Protocol for: Rapid, Optimized Interactomic Screening

BACKGROUND

This protocol utilizes cell powder, produced by cryogenic milling, and antibody conjugated magnetic beads. Full descriptions of procedures for preparing cell powder and antibody conjugated magnetic beads are located at http://www.ncdir.org/public-resources/protocols/.

We present two procedures for multi-well purifications. ***Protocol A*** *provides for twenty-four simultaneous affinity capture optimization experiments, carried out in a 96-well, deep-well microplate. We have optimized this procedure for E. coli and for human tissue culture – but it is likely adaptable to essentially any material that can be successfully pulverized and extracted. A primary feature of this procedure is the use of microtip probe sonication. For both E. coli and human cell lines, protein extraction may result in viscous solutions. Moreover, human cell powders often initially yield a somewhat inhomogeneous extract containing aggregates that require a more vigorous effort to homogenize than simple vortex mixing. Both of these attributes are detrimental to affinity capture quality. However, brief microtip probe sonication can cut viscosity and disperse aggregates resulting in cell extracts amenable to high quality affinity capture. Although we have implemented ice-water bath sonication using a microplate horn to successfully extract and homogenize S. cerevisiae cell powders (described subsequently in* ***Protocol B****), the problems associated with E. coli and human*

cell extracts were not readily resolved on a short time-scale by the same approach during our testing – necessitating a microtip probe. A second feature of this procedure is the use of high-speed centrifugation to clarify cell extracts. As such, established centrifugal clarification regiments may be applied while leveraging the throughput and convenience parallelization in the 96-well format. In principle, the only limiting factor in this procedure is the tedium of transferring more than ~twenty four reactions from the microplate to microfuge tubes for centrifugation and back for parallelized affinity capture; high-speed centrifugal clarification (e.g. 10 min at ~20k RCF, a typical regiment for thorough extract clarification prior to affinity capture) is not possible in microplates and standard bench top microcentrifuges accept only up to ~30 samples at a time. Using an 8-channel pipette with adjustable channel width simplifies and expedites the transfer from the microplate to 1.5 ml microcentrifuge tubes.

Protocol B *provides for up to ninety-six simultaneous affinity capture purifications in this same format. The primary feature that allows this throughput to be achieved is the parallel clarification of extracts by filtration in a 96-well format, as opposed to centrifugation. Filtration of cell extracts is non-trivial. Insoluble material and aggregates, a natural byproduct of cell breakage and extraction, rapidly foul most conventional filters of appropriate specification (e.g. pore size) to provide a clarified extract comparable in quality to that achieved by high-speed centrifugation. Hence, in this procedure we have implemented a multi-staged filtration approach (described in the main text) that addresses this*

common limitation. The filtration system presented was designed and optimized for S. cerevisiae cell extracts, but should be similarly effective on any extract generated from pulverized cell material in a similar fashion (i.e. extracted at comparable total mass and volume). Because yeast extracts do not typically exhibit issues related to viscosity, we chose sonication using a microplate horn in a chilled water bath to disperse and resuspend cell powders in the extraction solvents. Multi-microtip probe sonication can be equally applied here, as in ***Protocol A****, but during our tests we found that a brief water bath sonication with intermittent mechanical mixing provided rapid, simultaneous resuspension of all wells.*

Both procedures are elaborated below, including a multitude of alternative strategies we have tested at different steps to help facilitate implementation within most laboratories – where indicated, additional details may be found in the ***NOTES*** *section at bottom.*

SUPPLIERS

Here we indicate the suppliers of reagents and equipment we have used in the execution of these procedures. The product numbers are indicated where appropriate. However, in many cases an alternative supplier's (or self-made) product of comparable make and quality will equally suffice for these procedures. Naturally, initial testing should be carried out to ensure comparable performance.

Agilent Technologies (Santa Clara, CA); Beckman Coulter (Indianapolis IN); Corning (Corning, NY); Eppendorf (Hauppauge, NY); Life Technologies (Grand Island, NY); Rainin (Oakland, CA); Orochem Technologies (Naperville, IL); PHENIX Research Products (Asheville, NC); Promega (Madison, WI); QSonica (Newtown, CT); Roche Applied Science (Indianapolis, IN); Thermo Fisher Scientific (Waltham, MA); VWR (Radnor, PA);

REAGENTS

- Extraction solvents (see **Note 1**)
- Protease inhibitors cocktail (Roche #11 873 580 001, see **Note 2**)
- Liquid nitrogen (LN_2)
- 4x lithium dodecyl sulfate sample loading buffer (Life Technologies #NP0007)
- 500mM dithiothreitol (DTT)
- 4-12% Bis-Tris SDS midi-gels (Life Technologies #WG1403BX10) and appropriate running buffer

EQUIPMENT

Common to both protocols

- Dispensing manifold (**or** volumetric spoons, see **Note 3**)
- 2.5 ml 96-well deep-well microplates (square-well) with lids (VWR #37001-520)
- 96-well microplate cap mats (Thermo Scientific #4412-MTX)
- Sample rotator for microplates (Thermo Fisher #05-450-200, see **Note 4**)
- 96-well deep-well magnet (Promega #V3031)
- 96-well PCR plate magnet (Life Technologies #12331D).
- 250 µl 96-well PCR plate (Eppendorf #0030133390)
- PCR tube strip caps (Agilent Technologies #410096) or plate seals (Corning #3096)
- Vortex mixer w/ microplate adaptor (VWR # 58816-121 w/ #12620-878)
- Midi-gel system (Life Technologies #WR0100)

