Chapter 8

Mapping Interactomes with High Coverage and Efficiency Using the Shifted Transversal Design

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Abstract

"Smart-pooling" is a strategy to achieve high efficiency, sensitivity, and specificity in large-scale yeast two-hybrid screening. In smart-pooling, reagents are multiplexed in a highly redundant manner and the positives can be read out on the final selection plates without the requirement of any extra experimental steps. We have shown that the Shifted Transversal Design (STD), a powerful theoretical construction for smart-pooling, can be used in yeast two-hybrid interactome mapping. STD pooling can achieve similar levels of sensitivity and specificity as one-on-one array-based yeast two-hybrid, while the costs and workloads are much lower. This chapter focuses on the construction and usage of STD arrays for large-scale yeast two-hybrid interactome mapping.

Key words: Yeast two-hybrid, Protein–protein interaction, Interactome, Pooling, Smart-pooling, Multiplexing, Redundancy, Shifted transversal design

1. Introduction

"Smart-pooling" (or "group testing") aims to increase efficiency, accuracy, and coverage in high-throughput screening projects, including yeast two-hybrid (Y2H) interactome mapping ([1]). It involves the building and assaying of defined pools of specific items, including Y2H preys, such that each item is present in several pools and hence tested several times. In contrast to the traditional pooling approaches, positive preys can be read out from the pattern of positive pools on the final selection plates, without the need to perform a secondary step for identification, such as individually testing the preys from positive pools or colony-picking and sequencing the positive colonies in Y2H. The smart-pooling approach is general and has been applied recently to identify protein–protein

interactions (2, 3) and protein–DNA interactions (4), and combined with second-generation DNA sequencing to study rare sequence variants (5, 6).

Central to the smart-pooling procedure is the choice of the pooling design, i.e., which pools contain which preys. The Shifted Transversal Design (STD) is a flexible and powerful algorithm for designing smart-pools (7). It significantly outperforms other published combinatorial smart-pooling designs under a standard theoretical model (7, 8). In our previous study (3), we have shown that STD pooling is a method of choice for obtaining high-coverage protein–protein interactomes, and could prove effective in a wide range of high-throughput experiments.

Choosing an appropriate STD design requires estimating the maximum usable pool size (at the assay densities of interest), the expected rates of false positives and false negatives when using pools of that size, and the number of positives that one wishes to detect (accepting the fact that some positives may be missed for protein–protein interaction hubs that have more positives). Based on these estimates, computer simulations are performed to select the most appropriate STD parameters (redundancy, and batch size which is closely dependent on the pool size) as described (8). "Redundancy" is the number of pools that contain any given item. Part of the redundancy is necessary to identify a single positive item, and the remaining "extra redundancy" allows the system to deal with noise (false-positive and false-negative pools) and multiple positive items (within a particular batch of pools). To choose the most appropriate STD design, one needs to keep in mind that there is always a trade-off: a design with a higher redundancy and/or smaller batches will enable to detect more positives unambiguously and correct for more false positives and false negatives, but this comes at the price of a larger number of pools, hence increasing the screening workloads and costs.

