Detailed Protocol – rec-YnH

rec-YnH enables simultaneous many-by-many detection of direct protein-protein and protein-RNA interactions

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Abstract

Knowing which proteins and RNAs directly interact is essential for understanding cellular mechanisms. Unfortunately, discovering such interactions is still resource intensive and comes with considerable uncertainties. With the aim of creating a reliable, low-tech and affordable screen, we developed rec-YnH, a new yeast two- or three-hybrid-based screening pipeline capable of detecting interactions within protein libraries or between protein libraries and RNA fragment pools. rec-YnH combines batch cloning and transformation with intracellular homologous recombination to generate bait-prey fusion libraries with high efficiency. This protocol accompanies Yang, Garriga et al., Nature Communications, 2018.

Introduction

Revealing which proteins, protein domains and RNAs undergo direct, physical interactions is an essential prerequisite for understanding the principles by which c.ellular machines operate. Assays that can detect such interactions are in high demand as they function both to generate system-level views of cellular coordination but also to generate hypothesis for subsequent mechanistic studies[1]. Yeast two and three hybrid (Y2H, Y3H) screens[2,3], allow to detect direct protein-protein and protein-RNA interactions under physiological conditions. The Y2H assay was enhanced significantly in the past. Coupling the readout to next-generation sequencing (NGS)[4] and multiplexed generation of genetically fused bait-prey libraries[5,6] resulted in increased assay sensitivity and reproducibility while allowing screening many baits against many preys simultaneously. Improvements of Y3H, however, mostly focused on optimized RNA binding and presentation by linker RNA engineering and selection of stronger MS2-binding proteins[7]. To date, there is no method available allowing the many-by-many direct interaction detection of full-length RBPs and RNA-fragments.

With the development of rec-YnH, we aimed at creating one assay that further advances and integrates past developments in Y2H and Y3H technologies into a single, new assay pipeline allowing screening of protein libraries against protein libraries or RNA fragment libraries. At the same time, we focused on keeping the assay workflow as simple as possible to make it affordable and doable for the standard biomedical research lab. The assay pipeline combines batch-cloning and transformation with intracellular homologous recombination, liquid gel culturing, yeast two or three-hybrid based interaction detection and read out by next-generation-sequencing. This eliminates barcoding, arraying and plating steps needed for available methods. Three compatible vectors allow high-throughput interaction screening within protein libraries and, for the first time, between protein and RNA libraries. We demonstrate that the same assay pipeline can be used for rec-Y2H and rec-Y3H screening and thus create an assay for both, many-by-many protein-protein or protein-RNA interaction detection.

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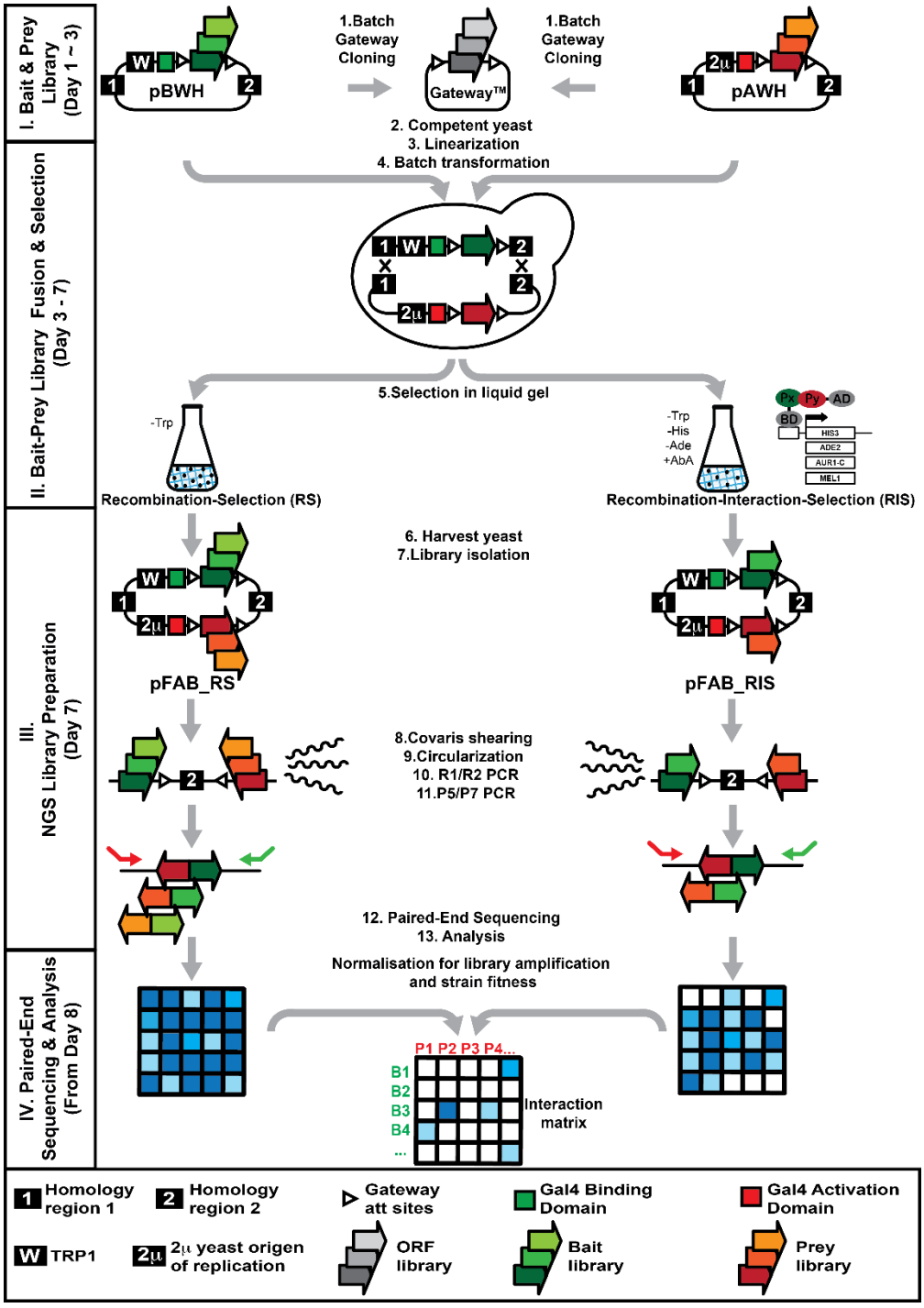
rec-YnH WORKFLOW