*24-well screens including centrifugation – **Protocol A***

- 8-channel pipette with adjustable channel width (Rainin #LA8-1200XLS)
- 96-well deep-well microplates (polypropylene, round or conical bottom) – 0.8 ml 96-well deep-well microplates (Thermo Fisher Scientific #AB-0765)
- 96-well microplate cap mats, pierced (see **Note 5**)

- Sonicator with 8-microtip probe (QSonica S4000 or Q700) **or** traditional microtip probe sonicator
- 1.5 ml microfuge tubes
- Bench-top refrigerated micro-centrifuge (Eppendorf #5417R)
- Bench top centrifuge with microplate carrier (Beckman Coulter Allegra 6R with GH 3.8 rotor and SX4750*μ* microplate carrier or Beckman Coulter Avanti J-26 XP with JS-5.3 rotor)

*96-well screens including filtration – **Protocol B***

- Standard 12-channel pipettes (**or** 96-channel pipette, see **Note 6**)
- 96-well deep-well microplates (polypropylene, round or conical bottom) – 1.2 ml 96-well deep-well microplates (PHENIX Research Products #M-0564)
- 96-well cell extract filtration unit (Orochem Technologies #OC21202DEPY)
- High-speed centrifuge with microplate carrier (Beckman Coulter Avanti J-26 XP with JS-5.3 rotor, see **Note 7**)
- (Optional) 2 mm Ø, stainless steel balls, Retsch part #22.455.0010 (see **Note 8**)
- Sonicator with Microplate horn sonicator (QSonica S4000 or Q700)
- (Optional) 300 µl 96-well 0.45 µm hydrophilic PVDF filter plate (Orochem Technologies #OC03PPT45) (see **Note 9**).

LEGEND

¡ATTENTION! – be careful here.

*HINT – added details that may make this easier.

✋REST – it is safe to stop here for some period.

These procedures use several multi-well plates indicated as they appear within the protocol:

SOLVENT PLATE – contains a matrix of extraction solvents.

PI PLATE – contains concentrated protease inhibitors.

BINDING PLATE – contains antibody-coupled magnetic media.

ELUTION PLATE – proteins are eluted from the affinity media using 1x LDS in this plate.

LOADING PLATE – Elutions are combined with 500 mM DTT (to ~50 mM final concentration) in this plate and subsequently loaded onto SDS-polyacrylamide gels.

Protocol A

Throughout this protocol a series of 24-wells within a 96-well plate (i.e. 3 consecutive columns) are used to hold extraction solvents, cell powders, cell extracts, magnetic beads, etc. in different microplates. The optimized procedure

implements 8-microtip probe sonication to homogenize extract volumes of ~500 µl in a 0.8 ml 96-well deep-well plate and an 8-channel adjustable-channel-width pipette for liquid manipulations. It has been successfully executed on E. coli and human cells (HEK 293 and HeLa Kyoto). It can also be carried out using a standard microtip sonication probe and a combination of a standard 8-channel and single-channel pipette. It is best to decide the orientation of the wells in advance – placing all e.g. solvents, cell powders, and magnetic beads in concordant wells of different plates will ensure that the orientation of the experiment is maintained throughout and the results will not be accidentally compromised. The 5 µl of magnetic beads slurry implemented in each affinity capture assumes a 15% w:v (300 mg : 2ml) slurry of antibody coupled Dynabeads M-270 or equivalent. In several steps, reagents must be dispensed to 24 wells from stock solutions, see ***Note 10*** *for recommended practices.*

1. Prepare an appropriate set of extraction solvents (see **Note 1**) to 2.2 ml each in a 2.5 ml 96-well deep-well microplate (SOLVENT PLATE) and set aside. This plate will be used in **Steps 4, 8, & 14-16**. After executing the following procedure ~400 µl will remain.

✋REST – We typically use 1x extraction solvents within ~24 hr of preparation. They may be held at RT if freshly made before use, or stored overnight, covered at 4°C. Prior to initiating the experiment, buffers should be equilibrated to RT for

the protein extraction, and then placed again at 4°C (or held on ice) for subsequent washing steps.

*HINT – Longer-term storage of solvents is possible, but remain vigilant of microbial contamination in non-sterile solutions and evaporative losses in unsealed storage plates.

2. Prepare a plate containing 2 µl of 500 mM DTT in each of 24 wells of a 250 µl 96-well PCR plate (LOADING PLATE). This plate will be included in the procedure by accepting fractions eluted in 1xLDS in **Steps 17 & 18** (see **Note 10**).

3. Prepare a 0.8 ml 96-well deep-well microplate with 5 µl of magnetic affinity medium slurry in each of 24 wells of a 0.8 ml 96-well deep-well microplate (BINDING PLATE). This will be used in **Steps 4 & 11-16**.

¡ATTENTION! – As beads may settle during dispensation, mix the stock periodically to ensure equivalent slurry is supplied to each well (see **Note 10**).

4. (OPTIONAL, see **Note 11**) Wash the beads in each well of the BINDING PLATE with 100 µl from the appropriate well of the SOLVENT PLATE and mix by pipetting up and down several times. Place the plate on the deep-well magnet, remove the washing solutions and hold covered at 4°C until ready for use (**Step 11**).

*HINT – Washed beads remain moist from the residual washing solutions and are stable for several hours. However, excessive drying of the beads should be avoided, as can occur due to evaporation on extended storage. If the BINDING PLATE is to be prepared more than several hours before the experiment, leave the beads within the storage or washing solutions, completing the preparation prior to executing the experiment.

5. Prepare protease inhibitors by dispensing 5 µl of 100x stock into each of 24 wells of a 0.8 ml 96-well deep-well microplate (PI PLATE, see **Note 12**). These inhibitors will be included in the procedure by combining with extraction solvent and transferring the mix to frozen cell powders in **Step 8**.

