Target Wise Association Studies (TWAS)

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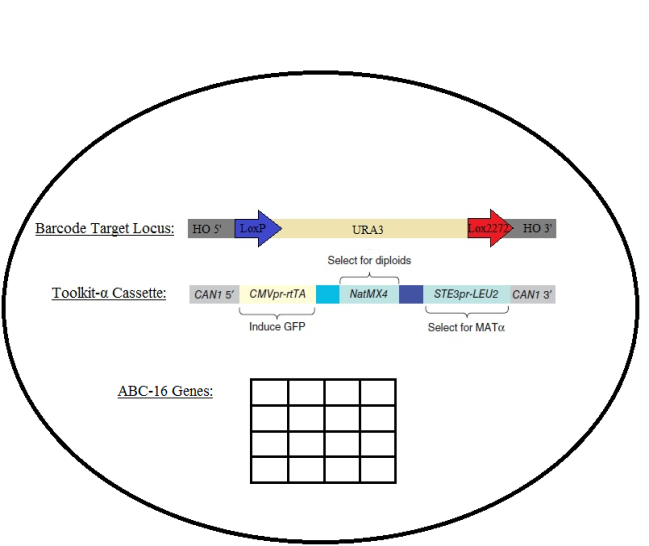
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# Project Outline:

The TWAS Project involves generating a pool of strains each carrying a unique barcode and then mating this pool of barcoded cells to a strain with multiple deletions (Green Monster Strain in this case). This generates a pool of sporulants which carry a unique barcode and possess different genotypes for those genes that were deleted. The genotypes associated with each strain barcode can be identified. This yields a pool of strains with varying genotypes at a desired set of genes each carrying a unique barcode. This pool can be studied under different conditions which allows for studying complex genotype-environment interactions that may underlie more complex phenotypes.

## Step 1: Constructing the Barcode Recipient Strain



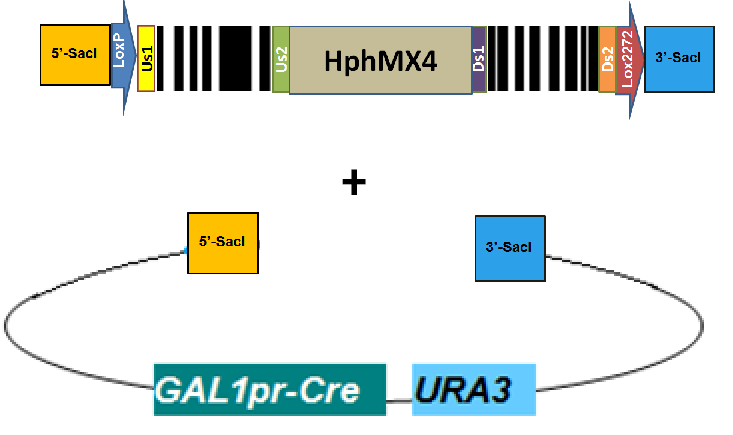
Homologous recombination has a low efficiency and isn’t suitable for creating a barcoder strain with enough complexity so we use Cre-Lox recombination to generate a complex barcoder strain pool.

Create a Backbone Strain(HO::LoxP-URA3-Lox2272) carrying a mating toolkit-alpha cassette and where the HO gene is replaced with a URA3gene flanked by a loxP and lox2272 site. This strain is created by using homologous transformation to insert a LoxP-URA3-Lox2272 cassette into the HO locus of Yo Suzuki’s RY0418 strain (Toolkit-alpha strain: MATalpha lyp1∆ his3∆1 leu2∆0 ura3∆0 met15∆0 can1∆::GMToolkit-alpha (CMVpr-rtTA KanMX4 STE2pr-Sp- his5).

Figure 1: HO::URALOX strain

This strain has a URA3 flanked by lox sites in the HO locus. A toolkit cassette for selection of type ‘α’ haploids and none of its ABC transporters are deleted.

## Step 2: Barcoder Strain Construction

A Cassette containing a hygromycin resistance cassette (HphMX4) flanked by 2 barcodes (UPTAG, DNTAG) and a LoxP and Lox2272 site on one end and overlap sequences to the pSH47 plasmid is constructed.

pSH47 is a plasmid containing Cre under the control of the Gal1 promoter. It also carries the URA3 gene and has a single SacI digestion site (see plasmid map in folder).

