Protocol for Membrane Yeast Two-Hybrid (MYTH) for analysis of targeted interactions or identification of novel interactors.

Adapted from

Voisin M, Vanrobays E, Tatout C (2020) Investigation of Nuclear Periphery Protein Interactions in Plants Using the Membrane Yeast Two-Hybrid (MbY2H) System. Methods Mol Biol. doi: 10.1007/978-1-4939-8691-0_16

Lund CH, Bromley JR, López-Marqués RL, Sakuragi Y (2020) DNA library screening to identify interacting proteins of Golgi-localized type II membrane proteins bioRxiv 2020.02.03.932111; doi: https://doi.org/10.1101/2020.02.03.932111

Dutta S, Teresinski HJ, Smith MD (2020) A split-ubiquitin yeast two-hybrid screen to examine the substrate specificity of atToc159 and atToc132, two Arabidopsis chloroplast preprotein import receptors. PLoS One. doi: 10.1371/journal.pone.0095026. PMID: 24736607

Graumann K, Vanrobays E, Tutois S, Probst AV, Evans DE, Tatout C (2014) Characterization of two distinct subfamilies of SUN-domain proteins in Arabidopsis and their interactions with the novel KASH-domain protein AtTIK. J Exp Bot. doi: 10.1093/jxb/eru368

i. Summary

Membrane Yeast Two Hybrid (MYTH) is a variation of standard yeast two hybrid that uses the split-ubiquitin system to allow the interrogation of interactions involving membrane bound proteins. This protocol has been used to study targeted interactions between pre-identified bait and prey proteins. In addition MYTH can use a known bait protein to screen a selection of unknown prey-proteins that have been generated from a cDNA library. This consensus methodology is for use of MYTH for both targeted screening as well as the identification of novel interactors.

ii. Keywords

Membrane Yeast Two Hybrid, MYTH, cDNA, Screening

1. Introduction

Yeast Two-Hybrid (Y2H) is a common technique for analysing *in vivo* protein interactions. Although Y2H has revealed myriad novel interactions it has limited efficacy for use with integral membrane proteins due to their obvious requirement to reside within a membrane (and not free in the nucleus). Use of the Split-Ubiquitin System (SUS) is able to solve this challenge as it removes the burden of interaction from an integral membrane motif to an attached cytoplasmically-localised portion of ubiquitin (**Figure 1**). In general Y2H relies on the interaction of 'bait' and 'prey' proteins and in SUS the membrane bound 'bait' is linked to both the C-terminal portion of ubiquitin (Cub) and also to the LexA-VP16 transcription factor (TF). Opposingly the 'prey' protein is linked to a modified N-terminal portion of ubiquitin (Nub). Whereas the wildtype Nub and Cub fragments will ordinarily spontaneously re-associate, a modified NubG (with a single isoleucine -> Glycine change) prevents this re-joining unless the fragments are in close proximity. Once the Nub and Cub fragments re-associate the full-length ubiquitin is recognised by the endogenous yeast machinery and cleaved by a deubquitinase enzyme (DUB). This releases the LexA-VP16 TF which will bind to a transgenic synthetic promotor to generate a product that can be selected by growth of yeast on different media.

This Membrane Yeast Two Hybrid (MYTH) can be used to investigate the interaction of either two pre-selected proteins or through library screening for unknown interactors. Importantly MYTH is able to identify proteins that integrate within any membrane; be it the plasma membrane, within the Golgi apparatus, endoplasmic reticulum or the nuclear envelope. One caveat with MYTH is that the selected bait protein must correctly fold and integrate into a yeast membrane. This ability needs to be empirically tested for each bait on a case-by-case basis. For some proteins this will depend on whether the N-terminal or C-terminal portion of the bait is attached to Cub-LexA-VP16 fragment. For example, if it is known that the C-terminal fragment resides within the cytosol then this would be the best candidate for attachment to Cub.

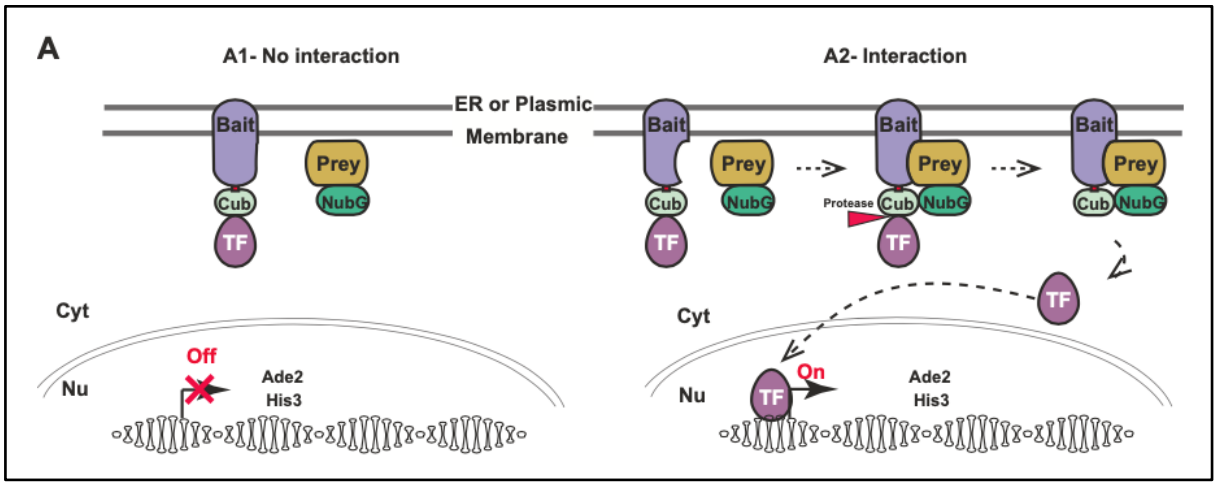

Figure 1: Schematic of MYTH (taken from Voisin *et al*, 2018)

