Studying protein-protein interactions in budding yeast using co-immunoprecipitation.

Running title: Co-immunoprecipitation in budding yeast cells

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SUMMARY

Understanding protein-protein interactions and the architecture of protein complexes in which they work is essential to identify their biological role. Protein co-immunoprecipitation (co-IP) is an invaluable technique used in biochemistry allowing the identification of protein interactors. Here, we describe in detail an immunoaffinity purification protocol as a one step or two-step immunoprecipitation from budding yeast *Saccharomyces cerevisiae* cells to subsequently detect interactions between proteins involved in the same biological process.

Key words: co-immunoprecipitation, immunoaffinity purification, magnetic beads, antibody, protein interactions, protein complexes, cell extract, Freezer/Mill grinder

1. INTRODUCTION

Proteins are important building blocks in cellular architecture and the vast majority of them interact with other proteins to form multi-protein complexes and perform particular cellular tasks. Biochemical analysis of protein complexes and identification of their components has been fundamental for the biological understanding of their function [1,2].

Protein-protein interactions are mediated by a variety of bonds, namely the combination of hydrophobic bonding, van der Waals forces, and salt bridges and might involve specific binding domains on each protein or the whole protein itself. Interaction between proteins might be stable or transient, which is reflected by whether such proteins bind in a strong or weak fashion. Stable interactions are those associated with proteins that are purified as subunits of multiprotein complexes, such as the core RNA polymerase I [3]. On the other hand, dynamic interactions are expected to control the majority of cellular processes and it is the dynamic nature of these protein-protein interactions that makes them more challenging to be detected.

Here, we describe a protocol based on immunoaffinity purification, as an efficient way to isolate protein complexes under physiological conditions and subsequently analyse their components. For this, budding yeast cells need to express the protein of interest with a purification tag, which is then used as a bait to capture the interacting proteins. One of the key feature of this protocol is that cell lysis is performed while cells are immersed under liquid nitrogen to preserve protein-protein interactions. Commercially available antibodies are coupled to magnetic beads and then antibody-coupled beads are used to immunoprecipitate the protein of interest, together with its binding partners. Finally, the purified material can be studied in different ways to characterise and identify all components of the protein complexes. The protocol can be divided into several steps:

- Growth, collection and subsequent freezing of cells in liquid nitrogen.
- Cell lysis and preparation of cell extract.
- Pre-clearing the protein extract using high-speed centrifugation steps.
- Protein immunoprecipitation from cell extract using antibodies coupled to magnetic beads.
- Extensive wash of the protein complexes bound to the magnetic beads.
- Recovery of protein complexes and downstream analysis, such as Western Blot or mass spectrometry.

The protocol we report has been extensively used to study protein complexes and their biological role. We have applied this co-immunoprecipitation to study different aspects of cellular biology, such as chromosome replication [4-10] or cell division [11]. This protocol can be efficiently adapted to study specific protein - protein interactions, for example, to study the weaker and transient interactions by crosslinking the proteins with formaldehyde [8] in order to preserve the transiently occurring interactions and enable their analysis.

2.MATERIALS

Prepare all solutions using ultra pure water and analytical grade reagents. Store all reagents at

room temperature unless specified otherwise.

2.1. Preparation of antibody-coupled magnetic beads

1. Magnetic rack.

2. Rotating wheel.

3. M-270 Epoxy magnetic beads (Life Technologies), store at 4˚C.

4. Dimethylformamide.

5. 0.1M Sodium phosphate pH 7.4.

6. 3M Ammonium sulphate.

7. Antibody of your choice to couple to the magnetic beads (*see* **Note 1**).

8. PBS.

9. 10% IGEPAL CA-360 (NP-40).

10. 20% Sodium azide.

2.2 One-step co-immunoprecipitation.

Store all reagents at room temperature unless specified otherwise.

1. Ice, dry ice, liquid nitrogen.

2. SPEX Sample Prep Freezer/Mill, grinding vials.

3. Ultracentrifuge and appropriate tubes.

4. High-speed centrifuge and appropriate tubes.

5. Bench-top centrifuge.

6. 100 ml plastic beakers (one per each sample).

7. Thin long spatulas (one per each sample).

8. 50 ml and 250 ml tubes.

9. 1.5 ml tubes.

10. Magnetic rack for 1.5 ml tubes.

11. Rotating wheel.

12. Heating block.

13. 5 ml syringes with 20.0 gauge needles.

14. Antibody-coupled beads (see section 3.1).

15. 1M HEPES-KOH pH 7.9, pH needs to be adjusted at 4˚C and solution stored at 4˚C.

16. 0.5M EDTA pH 7.9, store at 4˚C.

17. 50% Glycerol.

18. 10% IGEPAL CA-360 (NP-40).

19. 5M Potassium acetate.

20. 1M Magnesium acetate.

21. 1M Ditiothreitol (DTT), store at -20˚C.

22. 0.2M Sodium β-glycerophosphate pentahydrate, store at -20˚C.

23. 0.2M Sodium fluoride, store at -20˚C.

24. Protease inhibitor cocktail (Sigma), store at -20˚C.

25. Complete protease inhibitor cocktail, EDTA-free (Roche) – stock solution 25x made by dissolving 1 tablet in 1 ml of water, store at -20˚C.