Next, a detailed protocol for rec-Y2H, a protein-protein interactions screening between a library of baits and a library of preys, can be found. rec-Y3H screening, to identify RNA-protein interactions, follows the same protocol as rec-Y2H, with minor differences. A table with differences between rec-Y2H and rec-Y3H can be found in SECTION 2, as well as a detailed description of the steps that are specific for rec-Y3H



PARTS OF THE PROTOCOL (Figure 1)

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PROTOCOL AT A GLANCE, DAY BY DAY

DAY 1

Step 1. Batch Gateway cloning of ORF library into pDEST vectors
- Prepare pool of pENTR-ORF clones at 10 nM
- Prepare pDEST for Bait and Prey at 10 nM
- Set up LR reactions

Step 2. Prepare competent yeast
- Streak Y2HGold on a YPDA plate

DAY 2

Step 1. Batch Gateway cloning of ORF library into pDEST vectors
- Continue LR reaction
- Transform LR reaction into NEB stable cells
- Plate on LB-Agar with corresponding antibiotic

DAY 3

Step 1. Batch Gateway cloning of ORF library into pDEST vectors
- Colony counting
- Harvest colonies from LB-Agar plates
- Qiaprep DNA prep of Bait and Prey pDEST-ORF pool

Step 2. Competent yeast
- Grow 1 colony of Y2HGold during 8 hours
- Transfer to 50 ml YPDA media
- Grow overnight

Step 3. Linearization
- Digest Bait and Prey pDEST-ORF pool with I-SceI/I-CeuI
- Heat inactivation

DAY 4

Step 2. Competent yeast
- Transfer yeast to 100 ml YPDA
- Grow yeast to OD 0.5
- Wash cells
- Resuspend in TE/LiAc

Step 4. Batch yeast transformation
- Prepare Seaprep liquid-gel selection media
- Multiple transformations of yeast with linear Bait and Prey pDEST-ORF pool
- Pool together all transformations
- Plate dilutions for colony counting

Step 5. Growth in selection media
- Add transformations to liquid-gel selection media
- 1 hour on ice
- Grow at 30ºC for 60 hours

DAY 7

Step 6. Harvest yeast
- Colony counting
- Centrifuge liquid-gel selection media with yeast colonies
- Wash and resuspend in PBS
- OD_{660} to calculate concentration of cells

Step 7. Yeast library DNA isolation
- For each selection media, Zymoprep™ Yeast Plasmid Miniprep of recombined Bait and Prey fragments

Step 8. Covaris shearing
- Shear DNA to 1,500-bp fragments

Step 9. Circularisation
- End repair
- Mini Elute DNA purification
- Ligation
- Heat inactivation