Over the past few years MYTH has been used to identify many novel interactors; including AtTIK1 a component of the plant nuclear envelope (Graumann *et al*, 2014). This was discovered through interaction with the previously characterised AtSUN1 protein, which was used as the MYTH bait construct. In this case the MYTH prey constructs were prepared from a cDNA library extracted from 6-day old Arabidopsis seedlings.

More recently MYTH has been used to demonstrate interactions between members of the Arabidopsis galacturosyltransferase (GAUT) proteins that reside within the Golgi apparatus plasma membrane (Lund *et al*, 2020a). This research team has also have used MYTH to identity 25 novel interactors with the bait GAUT1 protein using a prey library generated from cDNA extracted from the leaves, stems or flowers from 6-week-old Arabidopsis plants (Lund *et al*, 2020b).

These studies demonstrate that MYTH can be used to identify novel components that interact with proteins that are localised to the nuclear envelope or membrane of the Golgi apparatus. This provides an exemplar that the technique can be used to identify interactors of proteins that sit within any sub-cellular membrane, assuming that the bait protein is correctly expressed in yeast.

This manuscript provides a consensus methodology for use of MYTH to either screen a cDNA library for novel interactors or to evaluate the interactions of previously identified bait and prey proteins. A workflow for this methodology is shown in **Figure 2**.

2. Materials

- pBT3-N is a bait vector allowing N-terminal tagging to Cub which is fused to the artificial transcription factor LexA-VP16 under the control of Cyclin1 (Cyc1) promoter (https://www.lifescience-market.com/plasmid-c-94/pbt3-n-p-108677.html). pBT3-N contains the yeast leucine (Leu2) marker, the *Escherichia coli* (*E. coli*) kanamycin resistance gene for bacterial selection.
- pPR3-N is a prey vector allowing N-terminal tagging to NubG (https://www.lifescience-market.com/plasmid-c-94/ppr3-n-p-108675.html). pPR3-N expresses the hemagglutinin A (HA) tag under the control of Cyc1 promoter and contains the yeast tryptophan (Trp1) marker, the *E. coli* ampicillin resistance gene for bacterial selection, and a *2 μ* multicopy origin of replication
- pOst1-NubI used as a positive control is a prey vector expressing an endoplasmic reticulum protein Ost1 fused to the wild-type Nub moiety of yeast ubiquitin (NubI) under the control of the Adh1 promoter (https://www.lifescience-market.com/plasmid-c-94/post1-nubi-p-108680.html). pOst1-NubI contains the yeast tryptophan (Trp1) marker, the *E. coli* ampicillin resistance gene for bacterial selection, and a *2 μ* multicopy origin of replication.
- pNubG-Fe65 used as noninteracting control is a prey vector expressing the cytosolic protein Fe65 fused to NubG under the control of Adh1 promoter (https://www.lifescience-market.com/plasmid-c-94/pnubg-fe65-p-108674.html). pNubG-Fe65 contains the yeast tryptophan (Trp1) marker, the *E. coli* ampicillin resistance gene for bacterial selection, and a *2 μ* multicopy origin of replication
- The yeast strain NMY51 (*MATa*, *his3Δ200*, *trp1-901*, *leu2- 3,112*, *ade2*, *LYS2::(lexAop)$_4$-HIS3*, *ura3::(lexAop)$_8$-lacZ*, *ade2::(lexAop)$_8$-ADE2*, *GAL4*) is used to express bait and prey vectors (https://www.lifescience-market.com/chemically-competent-cells-c-84_88/nmy51-chemically-yeast-express-competent-cells-p-63051.html).
- Ultra-electrocompetent or chemically competent *E. coli* for library transformation. Available from numerous suppliers.
- Standard chemically competent *E. coli* for routine transformations.
- Yeast extract-peptone-dextrose (YPD)-rich medium: 1% yeast extract, 2% peptone, 2% glucose, 2% Bacto Agar for solid medium, used for propagation of NMY51, are prepared in deionized water (dH$_2$O) and autoclaved (121 °C, 15 psi, 15 min).
- Glucose (20%) 10× solution is prepared by dissolving glucose in dH$_2$O, autoclaved and stored at room temperature.
- 3-Amino-1,2,4-Triazole (3-AT) 1M, a His3-competitive inhibitor, is prepared in dH$_2$O, and sterilized by filtration, and then stored at 4°C.
- Dropout (DO) mix 10× is a combination of amino-acid solutions diluted 10x lacking the appropriate supplement in sterile dH$_2$O and stored at room temperature.
- The following dropout solutions need to be prepared from the core mixture: DO-Leu, DO-Trp, DO-Trp-Leu (permissive medium) and DO-Trp-Leu-Ade-His (test medium high stringency). Core DO mixture is as follows:

Concentration (mg, 10x)	**Addition**
300	Isoleucine
1500	Valine
400	Adenine Sulfate dihydrate
200	Histidine
1000	Leucine
300	Lysine

1500	Methionine
500	Phenylalanine
2000	Threonine
400	Tryptophan
300	Tyrosime
200	Uracil
200	Arginine hydrochloride
Combine, omitting components as required. Bring to 1l w ddH20/ Autoclave and store at 4°C. http://cshprotocols.cshlp.org/content/2016/1/pdb.rec088195	

- Yeast nitrogen base (YNB) 10× solution: 1.7% yeast nitrogen base and 5% ammonium sulfate without amino acids or bases dissolved in dH_2O and autoclaved.
- Synthetic defined medium (SD medium): dilute 10x the YNB 10× solution, the glucose 10×, and the appropriate Dropout 10× mix in dH_2O with or without 2% Bacto Agar for solid medium. After mixing, the SD medium is autoclaved. The following SD medium solutions are prepared: SD-Leu (SD-L) for vector bait selection, SD-Trp- Leu (SD-LW) for prey and bait selection, and SD-Trp-Leu-Ade-His (SD-LWAH) to test interactions between baits and preys. 3-AT can be added to reduce the background growth
- 30ml and 150ml agar plates

- Salmon sperm DNA (ssDNA) at 10 mg/mL used as carrier for yeast transformations is dissolved in sterile dH_2O, then boiled for 5 min at 95°C, and stored at −20 °C.
- Lithium acetate (LiOAc) 10× solution (1M) is prepared by dissolving Lithium acetate in dH_2O. The final solution is autoclaved.
- TE/LiOAc 1× (LiOAc 0.1M, Tris 10 mM, EDTA 5 mM) is prepared by diluting 10x TE 10× solution and LiOAc 10× in dH_2O. The final solution is autoclaved.
- 50% polyethylene glycol (PEG) solution (PEG-3350) is dissolved in dH_2O and sterilized by filtration.
- PEG/TE/LiOAc (PEG 40%, LiOAc 1× TE 1×) is produced by diluting TE 10× solution and LiOAc 10× in dH_2O in 50% PEG solution and sterilized by filtration.
- 0.9% NaCl solution is prepared by dissolving NaCl in dH_2O. The final solution is autoclaved.
- 100% dimethyl sulfoxide (DMSO).
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- Kanamycin (100 mg/mL, 1000×) and ampicillin (100 mg/mL, 1000×) stocks are prepared in sterile dH_2O, and stored at −20 °C.
- LB Media, made by standard recipes.
- Standard Miniprep kit (available from numerous suppliers)
- Liquid nitrogen
- Mortar and pestle
- 50ml Falcon tubes
- 1.5ml Eppendorf tubes
- 0.5 mm metal beads (optional)
- Safe-Lock microfuge tube (optional)

- TRIZOL (https://www.thermofisher.com/order/catalog/product/15596026#/15596026)
- 95% ethanol
- 70% ethanol in RNase-free dH_2O
- DNAse I (available from numerous suppliers).
- Chloroform

- Phenol
- RNase-free dH_2O
- Reverse Transcriptase (available from numerous suppliers)
- Proof-reading polymerase (available from numerous suppliers)
- Standard Taq polymerase (available from numerous suppliers)
- PCR buffer and dNTPs (available from numerous suppliers)
- SfiI restriction enzyme and buffer (https://international.neb.com/products/r0123-sfii#Product%20Information)

- Nanodrop-1000 or similar micro-spectrophotometry
- Tissuelyser or similar bead-beater (optional)
- QIAGEN Plant RNeasy kit (optional) https://www.qiagen.com/gb/products/discovery-and-translational-research/dna-rna-purification/rna-purification/total-rna/rneasy-plant-mini-kit/
- QIAGEN Plasmid *Plus* Mega Kit (or similar) https://www.qiagen.com/de/products/discovery-and-translational-research/dna-rna-purification/dna-purification/plasmid-dna/qiagen-plasmid-plus-kits/#orderinginformation
- Bait and Prey Sequencing primers
  > pBT3-N Forward: 5′-CAGAAGGAGTCCACCTTAC-3′.
  > pPR3-N Forward: 5′-GTCGAAAATTCAAGACAAGG-3′.
  > pPR3-N and pBT3-N Reverse: 5′-AAGCGTGACATAACTAATTAC-3′.

  > pBT3-N Bait primer F: SfiI restriction site (GGCCATTACGGCC) and ~20 nucleotides from 5' of proposed bait sequence. Include ATG codon.
  > pBT3-N Bait primer R: SfiI restriction site (GGCCATTACGGCC) and ~20 nucleotides from 3' of proposed bait sequence. Include STOP codon.
  > pPR3-N Bait primer F: SfiI restriction site (GGCCATTACGGCC) and ~20 nucleotides from 5' of proposed bait sequence. Start ATG is optional as there is a 5' HA-tag in this plasmid
  > pPR3-N Bait primer F: SfiI restriction site (GGCCATTACGGCC) and ~20 nucleotides from 5' of proposed bait sequence. Include STOP codon.
  > SfiI-oligo-dT primer: AAGCAGTGGTATCAACGCAGAGTGGCCGAGGCGGCC$(T)_{20}$VN
  > SfiI-5'adaptor: AAGCAGTGGTATCAACGCAGAGTGGCCATTACGGCCGGGGG-P

3. Methods

Here we provide a consensus methodology for MYTH. The core components of the protocol are essentially the same whether investigating the targeted interaction of two known bait and prey proteins or for a screen for novel components. In each case a functional Cub bait construct is needed, which should be tested through interaction with the pOst1-NubI positive control (**Step 5**). If this interaction is *unsuccessful* then nothing useful will come from the remainder of the MYTH protocol so time and resources would be wasted in preparing prey constructs. Therefore we recommend that generation and testing of a Cub bait construct should be verified before committing to any further experiments.