The constructed barcode cassette and the SacI-HF digested pSH47 plasmid are transformed into the HO::URA3LOX strain where the plasmid and barcode cassette are assembled by in yeast assembly. The transformants are cultured in different conditions to select for barcode cassette integration in the HO locus and for loss of the plasmid.

Figure 2:The In Yeast Assembly Fragments

Figure 3: TWAS children Generation Process

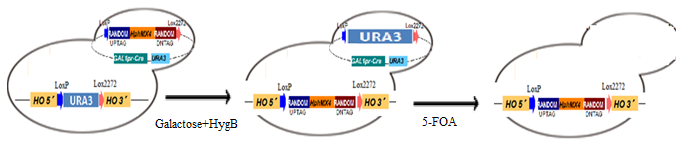
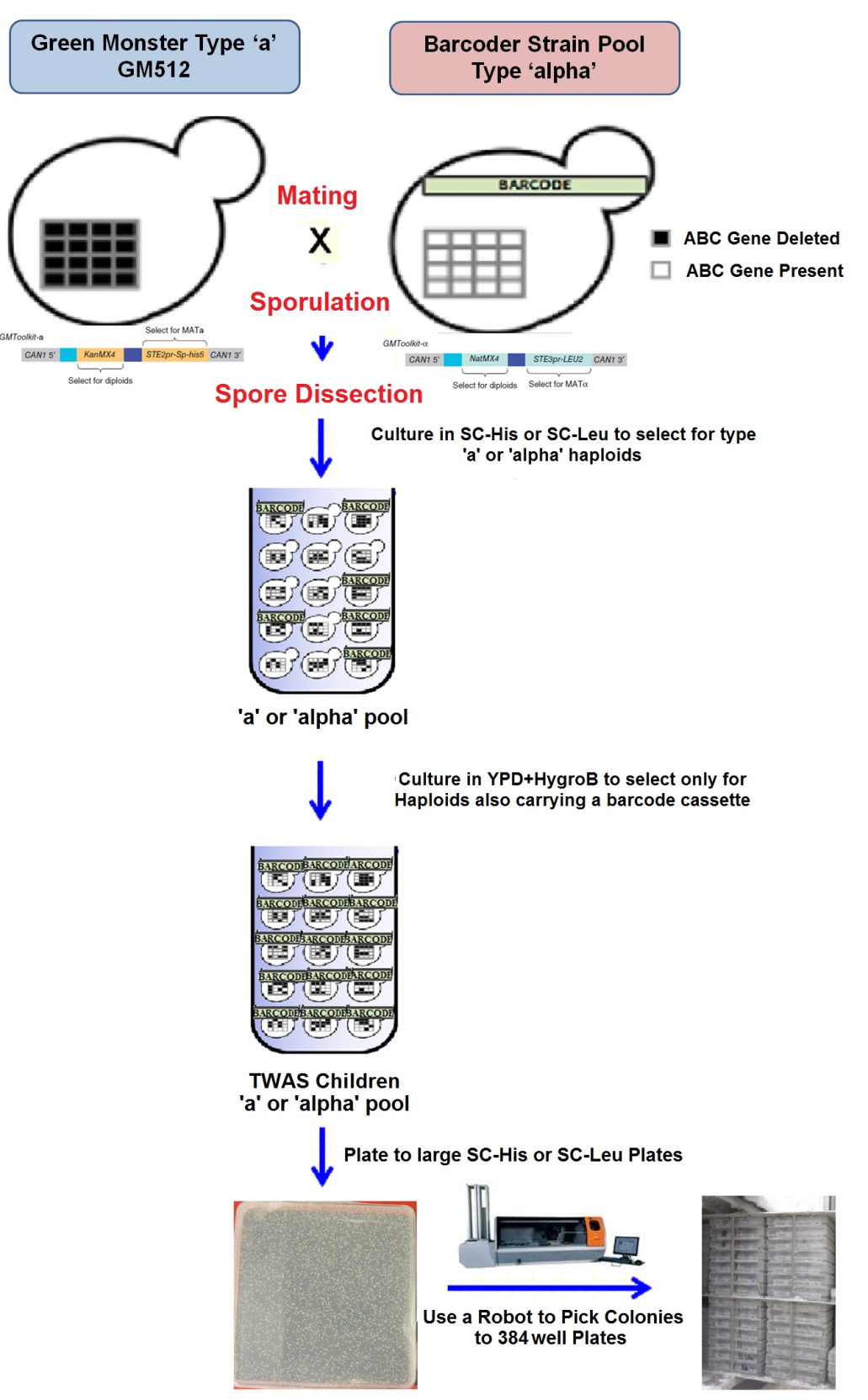
 After transformation/in yeast assembly, the strains are first plated on large YPG+HygB plates(contain Galactose and Hygromycin B).The Hygromycin selects for cells where in yeast assembly was successful. Galactose induces Cre activity and lox site recombination causing some of the cells to replace URA3 cassette at the HO locus with the HphMX4 cassette flanked by barcodes.

