Removing auto-activators from yeast-two-hybrid assays by conditional negative selection

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Yeast-two-hybrid (Y2H) is widely used as a strategy to detect protein–protein interactions (PPIs). Recent advancements have made it possible to generate and analyse genome-wide PPI networks en masse by coupling Y2H with next-generation sequencing technology. However, one of the major challenges of yeast two-hybrid assay is the large amount of false-positive hits caused by auto-activators (AAs), which are proteins that activate the reporter genes without the presence of an interacting protein partner. Here, we have developed a negative selection to minimize these auto-activators by integrating the pGAL2-URA3 fragment into the yeast genome. Upon activation of the pGAL2 promoter by an AA, yeast cells expressing URA3 cannot grow in media supplemented with 5-Fluoroorotic acid (5-FOA). Hence, we selectively inhibit the growth of yeast cells expressing auto-activators and thus minimizing the amount of false-positive hits. Here, we have demonstrated that auto-activators can be successfully removed from a Marchantia polymorpha cDNA library using pGAL2-URA3 and 5-FOA treatment, in liquid and solid-grown cultures. Furthermore, since URA3 can also serve as a marker for uracil autotrophy, we propose that our approach is a valuable addition to any large-scale Y2H screen.

The yeast two-hybrid (Y2H) assay is one of the most widely utilized approaches to identify protein–protein interactions (PPIs)⁵⁻⁶. Y2H data has been invaluable in revealing complexes regulating disease⁷, predicting gene function in plants⁸, and revealing protein complexes underlying different plant-pathogen infections⁹. However, genome-wide Y2H data generation is still challenging due to the cost and labor, needing to mate the specific constructs and to perform the necessary screening⁹. Recent approaches that combine next-generation sequencing (NGS) with Y2H are bringing us closer to obtaining genome-wide PPI networks¹⁰,¹¹. To avoid the manual, labor-intensive screening of bait proteins for tracking interactions, pools of baits are screened against pools of preys with multiplexed screening strategies¹¹–¹³. Barcode Fusion Genetics (BFG-Y2H)¹¹ utilizes intracellular DNA recombination of barcoded open reading frame (ORF) to identify interacting proteins, thus allowing pooling and simultaneous sequencing of Y2H-positive colonies. Another approach, Cre-reporter-mediated Y2H coupled with next-generation sequencing (CrY2H-seq), uses Cre recombinase as a Y2H protein–protein interaction reporter. Cre covalently and unidirectionally links the interacting bait and prey plasmids via specialized loxP sites that flank the protein-coding sequences¹³. The linked protein-coding sequences can be amplified by PCR and serve as interaction-identifying DNA molecules that can be sequenced by NGS approaches, thus allowing massively multiplexed screening for protein–protein interactions.

A common problem of the Y2H system is the autoactivation of Y2H-inducible reporter genes¹⁴, which can result in a large number of false positives. This occurs when either DNA binding domain (BD) or activation domain (AD) activates transcription of Y2H reporter genes irrespective of the presence of any PPI. Three types of autoactivators (AAs) can be observed: (i) transcription factors that natively contain a functional AD that is active when fused to BD, (ii) proteins that are not transcription factors but contain a cryptic AD, and (iii) proteins that are not transcription factors, that contain one or more cryptic ADs that are only functional as truncated fragments.

Regardless of their type, AAs remain a significant hindrance in obtaining a successful PPI. In smaller screens, the bait proteins are typically screened for self-activation before commencing the assay, whereas, in experiments

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where thousands of bait proteins are tested for interactions with thousands of prey proteins, this might not be feasible. Furthermore, while it is possible to bioinformatically identify AAs (as they will appear to be interacting with an empty vector), the number of AAs tend to vastly exceed the numbers of true protein–protein interactions. For example, in the CrY2H study, 95% (164,293 out of 173,000) of all interactions were false positives caused by AAs. This indicates that only 5% of the generated data can be used in such assays. Thus, novel methods that can effectively remove AAs from large-scale assays are needed, to save labor and sequencing costs.