Step 10. R1/R2 PCR
- 12 PCR cycles with Q5 polymerase
- Purification with AMPure beads

Step 11. P5/P7 PCR
- 12 PCR cycles with Q5 polymerase and NEBNext® Multiplex Oligos for Illumina
- Purification with AMPure beads

DAY 8 onwards

Step 12. Paired-end sequencing
- Library quality control
- MiSeq run

Step 13. Analysis

REAGENTS AND MATERIALS

Step 1. Batch Gateway cloning of ORF library into pDEST vectors

- pENTR-ORF clones
- TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA)
- 10 mM Tris, pH 8.0
- LR clonase II enzyme mix (ThermoFisher Scientific)
- NEB stable cells (New England Biolabs)
- SOC media
- 10-cm LB-agar plates
- Ampicillin
- Spectinomycin
- Kanomycin
- 1x PBS
- QIAprep Spin Miniprep Kit (Qiagen)

Step 2. Competent yeast

- Y2HGold (Clontech)
- Loops
- Plastic cuvettes
- YPDA–agar plates
- YPDA liquid media
- 50 ml tubes
- 250 ml flasks
- 500 ml flasks
- Sterile H2O
- TE buffer (10x)
- 1 M LiAc (10x)

Step 3. Linearization

- I-SceI ((New England Biolabs)
- I-CeuI (New England Biolabs)
- Agarose gel

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Step 4. Batch yeast transformation

- Yeast carrier DNA (Clontech)
- 50% PEG (Sigma)
- 1 M LiAc (10x)
- DMSO (Sigma)
- 0.9% (w/v) NaCl

Step 5. Growth in selection media

Yeast dropout agar plates

- Square BioAssay dishes, 245×245 mm (Corning) for screen
- 10-cm petri dish for colony counting
- Agar (Conda)
- Minimal SD base (Clontech),
- Amino Acid Dropout mixes (Clontech)
- Aureobasidin A (AbA) (Clontech) when indicated

Yeast dropout liquid-gel media

- 5 litre flask
- SeaPrep™ Agarose (Lonza) (0.5% (w/v) final concentration)
- Minimal SD base (Clontech),
- Amino Acid Dropout mixes (Clontech)

- Aureobasidin A (Clontech) when indicated

Step 6. Colony counting and harvesting yeast
- 500 ml centrifuge bottles
- 1xPBS
- Plastic cuvettes

Step 7. Yeast library DNA isolation
- Zymoprep™ Yeast Plasmid Miniprep II (Zymo Research)
- DNAse-free water

Step 8. Covaris shearing
- microTUBE AFA Fiber Pre-Slit Snap-Cap 6x16 mm (covaris)

Step 9. Circularisation
- NEBNext® End Repair Module (New England Biolabs)
- MinElute PCR Purification Kit (Qiagen)
- Quick Ligation™ Kit (New England Biolabs)
- DNAse-free water

Step 10. R1/R2 PCR
- Q5 High-Fidelity 2X Master Mix (New England Biolabs)
- Primer Pr4seq_F_TS_R1 (5'- ccctacacgacgctcttccgatctcgctgcaggtcgacggatc-3') (custom ordered from Integrated DNA Technologies)
- Primer Pr4seq_R_TS_R2 (5'- ttcagacgtgtgctcttccgatctgcagctcgagctcgatggatc-3') (custom ordered from Integrated DNA Technologies)
- Agencourt AMPure XP beads (Beckman Coulter)
- 10 mM Tris, pH 8.0

Step 11. P5/P7 PCR
- Q5 High-Fidelity 2X Master Mix (New England Biolabs)
- NEBNext® Multiplex Oligos for Illumina®, Index Primers Set 1 (New England Biolabs)
- DNAse-free water
- Agencourt AMPure XP beads (Beckman Coulter)
- 10 mM Tris, pH 8.0

Step 12. Paired-end sequencing
- MiSeq Reagent Kit v2 (Illumina)

EQUIPMENT

- PCR machine
- Microcentrifuge
- Clinical centrifuge
- Ultracentrifuge
- Water bath at 30ºC
- Water bath at 42ºC
- Spectrophotometer
- Nanodrop
- Shaker at 30ºC
- Shaker at 37ºC
- Incubator at 30ºC
- Incubator at 37ºC
- Horizontal Electrophoresis System for DNA
- S220 Focused-ultrasonicator with AFA Technology (Covaris)

SECTION 1: rec-Y2H – DETAILED PROTOCOL

DAY 1

Step 1. Batch Gateway cloning of ORF library into pDEST vectors

For each screen, both a bait and a prey library need to be built. Therefore, the following steps are performed independently but in parallel for both pDEST-bait and pDEST-prey libraries.

