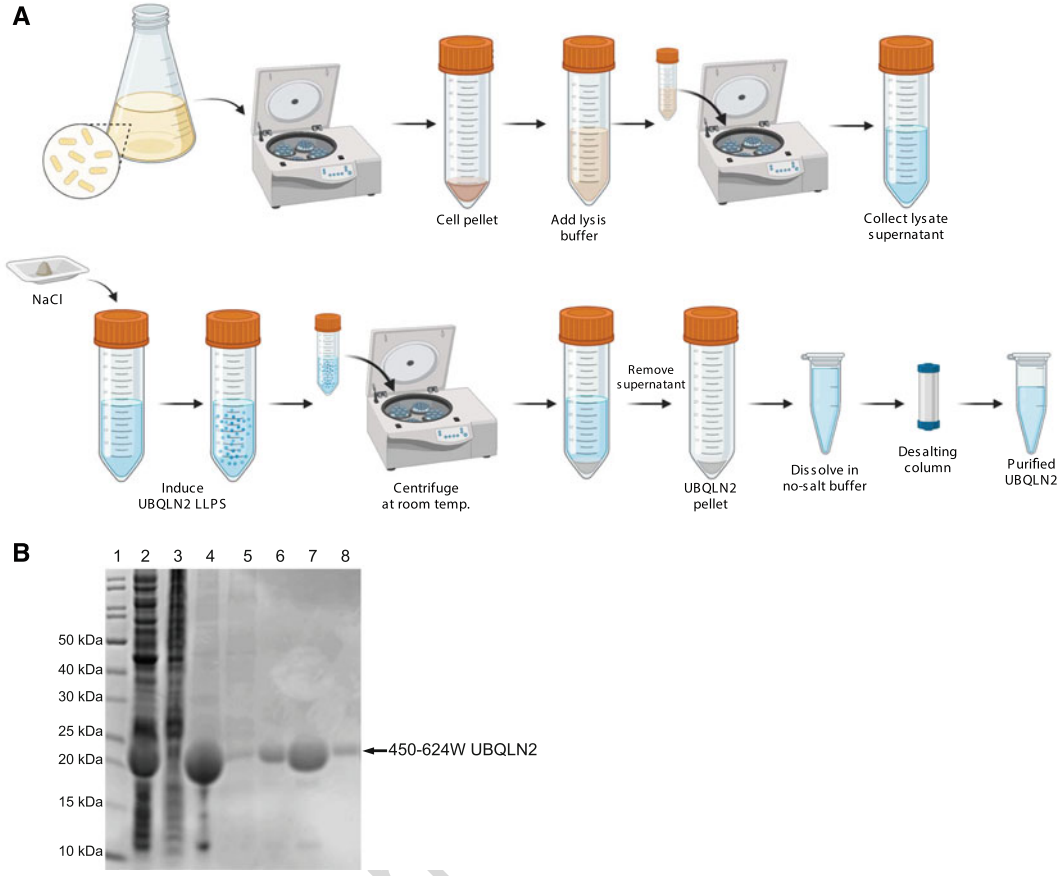

Fig. 2 (**a**) Schematic of "salting-out" UBQLN2 450-624 W purification method. Warm solution containing concentrated UBQLN2 450-624 W turns turbid when salt is added. Centrifugation of the turbid solution results in a pellet containing UBQLN2 450-624 W. Redissolving the pellet in fresh 20-mM $NaPO_4$ buffer and repeating salting-out steps purify the protein further. Please note that some protein is lost with each salting-out step. Created with Biorender.com. (**b**) SDS–PAGE gel of UBQLN2 450-624 W samples. (Lane 1) PageRuler Unstained Protein Ladder. (Lane 2) "Soluble" from step 9. (Lane 3) "Supernatant of 1st Salting Step" from step 11. (Lane 4) "Post-Salt" from step 12. (Lane 5) "Pre-Desalting Column" from step 14. (Lane 6) "Flow-Through" from step 17. (Lane 7) "E1" from step 17. (Lane 8) "E2" from step 17. The source of E1 is the fraction that is aliquoted and stored for later use. Refer to Subheading 3.1 for methods that produce these samples

8. Collect only the lysate supernatant into a 50-mL conical tube while not disturbing the pellet (*see* **Note 3**). Place 50-mL conical tubes into warm water for 10 min; before adding to the warm water, your solution should be translucent.
9. Obtain seven microfuge tubes to collect SDS–PAGE gel samples (*see* **Note 4**). Collect 10 μL sample from the soluble lysate fraction, and mix with 10-μL SDS buffer. This gel sample is labeled "Soluble" and kept refrigerated at 4 °C.