*HINT – Depending on the stability of the constituents of your protease inhibitors cocktail it may be feasible to prepare this plate and store frozen until use.

✋REST – In this way, it may be possible to execute steps 1-5 the day prior to the experiment. We typically carry out **Steps 2-5** the morning of the experiment and **Step 1** the night before or morning of.

6. Ensure that the SOLVENT PLATE and PI PLATE are equilibrated to RT.

7. Starting with cryomilled cell powder, dispense an appropriate amount to each well, using a dispensing manifold (described in the main text, Fig. 2, and Supplementary Fig. 1) or a volumetric spoon (see **Note 3**). Cell powders are

dumped onto the manifold surface and packed into all the wells using a packing tool (Supplementary Fig. 1). Excess powder is recovered using a spatula. A 0.8 ml 96-well deep-well microplate is placed on top of the manifold such that the openings of the wells of each device are aligned and face each other; and then the sandwich is inverted and given a firm tap from above (the underside of the manifold) – transferring the powders out of the wells of the manifold and into the wells of the 96-well plate (EXTRACTION PLATE). In case of any slight error or spillage during dispensing, a lint-free laboratory tissue paper or fine paintbrush can be used to brush away any powder that has accumulated between the wells of the deep-well microplate.

*HINT – We have found that ~25 mg of *E. coli* and ~50 mg of human cell powder can provide for robust colloidal Coomasie blue staining of all major components of a purified mixture. The procedures described here are optimized for the above given masses of cell powder. Very low molecular weight proteins (≳15 kDa) or proteins of very low abundance within the cell, or within the purified mixture, may fall below the limit of detection, however. In this case, utilizing a more sensitive stain may be appropriate. While using more cell powder is also possible, keep in mind this may require re-optimization of certain steps (e.g. **Step 9**).

¡ATTENTION! – Powders should always be held on LN_2 or dry ice when not in -80°C storage; dispensing occurs under LN_2 cooling and all utensils should be pre-cooled before initiating the procedure. It is useful to dispense your powders within three consecutive columns, excluding A1-H1 and A12-H12 (i.e. those

columns without a neighboring column on one side), of the deep-well microplate – we have observed that these edge columns can respond differently to sonication than inner rows.

✋REST – The microplate may be held on LN_2 until ready to proceed. Covering the plate with a cap mat may help reduce the accumulation of frozen condensate within the wells from moisture in the air during an extended pause – the mat will become rigid at subzero temperatures and does not need to be snuggly fitted.

8. Remove the EXTRACTION PLATE from LN_2 and allow it to stand ~2-3 minutes at RT. The powders will remain thoroughly frozen during this time. Add 475 µl (for 25 mg *E. coli* powder) or 450 µl (for 50 mg human cell powder) of each RT solvent from the SOLVENT PLATE to the PI PLATE – producing solvents including ~1x protease inhibitors. Transfer this entire mixture to the concordant wells of the EXTRACTION PLATE. Optionally, add one column (8-wells) of de-ionized water to the plate – this will allow the 8-microtip probe to be washed in between sonicating different samples (**Step 9**). Cover the plate with a cap mat that has had the caps from the appropriate columns pierced to allow the use of a multi-microtip probe sonication (see **Note 5**) and transfer the plate to 4°C.

¡ATTENTION! – Letting the plate stand briefly at RT and adding RT solvents helps ensure a more rapid and homogenous dispersal and extraction during the sonication, and results in a cold extract which should be subsequently maintained

at 4°C. Using solvents at 4°C for resuspension frequently results in the formation of ice at the powder / solvent interface and may significantly hinder subsequent resuspension. **Once RT buffers are added, all the remaining work prior to elution should be carried out within a controlled temperature room at 4°C.**

9. For ~50 mg of human cells, sonication is carried out for ~30 sec. using a QSonica S4000 with an 8-microtip probe using an amplitude setting of 15. For ~25 mg of *E. coli* use the same settings for 1 min. Apply sonication to one row, then proceed to the second row, and finally the third. By adding water to a column within the plate (**Step 8**), the 8-tip probe may be quickly and conveniently washed between sample columns to eliminate sample carryover (see **Note 13**).

¡ATTENTION! – This step may require empirical optimization for your particular sonication and microplate set-up. Please use these settings as a starting guide. Reasonable settings for human cell powder on most setups can probably be found between 15 sec – 1 min sonication at between 10 – 20 amplitude; similarly for *E. coli* at 1 – 3 min. We suggest keeping power low (below amplitude 20 on the QSonica S4000) and extending time as needed to avoid sample warming and loss due to excessive spraying or foaming. Complete resuspension can typically be assessed visually by holding the plate over head and looking at the bottom of the wells from underneath (to ensure materials is not trapped under ice). A thorough resuspension should appear translucent and homogenous when viewed

through the plate from underneath. Viscosity can be assessed by test pipetting selected wells – viscous wells will not pipette smoothly or completely and will appear snotty in consistency. Thus, in our procedure the minimum program applied to all samples should be that needed to resuspend and cut viscosity of the most demanding well – which varies according to extraction solvent – and thus some solvent matrices may be more or less demanding in sonication duration.

*HINT – This same procedure can be executed using a traditional sonicator and sequential sonication of each well – settings should be determined empirically. We have had success using a Misonix XL2020 with microtip probe on an amplitude setting of 2-3.

10. Using an 8-channel pipette with adjustable channel width, transfer crude extracts from the EXTRACTION PLATE to 24 x 1.5 ml tubes and centrifuge for 10 min, ~14k RPM at 4°C in a bench top microcentrifuge.