To remove AAs, we have designed and introduced a pGAL2-URA3 fragment to the yeast genome. URA3 encodes orotidine-5-phosphate decarboxylase (ODCase), that can transform 5-fluoroorotic acid (FOA, a uracil analogue) into a highly toxic compound, 5-fluorouracil (FU) Since URA3 is driven by a pGAL2 promoter that is activated by AAs, the addition of FOA should selectively harm yeast cells containing AAs. We validated our system by identifying and removing AAs from Marchantia polymorpha, a model bryophyte. Thus, our novel method has a potential to remove AAs from high-throughput yeast-two-hybrid assays.

Materials and methods

Nuclear transformation of yeast with pGAL2-URA3 fragment. To enhance the efficiency of the recombination, the DNA fragment containing the pRS305K-pGAL2-URA3 fragment and KanMX (kanamycin) selection marker was cleaved with AatII and KasiI, gel-purified and used for yeast transformation. Yeast cells were inoculated from a fresh pre-culture and grown to OD600 of 0.5–0.7 at 28 °C in YPD medium. The cells were harvested by centrifugation (3000 rpm for 3 min (min), 4 °C), washed twice with 50 ml of ice-cold, sterile water, once with 50 ml of ice-cold, sterile 1 M Sorbitol / 1 mM CaCl2. The cells were harvested by centrifugation (3000 rpm for 3 min, 4 °C) and resuspended gently in a 20 ml of ice-cold conditioning buffer (0.1 M Sorbitol/10 mM DTT). The cells were kept for 30 min at 28 °C with shaking (140 rpm) and then harvested by centrifugation (3000 rpm for 3 min, 4 °C), washed with 50 ml of ice-cold, sterile 1 M Sorbitol / 1 mM CaCl2. The cells were resuspended in 2 ml of ice-cold, sterile 1 M Sorbitol / 1 mM CaCl2, and used immediately for electroporation. A total of 300 ul of prepared yeast cells were incubated on ice with 500 ng of gel-purified pGAL2-URA3 DNA cassette for 5 min. The cells were transferred to a 0.2 cm gap electroporation cuvette and electroporation pulse was applied at 2.5 kV. The efficient transformation resulted in electroporation time of 4–5 ms. The electroporated cells were immediately diluted with 2 ml of YPD medium, incubated on a shaker (650 rpm) for 4 h (h) at 28 °C and spread on YPD plates containing Geneticin (200 µg/mL). Plates were grown for 48 h at 28 °C, and resistant colonies were transferred to a fresh selection plate and grown for 48 h at 28 °C.

Yeast strains and plasmids. CrY2H-seq yeast strains and AD and BD plasmids were obtained from Prof. Joseph Ecker’s lab, Salk Institute of Biological Sciences, La Jolla, USA. The strains and plasmids are also available from Arabidopsis Biological Resource Center (https://abrc.osu.edu/)13. The two yeast strains were transformed by the pRS305K-pGAL2-URA3 fragment and the resulting genotypes of the strains are:

- Y8800-URA3: P_{GAL2}-URA3 MATa leu2-3,112 trp1-901 his3-200 ura3-52 gal4Δ gal80Δ P_{GAL2}-ADE2 lys2::P_{GAL2}-HI53 MET2::P_{GAL2}-lacZ cyh2
- CRY8930-URA3: P_{GAL2}-URA3 MATα leu2-3,112 trp1-901 his3-200 ura3-52 gal4Δ gal80Δ P_{GAL2}-ADE2 lys2::P_{GAL2}-HI53 MET2::P_{GAL2}-CRE-HPHMX6 cyh2

Yeast growth media. Yeast strains were grown in YEPD media, pH 6.0, containing 1% yeast extract, 2% peptone, 200 mg/L adenine hemisulfate and 2% agar (only for solid) supplemented with 50 ml of 40% glucose and antibiotic, G418 (200 µg/mL) and G418 + hygromycin (125 µg/mL) for yeast strains Y8800 and CRY8930, respectively.

Yeast selection media. The selection for yeast strains harboring pDEST_AD_lox_TRP1 or pDEST_DB_lox_LEU2 plasmids was done using synthetic complete (SC) drop-out media containing 6.7 g/L yeast nitrogen base, 1.47 g/L (minus adenine (A), histidine (H), leucine (L), tryptophan (T)) or 1.37 g/L (minus AHLT and minus uracil) dropout mix, 200 mg/L adenine hemisulfate and 2% agar (only for solid) supplemented with 50 ml of 40% glucose, 8 mL/L of desired amino acid solution (100 mM leucine/histidine and 40 mM tryptophan stock solution). For SC-FOA media, 0.2% 5-FOA (5-Fluoroorotic acid hydrate, 100 mg/mL stock in DMSO) was added after autoclaving the media. The selection media was additionally supplemented with G418 (200 µg/mL) to avoid microbial contamination.