10. Induce phase separation of UBQLN2 by adding NaCl to a final concentration of 500 mM to the warmed conical containing lysate supernatant. Usually, you will have about 22-mL sample in the tube, so add 2.5-mL 5-M NaCl. Upon NaCl addition, solution should turn turbid.
11. Collect phase-separated UBQLN2 by spinning down conical tube in a room temperature centrifuge at 5000 × G for 15 min, and save the pellet (*see* **Note 5**). After spinning, quickly retrieve tubes and remove supernatant (take 10-μL sample from supernatant, and label this "Supernatant of 1st Salting Step" for SDS–PAGE gel). Collect and save the UBQLN2 pellet on ice.
12. Dissolve UBQLN2 pellet in 5-mL 20-mM $NaPO_4$ pH 6.8 buffer (without adding NaCl); this may take a while as protein solution may be gel-like. Use disposable pipette to mix while on ice; solution will be clear. Place tube in warm water for around 10 min. Take 10 μL of this "Post-Salt" sample for SDS–PAGE.
13. To further clean up the UBQLN2 sample, induce phase separation again by adding NaCl to a final concentration of 500 mM to the UBQLN2 Post-Salt solution. Solution should turn white. Centrifuge at room temperature at 5000 × G for 15 min.
14. Collect phase-separated UBQLN2 pellet on ice. Take 5-μL SDS–PAGE sample from supernatant ("Pre-Desalting Column"). Dissolve pellet in 1.5-mL 20-mM $NaPO_4$ buffer. This will be the longest dissolve during this protocol, so let the solution sit on ice for as long as it takes (*see* **Note 6**).
15. Transfer dissolved solution to 2-mL microfuge tube. Centrifuge for 3 min at 21,000 × g and 4 °C to remove any debris or aggregated protein. Keep sample on ice.
16. Prepare the 5-mL HiTrap Desalting/Exchange Column: After rinsing a 10-mL syringe with cold $NaPO_4$ pH 6.8 buffer, equilibrate column with this same buffer avoiding bubbles. Take up 3 mL of cold $NaPO_4$ pH 6.8 buffer into a 3-mL syringe and set aside.
17. Pour UBQLN2 sample (~1.5 mL) into a 3-mL syringe, and inject this sample into Desalting Column. Collect the 1.5-mL liquid in a microfuge tube labeled "Flow-Through." Next, inject 3-mL cold $NaPO_4$ pH 6.8 buffer into column. Collect the first 1.5–1.8 mL of eluent into a microfuge tube labeled "E1." Collect the remaining solution (1.5 mL) in "E2" microfuge tube. Leave all tubes on ice. Make 5-μL SDS–PAGE samples from each microfuge tube.
18. Check protein concentration using NanoDrop ND-1000. Record A_{280} and A260/A280. Use A_{280} to calculate protein

concentration in μM ((A_{280}*10^6)/5500). Check each microfuge tube three times to take average and standard deviation. Also, wash the Desalting Column with H_2O and then 5-mL 20% ethanol.

19. Flash-freeze 100-μL aliquots of "E1" using liquid N_2. Store at −80 °C.
20. Run a 15% SDS–PAGE gel to check protein purity and relative abundance. Visualize using Coomassie Blue Staining (*see* Fig. 2b).

3.2 Sample Preparation for Spectrophotometric Turbidity Assays

We explicitly describe preparation of turbidity assay samples for one point on the low-concentration arm of the temperature–concentration phase diagram, specifically the final concentrations of 100-μM UBQLN2 protein and 200-mM NaCl solution in 20-mM NaPhosphate buffer at pH 6.8. Note that the range of protein concentrations will need to be optimized for each protein and/or mutants (*see* **Note 7**). To ensure consistency and reproducibility, we recommend that turbidity assays be run with replicates of three for each protein concentration. As we typically load the cuvettes with 400-μL total solution, enough solution must be prepared at the correct protein and salt concentrations for a total of 1200 μL to account for the three replicates. We prepare turbidity assay samples by 1:1 mixing of 2X Protein Solution and 2X Salt Solution on ice (see "Materials" and **Note 8**). To account for potential pipetting errors, total amounts of 650-μL volume are prepared of each 2X Salt Solution and 2X Protein Solution for the three replicates. A summary of this protocol is presented in Fig. 3.

These preparatory steps can be used for other proteins that phase separate with increasing temperature (LCST phase transitions) and with increasing salt (e.g., systems whose phase separation is driven by hydrophobic interactions). It is important to prepare protein samples such that they are under conditions where phase separation does not occur (low salt and low temperature in the case of UBQLN2). For these reasons, we prepare UBQLN2 solutions on ice using a buffer without any added NaCl. UBQLN2 (and various constructs described in [23, 27, 33]) is expressed and purified from bacteria. During the last stage of purification as described in Subheading 3.1, UBQLN2 is exchanged into a 20-mM $NaPO_4$, 0.2% sodium azide, and pH 6.8 aqueous buffer solution (referred to as buffer from now on). In this buffer, UBQLN2 does not phase separate. All solutions for the turbidity assay will use buffer.

1. Using the protein stock concentration, estimate the volume necessary to make 2X Protein Solution in 650-μL buffer.
2. Frozen protein samples should be thawed on ice to minimize protein aggregation (*see* **Note 9**).

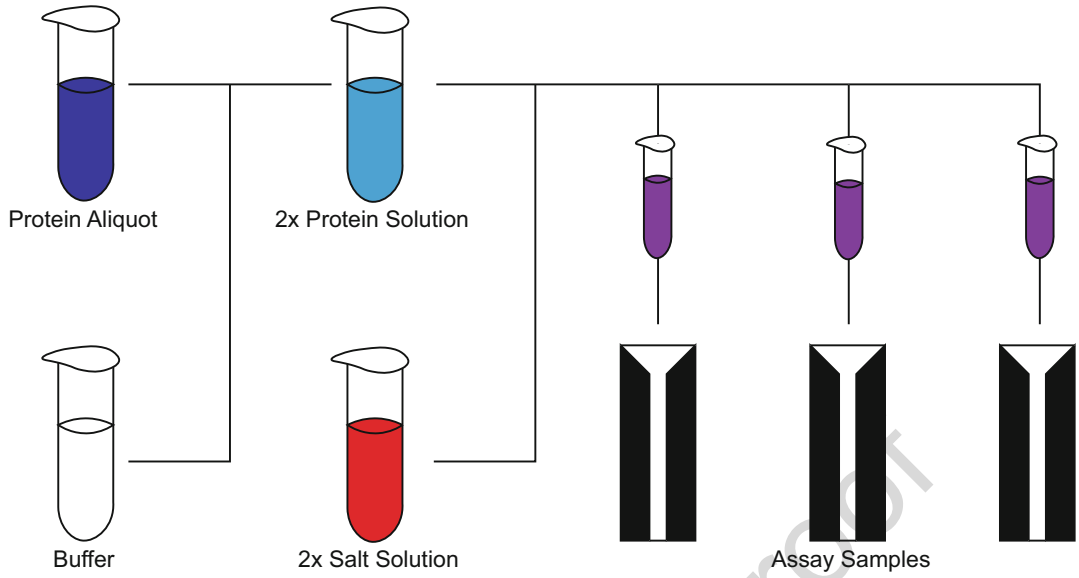

Fig. 3 Schematic for preparing turbidity assay samples. First, the concentrated protein aliquot is diluted with buffer to make the 2X Protein Solution (*see* Table 1). Then, 200 μL of 2X Protein Solution is mixed with 200 μL of 2X Salt Solution in microfuge tube before transferring to cuvettes for assay