26. PBS.

27. 20% Sodium dodecylsulfate.

28. 1M Tris-HCl pH 6.7.

29. β-mercaptoethanol.

30. Bromophenol blue.

31. Lysis buffer (store at 4˚C) (*see* **Note 2**): 100mM HEPES-KOH pH 7.9, 100mM potassium acetate, 10mM magnesium acetate, 2mM EDTA (*see* **Note 3**). Prior to use buffer needs to be supplemented with 1mM DTT and inhibitors: 2mM sodium β-glycerophosphate pentahydrate, 2mM sodium fluoride, 1% protease inhibitor cocktail, 1x complete protease inhibitor cocktail.

32. Glycerol mix buffer (store at 4˚C) (*see* **Note 2**): 100mM HEPES-KOH pH 7.9, 50% glycerol, 100mM potassium acetate, 10mM magnesium acetate, 2mM EDTA, 0.5 % IGEPAL CA-360 (*see* **Note 3**). Prior to use buffer needs to be supplemented with 1mM DTT and inhibitors: 2mM sodium β-glycerophosphate pentahydrate, 2mM sodium fluoride, 1% protease inhibitor cocktail, 1x complete protease inhibitor cocktail.

33. Wash buffer (store at 4˚C) (*see* **Note 2**): 100mM HEPES-KOH pH 7.9, 100mM potassium acetate, 10mM magnesium acetate, 2mM EDTA, 0.1% IGEPAL CA-360 (*see* **Note 3**). Prior to use buffer needs to be supplemented with inhibitors: 2mM sodium β-glycerophosphate pentahydrate, 2mM sodium fluoride, 1% protease inhibitor cocktail, 1x complete protease inhibitor cocktail.

34. 3x Laemmli buffer (dilute buffer with water to make corresponding 1.5x and 1x buffers that are required for the immunoprecipitation protocol): 5% (w/v) sodium dodecylsulfate, 666mM Tris-HCl pH 6.7, 30% glycerol, 715mM β-mercaptoethanol, 0.0125% bromophenol blue (*see* **Note 4**).

2.3 Large scale two-step TAP-MYC co-immunoprecipitation.

Store all reagents at room temperature unless specified otherwise.

1. Ice, dry ice, liquid nitrogen.

2. SPEX Sample Prep Freezer/Mill, grinding vials.

3. Ultracentrifuge and appropriate tubes.

4. High-speed centrifuge and appropriate tubes.

5. Bench-top centrifuge.

6. 100 ml plastic beakers (one per each sample).

7. Thin long spatulas (one per each sample).

8. 50 ml and 250 ml tubes.

9. 1.5 ml tubes.

10. Magnetic rack for 1.5 ml tubes.

11. Rotating wheel.

12. Heating block.

13. Shaking thermomixer.

14. 5 ml syringes with 20.0 gauge needles.

15. Antibody-coupled beads (see section 3.1).

16. 1M HEPES-KOH pH 7.9, pH needs to be adjusted at 4˚C and solution stored at 4˚C.

17. 0.5M EDTA pH 7.9, store at 4˚C.

18. Glycerol.

19. 10% IGEPAL CA-360 (NP-40).

20. 5M Potassium acetate.

21. 1M Magnesium acetate.

22. 1M Ditiothreitol (DTT), store at -20˚C.

23. 0.2M Sodium β-glycerophosphate pentahydrate, store at -20˚C.

24. 0.2M Sodium fluoride, store at -20˚C.

25. Protease inhibitor cocktail (Sigma), store at -20˚C.

26. Complete protease inhibitor cocktail (Roche) – stock solution 25x made by dissolving 1 tablet in 1 ml of water, store at -20˚C.

27. TEV protease (Life Technologies), store at -20˚C.

28. PBS.

29. 20% Sodium dodecylsulfate.

30. 1M Tris-HCl pH 6.7.

31. β-mercaptoethanol.

32. Bromophenol blue.

33. Lysis buffer 1 (store at 4˚C) (*see* **Note 2**): 100mM HEPES-KOH pH 7.9, 100mM

potassium acetate, 10mM magnesium acetate, 2mM EDTA (*see* **Note 3**). Prior to use buffer needs to be supplemented with 4mM DTT and inhibitors: 8mM sodium β-glycerophosphate pentahydrate, 8mM sodium fluoride, 4% protease inhibitor cocktail, 4x complete protease inhibitor cocktail (*see* **Note 5**).

34. Lysis buffer 2 (store at 4˚C) (*see* **Note 2**): 100mM HEPES-KOH pH 7.9, 100mM potassium acetate, 10mM magnesium acetate, 2mM EDTA (*see* **Note 3**). Prior to use buffer needs to be supplemented with 1mM DTT and inhibitors: 2mM sodium β-glycerophosphate pentahydrate, 2mM sodium fluoride, 1% protease inhibitor cocktail, 1x complete protease inhibitor cocktail.