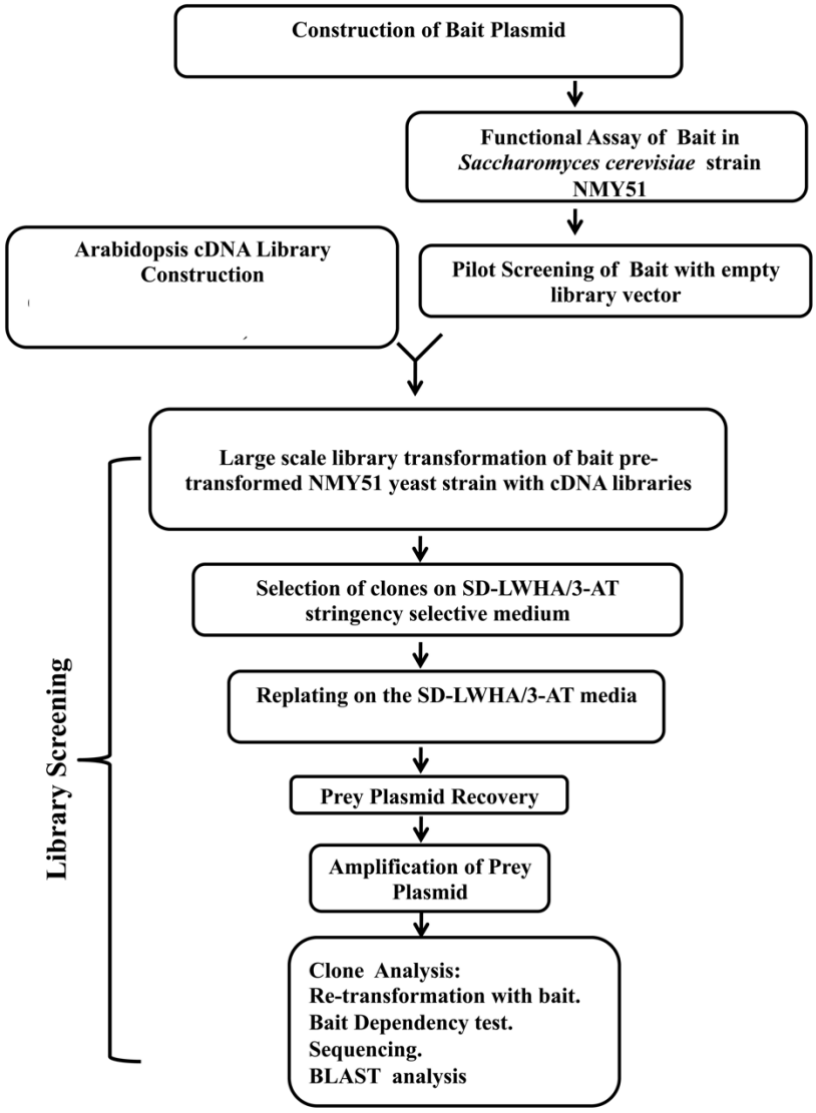

Figure 2: Workflow for screening Arabidopsis cDNA library for proteins interactors with selected bait-protein using MYTH. Adapted from Dutta *et al* (2014).

3.1 Generating and testing Cub bait construct.

1. Amplify bait cDNA from chosen template (from an ordered clone or amplified cDNA, see **Steps 10-25**) using primers with appropriate ends for cloning into the bait vector pBT3-N [**NOTE 1, NOTE 2**].

2. Digest both 1ug pBT3-N and an available amount of cDNA with SfiI at 50°C according to manufacturer instructions.

3. Ligate using T4 DNA ligase and transform appropriate *E.coli* strain [**NOTE 3**].

4. Test positive colonies by standard colony PCR using the 5' and 3' pBT3-N cloning primers.

5. Run samples on 1% agarose gel and if a band is at the predicted size then sequence selected clones using a local or commercial sanger sequencing service.

6. If successful clones are identified then co-transform *S.cerevisiae* strain NMY51 with pBT-N-Bait and positive control pOst1-NubI plasmid [**NOTE 4**]. This transformation is performed using standard lithium acetate transformation method [**NOTE 5**].

7. It is also necessary to test the self-activation of the bait-construct [**NOTE 6**]. The pBT-N-Bait should be transformed with the negative control prey vector pNubG-Fe65. This is performed by the standard lithium acetate transformation method [**NOTE 5]**. The difference from **Step 6** is that SD-LWHA test medium also includes an increasing concentration of the His3-competitive inhibitor, the 3-amino-1,2,4-triazole (3-AT) [**NOTE 7**]. Once the correct amount of 3-AT has been determined then is used during library screening to increase confidence that any positive colonies represent new interactions and are not due to bait self-activation [**NOTE 7**].