Figure 4: Barcoder Strain Generation:

1st Step: Perform in Yeast Assembly to obtain strains carrying the barcodes on a Cre expressing plasmid

2nd Step: Plate the strain on Galactose(YPG) and Hygromycin B plates to induce Cre activity and ensure only strains carrying barcodes survive.

3rd Step: Culture on 5-FOA plates, this counterselects against the URA3 gene, counterselecting for any strains that retain the Cre plasmid and for any strains where the URA3 gene in the HO locus is not replaced with a barcode

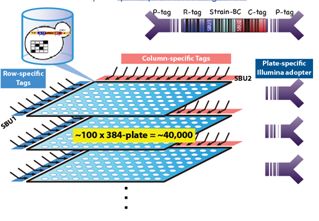
Following this step, the colonies are scraped and replated on 5-FOA plates which counterselects against URA3 strains. This counterselects strains that still retain the Cre plasmid and strains where recombination of the Barcode Cassette at the HO locus was not successful. Cells scraped from this 5-FOA now constitute the barcoder strain which can be mated to the Green Monster Strain.

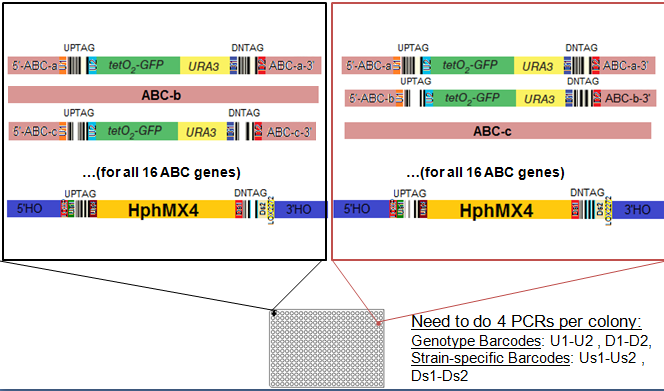
## Step 3: Generating the TWAS children

After the barcoder strain pool is generated and validated, it is mated to the type ‘a’ Green Monster strain (GM512). The barcoder strain pool carries the type ‘alpha’ mating cassette and is mating type ‘alpha’ while the Green Monster strain carries the type ‘a’ mating cassette and is mating type ‘a’(see Figure 4).

Following mating and sporulation, the sporulants are selected in liquid SC-His and SC-Leu media to select for mating type ‘a’ and ‘alpha’ haploids respectively. The type ‘a’ and type ‘alpha’ strains are then separately grown in YPD+Hygromycin B liquid cultures to select for strain carrying the HphMX4 cassette flanked by barcodes. This yields the TWAS children which are then plated to large SC-His plates (for Type ‘a’ TWAS children) and large SC-Leu plates(for Type ‘alpha’ TWAS children). Individual colonies are picked from these plates by a robot and arrayed to 348 well plates(>10,000 individual colonies). The genotypes for the ABC16 genes and the strain specific barcode for these strains are unknown, these are identified use row-column PCR.

## Step 4: Genotpying the TWAS children and Identifying Barcodes



To identify the ABC gene genotypes and the strain specific barcodes at the HO locus for all the TWAS strains we use Row-Column PCR. What this involves is performing 384 well PCRs on the single TWAS children colonies and using a primer having a unique row tag sequence for each row and a primer having a unique column tag sequence for each column. Unique plate tags can then be added to each Row Column PCR and the samples can be pooled and sequenced on a Miseq or Hiseq Flow cell. By using unique row, column and plate tags we can deduce which wells and therefore which strain each deletion and strain specific barcode comes from, thus identifying the strain’s genotype and strain specific barcode. After the genotypes of the TWAS children are identified the TWAS children of the same mating type can be pooled together and studied under different conditions. The fitness of each genotype can be assessed by tracking the abundance of its strain specific barcode. This makes it possible to examine multiple genotypes in a set of genes under multiple environmental conditions.