ORF pool	**pDEST**
Bait	pBWH (pBSWHhc or pBKWH)
Prey	pAWH

Table of compatibility between pDEST and pENTR

pDEST	**pENTR221 (Kanomycin)**	**pENTR223 (Spectinomycin)**
pAWH (Ampicilin)	Yes	Yes
pBSWHhc (Spectinomycin)	Yes	No
pBKWH (Kanomycin)	No	Yes

- Dilute each pENTR-ORF clone to 10 nM in 10 mM Tris, pH 8.0
- Mix together 5 µl of each pENTR-ORF clone at 10 nM to generate a pENTR-ORF pool at a final concentration of 10 nM. We recommend a maximum of 96 clones in each pool (see Note 1 for libraries with more than 96 clones).
- Dilute pDEST to 10 nM in 10 mM Tris, pH 8.0
- Set up 4 Batch Gateway LR reactions

Component	**Volumes for 5 µl reaction**	**x4 reactions**
pENTR-ORF pool 10 nM	1.5 µl	6 µl
pDEST 10 nMl	1.5 µl	6 µl
TE	1 µl	4 µl
LR clonase II	1 µl	4 µl
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	5 µl	Split in 4x 5 µl

- Incubate 8 hours at 25ºC
- Add to each tube

pDEST 10 nM	1.5 µl
LR clonase II	1 µl

- Incubate 8 hours at 25ºC

Step 2. Competent yeast

- Streak Y2HGold on a YPDA plate

DAY 2

Step 1. Batch Gateway cloning of ORF library into pDEST vectors

- Add to each tube

pDEST 10 nM	1.5 µl
LR clonase II	1 µl

- Incubate 8 hours at 25ºC
- Add 1 µl of Proteinase K to each LR reaction tube
- Incubate 10 minutes at 37ºC (if needed, DNA can be stored at -20ºC at this point)
- For each LR reaction, transform 1 µl into 27 µl of NEB stable cells
- Spread each transformation onto 2x 10 cm LB-agar-antibiotic plates (100 µl on each plate, total of 8 plates). See antibiotic marker in the above table.
- Spread 100 µl of 1/10, 1/100, and 1/1000 dilutions onto 10-cm LB-agar-antibiotic plates to count colonies
- Grow all plates for 16 hours at 37ºC

DAY 3

Step 1. Batch Gateway cloning of ORF library into pDEST vectors

- Count colonies
- Harvest and combine colonies from 8 plates by using 2x 5 ml of PBS for each plate and the aid of a cell spreader
- Make serial dilutions to measure OD
- Use 8 Qiaprep Spin Miniprep columns, with 15 OD units per column to prepare the pDEST-ORF pool, elute with 100 µl of EB buffer and pool together
- Dilute to 5 nM (to calculate the amount (ng) of the pDEST-ORF pool needed to make a 5 nM dilution, consider the average size of all ORFs in that pool)
- Summary

pDEST-ORF pool	**# LR reactions**	**Total # plates per pDEST**	**Total # Qiaprep spin columns**	**Final volume of Qiaprep pool**	**Dilute to**
pAWH ORF pool	4	8	8	800 µl	5 nM
pBSWHhc or pBKWH ORF pool	4	8	8	800 µl	5 nM

Step 2. Competent yeast

- In the morning, inoculate 1 fresh colony (2-3 days old) of Y2HGold into 3 ml of YPDA medium
- Grow at 30ºC with shaking at 200 rpm for 8 hours
- Transfer 10, 25 or 50 µl of yeast culture to 50 ml YPDA medium in a 250 ml flask
- Grow at 30ºC with shaking at 200 rpm for 16 hours

Step 3. Linearization

- When building a library containing more than 96 clones, different library sub-pools should be mixed at this stage. See NOTE 2.
- To set up the homing digest for linearization of pDEST-ORF pools, calculate the final amount of linear DNA needed for all transformations. For a typical screen with 96 clones and two types of selection media (recombination-selection (RS) and recombination-interaction-selection (RIS) media), see table below. Scale up accordingly when using larger libraries and/or more than one interaction-selection media. See NOTE 3.

pDEST-ORF pool	**□RF poolsee table below. Scale u**	**# of transformations for a library with 96 clones/ yeast selection media**	**# of selection medias used**	**Total volume of homing digest needed**	**# of 50 □5 homing digest reactions needed (in excess)**
pAWH-ORF pool	10 µl	12	2	240 µl	6
pBSWHhc or pBKWH ORF pool	10 µl	12	2	240 µl	6