¡ATTENTION! – Number your tubes in advance, and array them in a tube holder in the same orientation as the multi-well plate (e.g. such that H1 is on far left, A1 is on far right when the plate is facing you oriented as 8-well rows).

11. Transfer the supernatants (clarified extracts) to the BINDING PLATE and cap the plate with a cap mat or other liquid-tight plate seal. Resuspend the beads fully within each well by manually inverting the plate several times. Place the

plate on a rotating wheel at moderate speed (just enough to prevent the beads from settling) for 30 min – 1 hr (see **Note 4** & **Note 14**).

*HINT – Transferring the clarified extracts back to a 96-well plate can be done using the same adjustable width 8-channel pipette, but one must be careful not to recapture pellet from within the 1.5 ml tubes, which may be a different proportion depending on the extraction solvent. We prefer to rapidly and precisely transfer supernatants back to a microplate from the 1.5 ml tubes, one-at-a-time, using a standard pipette. This maximizes extract quality by minimizing pellet carryover.

12. Remove the BINDING PLATE from the rotating wheel and briefly spin down to collect all liquid within the bottom of the well (Beckman Coulter Allegra 6R with GH 3.8 rotor and SX4750 microplate carrier or Beckman Coulter Avanti J-26 XP with JS5.3 rotor – 1 min at 2k RPM and 4°C).

13. Place the BINDING PLATE on a 96-well deep-well magnet to retain the beads at the well sides. Then remove the depleted extracts and remove the plate from the magnet.

14. Add 500 µl of concordant extraction solvent to each well of the BINDING PLATE (wash #1). Place the plate back on the magnet and alter its position several times to wash and mix. Park the plate back in the original position on

the magnet to collect and hold the beads at the well side and remove the wash. Then remove the plate from the magnet.

*HINT – By altering the position of the plate on the magnet, one can cause the beads to slide across the well interior to an adjacent position as well as diagonally across the well. We use 9 moves to thoroughly mix beads within each well. This avoid problems with resuspension by pipetting including foaming, incomplete resuspension, and spillage due to the displacement of volume in the well by the pipette tip.

¡ATTENTION! – With a standard magnet (including the deep-well magnet recommended in this procedure) one row of the plate may not be under the influence of the magnetism when placed at certain positions. Ensure that the appropriate movements are made that all the wells receive comparable mixing.

15. Repeat **Step 14** (wash #2), then proceed to **Step 16**

16. Add 200 µl of concordant extraction solvent to each well of the BINDING PLATE (wash #3). Pipette the solution in each well up and down several times to fully resuspend the beads and transfer them to a 250 µl 96-well PCR plate (ELUTION PLATE) placed on a 96-well PCR plate magnet. Removing the wash solution once the beads have adhered to the well sides. Remove the plate from the magnet.

¡ATTENTION! – The beads in the BINDING PLATE reside mostly adhered to the sides of the wells after wash #3 is removed, their position alternating to opposite sides of the well with respect to neighboring columns. By pipetting up and down against the appropriate side of the wells for each column, the beads will be easily resuspended in 200 µl volume within the fewest repetitions.

17. Remove the wash solution and add 18 µl of 1.1x LDS loading buffer to the sample. Cover with a strip tube caps or a plate seal and incubate the plate 5-10 min at between RT and 70°C with vigorous shaking. Re-equilibrate the plate to RT if heated. Briefly spin the plate to recapture all beads and loading buffer at the bottom of the wells as in **Step 12** (operation at RT is fine). Place the ELUTION PLATE on the 96-well PCR plate magnet and transfer the elutions to a plate containing 2 µl 500 mM DTT (LOADING PLATE).

*HINT – You may need to determine the best elution regime empirically. RT incubation minimizes the amount of IgG that co-elutes from the beads (typically below detectable levels by colloidal Coomassie blue staining) – elevating the temperature enhances the amount of IgG polypeptide chains can be observed in the eluate. Hence, RT elution is preferred. However, some tag/antibody interactions are of sufficiently high affinity that they require elevated temperature to be effectively eluted by ~1x LDS. Our observation is that affinity capture using either a Protein A based tag in combination with Rabbit IgG or the 3xFLAG-tag in combination with the M2 anti-FLAG antibody, elution can effectively be achieved

upon incubation at RT with ~1x LDS and vigorous shaking; whereas a GFP-tag in combination with our in-house generated llama polyclonal antibody requires incubation at 70°C to effectively elute.

18. Cover the sample-containing wells of the plate with strip caps or thermal seal and reduce the samples by heating to 70°C for 10 min. Cool to RT. Briefly spin the plate to recapture all the solutions at the bottom of the wells (as in **Step 17**).

¡ATTENTION! – If you would like to carry out proteomic analysis by in-gel digestion of excised bands after SDS-PAGE / protein staining, block cysteines by alkylation with iodoacetamide prior to loading on the gel (see **Note 15**).

19. Load all samples on a 26-well 4-12% Bis-Tris midi-gel and stain with colloidal Coomassie or by your preferred method.

*HINT – using a 26-well gel leave 2 free lanes, one for a molecular weight marker and one for a protein quantity standard. We typically load 10-50 ng of BSA as a quantity standard to help assess our protein recoveries within the screen.

¡ATTENTION! – If you would like to carry out proteomic analysis by in-gel digestion of excised bands after SDS-PAGE / protein staining, use an MS-compatible protein stain and handle the gel in a keratin and contamination conscious way (i.e. use clean solutions and containers and observe best practices for MS) (see **Note 15**).