Table 1 t.1
Preparation of the 2X Protein Solution for a turbidity assay using $C_1V_1 = C_2V_2$

Desired protein concentration for assay	Volume of protein stock solution	Volume of buffer	
100 μM	$\frac{(200\mu M)(650\mu L)}{950\mu M} = 136.8\mu L$	$650\mu L - 136.8\mu L = 513.2\mu L$	t.3
20 μM	$\frac{(40\mu M)(650\mu L)}{950\mu M} = 27.4\mu L$	$650\mu L - 27.4\mu L = 622.6\mu L$	t.4

3. After thawing UBQLN2 aliquots, invert the tubes several times to homogenize protein solution (*see* **Note 10**).
4. Centrifuge the thawed protein solution sample at 4 °C 21,000 g for 3 min.
5. Transfer the supernatants to a single new tube using a P200 pipette. Be careful not to disturb the pellet if present (*see* **Note 11**).
6. Measure protein concentration using Nanopore ND-1000 Spectrophotometer. Take the average of three A280 and A260/A280 measurements for protein concentration calculations (*see* **Note 12**). AU5 AU6
7. Prepare 650 μL of 2X Protein Solution into a separate microfuge tube, and place on ice for at least 5 min. If the desired protein concentration is 100 μM, the concentration of 2X

Protein Solution should be 200 μM (*see* Table 1). In this example, 136.8 μL of the protein stock solution (950 μM) will be diluted into 513.2 μL buffer for a final 650 μL of 2X Protein Solution. The extra 50 μL is present to account for pipette errors.

8. Pipette 200 μL of 2X Salt Solution into three microfuge tubes (for each replicate), and incubate on ice.
9. Prepare the reference solution that will be used to zero cuvette readings on the spectrophotometer. In a separate microfuge tube, mix 200-μL 2X Salt Solution with 200-μL buffer.

3.3 Preparing Cuvettes and Loading Samples for Turbidity Assay

1. Prepare a total of four cuvettes (*see* **Note 13**): three will be protein samples, with the fourth being the reference solution.
2. Clean the cuvettes (*see* **Note 14**) and dry using pressurized air or nitrogen. This should be done before and after every assay. Set dried cuvettes in ice alongside the microfuge tubes.
3. Pipette 200-μL diluted protein sample into each of the microfuge tubes already containing 200 μL of 400-mM NaCl, and mix by gently pipetting up and down. Immediately transfer the mixed solutions into their respective cuvettes (being careful not to introduce bubbles), place lid on top, and keep the cuvettes on ice (*see* **Note 15**).

3.4 Spectrophotometer and Thermal Cycle Setup

These next steps describe our spectrophotometric temperature-ramp turbidity method, assuming that we will monitor an LCST phase transition (protein phase separation as temperature is increased). As the protein solution begins to phase separate, the absorbance will increase rapidly. While our protocol is specific for the *Cary UV-Vis Multicell Peltier*, the method can be adapted for other equipment.

In this method, we monitor absorbance at 600 nm; however, absorbance can be monitored at other wavelengths (e.g., 350 nm; *see* **Note 16**). After a wait step for 2 min at 16 °C, we ramp temperature between 16 °C and 60 °C at 1 °C/min (*see* **Note 17**). We do not increase beyond 60 °C to avoid protein denaturation; however, this is specific to the UBQLN2 system. We include the wait step to ensure that the entire system is equilibrated before starting the temperature-ramp experiment (*see* **Note 18**). To monitor reversibility of the phase transition, the method can be further modified to include a temperature-ramp between 60 °C and 16 °C at 1 °C/min (*see* **Note 19**).

1. Turn on the *Cary UV-Vis Multicell Peltier* spectrophotometer. Open the "Cary UV Workstation" program on the computer. Click the plug icon toward the top right corner of the menu to connect to the spectrophotometer.

Table 2
Parameters employed for turbidity assay using spectrophotometer

Wavelength (s)	600.00 nm
Averaging time (s)	1.000
Spectral bandwidth (nm)	2.00
Stirring	No
Multiple experiments	1 zone
Start (°C)	16.0
Return (°C)	16.0
Detector module	Multicell Peltier UV-vis
Applied temperature	16.0 °C
Number of stages	2
Stage 1 settings	
Collect data	Yes
Data interval	1.0 °C
Rate	1.0 °C/min
End	16.0 °C
Hold	2 min
Stage 2 settings	
Collect data	Yes
Data interval	0.2 °C
Rate	1.0 °C/min
End	60.0 °C
Hold	0 min

2. Select the method used for the desired assay, or design a new one from the home screen (*see* Table 2) (*see* **Note 20**).
3. Set up the eight-slot configuration for the spectrophotometer on the method setup screen. Check to ensure each box signifying a well is correctly selected as "Unused" or "Sample." We place our reference solution (buffer + salt solution; *see* Subheading 3.2) in slot 8. Check the boxes corresponding to the wells that will be used for the experiment, and label the contents appropriately (to be used in the output .csv file).
4. Cool the spectrophotometer to 16 °C so that the spectrophotometer reaches equilibrium prior to the start of the assay by clicking "Apply Temperature."

