A pipeline for systematic yeast 2-hybrid matricial screening in liquid culture.

Monachello Dario (dario.monachello@inra.fr)
INRA

Guillaumot Damien
INRA

Lurin Claire
INRA

Method Article

Keywords: Yeast 2-hybrid screen, high-throughput interaction mapping, liquid pipeline.

Posted Date: June 27th, 2019

DOI: https://doi.org/10.21203/rs.2.9948/v1

Abstract

Physical interactions mediated by proteins are a critical element of biological systems, and the analysis of interaction partners can provide valuable hints about unknown functions of a protein. Two major classes of experimental approaches are used for protein interaction mapping: analysis of direct interactions using binary methods such as yeast two-hybrid (Y2H) or split ubiquitin, and analysis of protein complexes through affinity purification followed by mass spectrometry. Thanks to his flexibility to low- and high-throughput approaches and a low operating cost the Y2H assay is widely used for high-throughput interaction mapping experiments. Moreover, it has now been shown that high-throughput methods can produce highly reliable interactome datasets[1 2 3 4]. Notably, in 2011 a proteome-wide binary protein-protein interaction map of the plant *Arabidopsis thaliana* [5] (Arabidopsis Interactome Mapping project – AIM) was described using a high-throughput binary interactome mapping pipeline based on the Y2H system and using a collection of ~8,000 open reading frames (8k_space). Here we describe a liquid pipeline for a high-throughput binary protein–protein Y2H screen of a pool of 50 proteins used as baits against a collection of ~12,000 Arabidopsis proteins encoded by sequence-verified ORFs (12k_space)[6 7].

Introduction

The protocol described here is an adaptation of the one presented in Dreze et al., 2010[7]. The same low copy number yeast expression vectors expressing DB-X and AD-Y hybrid proteins and the two Y2H strain backgrounds, *S. cerevisiae* Y8930 (for DB clones; MATα) and *S. cerevisiae* Y8800 (for AD clones; MATa) which harbor the following genotype: *leu2-3,112 trp1-901 his3-200 ura3-52 gal4D gal80D GAL2-ADE2 LYS2::GAL1-HIS3 MET2::GAL7-lacZ cyh2R* were used. The reporter genes *GAL2-ADE2* and *LYS2::GAL1-HIS3* are integrated into the yeast genome. Expression of the *GAL1-HIS3* reporter gene has to be tested with 1 mM 3AT (3-amino-1,2,4- triazole, a competitive inhibitor of the HIS3 gene product). A pool of 50 proteins of interest - POI used as baits is screened against the 12k_space AD-collection or the corresponding space DB-collection (without autoactivators) depending on the autoactivation state of the baits.

Reagents

Gateway® LR Clonase Enzyme Mix (Invitrogen)

Competent DH5a-T1R (Invitrogen)

HiFi Platinum Taq polymerase buffer (Invitrogen)

Lysogeny broth (LB) media

Super Optimal Broth (SOC) media

Salmon Sperm DNA

Tris-EDTA buffer (TE) 10 mM Tris–HCl (pH 8.0), 1 mM EDTA (pH 8.0),

Lithium acetate

Polyethyleneglycol (PEG) 3350

Yeast extract-peptone-dextrose (YEPD) media

Zymolase 20T (21,100 U/g, Seikagaku Corp.)

Synthetic complete (SC) media

3-Amino-1,2,4-triazole (3AT)

Equipment

Qpix2 XT (Molecular Devices)

MULTIDROP 384 (Thermo Ficher)

Liquid handling platform or Multichannel pipette

Tecan Infinite M200 PRO

Low profile microplates 96 (X6011) and 384 (X7001) well (Molecular Devices)

Water bath

Pipetman

Pipette tips

50 ml polypropylene conical tubes

Table top centrifuge

96 well PCR plate

Incubator

Standard microcentrifuge tubes, 1.5 ml and 2 ml

Petri dishes

Thermocycler with programmable temperature stepping functionality, 96 well

Procedure

A) Generation of expression plasmids

The 50 proteins used as baits were transferred into pDest-DB and pDest-AD vectors in simple single-step reaction by Gateway recombination. Completed recombination reactions are then transformed into Escherichia coli, grown for 18 h, and plasmids are isolated.