35. Glycerol mix buffer (store at 4˚C) (*see* **Note 2**): 100mM HEPES-KOH pH 7.9, 50% glycerol, 100mM potassium acetate, 10mM magnesium acetate, 2mM EDTA, 0.5 % IGEPAL CA-360 (*see* **Note 3**). Prior to use buffer needs to be supplemented with 1mM DTT and inhibitors: 2mM sodium β-glycerophosphate pentahydrate, 2mM sodium fluoride, 1% protease inhibitor cocktail, 1x complete protease inhibitor cocktail.

36. Wash buffer 1 (store at 4˚C) (*see* **Note 2**): 100mM HEPES-KOH pH 7.9, 100mM Potassium acetate, 10mM Magnesium acetate, 2mM EDTA, 0.1% IGEPAL CA-360 (*see* **Note 3**). Prior to use buffer needs to be supplemented with 1mM DTT and phosphatase inhibitors: 2mM sodium β-glycerophosphate pentahydrate, 2mM sodium fluoride (we do not add protease inhibitors as that would block TEV protease cleavage).

37. Wash buffer 2 (store at 4˚C) (*see* **Note 2**): 100mM HEPES-KOH pH 7.9, 100mM potassium acetate, 10mM magnesium acetate, 2mM EDTA, 0.1% IGEPAL CA-360 (*see* **Note 3**). Prior to use buffer needs to be supplemented with inhibitors: 2mM sodium β-glycerophosphate pentahydrate, 2mM sodium fluoride, 1% protease inhibitor cocktail, 1x complete protease inhibitor cocktail.

38. 3x Laemmli buffer (dilute buffer with water to make corresponding 1.5x and 1x buffers

that are required for the immunoprecipitation protocol): 5% (w/v) sodium dodecylsulfate, 666mM Tris-HCl pH 6.7, 30% glycerol, 715mM β-mercaptoethanol, 0.0125% bromophenol blue (*see* **Note 4**).

3. METHODS

3.1 Preparation of the antibody-coupled magnetic beads.

1. Add appropriate amount of dimethylformamide to the vial containing M-270 Epoxy beads as per the manufacturer's instruction (*see* **Note 6**).

2. Vortex the vial with beads vigorously and store at 4˚C (*see* **Note 7)**.

3. Remove 425 µl of beads to a 1.5 ml tube (*see* **Note 8**). Place the tube on a magnetic rack and wait 30 seconds for the magnet in the rack to gently capture the beads on the side of the tube. Remove the supernatant.

4. Add 1 ml of 0.1M sodium phosphate pH 7.4 and agitate the tube for 10 minutes on a rotating wheel.

5. Place on magnetic rack and remove the supernatant and repeat wash as in step 4.

6. Place on magnetic rack and remove the supernatant and add reagents and antibody in the following order:

PLACE TABLE 1 HERE

7. Leave the beads on rotating wheel for 2 days at 4˚C (*see* **Note 9**).

8. After the incubation, spin tubes briefly to collect any liquid in the cap and then place tubes containing beads in magnetic rack for 4 minutes, discard supernatant carefully and wash four times with 1 ml of ice cold PBS at room temperature (*see* **Note 10**).

9. Add 1 ml of PBS containing 0.5% IGEPAL, agitate the tube on rotating wheel for 10 minutes place in magnetic rack and remove supernatant (*see* **Note 10**).

10. Add 1 ml of PBS and leave on rotating wheel for 5 minutes, place in magnetic rack and then remove supernatant.

11. Repeat the wash with a further 1 ml of PBS for 5 minutes. Place in magnetic rack and discard the supernatant (*see* **Note 10**).

12. Resuspend the beads in 900 µl of PBS and add sodium azide to a final concentration of 0.02% (*see* **Note 11**). Store beads at 4˚C.

3.2 One-step co-immunoprecipitation.

3.2.1. Preparation of frozen cells pellet: "popcorn".

IMPORTANT: Please follow all health and safety rules relating to work using liquid nitrogen and always wear appropriate protective equipment (mask, gloves etc.).

Keep all the buffers and reagents on ice.

1. Grow 250 ml yeast culture of your choice (*see* **Note 12**) and centrifuge culture at 200 x g for 3 minutes using 250 ml tubes.

2. Resuspend cells with 50 ml of ice-cold 20mM HEPES-KOH pH 7.9 buffer, transfer to 50 ml tube and pellet cells at 200 x g for 3 minutes. Take off the supernatant.

3. Wash cells with 10 ml of ice-cold Lysis buffer WITHOUT the inhibitors and centrifuge cells at 200 x g for 3 minutes, discard supernatant.

4. Resuspend the cell pellet in three volumes of cold Lysis buffer (supplemented WITH inhibitors) (1 g of cell pellet equals 1 ml of buffer therefore, to resuspend 1g of cell pellet add 3 ml of cold lysis buffer WITH inhibitors).

5. Place an empty 50 ml tube on dry ice, fill with liquid nitrogen and freeze the resuspended pellet by dispensing dropwise into the liquid nitrogen (*see* **Note 13**). Leave the tubes with cells on dry ice until all remaining liquid nitrogen has evaporated. (*see* **Note 14**).