To limit the cost of building the STD pools and to increase their flexibility of usage, in our previous study (3) we took advantage of inherent STD symmetries by designing and building small intermediary micropools. In Fig. 1, an example illustrates the process of designing and building a simple STD: 18 preys are pooled according to a small STD design. Initially, the 18 preys are split into two groups of nine preys (groups A and B), and each group is pooled independently according to its corresponding STD subdesign to obtain two sets of micropools (sets A and B). Each micropool contains three different preys (pool size of 3), and each prey is contained in three different micropools (redundancy of 3), which form this prey's unique signature. In fact, any two micropools are sufficient to uniquely identify a prey, so these micropools have an extra redundancy of 1. Finally, each pair of same-numbered micropools from sets A and B are superposed to obtain one batch of STD pools (p1–p9). These STD pools still possess a redundancy of 3, but their pool size is now 6, and the nine STD pools accommodate
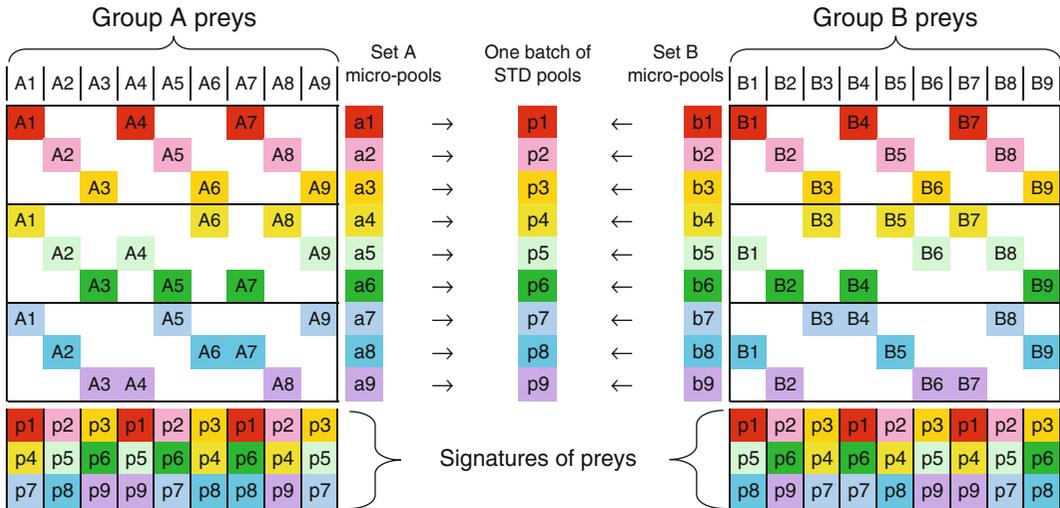
Fig. 1. A simple STD pooling design. Two groups of nine preys (group A: A1–A9 and group B: B1–B9) are separately pooled into nine color-coded micropools (set A: a1–a9 and set B: b1–b9), according to subdesigns of a larger STD design (which can accommodate all 18 preys). Each micropool contains three preys (e.g., a1 contains A1, A4, and A7), and each prey is present in a unique combination of three micropools (a unique signature, e.g., a1|a5|a9 for prey A4). The pairs of same-numbered micropools from sets A and B can then be superposed to generate STD pools (one batch: p1–p9), each containing six preys, and each prey still has a unique signature in the STD pools. Reproduced from ref. 3 with permission from Cold Spring Harbor Laboratory Press.

all 18 preys. Of note, each prey still has a unique signature, although the extra redundancy is now 0 because all three pools are required to identify each prey uniquely.

We built the worm STD pools in a similar manner but on a higher scale (Figs. 2 and 3). The prey library was conceptually split into smaller groups, e.g., 75 groups of 169 preys in our previous study (3). Each group was STD-pooled independently to obtain a set of micropools. Every 12 sets of micro-pools were built according to subdesigns of a larger STD design, so that the micropools are superposable to generate larger STD pools (Fig. 2). In practice, we superposed every two micropools to generate STD-1536, every six micropools to generate STD-384, and every 12 micropools to generate STD-96 (Fig. 3).

Once the STD arrays have been built, STD Y2H screening is performed similar to one-on-one array-based Y2H (1-on-1 Y2H) (i.e., mating, diploid selection, and final growth selection) (9) (see Note 1), with an additional in silico decoding step (Fig. 3).

