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semi-quantitative Yeast Two-Hybrid (sqY2H)

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We are still developing and optimizing this protocol

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Abstract

Assessment of the impact of missense variants on protein-protein interactions

Cloning into yeast two-hybrid vectors

1 LR reaction

- Put all plasticware (PCR plates, reservoir, tubes)  On ice before starting.
- All reagents should be thawed  On ice , thoroughly vortexed and briefly spun to remove any liquid from the cap.
- Assemble the following master mix:

	Reagent	Stock concentration	Final concentration	Volume (uL)/reaction	Volume (uL)/100 reactions
	TE buffer, pH 8.0	/	/	1	100
	Gateway™ LP Clonase™ II Enzyme mix	10x	1x	1	100
	pDEST-DUAL	100 ng/uL	20 ng/uL	1	100

LR master mix.

- Vortex the master and transfer into a pre-chilled reservoir.
- Aliquot  3 µL of the master mix into a new PCR plate using a automatic multichannel pipette.
- Using a multichannel pipette, add  2 µL of the purified entry products to the plates preloaded with the LR master mix.
- Seal the plates and spin  00:05:00 at  1000 rpm .
- Incubate the plates at  25 °C  Overnight . After incubation, BP reaction plates can be stored at  -20 °C .

2 Transformation into DH5a cells

- Put all plasticware (PCR plates, reservoir, tubes)  On ice before starting.
- Thaw *E. coli* DH5a cells  On ice .
- Gently mix tube by inverting (do not vortex!) to homogenize the suspension.
- Transfer cells to a pre-chilled reservoir and aliquot  10 µL of cells into each well of a pre-chilled empty PCR plate using an automatic multichannel pipette.
- Add  3 µL of BP product into the cell suspension using a multichannel pipette.
- Mix the cells by swirling the pipette tips in the wells after dispensing.

- Incubate  On ice for  00:30:00 .
- Using a 96well heating block, pre-heated at  42 °C , heat-shock the cells for  00:00:45 .
- Immediately return the cells  On ice for  00:02:00 .
- Carefully add  90 µL of complete SOC media to each well using an automatic multichannel pipette.
- Seal the plate with an airpore tape and incubate at  37 °C for  01:00:00 .
- Fill a costar plate with  120 µL of LB media containing  100 µg/mL ampicillin.
- Transfer  10 µL of the recovered cells into the costar plates and seal with airpore tape.
- Incubate at  37 °C  Overnight .

3 **Plating and spreading of bacterial culture**

Liquid bacterial cultures are spread onto solid selective media to isolate single colonies. This was conducted on 15 cm diameter petri dishes with a Tecan Evo robot, using a custom program.

Importantly, glycerol stocks of the liquid cultures are generated, in two copies, by combining  40 µL of  40 Mass Percent sterile glycerol and  40 µL of overnight culture. These glycerol stocks are sealed with aluminum foil and stored at  -80 °C .

4 **Colony picking and isolation**

- Fill costar plates with  150 µL LB media supplemented with  100 µg/mL ampicillin.
- One colony is picked and isolated by hand with sterile wooden toothpicks from each spot on solid media and transferred into the costar plates prefilled with LB+ampicillin.
- Seal plates with airpore tape and incubate at  37 °C  Overnight .
- The next day, make glycerol stocks, in three copies, by combining  40 µL of  40 Mass Percent sterile glycerol and  40 µL of overnight culture.
- Seal plates with aluminum foil and store at  -80 °C .

5 **DNA purification**

- Fill deep well plates with  1.2 mL LB media supplemented with  100 µg/mL ampicillin.
- Inoculate  10 µL glycerol stocks.
- Seal plates with airpore tape and incubate at  37 °C  Overnight .
- The next day, spin for  00:15:00 at  2000 rpm , or until cells form a pellet.



- Discard supernatant.
- DNA is extracted using a QIAGEN BioRobot 8000.

Plasmid pool sequencing

6 To confirm the successful transfer of the desired DNA insert from the entry vector into the destination vector, all purified DNA samples are sequenced. A pooled sequencing strategy is employed on the Illumina platform, where each pool contains one clone from different genes to ensure reliable identification.