- Digest each pDEST (pAWH or pBWH) with I-CeuI and I-SceI to linearize the vector at homology regions 1 and 2, respectively

Component	**Volumes for 50 µl reaction**	**Volumes x6 reactions**
pDEST ORF pool at 5 nM	20 µl	120 µl
H2O	23 µl	138 µl
NEB Buffer CutSmart	5 µl	30 µl
I-SceI	1 µl	5 µl
I-CeuI	1 µl	5 µl
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	50 µl	split in 6 x 50 µl

- Incubate at 37ºC for 16 hours in a PCR machine block with a heated lid
- Heat inactivate at 65ºC for 20 min

DAY 4

Step 2. Competent yeast

- Take the flasks with Y2HGold from day 3
- Measure OD_{600}, and choose the flask with an OD between 0.3 and 0.4
- Centrifuge 15 OD units of cells at 700 g for 5 min at room temperature
- Resuspend the pellet in 100 ml of YPDA medium and transfer to a 500-ml flask
- Grow at 30ºC with shaking at 200 rpm until OD reaches 0.4-0.5 (approximately 3 hours)
- Centrifuge cells in 2 x 50 ml tubes at 700 g for 5 min
- Wash each pellet with 25 ml of H2O
- cells at 700 g for 5 min

- Resuspend each pellet with 900 µl of 1.1xTE/LiAc solution and transfer to a 1.5 ml eppendorf
- Centrifuge at maximum speed for 30 s
- Resuspend each pellet with 600 µl of 1.1xTE/LiAc solution
- Store in ice (use the same day)

Step 3. Homing digest

- For each pDEST-ORF pool, mix together 6 homing digestion reactions
- Keep on ice

Step 4. Batch yeast transformation

A typical screen requires the libraries to be grown in RS media and RIS media. However, different stringencies for RIS media can be used (see NOTE 3). All the transformations need to be mixed together, and then split into the total number of selection medias to be used. Typically, we use one RS media and one RIS media for each replica.

- Prepare 250 ml of liquid gel RS and RIS media by adding 0.5% Seaprep agarose to the yeast media
- RS media: SD/-W
- RIS media: Choose one or more of the following (see NOTE 3)
- SD/-W/AbA (low stringency)
- SD/-W/-H/-A (medium stringency)
- SD/-W/-H/-A/AbA (high stringency)

  Note: Concentration of Aureobasidin A (AbA) can be varied for further altering stringency
- Autoclave liquid-gel media with a magnetic stirrer
- Stir at room temperature to cool down until for use
- Set up 12 small-scale transformations for each selection media
- Denature yeast carrier DNA at 95ºC for 5 min and chill on ice
- To pre-chilled, sterile tubes, add the following components in order

Component	Volumes for each transformation
pAWH-ORF pool Homing digest at 2 nM	10 µl
pBSWHhc- or pBKWH-ORF pool Homing digest at 2 nM	10 µl
Denatured yeast carrier DNA	5 µl
Competent Y2HGold	50 µl
PEG/LiAc	500 µl

- Mix by inverting
- Incubate 30 min at 30ºC, inverting tubes every 10 min
- 20 µl of DMSO to each transformation
- by inverting
- 15 min at 42ºC, inverting tubes every 5 min
- Centrifuge 30 seconds at maximum speed
- Resuspend each transformation in 200 µl of 0.9% NaCl

Step 5. Growth in selection media

- Mix together all transformations and measure total volume (approximately 10 ml)

- Take 100 µl to make serial dilutions (1/5, 1/25, 1/125, 1/625) for counting colonies
- Plate 100 µl of each dilution onto 10-cm RS agar plates to calculate total number of transformants screened
- Plate 100 µl of each dilution on 10-cm RIS media agar plates to calculate the percentage of positive interactions.
- Split the total volume of transformed yeast in two (or more if using more than one RIS media) and add to:
- 250 ml Seaprep liquid-gel RS media
- 250 ml Seaprep liquid-gel RIS media
- Stir
- For each liquid-gel selection media, transfer 250 ml to a 5 litre flask
- Place flasks on a tray with ice for 1 hour
- Carefully place flasks in a 30ºC incubator (this incubator should be as stable as possible to avoid the movement of the liquid-gel media)
- Incubate at 30ºC for 60 hours