Building an ORFeome-scale STD array with high redundancy requires a significant initial investment, but it results in a valuable resource that can be distributed and used repeatedly. Subsequently, the workload and cost for STD Y2H screening are much less than those for one-on-one Y2H. For example in our previous study (3), screening costs and workload were approximately threefold higher for one-on-one than for STD Y2H, while sensitivity and specificity were similar.
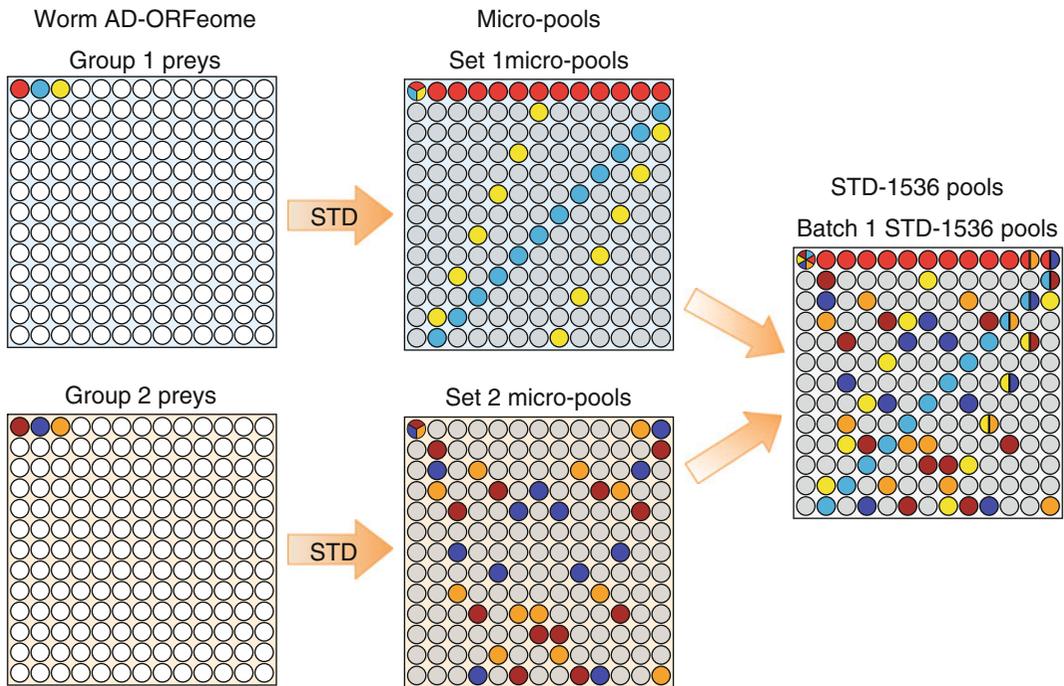
Fig. 2. STD pooling design described in this chapter. 12,675 Worm AD-ORFeome preys were split into 75 groups each containing 169 preys. Each group was STD pooled into a set of 169 micropools. Each micropool contains 13 preys (micropool size: 13), and each prey is contained in a unique combination of 13 micropools (a unique signature; redundancy: 13), as illustrated with three color-coded preys in sets 1 or 2. Two preys co-occur in at most one micropool, so that each prey is uniquely identified by any two of the 13 pools that contain it; therefore, these micropool designs have an extra redundancy of 11. In addition, the micropool signatures of preys with identical AD-ORFeome coordinates from groups 1 and 2 are very different (e.g., light red in set 1 and dark red in set 2): every two sets of micropools can be superposed to obtain one batch of STD-1536 pools, such that two preys from different groups co-occur in at most two common pools. Consequently, in STD-1536 each prey is uniquely identified by any 3 of the 13 pools that contain it: STD-1536 pools possess an extra redundancy of 10. Reproduced from ref. 3 with permission from Cold Spring Harbor Laboratory Press.

For details on STD design, please refer to (7, 8). In this chapter, we assume that an appropriate STD design has been selected, and we focus on the experimental protocols for constructing and screening the chosen STD pools. We illustrate the protocol with the designs that we used in our previous work (3), but all of the procedures would be very similar with other STD designs.

2. Materials

2.1. Media and Stock Solutions

1. 3-AT (3-amino-1,2,4-triazole, Sigma): Prepare 2 M solution in water, filter to sterilize, and store at 4°C (see Note 2).