Growth conditions for Marchantia polymorpha. Tissue culture grown Marchantia polymorpha was obtained from Prof. Chen Zhong from NIE, Singapore. The plant was propagated in the lab using half Gamborg B5 media without sucrose (pH 5.5, 1.4% agar) on deep tissue culture plates under 24 h light and 22–23 °C.

RNA extraction and construction of entry cDNA library for M. polymorpha. The total RNA was extracted from 2–3 weeks old thallus using ReliaPrep Promega kit as per manufacturer’s protocol. The integrity of the RNA samples was confirmed using bioanalyzer (Agilent 2100). The samples that passed quality control (RIN > 8.5) were pooled together, concentrated using Zymo RNA concentrator kit, and rechecked for the RIN value. cDNA was synthesised, and the entry library was prepared using CloneMiner II cDNA library construction kit (Invitrogen) using Gateway technology, as per manufacturer’s instruction with slight modifications. Briefly, the total RNA was used as an mRNA template, and reaction was primed by hybridizing Biotin-attB2-Oligo(dT) to
the poly(A) tail, followed by synthesis of first strand using SuperScript III Reverse transcriptase provided with the kit. The second strand was synthesized using the first strand as a template. The synthesized cDNA was extracted using phenol:chloroform:isoamyl alcohol (25:24:1) and precipitated using ethanol before finally dissolving in DEPC-treated water. The 5’ end of the cDNA is ligated with attB1 Adapter followed by size fractionation using columns provided with the kit to eliminate residual adapters and other low molecular weight DNA. Subsequently, the BP recombination reaction was performed using Gateway BP clonase II enzyme mix to facilitate recombination of attB-flanked cDNA into pDONR 222 entry vector. After that, the reaction was inactivated with proteinase K, and the cDNA was precipitated using ethanol. The cDNA pellet was resuspended in TE buffer.

The resuspended cDNA was divided into six aliquots and each aliquot was transformed into Electromax DH10B T1 phage resistant competent cells in a separate vial using a Gene pulsor Xcell electroporation system (Bio-Rad) as mentioned in the kit’s manual. After transformation, all the vials were pooled together, further aliquoted, and stored at −80 °C.

**Analysis of the cDNA entry library.** A small sample from the above library was utilised to perform the plating assay for titre determination as per kit’s manual. The sample was serial diluted and plated in duplicates on LB plates with kanamycin selection marker. Next day, colonies were counted and the titre for each plate was determined using the equation below:

\[
\text{cfu/mL} = \frac{\text{colonies on plate} \times \text{dilution factor}}{\text{volume plated}}
\]

The titre from each plate was used to calculate the average titre for the whole cDNA library. The total colony forming units (CFU) were determined using the following equation:

\[
\text{Total CFU} = \text{average titre (cfu/mL)} \times \text{total volume of cDNA library (mL)}
\]

The cDNA library was further qualified to determine the average insert size by setting up colony PCR reactions using M13 forward and reverse primers. We picked a total of 19 colonies and one vector control. The PCR reaction was set using Exten 2× PCR master mix (first base) as per manufacturer’s protocol.

**Generation of yeast destination expression library.** The cDNA library (entry) was shuttled into the Gateway destination vector (pADloxo or pDBloxo) to generate expression clones/library using the LR recombination reaction with Gateway LR Clonase II enzyme mix as per manufacturer’s protocol. The titre determination of each library was done using plating assay, as previously mentioned.

**Preparation of electro-competent cells and transformation of cDNA expression libraries into yeast strains using electroporation.** One vial of the glycerol stock from each destination expression library was thawed, inoculated in LB + Carbenicillin (100 μg/mL) and grown overnight at 37 °C at 220 rpm for plasmid isolation. Next day, plasmid DNA was isolated using Wizard Plus SV Miniprep purification kit from Promega and used for yeast transformation by electroporation as described in with modifications.