Protocol 1A: Gateway LR recombinational cloning

For each bait combine in 96-well PCR plate:

1)

- 1 µl of entry clone (10 ng/µl)
- 1 µl of destination vector (100 ng/µl)
- 1 µl clonase buffer 5x
- 1.6 µl TE 1x
- 0,4 µl of LR clonase enzyme mix (Invitrogen) (keep this mix on ice)

2) Homogenize by gently pipetting up and down.

3) Incubate at 25 °C for 18 h

4) Add 2 µl of Proteinase K (2µg/µl) solution

5) Incubate 15 min at 37°C

Protocol 2A: Bacterial transformation

1) Thaw competent DH5a-T1R (Invitrogen) cells on ice

2) Add 100 µl of competent cells into 96-well PCR plate containing 5 µl Gateway LR reaction mix

3) Incubate on ice water for 20 min

4) Heat shock cells in a water bath at 42 °C for 15 sec

5) Incubate on ice water for 5 min

6) Transfer the cells into a 96 deep well plate filled with 900 µl SOC

7) Incubate at 37 °C for 1 h

8) Plate out 300µl of reaction onto LB plates containing 100mg/l Ampicillin

9) Incubate over night at 37°C

10) Pick a single colony from the plate and inoculate a 96 deep well plate filled with 1 ml of LB containing 100mg/l Ampicillin

11) Incubate at 37 °C for 18 h

12) Remove 5 µl for subsequent analysis by PCR

13) Remove 100 µl of the overnight culture, mix with 100 µl of 40% (w/v) autoclaved glycerol and store at 80 °C into a 96-well Low profile microplates

14) Use the remainder of the overnight culture for plasmid isolation

Protocol 3A: Bacterial culture PCR

Dilute 5 µl of bacterial culture into 95 µl of sterile water and mix by pipetting up and down. Keep bacterial cultures at 4 °C until PCR results are determined.

For each reaction, prepare in a 96-well PCR plate on ice:

- 1.1 µl of HiFi Platinum Taq polymerase buffer 10x (Invitrogen)
- 0.4 µl of 50 mM MgSO4 (final concentration 1.8 mM)
- 0.11 µl of 40 mM dNTPs (final concentration 400 nM)

- 0.022 µl of 200 µM AD or DB forward primer (final concentration180 nM)
- 0.022 µl of 200 µM Term reverse primer (final concentration 180 nM)
- 0.066 µl of HiFi Platinum Taq polymerase (Invitrogen)
- 8.28 µl of filter-sterilized water
- Add 1 µl of the diluted bacterial culture as DNA template

Place the PCR plate on a thermocycler and run the following program:

Step 1: Denaturation at 94 °C for 4 min.

Step 2: Denaturation at 94 °C for 30 s.

Step 3: Annealing at 58 °C for 30 s.

Step 4: Elongation at 68 °C for x min (depending on the length of the longer ORF)

Repeat Steps 2-3-4, 34 times.

Step 5: Final elongation at 68 °C for 10 min.

Step 6: Hold at 10 °C.

Primer sequences:

AD: 5’-CGCGTTTGGAATCACTACAGGG-3’

DB: 5’-GGCTTCAGTGGAGACTGATATGCCTC-3’

Term: 5’-GGAGACTTGACCAAACCTCTGGCG-3’

Once PCR reactions are completed, analyze 5 µl of PCR product on a 1% agarose. Successful LR reactions will give rise at ORFs sizes to which ~280 bp of vector sequences are added due to the AD, DB,

and Term primer positions (the PCR amplicon from a destination vector containing the Gateway cassette has an expected size _1.9 kb). The remaining of the cultures are used for plasmid isolation.

B) Yeast transformation and identification of autoactivating DB-X hybrid proteins

Isolated destination clones were individually transferred into competent Y8930 (MATα) and Y8800 (MATa) strains by Lithium-Acetate based transformation. Identification of DB-ORF autoactivators is achieved in haploid yeast strain onto a Sc–Leu–His + 3-amino-1,2,4-triazole (3-AT) media.