3.5 Starting Data Collection

1. Remove each cuvette from the ice bucket, and wipe cuvette clean with lens paper (or Kimwipe). Set cuvette in its respective slot in the spectrophotometer. Ensure that the reference solution is in slot 8. Slide to close the lid, and click "Start a Collection" (triangle icon). Two prompts will appear: the first asks for a file name to save, and the second confirms sample names for each slot. Begin the assay by clicking "Save" and "OK," respectively.
2. Once the assay begins, ensure that data points are being properly collected according to the method and that the reference solution is properly zeroing the solution absorbances (*see* **Note 21**).
3. Upon completion, click the three dots toward the upper right corner, and select "Export to CSV"; this will place the .csv file in the "Downloads" folder. Use a USB stick to copy your data to a computer for data analysis.
4. Remove all cuvettes from the *Cary UV-Vis Multicell Peltier*, and clean them (*see* **Note 22**).

3.6 Data Analysis

This section will describe how to handle the data obtained from the turbidity assay. Please note that other methods will be required to confirm that the protein is indeed phase-separating and not aggregating (see Subheading 3.8 for verification via microscopy). We present two methods of obtaining the cloud point temperature at the midpoint (or inflection point) of the phase transition, either by GUI-based fitting to the data or by running a short script. We use MATLAB for data fitting, although other programs may be used.

1. Open the .csv file obtained from the assay. Data are organized in sets of two columns (temperature, absorbance reading) per stage of the method. If there is a wait stage in the method, delete the associated two columns (one column will be a stagnant temperature, and the other should be an unchanging absorbance value).
2. Normalize the data such that the minimum absorbance is zero for each sample. For each absorbance column, record the lowest value. Subtract this value from each of the absorbance values in the column.
3. Plot absorbance vs. temperature on a graph as shown in Fig. 4. Do note the decrease in absorbance at high temperatures (*see* **Note 23**).
4. Prepare for data analysis by importing the data file into MATLAB as "Column Vectors."
5. Remove row and column entries after the maximum absorbance value is reached for a given sample, to ensure a proper curve fit to the 4PL function in MATLAB.

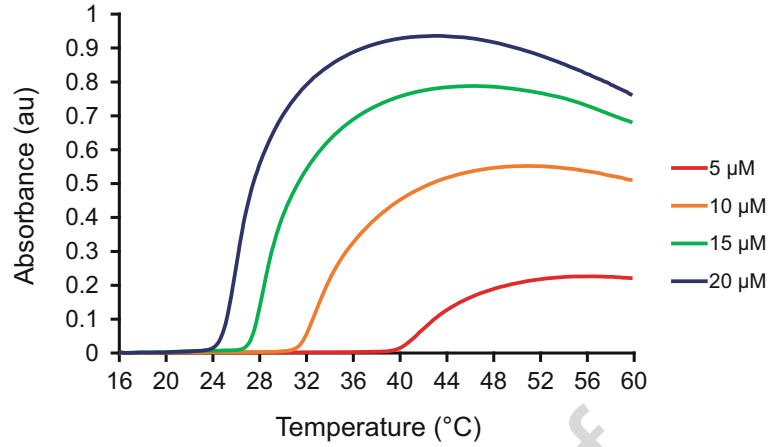

Fig. 4 Plot of four different turbidity assays for a UBQLN2 construct (not UBQLN2 450-624 W) varying in protein concentrations between 5 and 20 μM. All protein samples were prepared at pH 6.8, 20-mM NaPhosphate buffer with 200-mM NaCl. Absorbance (A_{600}) is used as a proxy for monitoring LLPS

6. Prepare for curve fitting by selecting the "Apps" tab and clicking "Curve Fitting" in the Apps window. Choose the temperature column as the x-axis and absorbance column as the y-axis.
7. Prepare fitting model by selecting" Custom Equation" and entering the following equation: f(x) = D+(A-D)/(1 + (x/C) ^B). This function is the four-parameter logistic regression eq. A and D are the minimum and maximum absorbance values, respectively, B is the slope of the transition, and C is the temperature at the inflection point of the curve (*see* **Note 24**).
8. Estimate the fitting variables (e.g., enter estimate for inflection point temperature and maximum absorbance value).
9. Record variable values and curve-fit statistics from the graph shown in Fig. 5. Check for curve fit accuracy. In some cases, you may need to decrease the range of temperature to improve the curve fit.
10. Alternatively, the code in Heading 4 may be used to analyze data in lieu of the Curve Fitting Tool App. The script prepares the data for analysis and executes the curve fit. Before running the code, delete the wait period columns from the .csv file if a wait period was used in the method. The code generates a .csv file containing variable values and goodness of fit statistics (*see* **Note 25**).

3.7 Construction of Low-Concentration Arm of Phase Diagram

This section describes the transformation of data from turbidity assays to assembling the low-concentration arm of a temperature-concentration phase diagram as shown in Fig 6.