Yeast strains, Y8800, and CRY8930 were streaked out on YEPD plates supplemented with G418 (200 μg/mL) and G418 + hygromycin (125 μg/mL), respectively, and incubated at 37 °C for 48 h. For each strain, 10–12 colonies were picked from the plates and grown in 20 mL of YEPD overnight at 30 °C and 220 rpm to attain a stationary phase. The next day, the OD600 was measured, and the cells were diluted up to a total volume of 100 mL of YEPD, aiming for an initial OD600 between 0.1–0.2. The cells were further grown until the OD600 reached approximately 1.0–1.5 and underwent at least two doublings, which usually takes 5–6 h. Yeast cells were collected by centrifugation at 3000 rpm for 3 min, media was removed, and pellet was washed twice with 50 mL ice-cold water and once with 50 mL of ice-cold electroporation buffer (1 M Sorbitol / 1 mM CaCl2). Yeast cells were conditioned by re-suspending the cell pellet in 20 mL 0.1 M Lithium acetate/10 mM DTT and shaking at 220 rpm in a culture flask for 30 min at 30 °C.

The conditioned cells were collected by centrifugation, washed once with 50 mL ice-cold electroporation buffer, and re-suspended in 100 to 200 μL electroporation buffer to reach a final volume of 1 mL. The electrocompetent cells were now ready to use and kept on ice until electroporation.

The electroporation reactions of 400 μL each were set by gently mixing electrocompetent cells with a library plasmid DNA. The reaction mixture was transferred to a pre-chilled electroporation cuvette (0.2 cm width, Bio-Rad) and kept on ice for at least 5 min before electroporation. The cells were electroporated at 2.5 kV and 25 μF and immediately supplemented with 1 mL of 1:1 mix of 1 M sorbitol:YEPD media. The entire content from each cuvette was further transferred to an additional 8 mL of 1:1 mix of 1 M sorbitol:YEPD media as soon as possible and incubated for 1 h at 30 °C and 220 rpm. Cells were collected, washed with sterile water once, and finally resuspended in 10 mL SC-Leu and SC- Trp media for bait and prey strains, respectively. An aliquot from each transformed cell mixture was serial diluted (1/100, 1/1000) and plated on SC-Leu and SC-Trp plates for bait and prey strain respectively and incubated at 30 °C. The remaining cells were grown for 2 days at 30 °C and 220 rpm, glycerol stocks were prepared, aliquoted, and stored frozen at −80 °C. The size of each library was determined from the colony counts after 3 days. This was the final bait and prey transformed library.

**PCR amplification of the transformed library and Next-generation sequencing (NGS).** One vial each of transformed bait and prey library was thawed and inoculated into 10–12 mL of SC-Leu and SC-Trp, respectively, at 30 °C and 220 rpm overnight, with the corresponding antibiotics.

The next day, yeast cells were collected, washed, and resuspended in 0.5–1 mL of yeast lysis buffer (50 mM Tris–HCl pH 7.5, 0.92 M sorbitol, 10 mM EDTA and 1% β-mercaptoethanol). Additionally, 200 U of lyticase...
the transformation of 5-fluoroorotic acid (FOA, a uracil analogue) into a highly toxic compound, 5-fluorouracil.

media (SC-Leu: no selection, SC-Leu + FOA: negative selection for auto-activators, and SC-LH: positive selec-
tion). After 3 days, the colonies were counted, and SC-HULA plates were scraped, resuspended in SC-Leu media, and
glycerol stocks were made to create archival BD auto-activator library. A vial from –HULA library was thawed,
serially diluted, and plated on –HULA plates. After 3 days, five independent colonies were picked and incubated
in liquid –LH + G418 media overnight at 30 °C. Glycerol stocks were prepared from the overnight culture as
representatives of auto-activators of BD library. Also, five overnight cultures were serially diluted (0, 1:10, and
1:100) and spotted on –L, –L + FOA, –HULA plates for further validation. The auto-activators genes were char-
acterized by Sanger sequencing, where isolated plasmids/PCR fragments were sent for forward sequencing by
using forward BD primer. After sequencing, the query sequences were matched with pDBlox plasmid sequences
using MultAlin multiple sequence alignment web tool19.