8. These tests will confirm both the activity of the bait with the pOst1-NubI positive control as well as the optimal concentration of 3-AT to add to the test medium. If these tests are successful then then the user can be confident that the bait is good to use to test the interaction with prey constructs.

3.2 Plant growth and tissue sampling

9. Plants are grown according to experimental needs. If researchers aim to screen the cDNA library from a specific tissue then this needs to be considered during planning so that they can extract an appropriate amount of RNA from the selected tissue. Voisin *et al* (2020) harvested twelve 10-15do Arabidopsis seedlings, corresponding to ~100mg tissue.

10. Tissue is frozen in liquid nitrogen and ground-up using a mortar and pestle [**NOTE 8**]. Alternatively depending on the required amount of tissue, samples can be a ground-up using a tissuelyser or similar bead-beater. In this case tissue is added to a 2ml Safe-lock eppendorf tube containing a metal bead before being frozen in liquid nitrogen and ground up using the specifics of the bead-beater [**NOTE 9**].

3.3 RNA Extraction and DNase treatment

Numerous options are available for RNA extraction from plant tissues. Column-based systems (such as the QIAGEN Plant RNeasy kit) are available for the isolation of clean samples. One drawback from these systems is that they usually remove the small RNA

fraction although this is not a problem when generating MYTH prey-constructs. Use of column-based extraction is more costly than using TRIZOL so the researcher must weigh-up what is best for their situation. Here we outline the protocol for RNA extraction using the TRIZOL method [**NOTE 10**].

11. Add 1m TRIZOL to the ground-up frozen tissue., Vortex well and leave at room temperature for 5minutes.

12. Add 200ul chloroform, vortex well and centrifuge at maximum speed for 10minutes.

13. Transfer upper phase (~600ul) to a new tube and add an equal volume of cold isopropanol. Store at -20C for >30minutes.

14. Centrifuge for 15minutes at 4°C. Pour off supernatant and wash with 1ml cold 70% ethanol. Centrifuge and repeat wash.

15. Remove all ethanol and air-dry for >10minutes.

16. Ensure pellet is dry before resuspending by pipetting in 50ul dH20 heated to 65°C. Voison *et al* (2018) report a total yield of 30ug from 100mg starting tissue.

17. Add 4ul DNaseI and 6ul 10x DNase buffer to RNA sample from **Step 16**. Incubate at 37°C for >1hour.

18. Add 140ul dH20 and 200ul phenol. Mix well by hand then centrifuge at max speed for 10minutes.

19. Remove upper phase and transfer to a fresh tube, add 200ul chloroform. Vortex well and centrifuge at max speed for 5minutes.

20. Remove upper phase (~200ul) to new tube containing 440 µL of 95% EtOH and 20µL 3M Sodium Acetate pH 5.2. Incubate at -20°C overnight.

21. Centrifuge for 15minutes at 4°C. Pour off supernatant and wash with 1ml cold 70% ethanol.

22. Remove all ethanol and air-dry for >10minutes.

23. Ensure pellet is dry before resuspending by pipetting in 50ul dH20 heated to 65°C. The RNA samples can be stored at -20°C but avoid freeze-thaw cycles.

24. Remove 1ul RNA to measure concentration and quality with a Nanodrop (or similar).

3.4 Reverse transcription

Numerous commercial versions of reverse transcriptase enzyme (RT) are available so the researcher should use the version that works well in their hands.

25. Use 1-5ug RNA in a RT reaction with 1ug Oligo dT 20mer primer using manufacturer instructions [**NOTE 11**].

26. cDNA samples can be stored at -20°C if needed.

3.5 Generating a cDNA library

Preparation of a cDNA library involves generation of a dsDNA population that has specific 5' and 3' adaptors that will allow for downstream cloning. Library preparation must be planned prior to the first stand synthesis RT reaction so that the oligodT primer has an appropriate 5' adaptor sequence. The single strand cDNA template (from **Step 26**) is used to generate a dsDNA population using DNA polymerase. Finally a 5'adaptor is ligated to dsDNA using DNA ligase. There are many kits available to generate a cDNA library [**NOTE 12**] that can be used to attach 5' and 3' adaptors that include SfiI sites that can be used for cloning into the pPR3-N prey construct (SfiI-oligo-dT primer and SfiI-5'adaptor).

Users should follow the guidelines for the cDNA preparation kit that is appropriate to use for their cloning procedure [**NOTE 13**]. Each library preparation protocol will involve the following general steps:

27. Second strand dsDNA synthesis using cDNA population (from **Step 26**) as template in a reaction with DNA polymerase and DNA ligase. This will generate a dsDNA population with a 3' SfiI-adaptor.

28. Phenol-chloroform or column-based extraction of dsDNA population.

29. Ligation of 5' SfiI adaptors using dsDNA population as template in a reaction with DNA ligase.

30. Size selection of adapted-ligated dsDNA population to remove fragments <400bp and unligated adaptors.

31. Storage at -20°C.

3.6 Cloning cDNA library into MYTH prey constructs

32. Digest both the pPR3-N prey-construct and the cDNA library with SfiI at 50°C according to manufacturers instructions.

33. Ligate using T4 DNA ligase and transform appropriate *E.coli* strain [**NOTE 14**].

34. For each aliquot of transformed *E.coli* perform three serial dilutions to obtain 1:10, 1:100 and 1:1000 concentrations of the original sample. Plate duplicate 100ul samples of each dilution onto pre-warmed LB+Ampicillin agar plates. Grow overnight at 37°C.