6. Store frozen yeast cells (hereafter referred to as "popcorn") at -80°C.

3.2.2. Co-immunoprecipitation.

IMPORTANT: Please follow all health and safety rules relating to work using liquid nitrogen and always wear appropriate protective equipment (mask, gloves etc.).

Keep all the buffers and reagents on ice. Pre-cool all centrifuges and centrifuge tubes.

1. Pre-cool the SPEX Sample Prep Freezer/Mill by filling it with liquid nitrogen (follow the manufacturer's instruction). Pre-cool the grinding vials in the filled freezer Mill (*see* **Note 15**).

2. Weigh 1 – 3.5 g of frozen popcorn for every sample and place into fresh 50 ml tube previously kept on dry ice (*see* **Note 16**).

3. Place popcorn into appropriate grinding vials, seal tightly and place securely inside the grinder (follow the manufacturer's instruction). To break efficiently budding yeast cells use two cycles of grinding, each one with the following settings:

2 minutes pre-cool

2 minutes run

2 minutes cool

Rate 14

4. After the run, collect the ground material carefully using a different spatula for each sample (*see* **Note 17**). Place the powder (ground yeast) into a plastic beaker and let it thaw at room temperature. This will take approximately 15-20 minutes.

5. Transfer thawed extract from the plastic beaker into the appropriate centrifuge tubes (we use 50 ml tubes). Take note of the volume of the extract transferred, as the volume of cell extract is important to calculate the amount of buffer needed in the next step.

6. Add one quarter volume of Glycerol mix buffer supplemented with inhibitors to the same plastic beaker from which you have collected the extract (therefore for each 1 ml of recovered cell extract add 0.25 ml of Glycerol mix buffer with inhibitors). Vortex well, collect the remaining liquid and transfer to the centrifuge tube with the rest of the extract. This allows recovery of all the remaining extract from the wall of plastic beaker (*see* **Note 18**).

7. Pellet the insoluble cell debris by centrifugation at 25000 x g for 30 minutes at 4˚C.

8. Collect the supernatant and transfer into an ultracentrifuge tube, top up with a thin layer of mineral oil ensuring the tubes are balanced and centrifuge at 100000 x g for one hour at 4˚C.

9. During the centrigugation wash the antibody-coupled beads (prepared previously, *see* section 3.1). Mix antibody-coupled beads thoroughly and aliquot 100 µl into 1.5 ml tubes (prepare two aliquots of beads for each extract). Wash beads twice with 1 ml of PBS at room temperature (*see* **Note 10**).

10. After the ultracentrifuge spin remove the tube carefully to avoid disrupting the separated phases. Using a 5 ml syringe with a needle, carefully pierce the ultracentrifuge tube just above the pellet. Make sure that the bevel of the needle is facing up to avoid disturbing the pellet. Collect the supernatant into a fresh 5 ml tube. At this stage prepare a cell extract sample for later analysis. Remove 50 µl of cell extract and add 100 µl of 1.5x Laemmli buffer. Mix and heat cell extract at 95°C for 5 minutes and store at -80°C.

11. Split remaining cell extract into two by adding half of the extract to one of the two tubes of antibody-coupled beads (prepared in step 9) after having removed any leftovers of remaining PBS from the beads (*see* **Note 9**).

12. Incubate at 4˚C for two hours on a rotating wheel.

13. After the incubation, spin tubes briefly to collect any liquid in the cap and then place tubes on a magnetic rack and remove the supernatant. Once beads are captured on the side of the tube, wash protein complexes bound to the antibody-coupled magnetic beads four times with 1 ml of Wash buffer (*see* **Note 10**).

14. After the last wash, discard the supernatant. IMPORTANT: make sure all the washing buffer is completely removed. Add 50 µl of 1x Laemmli buffer and heat samples at 95°C for 5 minutes.

15. Place tubes in a magnetic rack and collect supernatant that contains the purified material into a fresh 1.5 ml tube. Mix the eluates from the corresponding samples (split in step 11), together, aliquot the material accordingly (*see* **Note 19**) and snap freeze on dry ice and store at -80°C.

16. Run purified material on an SDS-PAGE gel to subsequently perform downstream analysis (*see* **Note 20**).

3.3 Large scale two-step TAP-MYC co-immunoprecipitation.

A protein can interact with different partners and be involved in different cellular processes. In order to try and isolate specific protein complexes we present one possible variation of the standard co-immunoprecipitation protocol, which consist of a two-step purification procedure. First, a protein of interest is immunoprecipitated together with its binding partners. One of these interactor proteins needs to be known, in order to be used as bait for second round of immunoprecipitation using the material purified in the first step. We have efficiently used TAP and MYC tags to perform this type of procedure. We have immunoprecipated TAP-tagged protein of interest and then we have used purified material to perform a second immunoprecipitation using a MYC tag that had been previously fused to another component of the complex of interest (*see* **Note 1**). Therefore, the final isolated material contains both bait proteins tagged with TAP and MYC and their interactors (Figure 1). This purified material might be then subjected to the biochemical application of your choice e.g. Western Blot or mass spectrometry. As we aim to enrich for the specific complexes that we are trying to isolate we recommend using initially higher amounts of cells and prepare what we term “concentrated popcorn”.