# Protocols and Results:

## Step 1: Constructing the Barcode Recipient Strain

### Creating HO::LoxP-URA3-Lox2272 Strain

Used PCR to add LoxP and Lox2272 sites around URA3, used pIS418 (a plasmid containing URA3) as the template

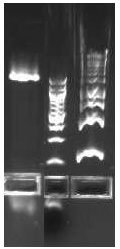
**PCR mix:**

Figure 5: Results PCR to add Lox sites to URA

Lane 1: PCR product(expected 1343 bp)

Lane 2:100 bp ladder

Lane 3: 1kb ladder(Genruler)

(15/12/2011)

1ul 10uM 5’HO-loxP-URA F primer

1ul 10uM URA-Lox2272-3’HO R primer

2ul of 10ng/ul pIS418 plasmid

6ul sterile DNA free H2O

10ul High Fidelity Phusion Master Mix(NEB)

20 ul Total

**PCR protocol:**

98˚C 30 sec

98˚C 10 sec , 60˚C 10 sec, 72˚C 90 sec (25 cycles)

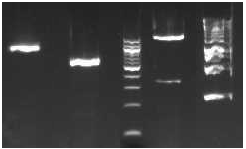
72˚C 5 min

4˚C forever

-Qiagen Qiaspin purify the PCR product

-Transform the linear (5’HO overlap-LoxP-URA3-Lox2272-3’HO Overlap) cassette into RY0148 strain (MATalpha lyp1∆ his3∆1 leu2∆0 ura3∆0 met15∆0 can1∆::GMToolkit-alpha (CMVpr-rtTA KanMX4 STE2pr-Sp- his5) using the EZ transformation kit. Plate onto SC-Ura plates

### Confirmation of HO::LoxP-URA3-Lox2272 Strain

-Picked colonies and performed colony PCR on them to confirm the expected cassette is present: picked a colony which gave expected bands for PCR(Figure 5). Gel extracted those bands and sent to sanger sequencing (see 10/1/2012 sanger seq data)

-Also confirmed the strain had the proper cassette by trying a small scale mock transformation with the barcoder cassette and SacI-HF digested pSH47 to confirm that the Lox sites are functional

-Made Glycerol stocks of this strain: **HO::URALOX strain**

Figure 6: Confirming URA-Lox Cassette Integration at HO (4/1/2012)

Lane1:(5’HO new seq-midURAw5’ PCR) (expect:988bp)

Lane 2:(midURAw3’-3’HO new seq PCR) (expect:677bp)

Lane 3:100 bp ladder

Lane 4:5’HO new seq-3’HO new seq PCR) (expect:~1.7kb)

Lane 5: 1kb Fermentas ladder

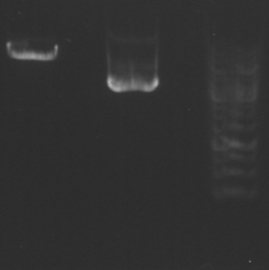
## Step 2: Barcoder Strain Construction

### Step 2a:Preparing the in yeast assembly pieces:

This entails preparing the SacI digested pSH47 and the Hygromycin cassette (HphMX4) flanked by barcodes and Lox sites.(see [Figure 2](#_Step_2:_Barcoder))

#### Part1:Restriction Digest the pSH47 plasmid

Digest the pSH47 plasmid which carries Cre under the control of the GAL1 promoter and URA3 at the SacI site.