Protocol 1B: Yeast transformation

1) Streak Y8800 and Y8930 on separate YEPD plates and incubate at 30 °C for 48–72h to obtain isolated colonies.

2) For each strain, inoculate 20 ml of YEPD with 10 isolated colonies. Incubate at 30 °C on a shaker for 14–18 h.

3) Measure and record the OD600, which should be between 4.0 and 6.0. Dilute cells into 100 ml of YEPD media to obtain a final OD600 ~ 0.1.

4) Incubate at 30 °C on a shaker until OD600 reaches 0.6–0.8 (4–6 h).

5) Boil carrier DNA (salmon sperm DNA) for 5 min then place on ice until needed.

6) Harvest cells by centrifugation at 800 x g for 5 min. Discard the supernatant and resuspend cells gently in 10 ml of sterile water.

7) Centrifuge as described in step 6 and discard the supernatant.

8) Resuspend cells in 10 ml of TE/LiAc solution, centrifuge, and discard the supernatant.

9) Resuspend cells in 2 ml of TE/LiAc solution, then add 10 ml of TE/ LiAc/PEG solution supplemented with 200 µl of boiled carrier DNA. Mix the solution by inversion.

10) Dispense 120 µl of this mix into each well of a round-bottom 96-well microtiter plate

11) Add 10 µl of plasmid DNA to the competent yeast and mix by pipetting up and down several times.

12) Incubate at 30 °C for 30 min

13) Subject to heat shock in a 42 °C water bath for 15 min.

14) Centrifuge 5 min at 800 x g. Carefully remove the supernatant

15) Add 100 µl of sterile water and resuspend cell pellets by pipetting up and down.

16) Centrifuge 5 min at 800 x g, then carefully remove 90 µl of water

17) Resuspend cell pellets by vortexing.

18) Spot 5 µl of cell suspension onto an appropriate selective plate (Sc-Trp for AD-Y, Sc-Leu for DB-X agar media).

19) Incubate at 30 °C for 72 h.

20) Pick transformed yeast colonies into a 96-well low profile microplates containing 100 µl of selective media (Sc-Trp for AD-Y, Sc-Leu for DB-X).

21) Incubate on a shaker at 30 °C for 72 h.

22) Remove 5 µl for subsequent lysis and analysis by PCR

23) Prepare archival stocks by combining 100 µl of the yeast culture with 100 µl of 40% (w/v) autoclaved glycerol into a 96-well Low profile microplate. Store at -80 °C.

Protocol 2B: Yeast cell lysis and lysate PCR

1) Prepare lysis buffer by dissolving 2.5 mg/ml zymolase 20T (21,100 U/g, Seikagaku Corp.) in 0.1M sodiumphosphate buffer (pH7.4). Keep on ice.

2) Aliquot 15 µl of lysis buffer into the wells of a 96-well PCR plate. Keep on ice.

3) Add 5 µl of yeast cells in the lysis buffer in the PCR plate

4) Place the PCR plate on a thermocycler and run the following program:

- Step 1: 37 °C for 15 min
- Step 2: 95 °C for 5 min
- Step 3: Hold at 10 °C

5) Add 100 ml of filter-sterilized water to each well.

6) Store at -20 °C

Protocol 3B: Yeast lysate PCR

For each reaction, prepare in a 96-well PCR plate on ice:

- 1.1 µl of HiFi Platinum Taq polymerase buffer 10x (Invitrogen)
- 0.4 µl of 50 mM MgSO4 (final concentration 1.8 mM)
- 0.11 µl of 40 mM dNTPs (final concentration 400 nM)
- 0.022 µl of 200 µM AD or DB forward primer (final concentration180 nM)
- 0.022 µl of 200 µM Term reverse primer (final concentration 180 nM)
- 0.066 µl of HiFi Platinum Taq polymerase (Invitrogen)
- 8.28 µl of filter-sterilized water
- Add 3 µl of the yeast cell lysate as DNA template

Place the PCR plate on a thermocycler and run the same program of Protocol 3A. Once PCR reactions are completed, analyze 5 µl of PCR product on a 1% agarose.