Protocol B

This procedure facilitates the utilization of each well of a 96-well plate. The optimized procedure implements a combination of water bath sonications and vortex mixing to homogenize extract volumes of ~750 µl in a 1.2 ml 96-well deep-well plate and a 12- or 96-channel pipette for liquid manipulations. It has been successfully implemented on cell powder derived from baker's yeast. The water bath sonication / vortex mixing implemented permits that all reactions are resuspended simultaneously; hence one can process an entire multi-well plate without significant time-staggering of sample extraction, facilitating a single tagged strain to be screened for optimized affinity capture with 96 different extraction solvents or e.g. two strains with 48 solvents, three strains with 32 solvents, and four strains with 24 solvents. Generally, if there is little or no information about the purification of the protein of interest with an interacting partner available, one should adopt a 48- or 96-condition screen for the broadest coverage of the purification space. However, if desired, several factors may be screened in parallel against a smaller matrix of conditions when some prior knowledge exists. The 5 µl of magnetic beads slurry implemented in each affinity capture assumes a 15% w:v (300 mg : 2ml) slurry of antibody coupled Dynabeads M-270 or equivalent. In several steps, reagents must be dispensed to 96 wells from stock solutions, see ***Note 10*** *for recommended practices.*

1. Prepare an appropriate set of extraction solvents (see **Note 1**) to 2.2 ml each in a 2.5 ml 96-well deep-well microplate (SOLVENT PLATE) and set aside (see **Note 16**). This plate will be used in **Steps 4, 8, & 15-17**. After executing the following procedure ~400 µl will remain.

✋REST – We typically use 1x extraction solvents within ~24 hr of preparation. They may be held at RT if freshly made before use, or stored overnight, covered at 4°C. Prior to initiating the experiment, buffers should be equilibrated to RT for the protein extraction, and then placed again at 4°C (or held on ice) for subsequent washing steps.

*HINT – Longer-term storage of solvents is possible, but remain vigilant of microbial contamination in non-sterile solutions and evaporative losses in unsealed storage plates.

2. Prepare a plate containing 2 µl of 500 mM DTT in each of 96 wells (LOADING PLATE). This plate will be included in the procedure by accepting fractions eluted in 1xLDS in **Steps 18 & 19** (see **Note 10**).

3. Prepare a 1.2 ml 96-well deep-well microplate with 5 µl of magnetic affinity medium slurry per well (BINDING PLATE). This will be used in **Steps 4 & 11-17**.

¡ATTENTION! – As beads may settle during dispensation, mix the stock periodically to ensure equivalent slurry is supplied to each well (see **Note 10**).

4. (OPTIONAL, see **Note 11**) Wash the beads in each well of the BINDING PLATE with 100 µl from the appropriate well of the SOLVENT PLATE and mix by pipetting up and down several times. Place the plate on the deep-well magnet, remove the washing solutions and hold covered at 4°C until ready for use (**Step 11**).

*HINT – Handled in this way, the beads remain moist from the residual washing solutions and are stable for several hours. However, excessive drying of the beads should be avoided, as can occur due to evaporation on extended storage. If the BINDING PLATE is to be prepared more than several hours before the experiment, leave the beads within the storage or washing solutions, completing the preparation prior to executing the experiment.

5. Prepare protease inhibitors by dispensing 5 µl of 100x stock into each of 96 wells of a 1.2 ml 96-well deep-well microplate (PI PLATE, see **Note 12**). These inhibitors will be included in the procedure by combining with extraction solvent and transferring the mix to frozen cell powders in **Step 8**.

*HINT – Depending on the stability of the constituents of your protease inhibitors cocktail it may be feasible to prepare this plate and store frozen until use.

✋REST –In this way, it may be possible to execute steps 1-5 the day prior to the experiment. We typically carry out **Steps 2-5** the morning of the experiment and **Step 1** the night before or morning of.

6. Ensure that the SOLVENT PLATE and PI PLATE are equilibrated to RT.

7. Starting with cryomilled cell powder, dispense an appropriate amount to each well, using a dispensing manifold (described in the main article and supplementary materials) or a volumetric spoon (see **Note 3**). Cell powders are dumped onto the manifold surface and packed into all the wells using a packing tool. If dispensing more than one tagged strain powder, dividers are inserted into grooves on the manifold to avoid cross-contamination (See **Note 17**). Excess powder is recovered using a spatula. A 1.2 ml 96-well deep-well microplate is placed on top of the manifold such that the openings of the wells of each device are aligned and face each other; and then the sandwich is inverted and given a firm tap from above (the underside of the manifold) – transferring the powders out of the wells of the manifold and into the wells of the 96-well plate (EXTRACTION PLATE). In case of any slight error or spillage during dispensing, a lint-free laboratory tissue paper or fine paintbrush can be used to brush away any powder that has accumulated between the wells of the deep-well microplate.

*HINT – We have found that 150 mg of yeast cell powder can provide for robust colloidal Coomasie blue staining of all major components of a purified mixture. Very low molecular weight proteins (≳15 kDa) or proteins of very low abundance

may approach or fall below the limit of detection, however. In this case using more material or a more sensitive stain may be appropriate.

¡ATTENTION! – Powders should always be held on LN_2 or dry ice when not in -80°C storage; dispensing occurs under LN_2 cooling and all utensils should be pre-cooled before initiating the procedure.

✋REST – The microplate may be held on LN_2 until ready to proceed. Covering the plate with a cap mat may help reduce the accumulation of frozen condensate within the wells from moisture in the air during an extended pause – the mat will become rigid at subzero temperatures and does not need to be fit snuggly.