3.3.1. Preparation of concentrated frozen cells pellet: “concentrated popcorn”.

IMPORTANT: Please follow all health and safety rules relating to work using liquid nitrogen and always wear appropriate protective equipment (mask, gloves etc.).

Keep all the buffers and reagents on ice. Pre-cool all centrifuges and centrifuge tubes.

1. Grow 1000 ml of yeast culture expressing proteins of interest tagged with TAP and MYC (*see* **Note 12**) and centrifuge culture at 200 x g for 3 minutes using a 250 ml tube (*see* **Note 21**).

2. Wash cells with 100 ml of ice-cold 20mM HEPES-KOH pH 7.9 buffer and pellet cells at 200 x g for 3 minutes. Remove the supernatant.

3. Resuspend cells with 20 ml of ice-cold Lysis buffer 1 WITHOUT inhibitors, transfer to 50 ml tube and pellet cells at 200 x g for 3 minutes and remove the supernatant.

4. Resuspend the pellet in one quarter of a volume of cold Lysis buffer 1 (supplemented WITH inhibitors) (1 g of cell pellet equals 1 ml of buffer WITH inhibitors, therefore, to resuspend 1g of cell pellet add 0.25 ml of cold Lysis buffer 1 WITH inhibitors) (*see* **Note 22**).

5. Place an empty 50 ml tube on dry ice, fill with liquid nitrogen and freeze the resuspended pellet by dispensing dropwise into the liquid nitrogen (*see* **Note 13**). Leave the tubes with cells on dry ice until all remaining liquid nitrogen has evaporated before closing the tubes (*see* **Note 14**).

6. Store frozen yeast cells (“ concentrated popcorn”) at -80°C.

3.3.2. Two-step TAP-MYC co-immunoprecipitation.

IMPORTANT: Please follow all health and safety rules relating to work using liquid nitrogen and always wear appropriate protective equipment (mask, gloves etc.).

Keep all buffers and reagents on ice. Pre-cool all centrifuges and centrifuge tubes..

1. Pre-cool the SPEX Sample Prep Freezer/Mill by filling it with liquid nitrogen (follow the manufacturer’s instruction) (*see* **Note 15**).

2. Weigh out equal amounts of frozen popcorn for every sample and place into fresh 50 ml tube previously kept on dry ice (*see* **Note 16**).

3. Place popcorn into appropriate pre-cooled grinding vials (*see* **Note 23**), seal tightly and place securely inside the grinder (follow the manufacturer's instruction). To efficiently break budding yeast cells use two cycles of grinding, each one with the following settings:

2 minutes pre-cool

2 minutes run

2 minutes cool

Rate 14

4. After the run collect the ground material carefully using a different spatula for each sample (*see* **Note 17**). Place powder (ground yeast) into a plastic beaker and let it thaw at room temperature. This will take approximately 15-20 minutes.

5. After thawing add 1 ml of Lysis buffer 2 supplemented with inhibitors and transfer melted extract from plastic beaker into the appropriate centrifuge tubes (we use 50 ml tubes). Vortex well, collect the remaining liquid and transfer to the centrifuge tube with the rest of the extract. Take note of the volume of the extract in the process, as the volume of cell extract is important to calculate the amount of buffer needed in the next step.

6. Add one quarter volume of Glycerol mix buffer supplemented with inhibitors to the cell extract (*see* **Note 18**).

7. Pellet the insoluble cell debris by centrifugation at 25000 x g for 30 minutes at 4˚C.

8. Collect the supernatant and transfer into an ultracentrifuge tube, top up with a thin layer of mineral oil ensuring the tubes are balanced and centrifuge at 100000 x g for one hour at 4˚C.

9. Wash the IgG antibody-coupled beads (to pull down the TAP-tagged protein) during the ultracentrifugation spin (prepared previously, *see* section 3.1). Mix the antibody-coupled beads thoroughly and aliquot 100 µl of antibody-coupled beads into 1.5 ml tubes (prepare two aliquots of beads for each 1000 ml of original yeast culture). Wash beads twice with 1 ml of PBS at room temperature (*see* **Note 10**).

10. After the ultracentrifuge spin remove the tube carefully to avoid disrupting the separated phases. Using a 5 ml syringe with a needle, carefully pierce the ultracentrifuge tube just above the pellet. Make sure that the bevel of the needle is facing up to avoid disturbing the pellet. Collect the supernatant into a fresh 5 ml tube. At this stage prepare a cell extract sample for later analysis. Remove 50 µl of cell extract and add 100 µl of 1.5x Laemmli buffer. Boil cell extracts at 95°C for 5 minutes and store at -80°C.

11. Split remaining cell extract into two adding each half of the extract to one of the two tubes of antibody-coupled beads (prepared in step 9) after having removed any leftovers of remaining PBS from magnetic beads (*see* **Note 9**).

12. Incubate at 4˚C for two hours on a rotating wheel.

13. After the incubation, spin tubes briefly to collect any liquid in the cap and then place tubes on a magnetic rack and remove the supernatant. Once the beads are captured on the side of the tube, wash the protein complexes bound to the antibody-coupled magnetic beads four times with 1 ml of Wash buffer 1 (NOT containing any protease inhibitors) (*see* **Note 10**).