6.1 Preparation of DNA pools

- Measure the concentration of each 96-well (containing DNA pool).
- *For this step, a Qubit dsDNA BR Assay Kit (catalog: Q32851) suffices.
- For each well, take an aliquot and dilute with PCR grade water in a new 96-well plate (label "sample plate") to the required concentration of **15 ng/μL**

6.2 Nextera Oligo Setup

Pre-mix i5 and i7 oligos for a concentration of **10 micromolar (μM)**

6.3 Illumina Tagment MasterMix prep

Reagent	1X (single reaction)	100X (one full plate)
Tagment DNA (TD) buffer	2μL	200μL
PCR grade water	6.5μL	650μL
Tagment DNA Enzyme (TDE1; catalog: 20034197) *	0.25μL	25μL

*Keep @ **-20 °C** , or on ice when in use!

- Use a multichannel pipette to add **8.75 μL** MasterMix to each well of a 96-well plate (label "reaction plate")
- Use a multichannel pipette to add **1.25 μL** DNA (**15 ng/μL** ; from the sample plate) to each corresponding well of the reaction plate
- Gently pipette to mix
- Cover the reaction plate with a BioRad PCR plate seal
- Incubate the reaction plate for **00:10:00** @ **55 °C** in a thermal cycler
- After, immediately place the reaction plate on ice
- Proceed immediately with the PCR

6.4 PCR amplification of tagmented DNA

Using a new 96-well plate (labeled amplification), add the following:



	A	B
	Reagent	1X (per well)
	2X Phusion MasterMix (catalog: M0530S)	10µL
	i5 & i7 oligo (10uM; see above/attached sheet)	2µL
	Tagmented DNA	8µL

- Using a multichannel pipette, pipette gently to mix
- Cover with a BioRad plate seal and placed in a thermal cycler

	Cycle(s)	Time	Temp
	1	3min	72°C
	10	10sec	98°C
		30sec	56°C
		3min	72°C

Thermal cycler Program

- Check 10 random wells confirming that the PCR worked by running  2 µL of each on a 1% EX e-gel along with a 1kb ladder.
- See a smear from ~100bp to ~5kb and each lane should have roughly equal amounts of product.

6.5 Pool amplified DNA

Using a multichannel, pool all wells ( 10 µL from each well)

6.6 Purifying for sequencing

- Vortex to mix the pooled, amplified DNA
- Load all wells of a single 1% EX e-gel from ThermoScientific (one plate per gel; catalog: A42100)
- Run using 50V until the 100bp band (1kb+ DNA ladder, catalog: N0469S) reaches the bottom of the gel.
- Cut the smear from 500bp-800bp
- Purified using the Qiagen gel purification kit (catalog: 28604)
- Elute the library in  15 µL
- Measure concentration using Qubit dsDNA HS Assay Kit (catalog: Q33230)



Yeast transformation

- 7 Haploid *S. cerevisiae* strains Y8800 (*MATa*) and Y8930 (*MATα*) are transformed with pDest-AD-prey vectors (carrying the *TRP1* gene) and pDest-DB-bait vectors (carrying the *LEU2* gene), respectively.
- Yeast cells are transformed following high-efficiency lithium acetate – polyethylene glycol protocol – salmon sperm carrier DNA (Yu *et al.*, *Science*, 2008), that was modified and adapted to a 96-well format for enhanced throughput. For each transformation reaction,  100 μL of exponential phase yeast cell culture (OD at 600 nm $\sim$ 0.6-1.0) and  100 ng of high-purity plasmid are used. Yeast cells are plated onto appropriate solid media (Yu *et al.*, *Science*, 2008), depending on the selective marker carried by the plasmid, to select for transformants. Transformants were picked after  120:00:00 of incubation at  30 $^{\circ}\text{C}$.