2. Glycerol: Prepare 40% (v/v) solution in water, filter to sterilize, and store at room temperature.

3. Glucose: Prepare 40% solution in water, autoclave, and store at room temperature.

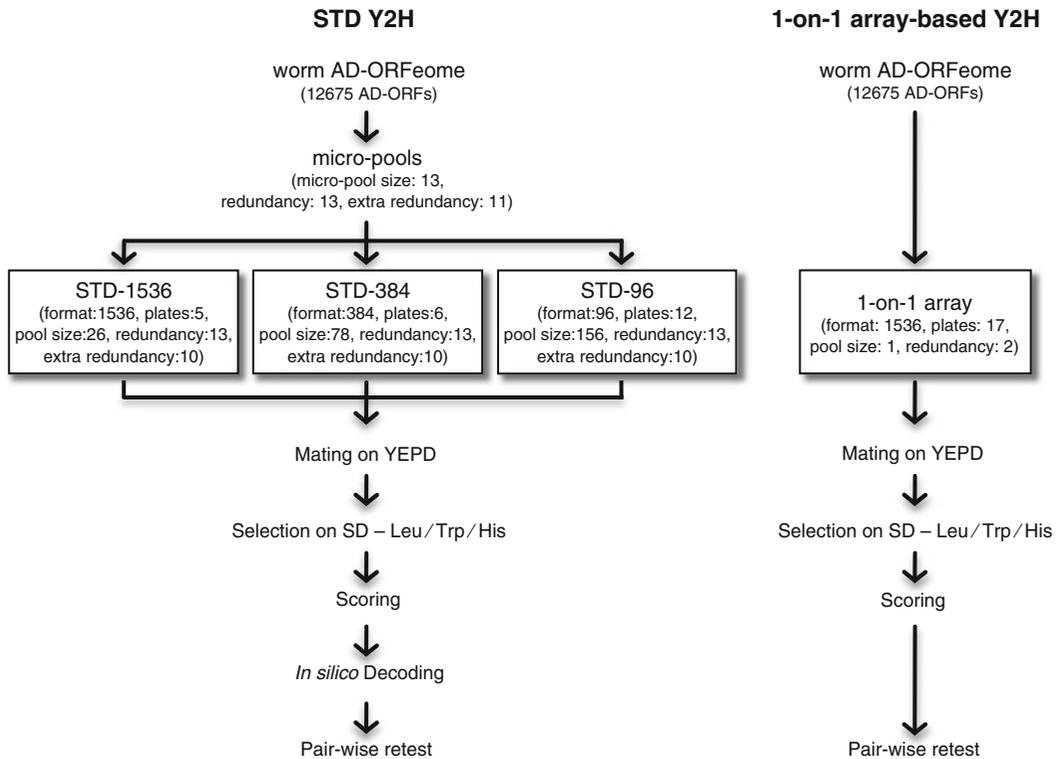**STD Y2H**   **1-on-1 array-based Y2H**

Fig. 3. Flowchart of STD Y2H side-by-side with one-on-one array-based Y2H.

4. Amino acid supplementary powder mixture for synthetic media (complete): 3 g adenine, 2 g uracil, 2 g inositol, 2 g *p*-aminobenzoic acid, 2 g alanine, 2 g arginine, 2 g asparagine, 2 g aspartic acid, 2 g cysteine, 2 g glutamic acid, 2 g glutamine, 2 g glycine, 2 g histidine, 2 g isoleucine, 10 g leucine, 2 g lysine, 2 g methionine, 2 g phenylalanine, 2 g proline, 2 g serine, 2 g theronine, 2 g tryptophan, 2 g tyrosine, and 2 g valine (Fisher). Dropout (DO) mix is a combination of the aforementioned ingredients minus the appropriate supplement (see Note 3). For example, to make DO – Trp, all ingredients except tryptophan need to be mixed; to make DO – Leu/Trp, all ingredients except leucine and tryptophan need to be mixed. The resultant DO mixture can be stored in tinted glass bottles and stored at room temperature. 2 g of the DO powder mixture is used per liter of medium.

5. SD – Leu liquid medium: Add 6.7 g yeast nitrogen base without amino acids (BD Difco), 2 g amino acid supplementary powder mixture lacking leucine (DO – Leu) and 950 mL water in a 2-L flask. Autoclave. Add 50 mL 40% glucose, and mix thoroughly (see Note 4).