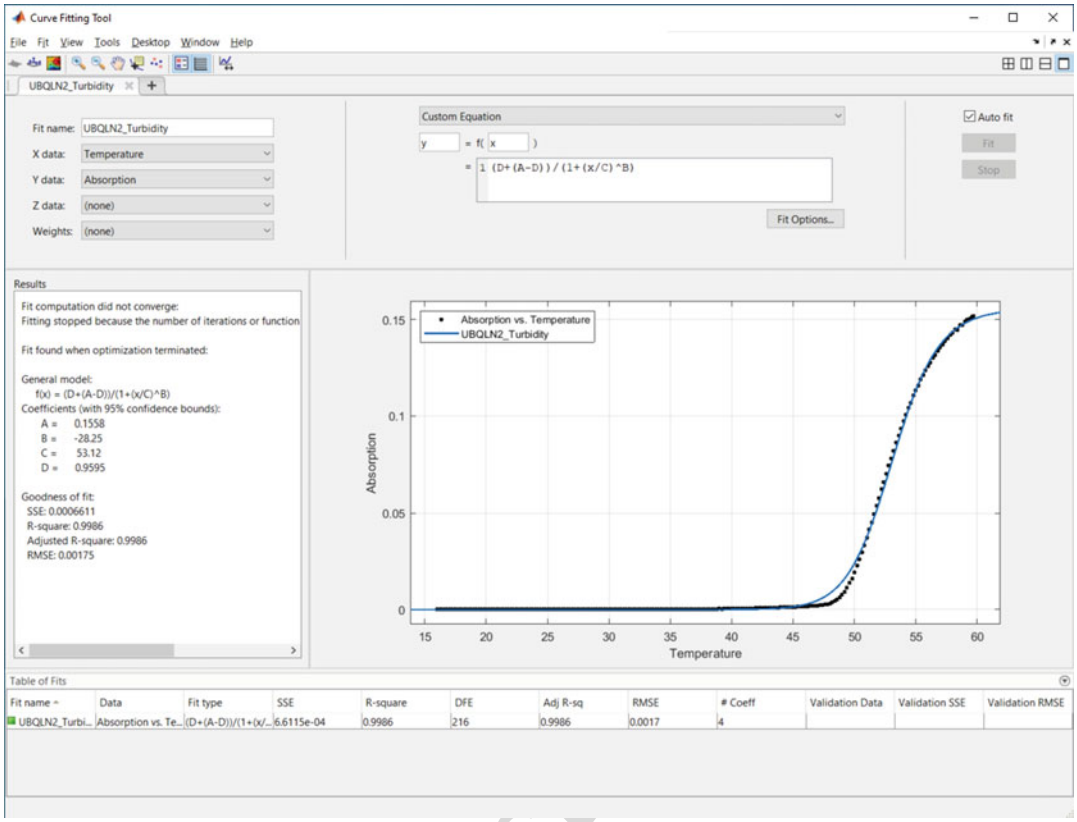

Fig. 5 Screen of the Curve Fitting Tool in MATLAB as applied to an example temperature-ramp spectrophotometric assay. Box A is where the 4PL equation is inputted. Box B contains the imported MATLAB objects to be used in the data fit. Box C is the graph and fit of data from Box B using the 4PL equation in Box A. Box D contains the fit parameter values in addition to statistical analysis based on the goodness of fit

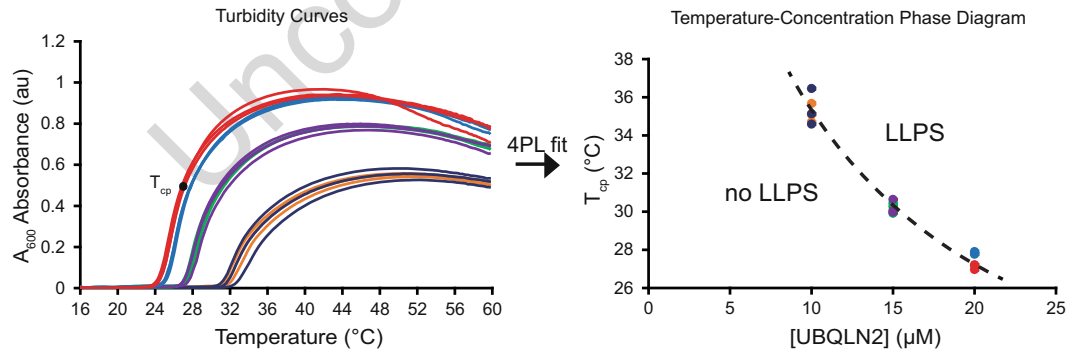

Fig. 6 Conversion of turbidity assay data into temperature–concentration phase diagram. Cloud point temperatures (T_{cp}) are extracted from individual turbidity assays for a UBQLN2 construct (not UBQLN2 450-624 W). Shown are actual data from two protein preparations (e.g., black and orange) with three replicates at each concentration. Dotted line on phase diagram indicates phase boundary with phase separation occurring above the line. Good practice requires running multiple turbidity assays and comparing the phase diagrams of at least two different preparations of the same protein

1. Temperatures at the inflection point/midpoint of phase transitions (C variable from Subheading 3.5) are plotted against the protein concentration. These values are used to construct the low-concentration arm of the temperature–concentration phase diagram.
2. Repeat turbidity assays for different protein concentrations and for at least two different protein purifications (*see* **Note 26**).

3.8 Confirming LLPS Behavior Using Bright-Field Microscopy

Turbidity assays monitor LLPS only by proxy, particularly as protein aggregation can also increase the turbidity of protein-containing solutions. Therefore, we use microscopy to image UBQLN2 droplet formation and monitor liquid-like behavior such as wetting and droplet fusion. Droplet sizes are on a micron scale. Sample preparation is conducted following the same steps as Subheading 3.2 except that 50-μL 2X Protein Solution is required per sample instead of 650 μL. Samples should remain on ice until ready for the experiment. Cavity slides may be reused, but we do not reuse glass coverslips (*see* **Note 27**).

1. Soak glass coverslip in 5% BSA solution for at least 1 h at room temperature up to overnight at 4 °C (*see* **Note 28**).
2. Use tweezers to remove glass coverslip from 5% BSA.
3. Fill two 50-mL conical tubes with 50-mL dH_2O, and label them "1" and "2." Using tweezers, dip coverslip into the conical tubes sequentially to wash the BSA off.
4. Tap corner of coverslip on dry Kimwipes several times until dripping stops.
5. Set on a slant in a closed container with Kimwipes lining the bottom and until dry.
6. Flush cavity slide with dH_2O at least ten separate times.
7. Set cavity slide on a slant in a closed container with one side touching a paper towel until dry.
8. Ready the microscope (we use an ONI Nanoimager). Turn on the computer connected to the microscope, and open the microscopy software to connect to the microscope. Set the chamber temperature to 37 °C. This may take up to an hour to equilibrate.
9. When both the coverslip and cavity slide are dry, mix the 2X Protein Solution with 2X Salt Solution 1:1. In this case, we mix 50-μL 2X Protein Solution with 50-μL 2X Salt Solution to make a 100 μL sample in a microfuge tube (*see* **Note 29**). Keep the sample on ice.
10. Pipette 50 μL sample into the cavity of the cavity slide. As it can be hard to tell which side of the cavity slide you are looking at, be sure to pipette the sample into the cavity instead of the flat