Protocol 4B: Autoactivator removal

1) Thaw glycerol stocks of the 50 individual DB-bait yeast strains and the glycerol stock of the yeast strains transformed with the AD encoding plasmid containing no insert (empty pDESTAD).

2) Using the colony picker Qpix2 XT replica-plate the 96-well Low profile microplate with the individual glycerol stocks of each DB-X yeast strain and the yeast strains transformed with the empty-AD into a new plates filled with 100 µl of fresh Sc-Leu and Sc-Trp media respectively.

3) Incubate at 30 °C for 72 h on a shaker

4) Replica-plate from the Sc-Leu-media and Sc-Trp media plates onto mating plates filled with YEPD media

5) Incubate at 30 °C for 24 h.

6) Replica-plate from mating plates onto Sc-Leu-Trp media plates to select for diploid cells and onto Sc-Leu-Trp-His + 1mM 3AT media.

7) Incubate at 30°C for 72 h. Score growth phenotypes. Non-autoactivating yeast cells are not able to activate the GAL1::HIS3 reporter gene hence should not grow on the Sc-Leu-Trp-His + 1mM 3AT media.

Yeast strains showing a growth phenotype on the Sc-Leu-Trp-His + 1mM 3AT media are considered autoactivators. They are physically removed from the collection of DB-X baits and screened against the 12k_space DB-collection using their AD-construction.

C) Primary Screening

Protocol 1C: Construction of DB baits pool

This protocol describes the construction of one pool of 50 different DB-hybrid constructs transformed into Y8930 yeast strain.

1) Thaw glycerol stocks of the 50 individual DB-bait yeast strains

2) Individually inoculate 50-ml polypropylene conical tubes that contain 5 ml of fresh Sc-Leu selective media, with 5 µl of the thawed glycerol stock.

3) Incubate at 30 °C for 72 h.

4) Transfer the contents of the culture tubes into a sterile trough.

5) Mix thoroughly to ensure equal representation of all DB-bait yeast strains in the pool.

6) On a liquid handling platform or with a multichannel pipette plated into the wells of ten 384-well Low profile microplates

Protocol 2C: 12k_space AD-collection culture

1) Thaw glycerol stocks of the 12k_space AD-collection corresponding to 127 96-well plates.

2) Using the colony picker Qpix2 XT replica-plate into 32 384-well plates filled with 50 µl of fresh Sc-Trp media

3) Incubate at 30 °C for 72 h

Protocol 3C: Mating

1) Using the colony picker Qpix2 XT replica-plate the 32 384-well plates corresponding to the 12k_space AD-collection 72 h cultures into the 384-well mating plates filled with YEPD media

2) Using the colony picker Qpix2 XT replica-plate the DB-baits pool into the 384-well mating plates precedently inoculated with the AD-collection. Use one DB-baits pool plate to inoculate five mating plates.

3) Incubate at 30 °C for 24 h

Protocol 4C: Screening

1) Using the colony picker Qpix2 XT replica-plate onto 384-well screening plates filled with 50 µl of fresh Sc-Leu-Trp-His + 1 mM 3AT media. Only diploid yeast with interacting couples can growth in this media.

2) Incubate at 30 °C for 5 days

3) Using the microplate-reader Tecan Infinite M200 PRO measure the OD600 of the 384-well screening plates in order to identify primary positives

Protocol 5C: AD-interacting proteins re-array and archival stock

1) Thaw glycerol stocks of the 12k_space AD-collection plates corresponding to the identified primary positives AD-interacting proteins

2) Using the colony picker Qpix2 XT re-array the primary positives AD-interacting proteins into 96-well plates filled with 100 µl of fresh Sc-Trp media.