DAY 7

Step 6. Harvest yeast

- Count the total number of colonies grown on the RS plates and RIS plates
  Total # colonies = # colonies * dilution factor * total volume / plating volume
  # colonies: number the colonies that appear on the plate
  Dilution factor: dilution used on the plate
  Total volume: final volume (in ml) in which the yeast cells were resuspended and added to one selection media
  Plating volume (in ml): if 100 µl were plated on a counting plate, the plating volume is 0.1
- Transfer 250 ml of Seaprep media with yeast colonies to a 500-ml centrifuge bottle
- Centrifuge cells at 1600 g for 10 min
- Wash pellet with 40 ml PBS and transfer to a 50-ml tube
- Centrifuge cells at 700 g 5 min
- Resuspend cells in 40 ml PBS
- Make serial dilutions and measure OD660 to determine the number of cells per ml. For calculations, use the dilution with an OD between 0.3 and 0.5

Step 7. Yeast library DNA isolation

For each selection media, prepare DNA independently and in parallel. Recombined yeast plasmid DNA for each selection media is referred to as pFAB_RS and pFAB_RIS

- Calculate the volume of cells in PBS needed to have $4x10^7$ cells
- Use 6 columns, with $4x10^7$ cells on each column, of Zymoprep™ Yeast Plasmid Miniprep kit
- Elute each column with 10 µl H_2O
- Pool together yeast DNA from 6 columns (about 55 µl)

Step 8. Covaris shearing

- Transfer DNA to a microTUBE-50 AFA Fiber (covaris)
- Shear DNA to 1,500 bp by Covaris ultra-sonication under the following conditions: duty cycle, 2%; intensity, 5; cycles per burst, 200; time, 25 seconds

- Save a 3-µl aliquot for running on a bioanalyzer to confirm the DNA quality

Step 9. Circularisation

- End repair

Component	Volumes for 60-µl reaction
Covaris-sheared yeast DNA	51 µl
10x NEBNext End repair buffer	6 µl
NEBNext End repair enzyme	3 µl
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	60 µl

- Incubate at 20ºC for 30 min
- Purify DNA with a MiniElute PCR Purification Kit column
- Elute with 10 µl H_2O
- Intramolecular ligation

Component	Volumes for 20-µl reaction
2xbuffer	10 µl
covaris/End repair	9 µl
Quick ligase NEB	1 µl
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	20 µl

- Incubate 5 min at 25ºC
- Heat inactivate for 20 min at 65ºC

Step 11. R1/R2 PCR:

- Set up 10 PCR reactions of 25 µl each

Component	Volumes for 25 µl reaction	x10 PCR each
Q5 High-Fidelity 2X Master Mix	12.5 µl	125 µl
Nuclease-Free Water	8 µl	80 µl
10 µM Pr4seq_F_TS_R1 (IDT)	1.25 µl	12.5 µl
10 µM Pr4seq_R_TS_R2 (IDT)	1.25 µl	12.5 µl
Template DNA 2 µl	2 µl	20 µl
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	25 µl	Split in 10x 25 µl

Step	Temp	Time
Initial Denaturation	98°C	30 seconds
12 Cycles	98°C	10 seconds
	72°C	60 seconds
Final Extension	72°C	5 minutes

- Pool together 10 PCR reactions (at this stage, DNA can be stored at -20ºC for further use)
- Measure volume (approximately 200 µl)
- Split sample in 50 µl aliquots (typically 4 aliquots for each selection media)
- Add 35 µl of AMPure XP beads to each 50 µl aliquot of R1/R2 PCR
- Incubate 5 min
- Quickly spin the tube (no more 400 g for 5 seconds)
- Place tubes on a magnetic rack
- After the solution is clear (5 min), carefully remove and discard the supernatant. Do not disturb the beads.
- Wash 3x 200 µl with 80% ethanol
- With the tubes on the magnetic rack, air dry the beads for 10 min
- Elute all aliquots from one same selection media with a total volume of 30 µl 10 mM Tris, pH 8.0 (by transferring beads and buffer from one aliquot to the next)
- Let the tube stand for 5 min at room temperature
- Quickly spin the tube (no more 400 g for 5 seconds)
- Place tubes on the magnetic rack
- After the solution is clear (5 min), carefully take 25 µl of supernatant containing the purified DNA

Step 12. P5/P7 PCR

- For each selection media, use a different index primer for multiplexing. Set up 6 PCR reactions of 25 µl each