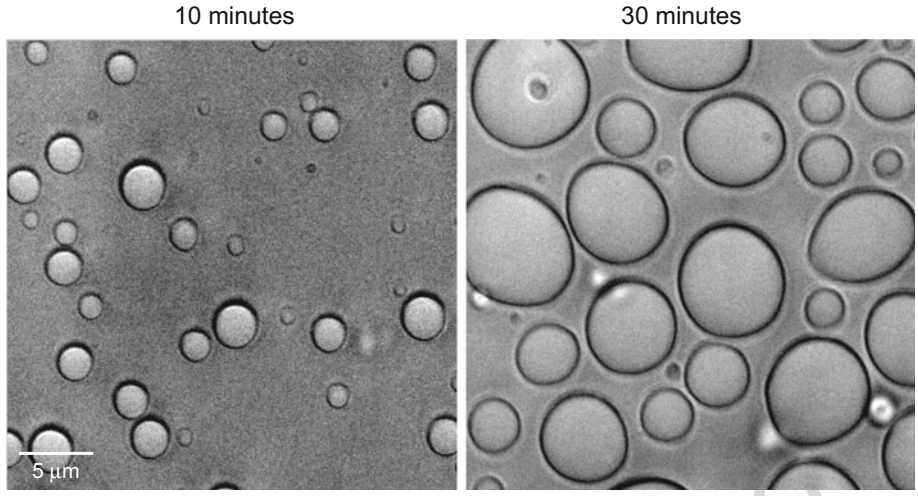

Fig. 7 Bright-field microscopy image of UBQLN2 450-624 W under phase-separating conditions. UBQLN2 droplets are round, fuse, and wet uncoated surfaces. Image is of 100-μM UBQLN2 450-624 W dissolved in 20-mM $NaPO_4$ buffer with 200-mM NaCl at 37 °C at indicated time points. Scale bar is 5 μm

surface on the opposite side. Practice can ensure you recognize the correct side without the need to touch the slide and risk contamination.

11. Set the coverslip over the cavity. The goal is to cover the cavity without introducing bubbles in the sample. Bubbles make imaging more difficult because they take up space where you could be imaging droplets (*see* **Note 30**). Gently press the edge of the coverslip with Kimwipes, and wipe off excess liquid to ensure that coverslip is set.
12. Set the covered slide into a 37 °C incubator for 15 min with coverslip facing down. We keep this incubator next to the microscope. As coverslip movement can displace the sample from the cavity, try to leave the coverslip undisturbed (*see* **Note 31**).
13. Immediately prior to imaging, place a small drop of oil immersion solution onto the coverslip (*see* **Note 32**).
14. Open the sample chamber on the microscope, and set the slide coverslip down so the oil droplet is above the objective (the ONI uses a 100X objective). Use the z-axis control to bring the stage down until the oil immersion solution contacts the objective.
15. Using z-axis control, adjust the stage until you see droplets that are not moving. This z-plane is the surface of the coverslip (*see* Fig. 7).
16. Move the x- and y-axes to image different parts of the slide. Keep in mind that most, if not all, protein-containing droplets

will sink to the coverslip as time goes on; thus droplet size will increase. As time continues, droplets may not be easily distinguishable as they will wet the entire coverslip surface.

17. Acquire and save images to a desired folder on the computer, and open TIFF files in Fiji.

3.9 Modifications to Monitor UCST Phase Transitions

Several systems undergo UCST phase transitions. Our UBQLN2 450–624 construct undergoes both LCST and UCST phase transitions over the 16 °C to 60 °C temperature range described in this protocol. To monitor the UCST phase transition, it is critical to prepare protein samples by mixing protein and buffer/salt solutions at temperatures above the UCST phase transition (e.g., incubation at 63 °C for at least 10 min). Cuvettes and microfuge tubes should be warmed to the incubation temperature to avoid any change that could result in phase separation prior to the assay. The UCST method for the spectrophotometer uses the same base parameters as shown in Table 1, with the exception of Stage 1 temperature set to 60 °C, and Stage 2 temperature range is set between 60 °C and 16 °C with a decreasing temperature rate of 1 °C/min.

4 Notes

1. Due to the nature of the preparation, these buffers contain approximately 20-mM Na^+. While UBQLN2 does not phase separate using this buffer, other systems may phase separate at these salt concentrations. Therefore, one can prepare a "no-salt" buffer, i.e., 20-mM HEPES pH 7.0 buffer. We have also prepared UBQLN2 solutions for turbidity assays using this alternative buffer. In preparing this buffer, we use ammonium hydroxide to adjust pH to 7.0. To prepare *20-mM $NaPO_4$ buffer pH 6.8*, obtain clean beaker and measure out 500 mL dH_2O. Place stirring magnet in beaker and set to 600 rpm. Transfer 12.0 mL of *1-M NaH_2PO4* into solution. Transfer 8.0 mL of *1-M Na_2HPO_4* into solution. Transfer 1.0 mL 20% sodium zzide into solution. Transfer solution to 1 L graduated cylinder. Fill to 950 mL with dH_2O. Measure pH to ensure pH is 6.8 and fill to 1 L. Filter using 0.2-μm filter paper. Store at 4 °C. *1 M NaH_2PO4:* Transfer 30 mL dH_2O into 50 mL conical tube. Weigh out 5.998-g sodium phosphate monobasic anhydrous, and dissolve in dH_2O. Fill to 50 mL using a graduated cylinder, and transfer back to the conical tube. Store at room temperature.*1-M Na_2HPO_4:* Transfer 30-mL dH_2O into 50 mL conical tube. Weigh out 7.098 g sodium phosphate dibasic anhydrous, and dissolve in dH_2O. Fill to 50 mL using a graduated cylinder, and transfer back to the conical tube. Store at room temperature. *20% sodium azide:*

Add 8-mL dH_2O to 15-mL conical tube. Weigh out and dissolve 2-g sodium azide in dH_2O. Bring volume up to 10 mL. Store at 4 °C.