6. SD – Trp liquid medium: Add 6.7 g yeast nitrogen base without amino acids, 2 g amino acid supplementary powder mixture lacking tryptophan (DO – Trp), and 950 mL water in a 2-L flask. Autoclave. Add 50 mL 40% glucose, and mix thoroughly.

7. YEPD agar medium: Add 120 mg adenine (Sigma), 10 g yeast extract, 20 g peptone, and 20 g bacto agar (BD Difco) to 950 mL water in a 2-L flask. Autoclave. Add 50 mL 40% glucose, mix thoroughly, cool to approximately 55°C, and pour plates.

8. SD – Leu agar medium: Add 6.7 g yeast nitrogen base without amino acids, 2 g amino acid supplementary powder mixture lacking leucine (DO – Leu), and 150 mL water in a 250 mL flask. Add 20 g bacto agar to 800 mL water in a 2-L flask. Autoclave separately. Combine the autoclaved solutions, add 50 mL 40% glucose, mix thoroughly, cool to approximately 55°C, and pour plates.

9. SD – Trp agar medium: Add 6.7 g yeast nitrogen base without amino acids, 2 g amino acid supplementary powder mixture lacking tryptophan (DO – Trp), and 150 mL water in a 250-mL flask. Add 20 g bacto agar to 800 mL water in a 2-L flask. Autoclave separately. Combine the autoclaved solutions, add 50 mL 40% glucose, mix thoroughly, cool to approximately 55°C, and pour plates.

10. SD – Leu/Trp/His agar medium: Add 6.7 g yeast nitrogen base without amino acids, 2 g amino acid supplementary powder mixture lacking leucine, tryptophan and histidine (DO – Leu/Trp/His), and 150 mL water in a 250 mL flask. Add 20 g bacto agar to 800 mL water in a 2-L flask. Autoclave separately. Combine the autoclaved solutions, add 50 mL 40% glucose, cool to approximately 55°C, add proper volume of 2 M 3-AT solution, mix thoroughly, and pour plates. For example, to make medium with a final concentration of 3-AT at 2 mM, add 1 mL of the 2 M 3-AT stock to the medium (see Note 2).

2.2. Plates and Accessories

1. 96-Format deep well plates (Nunc), Tecan Freedom EVO liquid handling robot, and Tecan Aquarius MultiChannel Pipetting robot (Tecan Group Ltd.) are used for building micropools and STD pools. 96-Well and 384-well plates (ABGene) are used for storing the resultant micropools and STD pools at –80°C.

2. The BioMatrix robot (S&P Robotics Inc., Toronto, ON) and OmniTrays (Nunc) are used for all replica pinning procedures with the 384- and 1536-format screens (see Note 5).

3. 150 mm Petri dishes (Fisher) are used for all procedures with the 96-format screens and pairwise retest. Yeast transferring from liquid culture to the agar plates is performed using the Tecan Aquarius MultiChannel Pipetting robot (Tecan Group Ltd.)

or a multichannel pipette (Fisher). The plate replication procedures are performed using velvet replica plating apparatus Cora Styles Lab Supplies, OR.

2.3. Strains, Plasmids, and AD-ORFeome Libraries

1. Yeast two-hybrid (Y2H) host strains: prey strain Y8800 (*MATa trp1-901 leu2-3,112 his3-200 ura3-52 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ cyh^R*) and bait strain Y8930 (*MATα trp1-901 leu2-3,112 his3-200 ura3-52 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ cyh^R*).

2. pPC97 (*GAL4-DBD, LEU2*) is the *GAL4* DNA-binding domain (DBD) plasmid for Y2H bait construction.

3. pMV257 (also called pPC86-CYH, *GAL4-AD, TRP1*) is the *GAL4* activation domain (AD) plasmid for Y2H prey construction.

4. AD-ORFeome libraries: Many AD-ORFeome collections (and other ORFeome collections) are available through the ORFeome Collaboration (http://www.orfeomecollaboration.org/). We used the *C. elegans* AD-ORFeome v1.1 and v3.1 in our previous study (3), which consists of 12,675 worm AD-ORFs.