8. Remove the EXTRACTION PLATE from LN_2 and allow it to stand ~2-3 minutes at RT. The powders will remain thoroughly frozen during this time. Add 600 µl of RT solvents from the SOLVENT PLATE to the PI PLATE – producing solvents including ~1x protease inhibitors. Transfer this entire mixture to the concordant wells of the EXTRACTION PLATE. See **Note 18**.

¡ATTENTION! – Letting the plate stand briefly at RT and adding RT solvents helps ensure a more rapid and homogenous dispersal and extraction during the sonication, and results in a cold extract which should be subsequently maintained at 4°C. Using solvents at 4°C for resuspension frequently results in the formation of ice at the powder / solvent interface and may significantly hinder subsequent resuspension. **Once RT buffers are added, all the remaining work prior to**

elution should be carried out within a controlled temperature room at 4°C if working with a 12-channel pipette.

9. (OPTIONAL, see **Note 8**) – add 2 x 2 mm Ø steel balls to each well.

*HINT – For protein extraction within each well, we utilize a sequence of water bath sonication and vortex mixing, described below; we include 2 x 2 mm Ø stainless steel balls (e.g. Retsch part #22.455.0010) in each well to expedite resuspension of the powder and homogenization of the resulting extract during this process.

10. Cover the plate with a clean cap mat and make sure all the caps fit snuggly inside the wells. Perform a sequence of 1 min ice bath sonication (QSonica S4000, microplate horn, amplitude: 95) and 1 min vortexing (we use an adapter for microplates). Usually 3 cycles are enough to achieve complete and homogenous resuspension, which can be monitored visually by looking at the bottom of the plate (the steel balls sink to the bottom of the well when the mixture has been homogenized).

¡ATTENTION! – This step may require empirical optimization for your particular sonication and microplate set-up. Please use these settings as a starting guide. When resuspending without steel balls more sonication and vortexing cycles may be necessary. Resuspension is complete when the crude extract appears

translucent and homogenous when the plate is viewed from underneath with a light source above.

11. Using a 12-channel pipette transfer the crude lysate onto the filter plate sitting on top of the BINDING PLATE. Spin 5 min at 3.5k RPM in Beckman Coulter Avanti J-26 XP with JS5.3 rotor. See **Note 7**.

¡ATTENTION! – With this step we have experienced problems using the VIAFLO 96; some channels do not retain the extract solution well. Use a 12-channel pipette if you observe inconsistent pipetting at this step.

12. Seal the plate with a cap mat or other liquid-tight plate seal. Resuspend the beads fully within each well by manually inverting the plate several times. Place the plate on a rotating wheel at moderate speed (just enough to prevent the beads from settling, but not too fast as to precipitate the beads) for 30 min – 1 h (see **Note 4** & **Note 14**).

13. Remove the BINDING PLATE from the rotating wheel and briefly spin down to collect all liquid within the bottom of the well (Beckman Coulter Allegra 6R with GH 3.8 rotor and SX4750 microplate carrier or Beckman Coulter Avanti J-26 XP with JS5.3 rotor – 1 min at 2k RPM and 4°C).

14. Place the BINDING PLATE on a 96-well deep-well magnet to retain the beads at the well sides. Then remove the depleted extracts and remove the plate from the magnet.

*HINT – when using a 96-well pipette, the procedure is fast enough to be performed at RT. Otherwise use a 12-channel pipette and work in the cold room.

15. Add 500 µl of concordant extraction solvent to each well of the BINDING PLATE (wash #1). Place the plate back on the magnet and alter its position several times to wash and mix. Park the plate back in the original position on the magnet to collect and hold the beads at the well side and remove the wash. Then remove the plate from the magnet.

*HINT – By altering the position of the plate on the magnet, one can cause the beads to slide across the well interior to an adjacent position as well as diagonally across the well. We use 9 moves to thoroughly mix beads within each well. This avoid problems with resuspension by pipetting including foaming, incomplete resuspension, and spillage due to the displacement of volume in the well by the pipette tip.

¡ATTENTION! – With a standard magnet (including the deep-well magnet recommended in this procedure) one row of the plate may not be under the influence of the magnetism when placed at certain positions. Ensure that the appropriate movements are made that all the wells receive comparable mixing.

*HINT – using a 96-well pipette, the beads can also be pipetted up and down to wash. However, for the second wash (**Step 16**), after resuspending the medium in the wash, place the BINDING PLATE on the magnet and pipette up and down to wash the tips of residual affinity medium before removing the wash from the well.

16. Repeat **Step 15** (wash #2), then proceed to **Step 17**

17. (a) *Without filter plate* - add 200 µl of concordant extraction solvent to each well of the BINDING PLATE (wash #3). Pipette the solution in each well up and down several times to fully resuspend the beads and transfer them to a 250 µl 96-well PCR plate (ELUTION PLATE) placed on a 96-well PCR plate magnet. Removing the wash solution once the beads have adhered to the well sides. Remove the plate from the magnet.

(b) (OPTIONAL) *With filter plate* - alternatively add 200 µl of concordant extraction solvent to each well of the BINDING PLATE (wash #3). Pipette the solution in each well up and down several times to fully resuspend the beads and transfer them to a 0.45 µ filter plate (e.g. Orochem Technologies, OC03PPT45). Put the filter plate on top of a microplate with > 300 µl well size for flow-through collection and spin down for 5 min at 3k RPM and 4°C

(Beckman Coulter Allegra 6R with GH 3.8 rotor and SX4750 microplate carrier or Beckman Coulter Avanti J-26 XP with JS5.3 rotor).