35. Count colonies on agar plates to determine overall titer of original transformation mix. A well represented library should contain $5x10^6$ -> $1x10^7$ primary clones.

36. Randomly select 20 colonies to grow overnight and extract plasmid DNA with a standard miniprep kit. Digest with SfiI to release prey-insert and run on 1% agarose gel to assess the size of any insert.

37. The average size of an insert should be ~1.5kb and over 95% of colonies should contain an insert. If this is not the case then the library preparation may have been unsuccessful.

38. The remaining transformation from **Step 33** can be stored at 4°C or (ideally) can be immediately processed in **Step 39** if the user is confident that the titer will be sufficient for their needs.

39. Grow transformation (from **Step 33**) overnight in a 500ml culture of LB+Ampicillin.

40. Perform a large-scale plasmid preparation from this culture using an appropriate kit (such as the QIAGEN Plasmid *Plus* Mega Kit). A total amount of 40ug plasmid DNA is required for a single library transformation as outlined in **Step 47**.

41. The plasmid DNA represents the library of prey-constructs that is ready to test for interaction with the pBT3-N-bait construct that was constructed and verified in **Steps 1-8**.

3.7 Generation and testing of interactions between known bait and prey

Firstly the targeted prey-construct must be generated in pPR3-N prior to co-transformation with pBT-N-Bait into yeast before selection on test media.

42. Amplify prey cDNA from chosen template (from an ordered clone or from cDNA library generated in **Step 26**) using primers with appropriate ends for cloning into the prey vector pPR3-N.

43. Digest both 1ug pPR3-N and an available amount of cDNA with SfiI at 50°C according to manufacturers instructions.

44. Ligate using T4 DNA ligase and transform appropriate *E.coli* strain.

45. Test colonies by standard colony PCR using the 5’ and 3’ cloning primers.

46. Run PCR samples on 1% agarose gel and if resulting bands are at the predicted size then sequence selected clones using local or commercial sanger sequencing service.

47. If prey-construct has been successfully constructed then co-transform NMY51 with pBT-N-Bait with pPR3-N-Prey using Lithium Acetate method [**NOTE 4, NOTE 15]**.

48. If the bait and prey proteins interact then transformed yeast will grow on SD-LWHA test plates. Any colonies can be tested by PCR using bait and prey-specific primers. Colonies that have both bait and prey clones can be streaked and maintained onto a fresh SD-LW plate. The strength of their interaction can then be evaluated by drop-test as outlined in **Step 67**.

3.8 Screening Prey library for novel interactions.

This portion of the protocol outlines how the Cub-bait protein is co-transformed with the library of Nub-prey proteins with the aim of identifying new interactions.

49. Grow 5ml of NMY51 containing pBT3-bait (from **Step 8)** for 8hr in DO-L at 30°C with shaking to generate a starter culture.

50. Use starter culture to inoculate 100ml DO-L at 30°C with shaking overnight.

51. Measure OD_{600} and remove the yeast that is equivalent to 30U OD_{600}. Pellet and add to 200ml of 2xYPD (OD_{546}= 0.15)

52. Grow at 30°C with shaking until OD_{600}=0.6.

53. Separate cultures in 4x50ml falcon tubes and pellet the cells. Wash on 30ml sterile water before resuspension in 600ul of 100mM LiAOAc in TE pH7.5.

54. Transfer each culture to a fresh 50ml tube and add a mixture of: 100ul salmon sperm DNA (20mg/ml), 10ug of pPR3-prey-cDNA library (from **Step 41**), 2.5ml of PEG/LiOAc mix (100mM LiOAc, 40% PEG 3350 in TE pH 7.5). Vortex and hold at 30°C for 45minutes without shaking.

55. Add 160ul DMSO, gently mix and heat shock at 42°C for 20minutes.

56. Gently centrifuge cells and harvest pellet, resuspend each in 3ml of 2x YPD and pooled to give a final mix of ~12ml co-transformed NMY51. Allow cells to recover at 30°C for 90minutes with slow shaking.

57. Pellet cells, wash and resuspend in 4.8ml 0.9% NaCl.

58. Transformation efficiency is determined by using 100ul to make a serial dilution to 1:100, 1:1000, 1:10000 in 0.9%Nacl, which are then plated on SD-LW and grown at 30°C. After 4days the number of colonies can be counted to determine transformation frequency [**NOTE 16**].

59. The remaining suspension from **Step 57** is plated on fifteen 150ml agar plates with SD-LWHA/3-AT media [**NOTE 15**].

60. Positive colonies from **Step 59** are transferred onto fresh SD-LWHA/3-AT plates to verify growth.

61. Plasmids are recovered from confirmed positive clones (from **Step 60**) using the method of Hoffman and Winston (1987)(**Steps 62-64**).

62. Grow 2ml yeast o/n at 30°C in SD-LWHA, pellet and add 200ul of Lysis buffer (2% Triton X-100, 1% SDS, 100mM NaCl, 10mM Tris pH8, 1mM EDTA) then 200ul phenol:choloform (1:1).