14. After the last wash discard supernatant. IMPORTANT: make sure that all the washing buffer is removed and there is no liquid remaining on the side of the tube (*see* **Note 24**).

15. Add 50 µl of Wash buffer 1 and 10 U of TEV protease for each 1000 ml of original yeast culture (*see* **Note 25**).

16. Incubate for 2 hours with agitation at room temperature e.g. in shaking thermomixer.

17. Wash the MYC antibody-coupled beads (to pull down the MYC-tagged protein) during the TEV incubation (prepared previously, *see* section 3.1). Aliquot 100 µl of antibody-coupled beads into 1.5 ml tubes (prepare two aliquots of beads). Wash beads twice with 1 ml of PBS at room temperature (*see* **Note 10**).

18. After incubation with TEV protease, combine the extracts together, adjust with Lysis buffer 2 to a final volume of 2 ml and split the extracts again into the tubes containing the MYC antibody-coupled magnetic beads.

19. Incubate at 4˚C for two hours on a rotating wheel.

20. After the incubation, spin tubes briefly to collect any liquid in the cap and then place the tubes on a magnetic rack and remove the supernatant. Once the beads are captured on the side of the tube, wash protein complexes bound to the antibody-coupled magnetic beads four times with 1 ml of Wash buffer 2 (*see* **Note 10**).

21. After the last wash, discard the supernatant. IMPORTANT: make sure all the washing buffer is removed and there is no liquid remaining on the side of the tube. Add 50 µl of 1x Laemmli buffer and heat samples at 95°C for 5 minutes.

22. Place tubes in a magnetic rack and collect supernatant that contains the purified material into a fresh 1.5ml tube. Mix the eluates from the corresponding samples (split in step 18) together, aliquot the material accordingly (*see* **Note 19**) and snap freeze on dry ice and store at -80°C.

23. Run purified material on a SDS-PAGE gel to subsequently carry out Western Blot analysis (*see* **Note 20**). Alternatively, to identify components of protein complexes, mass spectrometry can be performed on purified material (*see* **Note 26**).

4. NOTES

1. We do not recommend using the polyclonal antibodies raised against particular proteins. In our experience the most efficient way to pull down the protein of your choice is to use widely available epitopes, we have extensively used: TAP, MYC, HA and FLAG. When performing two-step purification including TAP tag on one of the protein it is important to use TAP purification ALWAYS as a first step since TAP tagged proteins can bind any antibody coupled beads and would ruin second step purification by binding unspecifically.

2. We find that it is best to prepare this fresh each time.

3. When calculating the amount of water that needs to be added to the buffer, the volume of inhibitors that you need to add prior to use needs to be taken into account.

4. SDS precipitates at 4°C therefore the buffer needs to be warmed up to room temperature prior to use.

5. Note that the amounts of inhibitors used when making popcorn and concentrated popcorn (from concentrated extracts) is different.

6. Add dimethylformamide in a fume hood and follow all waste disposal regulations accordingly.

7. You can store beads for up to 6 months at 4°C.

8. We use 3.4×10^9 of antibody-coupled magnetic beads for each cell extract.

9. Placing parafilm around the tube lid will reduce the risk of the tube suddenly opening during the incubation.

10. To perform an extensive wash each time place tube on magnetic rack, remove magnet from rack and shake the rack for a minimum of twenty times. Place back the magnet and allow it to capture the beads on the side of the tube.

11. Antibody-coupled beads can be stored for up to 3 - 4 months at 4°C. It is possible to omit sodium azide and store the coupled beads up to one week at 4°C.

12. We have experience using yeast cultures with a density ranging from 0.7×10^7 cells/ml up to 2×10^7 cells/ml. We grow strains of interest together with corresponding controls.

13. The most efficient way to create frozen cell extract is to use a 1 ml pipette and keep adding drops of yeast extract into a 50 ml tube placed on dry ice that has been filled with liquid nitrogen, they will freeze instantly. It is important to do this slowly in order to obtain separate drops of frozen yeast cells. Add drops from above and in the middle of the 50 ml tube so drops do not stick to the sides of the tube. Avoid placing the pipette tip too close to

the liquid nitrogen otherwise the extract will freeze inside the pipette tip.

14. Make sure all liquid nitrogen is evaporated before closing the tube. Secure caps loosely on the tubes overnight at -80°C whilst all traces of liquid nitrogen evaporate to prevent the tube from 'popping'. Tighten the lids fully the next day.

15. Depending on the type of the cryogenic grinder and the number of samples to be lysed, more liquid nitrogen might be needed to be added to the grinder, always to be filled up in between the cycles of grinding. Always follow the health and safety rules when handling liquid nitrogen.

16. When analysing few extracts that belong to the same experiment it is important to lyse the same amount of popcorn for all of them. Start weighing the samples from the one that weighed the least (you will know which one as you have to calculate the weight of cell extracts before making the popcorn) and adjust the weight of remaining samples accordingly.