3. Methods

3.1. STD Designs

1. Before designing the STD arrays, we performed dilution tests at two different densities, 384 and 1,536 spots per plate, in order to identify the maximum pool sizes that enable detection of positive controls. In conjunction with simulations performed with interpool (8), we choose pool sizes of 78 for the 384-format and 26 for the 1536-format arrays.

2. We did not investigate the maximum pool size as thoroughly for 96-format arrays, because we only screened the *C. elegans* STD pools in 384- and 1536-formats. However, our preliminary experiments in 96-format with human ORFs indicated that pool size of up to 500 may be used (3), employing a screening protocol similar to pairwise retest steps (see Subheading 3.5 below). We still built 96-format worm STD pools, because due to our micropool strategy they were an essentially free side-product. Since our focus was on high-density formats, we did not optimize these pools but chose a pool size of 156 for 96-format (see Note 6).

3. The prey library (worm AD-ORFeome v1.1 and v3.1), which contains 12,675 unique AD-ORFs, was conceptually split into 75 groups of 169 preys ($75 \times 169 = 12{,}675$). Each group was STD-pooled independently to obtain a set of 169 worm

micropools containing 13 preys each (micro-pool size of 13). Every 12 sets of micropools were built according to sub-designs of a larger STD design, so that the micropools are superposable to generate larger STD pools (Fig. 2).

4. Based on the chosen 1536-, 384- and 96-format pool sizes, the micropools were (1) superposed in pairs (as shown in Fig. 2), to produce the STD-1536 pools containing 26 preys per pool, or (2) superposed in sextuplets, to generate the STD-384 pools with 78 preys per pool, or (3) superposed in duodecaplets, to generate the STD-96 pools with 156 preys per pool. In the resulting STD pooling designs, the 12,675 preys are either split into 40 batches of STD-1536 pools with up to 338 preys per batch, or 13 batches of STD-384 pools with up to 1,014 preys per batch, or seven batches of STD-96 pools with up to 2,028 preys per batch. The STD-1536, STD-384, and STD-96 batches each contain 169 pools. All batches within an STD design are arrayed as spots on a series of plates, but the batches are disjoint and decoded independently. These three designs possess an extra redundancy of 10, which provides high noise-correction capabilities.

In fact, every 12 sets of worm micropools (169 preys per set) is a collection of subdesigns of an STD design with 2,028 preys per batch (whose extra redundancy is 10). They can therefore be superposed to obtain different STD designs. Each individual set of micropools is isomorphic to a smaller STD design and can be used as an STD pooling batch in its own right, with an extra redundancy of 11. When 2, 6, or 12 consecutive micropool sets are superposed to obtain STD-1536, or STD-384, or STD-96, the resulting designs are again isomorphic to an STD design with 228, 1,014, or 2,028 preys per batch, respectively, and therefore they all have an extra redundancy of 10. More specifically, worm micropools are subdesigns of STD(2,028;13;13) isomorphic to STD(169; 13;13), and were superposed to obtain designs isomorphic to STD(338;13;13) for STD-1536, STD(1,014;13;13) for STD-384, and STD(2,028;13;13) for STD-96 (see ref. 8 for details).

3.2. Building STD Pools

1. The sources were all worm ORFeome v1.1 and v3.1 AD plates (11,001–11,114 and 31,001–31,022).

2. The source plates were thawed at room temperature, inoculated in 96-format deep well plates containing SD – Trp liquid media, and incubated at 30°C for 2 days.

3. Before pooling, the yeast cells in the freshly cultured source plates were mixed thoroughly with a plate shaker or by pipetting with a Tecan Aquarius MultiChannel Pipetting robot.