¡ATTENTION! – The beads in the BINDING PLATE reside mostly adhered to the sides of the wells after wash #3 is removed, their position alternating to opposite sides of the well with respect to neighboring columns. By pipetting up and down against the appropriate side of the wells for each column, the beads will be easily resuspended in 200 µl volume within the fewest repetitions.

*HINT – in our experience collecting the beads on the filter plate is the quickest and most efficient way. When choosing a filter of a different make one has to be aware of the protein binding capacity of the filter (i.e. use low binding materials).

18. (a) *Without filter plate* - add 18 µl of 1.1 x LDS loading buffer to the beads. Cover with a strip tube caps or a plate seal and incubate the plate 5-10 min at between RT and 70°C with vigorous shaking. Re-equilibrate the plate to RT if heated. Briefly spin the plate to recapture all beads and loading buffer at the bottom of the wells as in **Step 13** (operation at RT is fine). Place the ELUTION PLATE on the 96-well PCR plate magnet and transfer the elutions to a plate containing 2 µl 500 mM DTT (LOADING PLATE).

(b) *With filter plate* – Using a multi-channel pipette add 10 µL of 1.1x LDS to the beads on the surface of the filter in each well. Cover with a cap mat or a plate seal and incubate the plate 5-10 min at between RT and 70°C with

vigorous shaking. Re-equilibrate the plate to RT if heated. Place the filter on top of the LOADING PLATE and collect the eluate by briefly spinning the plate (Beckman Coulter Allegra 6R with GH 3.8 rotor and SX4750 microplate carrier or Beckman Coulter Avanti J-26 XP with JS5.3 rotor – 1 min at 2.5k RPM, RT). Repeat the incubation with 10 µl fresh 1.1x LDS and repeat the centrifugation to capture a second eluate fraction also in the LOADING PLATE, giving ~20 µl final combined elution.

*HINT – You may need to determine the best elution regime empirically. RT incubation minimizes the amount of IgG that co-elutes from the beads (typically below detectable levels by colloidal Coomassie blue staining) – elevating the temperature enhances the amount of IgG polypeptides can be observed in the eluate. Hence, RT elution is preferred. However, some tag/antibody interactions are of sufficiently high affinity that they require elevated temperature to be effectively eluted by ~1x LDS. Our observation is that affinity capture using either a Protein A based tag in combination with Rabbit IgG or the 3xFLAG-tag in combination with the M2 anti-FLAG antibody, elution can effectively be achieved upon incubation at RT with ~1x LDS and vigorous shaking; whereas a GFP-tag in combination with our in-house generated llama polyclonal antibody requires incubation at 70°C to effectively elute.

*HINT – when using the optional filter plate for elution, eluting twice with 10 µl gives a more consistent and effective elution than a single 20 µl volume.

19. Cover the LOADING PLATE with thermal seal and reduce the samples by heating to 70°C for 10 min. Cool to RT. Briefly spin the plate to recapture all the solutions at the bottom of the wells (as in **Step 13**; operation at RT is fine).

¡ATTENTION! – If you would like to carry out proteomic analysis by in-gel digestion of excised bands after SDS-PAGE / protein staining, block cysteines by alkylation with iodoacetamide prior to loading on the gel (see **Note 15**).

20. Load all samples on a 26-well 4-12% Bis-Tris midi-gel and stain with colloidal Coomassie blue or by your preferred method.

*HINT – using a 26-well gel leave 2 free lanes, one for a molecular weight marker and one for a protein quantity standard. We typically load 50-100 ng BSA to help assess our protein recoveries within the screen.

¡ATTENTION! – If you would like to carry out proteomic analysis by in-gel digestion of excised bands after SDS-PAGE / protein staining, use an MS-compatible protein stain and handle the gel in a keratin and contamination conscious way (i.e. use clean solutions and containers and observe best practices for MS) (see **Note 15**).

NOTES

1. Extraction solvents can be prepared by hand or by using robotic automation. We have successfully automated this procedure using The Formulator liquid handler, from Formulatrix (Waltham, MA), a device typically used for preparing protein crystallization screening matrices, and also using a Hamilton (Reno, NV) STAR liquid handling workstation (an example solvent mixing program is included within the Hamilton Methods Supplement accompanying this publication). Extraction solvent formulations used in the present study are provided in the supplementary materials.

2. Use an EDTA-free protease inhibitor cocktail if you plan to explore the effect of this reagent or of divalent cations on affinity capture optimization. Although chelation will restrict the activity of metalloproteases, divalent cations are also important to many protein classes and chelation may affect interaction stability and co-purification profiles. Also, keep in mind that other reagents may chelate or sequester metal ions (e.g. phosphate or citrate).

3. In the absence of a dispensing manifold, volumetric spoons may be used to add powder to each well of a liquid N_2 cooled multi-well plate, one well at a time. Our favorite: Norpro 3080 Mini Measuring Spoons, 5 Piece Set available though www.amazon.com. Using this set we have found that, e.g. one heaping scoop using ‘smidgen’ gives ~50 mg and one level scoop with ‘dash’

gives ~250 mg. The user must achieve an initial feel for the 'size' of the scoop, but dispensing powders within +/- 20% by mass can generally be achieved with practice.

4. The product indicated is a wheel intended for mixing solutions within test tubes. However, a microplate fits securely on this wheel between the metallic test tube holders and the central knob. A setting of 40 RPM provides excellent mixing for batch binding.