2. We previously used a Beckman DU-640 instrument with a six-slot thermal unit for turbidity assay measurements; however this instrument is no longer in production. It is not expected that exact turbidity measurements will be transferable across spectrophotometers as each uses different heating elements, different cuvettes, and detectors. It is important to use the same spectrophotometer and cuvettes to ensure reproducibility of experiments. Periodically, it is recommended to rerun protein samples to ensure that the spectrophotometer is operating properly.
3. This separates other cell debris from the fraction containing UBQLN2 450-624 W. You may have to transfer into another centrifuge tube to spin again because spinning again removes even more cell debris from the desired fraction with a less likely chance of transferring unwanted debris into the 50-mL conical tube. The soluble fraction should be translucent.
4. These samples will be used for an SDS–PAGE gel to check protein purification at each step. This can be useful for troubleshooting any issues during the process. Samples are as follows: Soluble, Supernatant of first Salting Step, Post-Salt, Pre-Desalting Column, Flow-Through, Elution 1, and Elution 2.
5. This centrifuge step is done at room temperature to keep UBQLN2 under phase-separating conditions. If UBQLN2 is centrifuged at cold temperatures such as 4 °C, then it will dissolve in solution and no pellet will form.
6. Dissolving UBQLN2 in 1.5 mL is the last step before the Desalting Column. We have found that a single round of dissolving and salting-out (*see* Subheading 3.1, **steps 13–14**) yields clean samples of UBQLN2 450-624 W after elution from the Desalting Column. We define "clean" as A260/A280 $\leq$ 0.9 and >95% pure by SDS–PAGE. If a single round of salting-out does not clean the sample enough, you should repeat steps 13–14 in Subheading 3.1 at least once before desalting using the column again.
7. To find an initial range of protein concentration needed for this assay, you will likely need to test several initial concentrations over a broad range. For instance, assay a pure concentrated solution, then dilute by an order of magnitude repeatedly while assaying each dilution. For example, if the protein stock concentration was 1 mM, prepare protein solutions for turbidity assays at 100-μM, 10-μM, and 1-μM protein solutions.

8. The rationale for preparing 2X Salt Solutions for UBQLN2 is as follows. First, titrating in salt from a higher stock concentration (e.g., >5X) may induce localized phase separation in a small portion of the sample when salt is added. This could trigger nucleation events for LLPS if the sample is not subsequently properly mixed. Second, using a 2X solution ensures accuracy when pipetting as opposed to potentially pipetting small volumes from a 10X solution. Therefore, we aim for consistency and reproducibility across these experiments.
9. After purifying UBQLN2, the concentrated solution is divided into 100 μL or 200 μL aliquots and snap frozen in liquid nitrogen. The frozen samples are then stored at −80 °C. The protein is aliquoted to avoid repeated freeze-thaw cycles. Note that this procedure is specific to the protein of interest, and other proteins may be stored differently.
10. Concentrated protein solutions must be made homogeneous by inverting the tube or by gently pipetting up and down. Be careful not to introduce bubbles. This step is to eliminate locally concentrated portions of the solution from cryostorage.
11. After centrifuging, inspect the microfuge tube for any protein precipitant at the bottom. In our experience, we have seen little or no protein precipitant.
12. Use Beer–Lambert Law $A = \varepsilon cl$ to calculate the protein concentration. A is unitless absorbance at 280 nm, ε is the extinction coefficient specific to each protein in M^{-1} cm^{-1}, c is the concentration of protein in M, and l is the light path length in cm. The NanoDrop typically reports A280 assuming a 1 cm path length. Additionally, the 260/280 ratio should be noted. For a turbidity assay, we do not use protein with a 260/280 ratio greater than 0.90, as this indicates possible DNA/RNA contamination or microphase separation. We have noted that samples with higher 260/280 ratio (>1.0) phase separate more readily, possibly due to these contaminants.
13. We store our cuvettes in conical tubes containing 20 mL of cuvette cleaning solution.
14. We have found that the best way to clean these specific cuvettes is to use a stack of litmus pH paper that can be made into a brush. Use a stack as thick as can fit into the cuvette to scrub the insides. This is necessary as phase-separated protein may occasionally stick to the cuvette walls. Rinse cuvette with dH_2O to remove any leftover soap in the cuvette. After rinsing with dH_2O, use 100% ethanol to rinse the cuvettes. Let the ethanol sit for about 1 min before removing by flicking the cuvette gently. Use pressurized air for about a minute to dry the cuvette completely. Ensure ethanol is completely removed

as even trace amounts will disrupt the assay. Be sure to hold the cuvette up to a light source to spot any residual trace moisture inside.

15. We recommend recording the amount of time between preparing a protein sample and starting the assay. If there are differences in reproducing turbidity assay experiments, we suggest keeping this time consistent across sample preparations and turbidity assays.

16. In our experience, we have used a wavelength of 600 nm, but other labs have used wavelengths such as 400 nm or 350 nm. These lower wavelengths offer greater signal to noise, but these wavelengths should not be used if chromophores in the sample absorb light in this range (e.g., if the protein is fluorescently labeled). You may choose alternative wavelengths as long as you stay consistent across all turbidity experiments.

17. We choose a temperature-ramp rate of 1 °C/min as a tradeoff between keeping conditions at pseudo-equilibrium and minimizing protein sticking to the walls of the cuvette. Ideally, a slower temperature-ramp rate should be used to attain equilibrium conditions. However, we have noticed that the absorbance steadily decreases if the solution is left sitting at a given temperature (*see* Fig. 6). We notice this is a result of droplets fusing and sticking to the sides of the cuvette.

18. We have found that 2 min in the Agilent 3500 spectrophotometer is the minimal amount of time for our UBQLN2 sample to equilibrate; however, this will need to be optimized for each system.