Component	Volumes for 25 µl Reaction	6 replicates each => x6
Q5 High-Fidelity 2X Master Mix	12.5 µl	75 µl
Nuclease-Free Water	6 µl	36 µl
Template DNA	4 µl	24 µl
10 µM NEBNext Index Primer for Illumina	1.25 µl	7.5 µl
10 µM NEBNext Universal PCR Primer for Illumina	1.25 µl	7.5 µl
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	25 µl	Split in 6x 25 µl

Step	Temp	Time
Initial Denaturation	98°C	30 seconds
Cycles 12	98°C	10 seconds
	65°C	10 seconds
	72°C	45 seconds
Final Extension	72°C	5 minutes

- Pool together 6 PCR reactions
- Measure volume (typically 120 µl)
- Split sample in 50-60-µl aliquots
- Add 30 µl beads per 50 µl of each aliquot of R1/R2 PCR
- Incubate 5 min
- Quickly spin tubes (no more than 400 g for 5 seconds)
- Place tubes on magnetic rack
- After the solution is clear (5 min), carefully remove and discard the supernatant. Do not disturb the beads
- Wash 2x 200 µl with 80% ethanol
- Air dry 10 min
- For each selection media, elute all tubes with a final volume of 30 µl of 10 mM Tris, pH 8.0
- Recover 25 µl (at this stage, DNA can be stored at -20ºC, if needed)
- Run 2 µl of purified PCR product in a 0.8% gel to check quality. A smear of sizes between 600 and 1,200 bp should be seen. The presence of discrete bands indicates biased amplification of some fragments, and a bad quality of the final product

DAY ~8

12. MiSeq sequencing

To identify those protein (or RNA) pairs selected for in the RS or RIS media, next generation sequencing is performed in the 2x150-bp paired-end reads mode. We used MiSeq sequencing simply because of its fast turnaround time. It gives about a 10 million read depth, and in our experiments, we generally used over 100 x sequencing depth of all theoretical possible pair-wise combinations. However, with simulation results, over 8x sequencing depth of all possible combinations should be enough to get reliable results (**Supplementary Fig. 17**).

- Analyse DNA after covaris, R1/R2 PCR and P5/P7 PCR with High Sensitivity DNA Analysis kit and bioanalyser.
- Quantify by qPCR
- 2x150-bp paired-end sequencing with MiSeq Reagent Kit v2

13. Processing of sequencing data

Below is a brief description of how the pipeline processes the raw data. Inputs to the pipeline are FASTQ files from one sequencing run and the FASTA file for input proteins or RNAs.

- Reads are trimmed by cutadapt (v.1.14) with the proper common sequences depending on whether it is a PPI or RPI experiment.
- For the protein part, the trimmed read sequences are aligned to the reverse complement 3' coding sequence (for the last 100 nts) of the target library using the blastn (v.2.3.0) program with the blastn-short option and an E-value cut-off of 1e–8.
- For the RNA part, due to the short length of the some of RNAs (< 20nt), an exact motif search is conducted.
- Reads are considered usable reads only if the pair of reads are mapped to target proteins or RNAs.
- PPI/RPI pair reads, are first normalized by the all useable read counts to make frequency matrixes
- Frequency matrixes obtained from RS media are further converted into a null matrix by multiplying marginal distributions of row and column.
- fitting Gaussian mixture models, noise is removed from the RIS media frequency matrixes.
- By dividing the noise-filtered frequency matrix with a null matrix, an interaction score matrix (IS) is generated.
- If more than one experiments is performed, the IS matrixes are averaged to generate an average IS.
- To reduce the basal auto-activation signal, the upper quartile of the average IS for all baits is subtracted from the average IS scores.

NOTES

NOTE 1: When screening a library with more than 96 ORFs, split the library into several sub-pools, each with a maximum of 96 ORFs (pENTR-ORF pool A, B, C, etc…)

NOTE 2: When building a library with more than 96 clones, multiple pDEST-ORF pools are generated (pDEST-ORF pool A, B, C…). To obtain the final pDEST-ORF pool, sub-pools can be mixed according to the table below:

	# clones in pool	**Concentration**	**Mix**
pAWH-ORF pool A	X clones	5 nM	X µl
pAWH-ORF pool B	Y clones	5 nM	Y µl
pAWH-ORF pool C	Z clones	5 nM	Z µl

Final Concentration of pDEST-ORF pool: 5 nM
Final µl of pDEST-ORF pool: X+Y+Z µl

NOTE 3: A typical screen requires the growth of the libraries in RS media and RIS media. However, different stringencies for interaction-selection media can be employed by using different concentrations of Aureobasidin A and/or selecting for more than one reporter gene (e.g., media lacking histidine and adenine).