Removal of auto-activators by 5-FOA in liquid cultures. Five AAs and one empty vector strain were grown
individually overnight in SC-Leu media. The initial ODs of the overnight grown cultures were set to 0.001 each,
and the cultures were mixed to achieve the final OD of 0.006 (0.001 each for 5 AAs and 1 empty vector (EV)) and
grown overnight in SC-Leu media. The next day, the mixed culture was inoculated in SC–Leu (selection for BD
plasmid only), SC-Leu + FOA (to remove AAs), and SC-Leu-His (to remove non-AAs) liquid culture for 24 h in
culture tubes. The next day, the liquid cultures from the SC–Leu, SC-Leu + FOA, and SC-Leu-His culture tubes
were plated on individual SC–Leu plates. After 2 days, 48 colonies from each SC-Leu plate were grown in liquid
SC–Leu media overnight, and finally spotted (1:10 dilution) on –Leu + FOA and –Leu-His plates and the num-
ber of growing colonies was counted after three days (Fig. 3A).

Results
Designing conditional negative selection in yeast-two-hybrid with URA3. In our pursuit to
develop a genetic tool that can remove auto-activators from the yeast two-hybrid assay, we had to establish two
components of the tool. First, we needed a negative selection marker that can inhibit the growth or kill the yeast
cells. Second, we needed a means to conditionally activate the negative selection marker only when an auto-
activator is present in the yeast cell.

To identify a suitable negative selection marker, we evaluated ten markers available for yeast20. The most
well-established negative selection marker, URA3, has been used for decades in positive and negative selection
in Saccharomyces cerevisiae. URA3 encodes orotidine-5-phosphate decarboxylase (ODCase), which can catalyze
the transformation of 5-fluoroorotic acid (FOA, a uracil analogue) into a highly toxic compound, 5-fluorouracil
(FU)35. Thus, the activity of URA3 confers sensitivity to FOA, and cells not expressing URA3 are FOA resis-
ant. Alternatively, URA3 can be used as a positive selection marker, as it can complement uracil auxotrophy of a
URA3 negative strain. To implement the conditional activation of URA3, we assessed systems that would
induce the expression of URA3 only when an auto-activator is present. Since auto-activators induce expression
of positive selection makers (His, Ade) that are driven by pGAL-family promoters, we chose to use pGAL2
promoter to drive URA3.

We synthesized pGAL2-URA3 fragment and cloned it into pRS305 plasmid, which can be used to integrate
the fragment into Leu2 chromosomal locus and select for the transformation by using kanamycin (Fig. 1A). The
sequence of the vector is available as Supplemental Data 1. We have integrated the fragment into the genomes

of yeast strains used in CrY2H-seq, which use a Cre recombinase to fuse in vivo the coding sequences of two interacting proteins, allowing to identify these interactions with next-generation DNA sequencing.

A yeast strain containing this new fragment in the presence of FOA, but with the absence of an auto-activator, would not express HIS3 and URA3 and would be FOA insensitive and histidine auxotrophic (Fig. 1B). Conversely, in the presence of an auto-activator, both HIS3 and URA3 would be expressed, and the yeast would be histidine and uracil autotrophic and sensitive to FOA (Fig. 1C).

We hypothesize that by adding FOA to the media before the mating, growth of haploid yeast cells that express URA3 should be inhibited (Fig. 1D), due to the accumulation of the toxic FU in the yeast cells. This should reduce...
or eliminate the number of auto-activators in our library population, and subsequently reduce the number of false-positive hits when used for mating in a yeast two-hybrid assay.

Construction of AD and BD libraries for *Marchantia polymorpha*. To test our AA-removal method, we set to identify genes that are AAs in the activation domain (AD) and the DNA binding domain (BD) libraries. We chose *Marchantia polymorpha* as a source for AAs, as it is an emerging model for early diverging plants, has a low number of genes, and minimal genetic redundancy.

The cDNA library was successfully generated from *Marchantia* thallus mRNA, moved into *E. coli*, where a plate assay revealed that 6.276 × 10⁵ entry clones were produced. Colony PCR analysis of 19 individual colonies confirmed inserts of variable size ranging from > 1 kb to < 3 kb (Figure S1). Following this, the entry library was transferred into destination vectors using LR recombination reaction to generate expression clones. The final titre of the cDNA expression library for *M. polymorpha* was determined to be 5.48 × 10⁵ and 6.12 × 10⁵ for AD and BD clones, respectively.