63. Add 0.3g acid-washed beads and vortex for 2minutes. Centrifuge at max speed for 10minutes.

64. Remove 1-5ul from the aqueous phase to transform *E.coli* DH5a using standard protocols with selection for colonies containing pPR3-prey on LB+Ampicillin.

65. To confirm bait-prey interactions, co-transform pPR3-prey constructs from **Step 64** with pBT3-bait as in **Step 47** [**NOTE 17**].

66. Use standard techniques to verify size of pPR3-N-prey insert and sequence using pPR3-N specific primers. The identity of novel prey clones can be revealed using standard bioinformatic software.

3.9 Evaluate Interaction by Drop Test

Once a positive bait-prey interaction has been identified (in **Step 48** or **Step 66**) a more detailed picture of the interaction can be obtained using a drop test.

67. Select a colony from a positive bait-prey interaction from selective SD-LWHA/3-AT plates. In addition select a positive colony (from pBT3-bait + pOst1-NubI) and a negative colony (from pBT3-bait + pNubG-Fe65).

68. Resuspend in 1ml SD-LW, adjust OD_{600} to 2.0.

69. Prepare 1:10, 1:100, 1:1000, 1:10000 dilutions in 0.9% NaCl.

70. Spot 8x 1ul drops on a SD-LW test plate and SD-LWHA/3-AT selective plate [**NOTE 18**]. Allow drops to dry until liquid is absorbed into the media plate

71. After 3-5days of growth at 30°C examine plates. The strength of interaction is defined by the difference in growth; as shown in Figure 3.

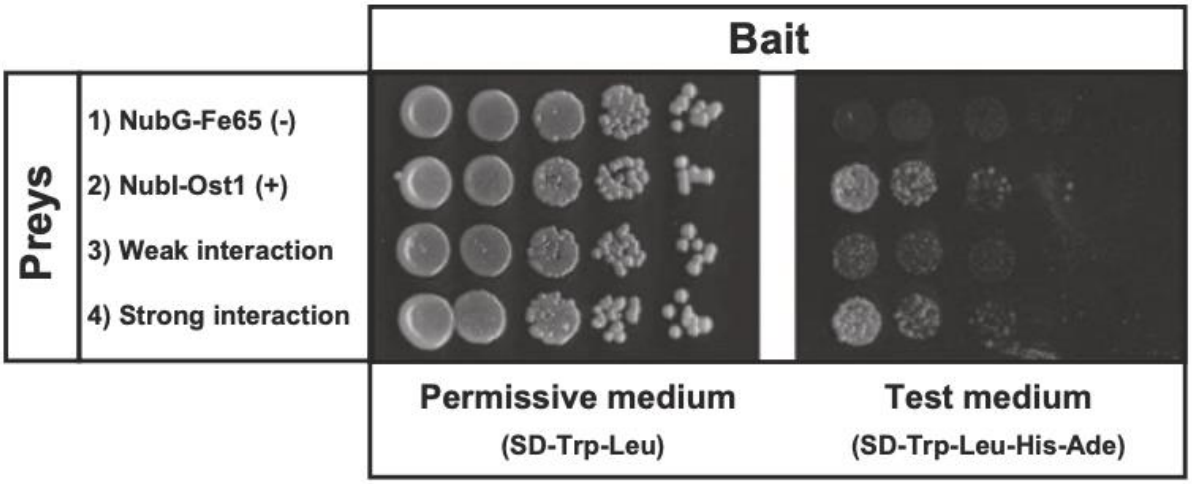

Figure 3: Example of a drop-test (taken from Voisin *et al*, 2018). Stronger interactions show more growth at lower dilutions (moving left to right).

4 Notes

NOTE 1: Users should use a cloning scheme with which they are familiar. The pBT3-N plasmid includes a SfiI restriction site that can be used for directional cloning of your bait cDNA (https://www.lifescience-market.com/plasmid-c-94/pbt3-n-p-108677.html). Alternatively cloning can be directly performed in *S. cerevisiae* by gap-repair. This involved co-transforming yeast with a linearalised pBT3-N and a bait cDNA sequence that is flanked by 35-40 extra nucleotides. These nucleotides provide a template for homologous recombination to generate a new clone. The gap-repair strategy is outlined in Voisin *et al* (2018) but the methodology outlined in this article will use directional cloning using SfiI (in *E.coli).*

NOTE 2: The suitability of a protein for use as a MYTH bait must be determined on a case-by-case basis as there is no easy way to predict how a given protein will perform as a bait. A major consideration is the membrane topology of the protein of interest, and so when selecting a potential bait, it is critical to check that its N and/or C terminus is predicted to reside in the cytosol of the cell. Correct functioning of the system requires that the endogenous yeast DUBs have access to the Cub-LexA-VP16. If the bait topology is unknown, the user can generate constructs tagged at both N- and C-termini and empirically assess their performance to interact with the pOst1-NubI positive control.

NOTE 3: This protocol does not require unusual strains of *E.coli* so DH5α or similar is sufficient. Remember to transform an appropriate no-insert control to determine the level of background transformation of the SfiI-digested vector.

NOTE 4: pOst1-NubI used as a positive control is a prey vector expressing an endoplasmic reticulum protein Ost1 fused to the wild-type Nub. This is under the control of the Adh1 promoter and in the presence of an interacting bait will cause release of the TF LexA-VP16 and activation of the reporter genes His3 and Ade2 allowing yeast growth on SD-LWHA test medium.