SECTION 2: rec-Y3H – DETAILED PROTOCOL

For RNA-protein interaction screening, rec-Y3H is used. It follows the same protocol as for rec-Y2H, except for the minor differences detailed in the following table. N/A: no difference between rec-Y2H and rec-Y3H. A detailed protocol for these rec-Y3H-specific steps are given below.

	Part of the protocol	Step	rec-Y2H	rec-Y3H
I.	**Bait and Prey library preparation**	1. Batch cloning of libraries	pAWH and pBWH by Gateway	pAWH by Gateway, pMS22H by gibson
II.	**Bait and Prey library fusion & Selection in liquid gel**	2. Competent yeast	Y2HGold	YBZ-1
		3. Linearisation	N/A	N/A
		4. Batch yeast transformation	N/A	N/A
		5a. Recombination-Selection liquid gel media	N/A	N/A
		5b. Recombination-Interaction-Selection in liquid gel	Low Stringency: SD/-Trp/Aba, Medium stringency: SD/-Trp/-His/-Ade, High stringency: SD/-Trp/-His/-Ade/AbA*	Low Stringency: SD/-Trp/-His, High stringency: SD/-Trp/-His/3-AT*
III.	**NGS library preparation**	6. Harvest yeast	N/A	N/A
		7. Yeast library DNA isolation	N/A	N/A
		8. Covaris shearing	N/A	N/A
		9. Circularisation	N/A	N/A
		10. R1/R2 PCR	Pr4seq_F_TS_R1/ Pr4seq_R_TS_R2	Pr4seq_F_TS_R1/ Pr4seq_MS22_R2_rev
		11. P5/P7 PCR	N/A	N/A
III.	**Paired–end sequencing**	12. Library quality control and MiSeq	N/A	N/A
		13. Analysis	N/A	N/A

rec-Y3H SPECIFIC REAGENTS AND MATERIALS

- YBZ-1
- Primer Pr4seq_MS22_R1_rev
    - (5’ ccctacacgacgctcttccgatctgcaggcatgcaagctgcc -3’)
- Oligo_giv_rev
    - (5’-CAGGCATGCAAGCTG-3’)

DETAILED PROTOCOL FOR rec-Y3H

Step 1. Gibson cloning of RNA sites

- For each RNA site, a single-stranded oligonucleotide with Gibson overhangs is ordered, where XXX represents an RNA motif.
  5'-gaactagtggatcccXXXccgggcagcttgcatgcctg-3'
- Set up annealing reaction

	25-µl reaction	**Final concentration**
RNA site oligonucleotide	0.5 µl	1 µg
Oligo_giv_rev	0.5 µl	0.2 µg
1x NEB buffer 2.1	2.5 µl	1x
H2O	21.1 µl	

- Heat at 95°C for 2 min
- Slowly cool down
- Add:

	25-µl reaction	**Final concentration**
dNTPs 10 mM	0.1 µl	33 µM
DNA Polymerase I, Large (Klenow) Fragment 5 U per µl	0.3 µl	1 U per µg DNA

- Incubate 15 min at 25°C
- Purify DNA with a MiniElute PCR Purification Kit column
- Elute DNA with 10 ul of H_2O
- Cut 1 µg of pMS22KH with XmaI for 1 hour at 37°C
- Run on a 0.7% agarose gel
- Cut the band corresponding to the linearized vector
- Purify with QIAquick Gel Extraction kit using one Minielute column
- Elute with 10 µl Elution buffer
- Set up Gibson reaction:

Klenow filled-in DNA	1 µl
XmaI linearized pMS22KH vector	1 µl
Gibson mix (CRG facility)	10 µl

- Incubate 1 hour at 50°C
- Transform 2 µl of Gibson reaction into 50 µl of Stellar Competent Cells (Clontech)
- Pick one colony for each RNA site
- Prepare plasmid DNA with Qiagen spin prep
- Dilute each pMS22H RNA motif to 5 nM
- Mix equal amounts of each pMS22H-RNA, to obtain an equimolar pMS22H-RNA pool with a final concentration of 5 nM

Step 2. Competent yeast

Same as for rec-Y2H, but the YBZ-1 yeast strain is used instead of Y2HGold

Step 5. Growth in selection media

Recombination media is SD/-Trp, as for rec-Y2H
Recombination-interaction-selection media is:

- Low Stringency: SD/-Trp/-His
- Medium-high stringency: SD/-Trp/-His/3-AT*

Step 11. R1/R2 PCR

Same as for rec-Y2H Gold, but use the Pr4seq_MS22_R1_rev primer instead of Pr4seq_R_TS_R1.

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