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- 8 **Experimental rearraying of the strains**
- Inoculate the yeast strains into  200 μL appropriate selective media in round bottom plates and incubate at  30 $^{\circ}\text{C}$ for  48:00:00
 - After this incubation period, the yeast strains are re-arrayed with a Tecan Genesis robot, using a customized protocol
 - Cells are incubated at  30 $^{\circ}\text{C}$ for  72:00:00.
 - After incubation, glycerol stock are made in two copies, by combining  40 μL of [M] 40 Mass Percent sterile glycerol and  40 μL of culture.
 - The rest of the culture is used in the mating step
- 9 **Mating**
- Dispense  200 μL YPD into round bottom plates
 - Combine equal volumes of the corresponding rearrayed DB and AD cultures ( 5 μL each) into these prefilled plates
 - Seal the plate with an airpore tape and incubate at  30 $^{\circ}\text{C}$ for  24:00:00.
- 10 **Diploid selection**
- Dispense  200 μL SC-LW into round bottom plates
 - Transfer  10 μL mated culture
 - Seal the plate with an airpore tape and incubate at  30 $^{\circ}\text{C}$ for  48:00:00
- 11 **Interaction selection**

- 5ul of diploid selection cultures are spotted in quadruplicates onto the following selective media:
- 1. SC-LW: selection for diploid cells. This controls for the presence of diploid cells harboring both AD and DB plasmids.
- 2. SC-LWH + [M] 1 millimolar (mM) or [M] 10 millimolar (mM) or [M] 25 millimolar (mM) 3-Amino-1,2,4-triazole (3-AT)
- Let the plates dry at [T] Room temperature for [D] 01:00:00
- Move the plates to [T] 30 °C and incubate for [D] 120:00:00 .
- Take pictures at day 3, day 4 and day 5.

12 Scoring

- Pictures are scored with the CellProfiler software, using a customized script

Sequencing confirmation using SWIM-Seq

13 Lysates

In addition to being spotted on solid selective media, cultures of diploid yeast cells are processed as previously described (Dreze *et al.*, 2010) to generate cell lysates

14 Duplex PCR

PCR is conducted on each lysate sample to amplify the DB-X and AD-Y fusion constructs.

Barcoded primers are used for AD and DB primers, to assign well identity to the amplicons

- Thaw the primers and the diluted miniprep DNA plates at room temperature, vortex, and spin quickly to remove liquid from the lid.
- In a tube [T] On ice , assemble a master mix as described in substep 13.1.
- Aliquot [V] 29 µL of the master mix with an automatic multichannel pipette into the PCR plates.
- Using a 12 channel pipette, distribute [V] 1 µL of the lysates to the PCR plates preloaded with master mix.
- Seal plates, vortex and spin [D] 00:05:00 at [R] 1000 rpm .
- PCR is conducted for 35 cycles, using the following cycling conditions:

	A	B
	98 °C	5 min
	98 °C	30 sec

	A	B
	57 °C	30 sec
	72 °C	5 min
	72 °C	10 min
	10 °C	Hold

- After PCR, spin plates quickly to remove droplets from the lid.
- The first row of each PCR plate is run on a 1% agarose gel to check for successful DNA amplification
- 96well plates are pooled into a single well
- Pooled samples are purified using a QIAquick 96 PCR Purification kit from Qiagen
- During sequencing library preparation, Illumina adapter sequences, i7 and i5, were incorporated as SWIM plate indices. The library was then sequenced using an Illumina platform (MiSeq or NextSeq 500).

14.1 PCR master mix

	Reagent	Stock concentration	Final concentration	Volume (uL)/reaction	Volume (uL)/100 reactions
	Dream Taq HS Mix	2x	1x	15	1500
	Primer AD	10 uM	0.2 uM	0.6	60
	Primer DB	10 uM	0.2 uM	0.6	60
	Primer term	10 uM	0.2 uM	0.6	60
	Water	/	/	12.2	1220

PCR master mix

Protocol references

Yu et al, Science, 2008: <https://www.science.org/doi/10.1126/science.1158684>

Dreze et al, Methods Enzymol, 2010: [https://doi.org/10.1016/s0076-6879\(10\)70012-4](https://doi.org/10.1016/s0076-6879(10)70012-4)