17. The ground material should form a fine powder. If not fine, white powder is observed after grinding, consider extending the number or the length of grinding cycles. You can check efficiency of the lysis by placing 2 µl of material under the microscope and checking the breakage of cells. Conditions that we use give us 90 - 95% breakage efficiency. When collecting the ground material it is essential to work quickly as once the powder starts to thaw it becomes more difficult to recover it from the vials.

18. The study of protein interactions within complexes built around DNA requires digestion of DNA in order to release proteins. Add 400 U/ml Universal Nuclease (Fisher) (or 1600 U/ml when preparing "concentrated popcorn") at this point of the protocol and incubate for 30 minutes at 4˚C while mixing on rotating wheel prior to the first centrifugation step.

19. We routinely make aliquots of 1x 50 µl and 2x 25 µl but depending on the downstream application you might need to reconsider these amounts. We do not recommend the repeated freeze thaw of immunoprecipitated extracts.

20. To identify proteins present in the purified material perform Western Blot analysis. We routinely load 5 µl of the cell extract sample and 12 µl of the purified immunoprecipitated complex on a SDS-PAGE gel in order to resolve the proteins and we transfer them routinely onto nitrocellulose membrane.

21. In our experience, to improve visualisation and increase the yield of purified complexes we have grown anything between 1-5 litres of a single yeast culture.

22. For large scale experiments we have adapted the protocol so we produce more concentrated cell extracts by adding a quarter of a volume of a lysis buffer compared to the three times volume of lysis buffer for the standard protocol.

23. Depending on the manufacturer's instruction for the cryogenic grinder of your choice there might be a weight limit for the amount of sample that can be ground at once. More than one vial per sample might be used, depending on the scale of the original culture (*see* **Note 21**).

24. Make sure that the entire amount of washing buffer is collected and there is no remaining liquid on the side of the tube.

25. TEV protease cuts within TEV recognition site that is included in the TAP tag (Figure 1). Therefore, the tagged protein and its interacting partners will be released from the magnetic beads. It is important to remember that TAP tag will be lost after this step and thereafter the protein of interest will be fused only to the remaining Calmodulin Binding Protein (CBP) (details in Figure 1), thus anti-CBP or native antibodies against the protein must be used in order to detect the protein on Western Blots. Efficiency of TEV protease digestion will depend on the protein thus more or less TEV protease might be required.

26. When more concentrated protein samples are required you can elute your protein complexes bound to magnetic beads in 30 µl of 1x Laemmli buffer. We have routinely eluted the proteins in 30 µl and used the whole eluate to run in SDS-PAGE gels. To identify

components of the purified protein complexes in an unbiased fashion mass spectrometry can be used.

Figure legends

Figure 1. Overview of a two-step TAP-MYC co-immunoprecipitation.

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References:

1. Gavin AC, Bosche M, Krause R, Grandi P, Marzioch M, Bauer A, Schultz J, Rick JM, Michon AM, Cruciat CM, Remor M, Hofert C, Schelder M, Brajenovic M, Ruffner H, Merino A, Klein K, Hudak M, Dickson D, Rudi T, Gnau V, Bauch A, Bastuck S, Huhse B, Leutwein C, Heurtier MA, Copley RR, Edelmann A, Querfurth E, Rybin V, Drewes G, Raida M, Bouwmeester T, Bork P, Seraphin B, Kuster B, Neubauer G, Superti-Furga G (2002) Functional organization of the yeast proteome by systematic analysis of protein complexes. Nature 415 (6868):141-147

2. Ho Y, Gruhler A, Heilbut A, Bader GD, Moore L, Adams SL, Millar A, Taylor P, Bennett K, Boutilier K, Yang L, Wolting C, Donaldson I, Schandorff S, Shewnarane J, Vo M, Taggart J, Goudreault M, Muskat B, Alfarano C, Dewar D, Lin Z, Michalickova K, Willems AR, Sassi H, Nielsen PA, Rasmussen KJ, Andersen JR, Johansen LE, Hansen LH, Jespersen H, Podtelejnikov A, Nielsen E, Crawford J, Poulsen V, Sorensen BD, Matthiesen J, Hendrickson RC, Gleeson F, Pawson T, Moran MF, Durocher D, Mann M, Hogue CW, Figeys D, Tyers M (2002) Systematic identification of protein complexes in Saccharomyces cerevisiae by mass spectrometry. Nature 415 (6868):180-183. doi:10.1038/415180a

3. Knutson BA, Luo J, Ranish J, Hahn S (2014) Architecture of the Saccharomyces cerevisiae RNA polymerase I Core Factor complex. Nature structural & molecular biology 21 (9):810-816. doi:10.1038/nsmb.2873