The expression libraries were successfully electro-transformed into their respective host yeast strains. The plating assay performed with transformed libraries revealed a total of 3.4 × 10⁵ and 2.86 × 10⁵ transformants from AD and BD libraries, respectively.

To estimate the percentage of *Marchantia* coding sequences that are captured by our AD and BD libraries, we amplified the cloned CDSs by PCR, which revealed an expected smear on the gel (Figure S2). The purified DNA was subjected to Illumina sequencing, which revealed that 7,325 and 7,035 genes were present in AD and BD libraries, respectively, while 5,926 genes were present in both. This implies that our libraries contain ~ 1/3 of the total CDSs (*Marchantia* contains 19,287 genes, https://plants.ensembl.org/Marchantia_polymerphla/Info/Index), indicating reasonably good coverage.

Quantification of AAs in AD and BD libraries. To quantify the amount of AAs in AD and BD libraries, we used a plate assay to compare the number of yeast colonies that grow on media selecting for activation of *His* (H), *Ura* (U) and *Ade* (A) markers (Fig. 1C), versus the total number of colonies. BD library was grown on –L (selecting for presence of pDBlox plasmid) and –HULA (additional selection for expression of *His*, *Ura* and *Ade* markers), while AD library was grown on -Trp (T, selecting for presence of pADlox plasmid) and –HUTA

![Figure 2.](image-url)
After 3 days, the colonies were counted, and the average percentages of auto-activators were calculated by dividing the number of colonies on –HULA plates against the number of colonies on –L plates.

We found that the BD library contains 0.13% (448 colonies on –HULA plates, 345,000 colonies on –L plates) auto-activators (Fig. 2A). Conversely, we rarely found any AD library colonies on –HUTA plates, and only 0.0029% (13 colonies on –HUTA plates, 484,000 colonies on –T plates) of auto-activators were found (Fig. 2A).

To reveal the identities of the AAs from the BD library, we chose five colonies (AA1-AA5) growing on –HULA plates and subjected them to Sanger sequencing. Subsequent BLAST analysis against Marchantia polymorpha genome revealed that AA1 best matched with a hypothetical protein MARPO_0107s0034 (putative histone deacetylase), AA2 matched MARPO_0055s0078 (putative ubiquitin-protein ligase), AA3 and AA4 both matched MARPO_0101s0042 (putative NEDD8, ubiquitin-dependent protein catabolic process) and AA5 matched AXG93_4751s1100 (putative 40S ribosomal protein) (Table S1).

Since AAs were more frequent in the BD library, we decided to use the five colonies growing on –HULA as a validation of our AA-removal method.

**FOA can suppress the growth of AAs on solid and liquid media.** To test whether FOA can inhibit the growth of AAs, we plated the five AAs on solid media containing –L (selection for pDBlox plasmid), –HULA (selection for AAs), –L + FOA (0.2% 5-FOA). As expected for the AAs, we observed the growth of AA1-5 on –L and –HULA (Fig. 2B). Conversely, we did not see any growth on FOA plates, suggesting that FOA suppressed the growth of AAs.

To further confirm this result and see whether this system applies to liquid-grown cultures, we have compared growth rates of AAs, and empty vector (EV) controls in –L, –LH and –L + FOA. In –L media, since there was no positive or negative selection for the pGAL2-activated genes, the auto-activators (AA, red) and empty vector strains (EV, black) showed similar growth pattern for all three days (Fig. 2C, left). On the other hand, in the presence of –LH media, as expected for a scenario where pGAL-driven genes are selected for, AAs grow normally, reaching saturation on the second day, while EV did not show any significant growth even after 3 days (Fig. 2C, middle).

However, when SC-Leu media was supplemented with 0.2% FOA, AAs did not show observable growth after 1 day, while the EV showed a slower growth (Fig. 2C, right). On day 2, AAs showed minor growth, and their OD was 5.3 times lower compared to EV, indicating that the growth of AAs is penalized in FOA. On day 3, auto-activators and EV showed similar growth. This indicates that FOA can inhibit the growth of AAs, but the negative selection loses its potency after day 2 in liquid media. Consequently, we recommend to limit the FOA treatment to two days.
Removal of auto-activators from a mixed culture. After demonstrating the inhibiting effect of FOA on AAs grown in solid and liquid culture, we investigated whether we can remove AAs from a mixture of yeast cells containing AAs and non-AAs (EV, empty vector controls). To this end, we have mixed AA:EV liquid cultures in a 5:1 ratio, resulting in a culture containing 83% (5/6) AAs. The mixed culture was grown in liquid medium resulting in no selection (–L), enrichment for AAs (–LH), and removal of AAs (–L + FOA). After 24 h of incubation, 48 colonies from each of the three cultures were parallel-spotted on plates selecting non-AAs (–L + FOA) or selecting AAs (–LH, Fig. 3A). As a control, we have spotted two colonies of AAs (red square) and non-AAs (green square, Fig. 3B).