19. Please note that the phase transition as temperature is decreased is unlikely to overlap with the phase transition when temperature was increased. This is due to system hysteresis. Droplet disassembly typically occurs at a different rate than droplet assembly. Hysteresis can be diminished if a slower temperature-ramp rate is used (e.g., 0.5 °C/min), but this is not ideal if protein droplets adhere to the cuvette surfaces.

20. Two stages are used here. The first stage is designed to equilibrate the cuvettes at 16 °C prior to the start of the turbidity experiment. The second stage is the actual temperature-ramp turbidity experiment.

21. The absorbance values should be zero or very close to zero at the start of the experiment. If they significantly deviate from this value, this means that the sample may already be phase-separating at these temperatures or the cuvette exteriors were not properly dried before placing in the spectrophotometer. If the former, the turbidity assay cannot be used to reliably determine the cloud point temperature. You will need to repeat the

assay by adjusting the starting temperature to a value below which the sample does not phase separate.

22. Rinse the cuvettes first with dH_2O, and let sit with dH_2O for a few minutes. This reduces the chance for residual protein to precipitate inside the cuvette. Next, use ethanol washes to remove residual protein, followed by dH_2O washes and rinses with cuvette cleaning solution. Store the cuvette in 50 mL conical tubes containing at least 20 mL of cuvette cleaning solution.
23. Note that the absorbance begins to decrease as temperature is further increased to 60 °C. This is a result of protein droplets sticking to the walls of the cuvette. For some systems, this behavior can be eliminated by coating the cuvettes, but this is system dependent.
24. The C value obtained from this method is equivalent to the midpoint of the curve. Other methods for T_{cp} calculation exist such as using the temperature at 10% the maximum absorbance or the inflection point. The most important thing is to be consistent throughout experiments when calculating T_{cp}.
25. MATLAB code to process turbidity assay data and fit the 4PL model to the data. This code is specific to modified data output from the Agilent 3500 spectrophotometer. With modifications, the code can be adapted to other instruments. Highlighted are file names to be changed.

```
% extract_Tcp_from_turbidity_assay.m

clear;
data_raw = xlsread ('Turbidity_Data_File.xlsx');
data = data_raw;


% Correct baseline.
[numRows,numCols]=size(data_raw)
    for Col=[2:2:numCols]
    %Find the min value in a columnm
    M=min(data_raw(:,Col))
    %Subtract the value from all elements in the column
    data(:,Col)= bsxfun(@minus,data_raw(:,Col),M)
end

% Delete data after maximum absorbance value is reached
for Col=[2:2:numCols]
    % Find max value in the absorbance column.
    [val,idx] = max(data_raw(:,Col))
    % Delete all absorbance readings past the max value.
    data(idx+1:end,Col) = [NaN]
    % Delete all temperatures past the max value.
    data(idx+1:end,Col-1) = [NaN]
end

% 4-parameter non-linear curve fitting.
ncol=size(data,2)
for n=1:2:ncol
    temp=data(:,n)
    abs=data(:,n+1)
[xData, yData] = prepareCurveData( temp, abs );

% Set up fittype and options.
% a and d are minimum and maximum absorbance values;
% b is the Hill slope, reflecting steepness; c is the temperature at the inflection point/ midpoint.
ft = fittype( 'd+(a-d)/[1+(x/c)^b]', 'independent', 'x', 'dependent', 'y' );
opts = fitoptions( 'Method', 'NonlinearLeastSquares' );
opts.Display = 'Off';
% adjust these values if necessary.
opts.Lower = [-Inf -Inf 16 -Inf];
opts.Upper = [Inf Inf 60 Inf];
opts.StartPoint = [0.01 10 30 1];

% Fit model to data.
[fitresult, gof] = fit( xData, yData, ft, opts );

% Plot fit with data.
figure( 'Name', '4PL' );
h = plot( fitresult, xData, yData );
legend( h, 'abs vs. temp', '4PL', 'Location', 'NorthEast', 'Interpreter', 'none' );
% Label axes
xlabel( 'temp', 'Interpreter', 'none' );
ylabel( 'abs', 'Interpreter', 'none' );
grid on

% Write fitted parameters and goodness of fit to result.
result(:,n)=coeffvalues(fitresult);
result(1,n+1)=gof.rsquare(1,1);
result(2,n+1)=gof.dfe(1,1);
result(3,n+1)=gof.adjrsquare(1,1);
result(4,n+1)=gof.rmse(1,1);

disp(fitresult)
end

csvwrite(' Turbidity_Data_File .csv', result)
```

26. It is very important to repeat turbidity assays using at least two or three separate protein purifications. This is a standard practice in our lab as we find it ensures consistency and reproducibility while also identifying problems with protein purifications.
27. Cavity slides may be reused using the following method: Soak cavity slide in 0.5 M KOH and set in a sonicator for 15 min. Flush both sides of the cavity slide with running dH_2O at least ten times each. It is recommended to repeat the wash at least once to ensure the cavity slide is cleaned. Dry using the same method as in Subheading 3.8, and store in a closed container where slides are not touching any other surfaces, including each other.
28. 5% BSA is used to prevent interaction of UBQLN2 with the coverslip surface.
29. When using microscopy to confirm protein LLPS, concentration can become an issue as LLPS is dependent on LLPS. In this example, we use 100-μM UBQLN2 for microscopy. That means the 2X Protein Solution contains 200-μM UBQLN2.
30. There are a few helpful considerations to avoid bubbles. Ensure the coverslip is completely dry and free of dust or other small particles as bubbles may nucleate on them when setting the coverslip. Try to set the coverslip at an angle to allow air to escape from one side as the coverslip lowers. Finally, care when pipetting the sample can reduce the chance that bubbles are introduced before setting the coverslip over the sample.
31. The cavity acts as a well for the sample. If the coverslip moves while in contact with the sample, it can drag sample out of the well and onto the flat surface of the slide. The droplets can wet the surface of the slide which prevents imaging of circular droplets as they would be in solution.
32. Oil should only be used with oil immersion lenses. Oil can ruin a lens that is not compatible with oil immersion technique.

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