4. Micropools were assembled in 96-format deep well plates by cherry-picking from the source to the target plates using a

Tecan Freedom EVO liquid handling robot. The robot was programmed directly in GWL (rather than using CSV files), which optimized the process. Indeed, each column of eight source wells was aspirated (and dispensed to the appropriate destination plates and wells) 13 times in a row, before moving on to the next column of source wells. With GWL we were able to resuspend and thoroughly mix the yeast in source wells by performing a series of up-and-down operations before the first aspiration (using a specific "liquid class"); while the 12 subsequent aspirations were performed without any up-and-down cycles, which gained a lot of time. All scripts are available (see Note 10).

5. STD-1536, STD-384, and STD-96 pools were generated in 384-format and 96-format by superposing the appropriate micropool plates with a Tecan Aquarius MultiChannel Pipetting robot in 384-well and 96-well storage plates.

6. All pools were frozen and stored at –80°C with 20% glycerol, by adding an equal volume of 40% (v/v) glycerol solution to the yeast culture, and mixing thoroughly.

3.3. Handling STD Arrays

1. Before Y2H screening, STD arrays glycerol stock plates were thawed at room temperature, mixed thoroughly with a plate shaker and transferred to SD – Trp agar plates with a BioMatrix robot, and incubated at 30°C for 2 days.

2. After incubation, this set of "master" agar plates was replicated into multiple copies (up to eight copies), which could be used either for screening or as a source for further replications (see Note 7).

3.4. Y2H Screening with STD Arrays

The Y2H screening with STD arrays was performed following the steps below. For 1536-format and 384-format screens, all pinning steps are performed with a BioMatrix robot, using a 1536-format pinhead with 0.7 mm diameter pins or a 384-format pinhead with 1 mm diameter pins. For 96-format, cell transfer from liquid culture to agar plates is performed using a Tecan Aquarius Multi-Channel Pipetting robot, and plate replication is performed using velvet replication.

1. Bait culture: Bait is inoculated in 5 mL SD – Leu liquid medium and cultured at 30°C, 200 rpm, for 2 days. Bait culture is then transferred to SD – Leu plates. One bait plate is sufficient for up to eight mating plates in step 3 below. The bait plates are bagged and incubated upside-down at 30°C for 2 days.

2. Prey culture: Prey plates are replicated from STD stock array plates from Subheading 3.3 above. The prey plates are bagged and incubated upside-down at 30°C for 2 days.

3. Mating: Baits are first transferred to YEPD plates by robotic pinning (see Note 8). Preys are then transferred to the mating plates on top of the bait spots. The mating plates are bagged and incubated upside-down at 30°C for 16–24 h.

4. Final selection: The incubated mating plates are replicated to SD – Leu/Trp/His + 3-AT final selection plates. The selection plates are bagged and incubated upside-down at 30°C for 7–14 days (see Note 9). Of note, the diploid selection step on SD – Leu/Trp, which is normally used in similar array-based Y2H screens, was skipped (see Note 1).

5. Scoring: STD spots were scored manually using the in-house ColonyImager image-processing program using four discrete levels for each spot: strong (clear positive) or weak (smaller than strong but well above background) for positives, and none (no detectable signal) or faint (barely above background, most likely negative) for negatives. These results were transformed into a suitable XML format with Perl scripts, and decoded with interpool (8). The "distance" parameter δ was chosen to fit the experimental conditions. For example, in our previous work (3), false positives were relatively rare while false negatives were common, leading us to use a very sensitive distance ($\delta_{NONE} = 2, \delta_{FAINT} = 1, \delta_{WEAK} = 4, \delta_{STRONG} = 6$). This choice did not compromise specificity. All relevant scripts, programs, and data files are available (see Note 10). A confidence score was attributed to each STD hit, depending solely on the number of putative false-negative spots for the hit. Specifically, "none" spots carry a cost of 2 and "faint" spots 1, and summing over all false negatives for a hit yields a total cost; if this total cost is at most 4 the confidence score is 5, if it is up to 8 the score is 4, and so on until reaching the lowest confidence score of 1 if the total cost is between 17 and 20.

3.5. Pairwise Retest

1. Pairwise retests were performed in quadruplicate by scoring a single phenotype of the *HIS* reporter in 96-format on agar plates.