The AA:EV culture that was passed through –L, which imparts no positive or negative selection, we observed 26 colonies growing on the –LH plate, indicating that roughly half of the 48 colonies are AAs (Fig. 3B, left-bottom plate). As expected, AA controls (red square) grew well on –LH, but poorly on –L + FOA, while non-AA control showed an opposite behavior (green square).

Growing the AA:EV culture in –LH should have resulted in a culture composed predominantly by AAs. Indeed, 47 out of 48 colonies were growing well on –LH (Fig. 3B, middle-bottom plate), but poorly on –L + FOA (Fig. 3B, middle-top plate). Interestingly, the one colony growing poorly on –LH (red arrow), grew well on –L + FOA, indicating that this colony is a non-AA that escaped the –LH selection. Finally, the tested whether the AA:EV culture grown in –L + FOA was depleted of all AAs. Indeed, we observed no colonies on –LH (Fig. 3B, bottom-right), indicating that our method has successfully removed AAs from the mix.

Taken together, based on results from this and previous sections, we can conclude that the negative selection using pGAL2-URA3 and FOA screening works well in liquid and solid media, preventing the growth of AAs. Our method successfully removed all auto-activators from a culture containing 83% AAs.

Discussion
Since AAs are a large problem in Y2H screens and are difficult to remove by screening when performing a large-scale assay where millions of interactions are tested, we have developed a method that removes AAs by using conditional negative selection with URA3 gene, driven by pGAL2 promoter. The pGAL2 promoter activates URA3 only in the presence of an AA or a genuine PPI, allowing URA3 to convert 5-FOA into a toxic FU. When applied to haploid AD and BD cultures, the method has thus potential to selectively minimize or outright eliminate yeast cells harboring AAs.

The previous study has shown that AAs comprise 16% of baits, which in turn comprised 95% of detected interactions in their large-scale CrY2H assay. In our study, we observed that BD and AD libraries contained 0.13% and 0.0029% AAs, respectively (Fig. 2A). We speculate that the higher number of AAs observed in the CrY2H study is because the authors focused on transcription factors, which are likely to contain bona fide activation domains. Conversely, since we have studied all coding sequences in Marchantia polymorpha, the proportion of transcription factors containing activation domains should be lower, explaining the lower number of observed AAs. Despite the lower numbers in Marchantia, removal of AAs is still a worthwhile pursuit as it will result in a higher number of detectable true positive interactions.

We observed that the FOA treatment effectively suppresses the growth of AAs on solid media (Fig. 2B and 3B), and liquid media (Fig. 2C). However, the efficacy of the assay in liquid media drops after three days of incubation, as the growth of AAs equals that of the non-AAs (Fig. 2C, right panel). This indicates that our approach is only effective in the first two days. While the molecular basis for the loss of efficacy is unclear, we speculate that the unwanted growth could be caused by spontaneous mutations of the pGAL2-URA3, which either inhibit the activation of URA3 or inactivate the enzyme. Further work should attempt to introduce another copy of pGAL2-URA3 into the genome to provide a genetic backup for the assay.

We were able to remove all AAs from the panel of 48 colonies, indicating that despite the loss of efficacy after two days of cultivation in FOA, the assay is still able to deplete the AAs from a yeast culture (Fig. 3B). Furthermore, since the URA3 gene is also activated by true interactions (Fig. 2B), including pGAL2-URA3 into Y2H assays will have a valuable dual role of removing AAs and selecting for true PPIs.

To conclude, our method can remove the AAs before the resource-consuming mating, selection and sequencing experiments. Consequently, our method should be used in conjunction with large-scale yeast-two-hybrid assays, to generate more comprehensive protein–protein interaction networks with fewer false-positive interactions.

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