5. The sonication of ~0.5 ml volumes in a 0.8 ml deep-well microplate can result in some sample spraying from the well depending on the power transmitted and the probe depth within the sample. An excellent implementation will minimize these effects, and best results are observed when the probe can be submerged to a depth at the mid-point or below within the sample. Using the 8-microtip probe described in **Protocol A**, the probe depth with respect to sample is suboptimal (a limitation in the current design of the 8-microtip probe) and it is therefore useful to have a splashguard to protect against inadvertent cross mixing of samples during sonication. A splashguard can be constructed with a scalpel by manually cutting an "X" into each cap on the mat corresponding to a well that you would like to sonicate. In a first test, fill the corresponding wells of a 96-well plate with water and then cover with the pierced cap mat. Gently lower the multi-microtip probe into the wells that have

been pierced and run at the maximum intended power and duration of use. This will result in the “X’s” cut into the caps becoming small holes of roughly the diameter of the sonication probe microtips. Each well of a subsequent experiment can now be sealed at the perimeter by using this mat, providing a reduced diameter orifice that allows probe insertion while limiting liquid escape.

6. We have successfully executed **Protocol B** using a VIAFLO 96, 96-channel pipette (Integra Biosciences AG, Zizers, Switzerland) – with the exception of **Step 11**. The use of a 96-channel pipette greatly expedites the processing of samples and is fast enough to be performed at RT without apparent deterioration of protein complexes.

7. When the 96-well cell extract filtration unit (Orochem Technologies #OC21202DEPY) is stacked upon the 1.2 ml 96-well deep-well microplates (PHENIX Research Products #M-0564) the resulting assembly is too tall to fit in carriage of the Beckman SX4750µ microplate carrier. Although SX4750µ microplate carrier can be operated without the carriages, we recommend using the Beckman Coulter JS-5.3 rotor for ease of handling and surety of operation.

8. These expedite the resuspension process initiated in **Step** 10. The balls may be added en masse at precisely 2 per well using a precision machined at manifold (similar to the one for dispensing powders – see Supplementary Figure 1c).

9. We have found that SpA-tagged proteins can be efficiently eluted from IgG coupled magnetic beads in a small volume of ~1x LDS at RT from within the well of a filter plate. Working from within this filter plate simplifies the elution stage of this procedure as described in **Step 18** of **Protocol B**.

10. When dispensing a single stock solution to multiple wells, it is helpful to first dispense this stock to a reservoir or strip tubes and then use a multi-channel pipette to dispense to the many wells of a 96-well plate. A reservoir requires excess reagent be added whereas the use of strip tubes necessitates pre-allocation of the reagent to the individual tubes on the strip, but creates less waste and greater storage flexibility for the remaining reagent. Our tendency is to dispense inexpensive reagents, e.g. 1x LDS (**Step 17, Protocol A; Step 18, Protocol B**), from a reservoir (Thermo Fisher #8075) and to dispense expensive reagents, e.g. magnetic affinity media (**Step 3**) from PCR strip tubes (Agilent Technologies #410092). The latter approach ensures that no beads are sacrificed and that the remainders can be returned to storage; the former is simply less effort – and remainders can often still be recaptured from

the reservoir as desired. In the case of dispensing magnetic beads, PCR strip tubes are especially recommended because the tubes enable the slurry to be maintained as a suspension by pipetting up and down periodically, whereas the beads tend to settle within a trough.

11. The 5 µl beads slurry is only ≲1% the total cell extract volume, and therefore the residual storage buffer is not likely to exert any discernable impact on the resulting purification profile. However, pre-equilibrating the beads is a best practice.

12. E.g. dissolve one Roche complete EDTA-free protease inhibitor tablet (product #11 873 580 001) in 500 µl of Milli-Q water (18 MΩ cm deionized water), giving a 100x solution.

13. It may be important to eliminate carryover between neighboring columns during probe sonication. Adding one column of water to the plate allows the multi-tip probe to be washed between samples by brief sonication (~5 sec) within the water, eliminating potential extract carryover. Also adding a row of cotton wool can provide for drying of the washed tips by brief touching to the tips, wicking away the water – although we do not implement this latter measure for our work.

14. We routinely use 1 hr binding for preliminary tests, but typically restrict binding to the shortest time giving ≥ 70% recovery of the tagged protein. Within limits, as the binding time increases the yield of the handle and co-purifying proteins also typically increase, however the non-specific background may increase disproportionately during long incubations. The optimal binding time has to be determined empirically.

15. While MS data can often be obtained directly from gel produced as a result of multi-well screening, promising results generated in multi-well format should be repeated in single tube format to verify the reproducibility of the result. If the reaction scale needs to be increased to promote detection by MS, we recommend pooling multiple identical affinity capture experiments rather than increasing scale for a single reaction.

16. Adding 1:1000 Antifoam B per well (right before use, as it is not sterile) will help reduce the foaming during sonication steps and we have not observed it to negatively affect the affinity capture.

17. Keep in mind that when processing different strains within the same plate, care must be taken not to cross-contaminate the samples, this is achieved by pre-weighing the powder to just in excess of that required to fill the cylinders within the manifold. For example, to fill 24 cylinders at 150 mg per purification

(for processing 4 strains in parallel across 24 extraction solvents), 3.8 g may be applied to each section of the manifold (consisting of 200 mg surplus powder to ensure each well is equally and completely filled). Carefully recover the small amount of remaining excess powder in each section, brush to the sides any minute remainders, before removing the dividers (see Supplementary Fig. 1) and transferring the powder to the 96-well plate.

18. We use 1.2 ml deep-well plates to combine 150 mg of cell powder per well with 600 µl of buffer. The 1:4 ratio of cell material to extraction solvent is typically enough to stabilize the pH of the extract and has frequently exhibited improved yield when compared to more dilute proportions (such as our prior standard of 1:9 for bench-scale preparative work). The final lysate volume is ~750 µL.