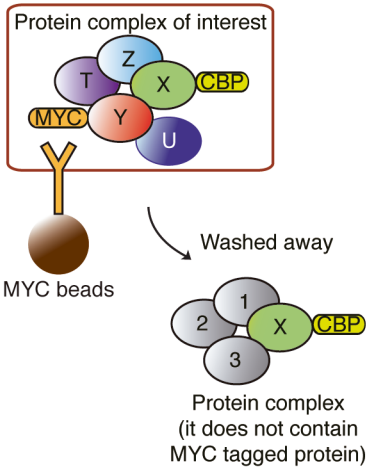
4. Foltman M, Evrin C, De Piccoli G, Jones RC, Edmondson RD, Katou Y, Nakato R, Shirahige K, Labib K (2013) Eukaryotic replisome components cooperate to process histones during chromosome replication. Cell Rep 3 (3):892-904. doi:10.1016/j.celrep.2013.02.028
5. Gambus A, Jones RC, Sanchez-Diaz A, Kanemaki M, van Deursen F, Edmondson RD, Labib K (2006) GINS maintains association of Cdc45 with MCM in replisome progression complexes at eukaryotic DNA replication forks. Nat Cell Biol 8 (4):358-366
6. Gambus A, van Deursen F, Polychronopoulos D, Foltman M, Jones RC, Edmondson RD, Calzada A, Labib K (2009) A key role for Ctf4 in coupling the MCM2-7 helicase to DNA polymerase alpha within the eukaryotic replisome. EMBO J 28 (19):2992-3004. doi:emboj2009226 [pii]
10.1038/emboj.2009.226
7. Sengupta S, van Deursen F, de Piccoli G, Labib K (2013) Dpb2 integrates the leading-strand DNA polymerase into the eukaryotic replisome. Current biology : CB 23 (7):543-552. doi:10.1016/j.cub.2013.02.011
8. van Deursen F, Sengupta S, De Piccoli G, Sanchez-Diaz A, Labib K (2012) Mcm10 associates with the loaded DNA helicase at replication origins and defines a novel step in its activation. The EMBO journal 31 (9):2195-2206. doi:10.1038/emboj.2012.69
9. De Piccoli G, Katou Y, Itoh T, Nakato R, Shirahige K, Labib K (2012) Replisome stability at defective DNA replication forks is independent of S phase checkpoint kinases. Molecular cell 45 (5):696-704. doi:10.1016/j.molcel.2012.01.007
10. Maric M, Maculins T, De Piccoli G, Labib K (2014) Cdc48 and a ubiquitin ligase drive disassembly of the CMG helicase at the end of DNA replication. Science 346 (6208):1253596. doi:10.1126/science.1253596
11. Sanchez-Diaz A, Marchesi V, Murray S, Jones R, Pereira G, Edmondson R, Allen T, Labib K (2008) Inn1 couples contraction of the actomyosin ring to membrane ingression during cytokinesis in budding yeast. Nature cell biology 10 (4):395-406. doi:10.1038/ncb1701

Table 1

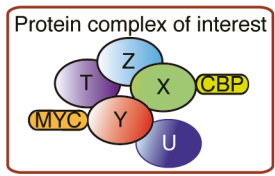
Reagent	Volume	Comment
3M Ammonium sulphate	300 µl	
0.1M Sodium phosphate pH 7.4	X µl	Adjust depending on volume of antibody
Antibody	300 µg	Calculate volume that contains 300µg antibody
Total volume	900 µl	

2nd step purification MYC IP

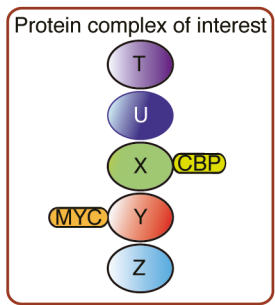
4. MYC immunoprecipitation to isolate protein complex of interest.



5. Recovery of protein complex from magnetic beads by boiling of the sample.



6. Downstream analysis to identify components of the complex of interest.

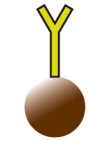


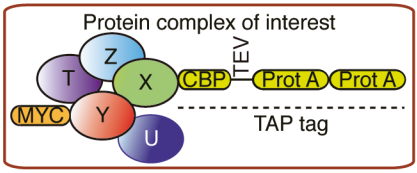
Isolation of specific protein complex

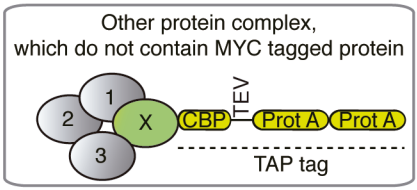
1. Yeast cells need to express two components of a complex of interest, both tagged with epitopes (TAP and MYC).
The TAP tag consists of calmodulin binding peptide (CBP), followed by TEV protease cleavage site and Protein A, which binds tightly to IgG.

1st step purification: TAP IP

2. Yeast cell lysis and subsequent mixing of cell extracts with magnetic beads coated with IgG that recognizes the TAP tag.

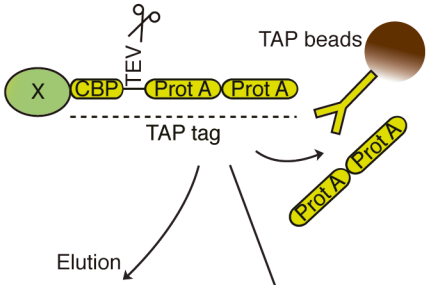


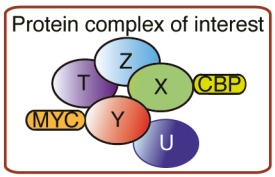




TEV protease cleavage

3. Cleavage within TAP tag with TEV protease to elute protein complexes bound to magnetic beads (CBP will be still fused to protein of interest X).





Elution