3) Incubate at 30 °C for 72 h

4) Prepare an archival glycerol stock by adding 100 µl of 40% (w/v) autoclaved glycerol

D) Deconvolution and targeted matricial assay

Protocol 1D: DB-baits and AD-interacting proteins cultures

1) Thaw glycerol stocks of the 50 individual DB-bait yeast strains and of the identified primary positives AD-interacting proteins

2) Individually inoculate 50 distinct 50-ml polypropylene conical tubes that contain 10 ml of fresh Sc-Leu selective media, with 5 µl of the 50 individual DB-baits

3) Using the colony picker Qpix2 XT replica-plate the glycerol stocks of the identified primary positives AD-interacting proteins into ten 96-well plates filled with 100 µl of fresh Sc-Trp selective media

4) Incubate at 30 °C for 72 h.

5) On a liquid handling platform or with a multichannel pipette plate the 50 individual DB-bait cultures into the wells of 50 distinct 96-well Low profile microplates

Protocol 2D: Mating

1) Using the colony picker Qpix2 XT replica-plate the identified primary positives AD-interacting proteins cultures into the 96-well mating plates filled with 100 µl of YEPD media. Use one AD-interacting proteins plate to inoculate five mating plates.

2) Using the colony picker Qpix2 XT replica-plate the individual DB-baits cultures into the 96-well mating plates precedently inoculated with the AD-interacting proteins.

3) Incubate at 30 °C for 24 h

Protocol 3D: Verification and interacting proteins identification

1) Using the colony picker Qpix2 XT replica-plate onto two phenotyping 96-well plates:

- Verification-plates filled with 100 µl of fresh Sc-Leu-Trp-His + 1 mM 3AT media. Only diploid yeast with interacting couples can growth in this media.

- *de novo* autoactivators-plates filled with 100 µl of fresh Sc-Leu-His + 1 mM 3AT + CHX (1 mg/l) media. Only truncated fragments that can act like transcription factors can growth in this media

2) Incubate at 30 °C for 5 days

3) Using the microplate-reader Tecan Infinite M200 PRO measure the OD600 of the 96-well plates in order to identify positives pairs and *de novo* autoactivators

4) Using the colony picker Qpix2 XT re-array the identified positives pairs into 96-well plates filled with 100 µl of fresh Sc-Leu-Trp-His + 1 mM 3AT

5) Incubate at 30 °C for 72 h

6) Lyse cells according to Protocol 2B

7) Amplify the inserts of the DB-X and AD-Y inserts of positive cultures by yeast colony PCR according to Protocol 3B for subsequent ORF identification by end-read sequencing.

8) Prepare an archival glycerol stock by adding 100 µl of 40% (w/v) autoclaved glycerol

Troubleshooting

- Y2H auto-activator removal **(Protocol 4B)**: All techniques used to map protein interactions can give rise to artifacts. Ensure all the autoactivators are physically removed before the primary screen.

- Minimizing false negatives: Run replicates of the experiment to increase the number of detected interaction pairs.

Time Taken

The time required to entirely execute this protocol is approximately two months.

Anticipated Results

Identification of protein interaction partners yields crucial biological insights and assign functions to both uncharacterized and well-studied gene products.

References

1 Braun et al., An experimentally derived confidence score for binary protein–protein interactions. *Nat. Methods* 6, 91–97. (2009)

2 Cusick et al., Literature-curated protein interaction datasets. *Nat. Methods* 6, 39–46. (2009)

3 Simonis et al., Empirically controlled mapping of the Caenorhabditis elegans protein–protein interactome network. *Nat. Methods* 6, 47–54. (2009)

4 Venkatesan et al., An empirical framework for binary interactome mapping. *Nat. Methods* 6, 83–90. (2009)

5 Arabidopsis Interactome Mapping Consortium. Evidence for Network Evolution in an Arabidopsis Interactome Map. *Science* 333(6042):601–606. July (2011)

6 Ralf Weßling et al., Convergent targeting of a common host protein-network by pathogen effectors from three kingdoms of life. *Cell Host Microbe*. September 10; 16(3): 364–375. 2014

7 Dreze et al., High-Quality Binary Interactome Mapping. Methods in Enzymology. Chapter twelve. (2010*)*

Acknowledgements