2. Put 5 µL bait fresh culture on the YEPD plates using a Tecan Aquarius MultiChannel Pipetting robot or a multichannel pipette, let the plate dry at room temperature. Put 5 µL prey fresh culture on top of the bait spots, dry at room temperature. Incubate at 30°C for 16–24 h.

3. Replicate the YEPD plates to SD – Leu/Trp/His plates using velvet replica plating. Incubate the target plates at 30°C for 2 days.

4. Remove most of the yeast cells on the plates using velvet replica until the yeast cell spots can be barely see on the plates. Incubate at 30°C for 2 days.

5. Each retest was scored as negative, weak, or strong (0, 1, or 2, respectively). Summing over the four replicates, one can obtain a retest score between 0 and 8 for each hit. Positive hits are those whose retest score was at least six, while hits with scores at most two were classified as false positives and the remaining hits with intermediate retest scores were classified as dubious, which can either be retested or be grouped as true positives or false positives depending on one's discrimination and screening stringency.

4. Notes

1. Diploid step skipping: Preliminary experiments showed that, in our hands, including a diploid selection step (on SD – Leu/Trp between mating and final selection) did not result in any improvements, perhaps because the robotic replication step may not be fully effective in transferring all components of a colony spot, so that the additional replication step compensates any gains from the diploid selection.

2. 3-AT can also be added as powder, but it will take longer to dissolve. We recommend to use 2 M 3-AT solution for media with 3-AT concentrations up to 20 mM, and use 3-AT powder for media with >20 mM 3-AT.

3. When making up the amino acid supplementary mixture (complete or dropout), the solid ingredients should be combined and mixed very thoroughly by turning end-over-end for at least 15 min, while adding a couple of clean marbles or glass beads helps the mixing.

4. The yeast medium used in this work can be made using any generic method for making yeast medium, not limited to the one described in Subheading 2 of this chapter.

5. Other robotic pinning systems can also be used. For example, the Singer RoTor DHA benchtop robot uses disposable replicators RePads, and PlusPlates (Singer Instruments, UK). Although manual pinning should also work in theory, to guarantee high quality and high reproducibility of the screening results, robotics pinning is highly recommended for high-density formats such as 384-format, 768-format, 1536-format, or higher density formats.

6. Users focusing on 96-format assays should choose an STD design optimized for this density. In particular, dilution tests should be performed to find the largest reliable pool size in their hands. In our experience, pool size is often the limiting

factor in smart-pooling, and using pools as large as possible should yield the most efficient STD pools without compromising sensitivity – although this also depends on the expected fraction of positives and error-rates, and should be confirmed through simulations as described. The micropools described here can produce pools of size at most 156 (2,028/13), since they are subdesigns of STD(2,028;13;13), but the approach can be easily extended to allow the production of larger pools. For example micropools constructed as subdesigns of STD(12,675;13;13) can be superposed to obtain STD pools of size up to 975, while smaller pools for high-density formats, obtained by superposing two or six sets of these new micropools, would be equivalent to those produced in this study.

7. However, fresh arrays should still be occasionally remade from glycerol stock, because the STD arrays begin losing representativity after more than five sequential replications. The arrays appear fully functional after being stored at 4°C for at least 2 months, though in our previous study we used them within 1 week after replication to avoid confounding factors and guarantee the highest data quality.

8. We notice the wetness of agar plates significantly contributes to the screening variation. Wet plates could lead to too much yeast cells been transferred and result in high background; dry plates could lead to too few and uneven transferring and result in unreadable final plates. It would be a good idea to make and process the plates in a uniform matter, and test plates with different wetness before large-scale screening.

9. The difference of incubation time might vary for different baits. We suggest starting to check the plates after 5 days incubation, and continue the monitoring every 2 days afterward. Multiple sets of pictures might be taken for choosing the best scoring time.

10. The scripts and programs mentioned in this chapter are freely available online: they have been published as a Supplementary Data file associated to our previous study (3), and can also be downloaded from NT-M's website (http://www-timc.imag. fr/Nicolas.Thierry-Mieg).

Acknowledgment

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