NOTE 5: Lithium acetate/single-stranded carrier DNA/polyethylene glycol method:
1: Grow 50ml yeast strain NMY51 overnight in YPD at 30°C.
2: At OD_{600} 0.4-0.6 transfer cells to 50ml falcon tube and centrifuge to pellet.
3: Resuspend in 1ml TE/LiOAc 1× in a 1.5ml eppendorf.
4: Wash twice by centrifuging at 11000*xg* and resuspending in 1ml TE/LiOAc 1×
5: After second wash resuspend in 500ul TE/LiOAc 1× (sufficient for at least ten transformations).
6: Mix 40 µL NMY51/TE/LiOAc 1×, 300 µL PEG/TE/ LiOAc and either **1**, 1µL pBT3-N-Bait only; **2,** 1µL pBT3-N-Bait + 1µL pOst1-NubI, **3**, 1µL pOst1-NubI only. All plasmids at 100ng/ul.
7: Briefly vortex then incubate 30°C for 30 min, add 12 µL DMSO and heat shock at 42°C for 15 min.
8: Plate half of the yeast cells on SD-LW selective medium and the other half on SD-LWHA test medium. Growth on SD-LW selects for colonies that contain each plasmid but makes no determination of the level of interaction between bait and prey.
9: Grow for >3d at 30°C then estimate the %tage growth on SD-LWHA versus SD-LW. If the bait is functional, 20% more growth will be observed on the test medium SD-LWHA.

NOTE 6: In two-hybrid screens, background can be caused by self-activation of bait proteins that provokes growth on selective medium without any interaction. The aim of the bait self-activation test is to estimate the background when the bait is co-expressed with a non-

interacting prey control. This will allow for adjustment of selection conditions used for the screen by adding an His3-competitive inhibitor, the 3-amino-1,2,4-triazole (3-AT) on test medium plates.

NOTE 7: The background of the bait on test medium can be removed by the addition of 3-AT. As different baits do not require the same amount of 3-AT, the concentration has to be adjusted depending on the bait used. Higher concentrations of 3-AT will only select for yeast strains that have higher expression of the HIS3 gene due to a stronger interaction between bait and prey. Therefore adjusting the amount of 3-AT will increase the stringency of the MYTH reaction. To find the optimal 3-AT concentration, SD-LWHA test medium is supplemented with 0, 1, 2.5, 5, 10, 50 and 100 mM 3-AT. For weak interactions, the stringency of the test medium plate can be adjusted either by decreasing the level of 3-AT in the SD-LWHA test medium.

NOTE 8: Grinding tissue under liquid nitrogen is most effective when tissue is initially ground up, scraped to the bottom of the pestle using a cold spatula before carefully adding new liquid nitrogen to grind up the tissue again. Ensure the metal spatula is cooled in liquid nitrogen prior to scrapping tissue as otherwise the tissue can melt before transfer to RNA extraction buffer.

NOTE 9: In personal experience plant tissue is better disrupted using a mortar and pestle compared to a beadbeater. However the beadbeater allows for much more rapid extraction. Therefore a user needs to weigh-up the difference between extraction quality and time available.

NOTE 10: Whether a column-based or TRIZOL method is used it is essential that the initial RNA extraction buffer is added to the tissue whilst still frozen. If the sample begins to melt out of extraction buffer then the resulting RNA quality will diminish.

NOTE 11: The specific reagent mix will vary depending on the RT kit that is used. The protocol will involve a denaturation step at 70°C and an incubation at 42°C >1hr. The oligodT primer will include a 5' addition of the relevant adaptor for downstream cloning.

NOTE 12: Many published protocols for using MYTH to generate a cDNA library and to screen a library of prey-clones reference protocols from Dualsystems Biotech, Switzerland. Unfortunately this portion of company activities was sold to Hybrigenics in 2013 and the protocols and kit are now unavailable for purchase (https://www.dualsystems.com/2013/07/01/dualsystems-biotech-sells-its-yeast-two-hybrid-y2h-activities-to-hybrigenics/). As noted in the Materials section the plasmids and yeast stocks can be obtained from other sources but a single yeast library screening kit is not available.

NOTE 13: There are many cloning solutions available and if users routinely use Gateway cloning then Gateway-compatible versions of pPR3-N are available. In this case cDNA library preparation can be performed using the Gateway compatible CloneMiner kit (https://www.thermofisher.com/order/catalog/product/A11180?uk&en#/A11180?uk&en)

NOTE 14: *E.coli* transformation of an cDNA library involved use of ultra-competent cells either by electroporation or standard chemical transformation. Split pPR3-N prey library into 6 fractions and independently transform an aliquot of competent cells. It is important to include all of the transformation controls, which include known concentrations of **1,** Undigested pPR3-N plasmid; **2,** Digested pPR3-N plasmid; **3,** cDNA-only control.
To test the titer of the transformation 100ul aliquots are removed as outlined in **Step 34-37.**

NOTE 15: Use a concentration of 3-AT determined during the pBT3-bait self-activation test outlined in **Step 7**.

NOTE 16: Growth on SD-LW selects for colonies that contain each plasmid but makes no determination of the level of interaction between bait and prey.

NOTE 17: This is an optional step but will provide additional confirmation that the bait-prey interaction is legitimate.

NOTE 18: Carefully spot 1ul aliquots as they can easily spray across the agar surface.

5 References

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