Perform 10 X 100ul SacI-HF restriction digests of pSH47

**Protocol:**

100ul 250ng/ul pSH47

100ul NEB Buffer 4

10ul BSA

10ul SacI-HF

780ul Sterile DNA free Water

1 ml Total

Figure 7: Restriction Digest Results:

Lane 1:pSH47 SacI-HF Digest (expect 6.9kb)

Lane2: uncut pSH47 plasmid

Lane3: 1kb Genruler Ladder(Fermentas)

6/6/2012

-Vortex to mix well

-Aliquot 100ul of Restriction Digest mix to 10 1.5ml Epitubes

-Incubate at 37C for 2.5 hrs

-Inactivate at 65C for 20min

-Qiagen Qiaspin(not qiaprep) purify the restriction Digests and Nanodrop

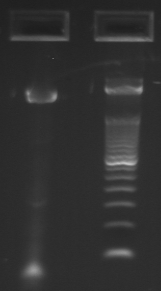
-Run a 0.8% gel to confirm that your digest was a success (see Figure 4)

#### Part 2: Prepare the Barcode Cassette

Figure 8:3 consecutive PCR reactions are used to produce the desired Barcode Cassette

This entails constructing a DNA piece made of a Hygromycin-B resistance cassette, flanked by an UPTAG and DNTAG barcode cassettes, which are in turn flanked by LoxP and Lox2272 recombination sites and regions homologous to the regions surrounding the SacI digest locus in the pSH47 plasmid( see Figures 2,7). The cassette is constructed in 3 consecutive PCR reactions. In the 1st PCR reaction, the common Us2 and Ds1 regions of the barcode cassettes are added to the HphMX4 cassette. In the 2nd PCR reaction the barcode regions(25 random bases(N’s)) and the remaining constant regions of the barcode(Us1,Ds2) are added to the cassette. In the 3rd PCR reaction the LoxP and the Lox2272 sites are added as well as regions homologous to the pSH47 plasmid sequence flanking the SacI site.

##### Step 1 PCR: Add the Us2 and Ds1 to the HphMX4 Cassette:



20ul 1ng/ul pIS420 plasmid( a plasmid carrying the HphMX4 cassette)

7.5 μL of 10uM sBC-STEP1F primer

7.5 μL of 10uM sBC-STEP1R primer

30 μL of 5X HF Buffer (from NEB)

82.3 μL of PCR quality H2O

1.2 ul 25mM dNTP

1.5 μL Phusion Polymerase(Anna’s Stock)

150 μL Total PCR mix

-Aliquot 30 or 40ul of this PCR mix to PCR tubes

PCR program for Step 1 PCR:

98C for 30sec

98C for 10sec, 59C for 15sec, 72C for 60sec [25 cycles]

72C for 5min

Figure 9: Step 1 PCR products

Lane 1: Step 1 PCR product (~1732 bp)

Lane 2: 50bp ladder(Invitrogen)

5/6/2012

4C forever

-Qiagen Qiaspin Purify the PCR products and Nanodrop(got yield ~3ug)

-Run a 2% gel to confirm the products( Expected Size: 1732bp)

##### Step 2 PCR: Add the remaining parts of the UPTAG and DNTAG:

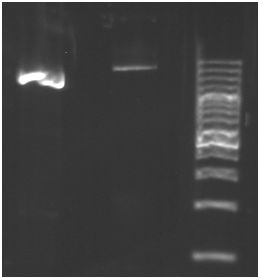


Figure 10: Step 2 PCR

Lane 1: Step 1 PCR product

Lane 2: Step 2 PCR product

Lane 3: 100 bp ladder(Fermentas)

(5/6/2012)

2.5 ul 100 ng/ul Step 1 PCR Product(S1)

12.5 μL of 10uM STEP2F N ver primer

12.5 μL of 10uM STEP2R N ver primer

97.5 μL of PCR quality H2O

125 μL High Fidelity Phusion Master Mix(NEB)

250 μL Total PCR mix

-Aliquot 25ul of this PCR mix to 10 PCR tubes

PCR program for Step 2 PCR:

98C for 30sec

98C for 10sec, 63C for 10sec, 72C for 60sec [25 cycles]

72C for 5min

4C forever

-Qiagen Qiaspin Purify the PCR products and Nanodrop(got yield of ~ 4ug)

-Run a 2% gel to confirm the products( Expected Size: 1826bp)

##### Step 3 PCR: Add the Lox Sites and the pSH47 overlap regions:

70 ul 30 ng/ul Step 2 PCR Product(S2 Nver)

25 μL of 10uM IT rgnt- HphMX4-LoxP Reamp primer

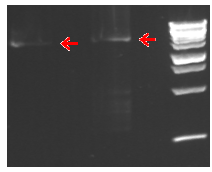
25 μL of 10uM IT rgnt- HphMX4-Lox2272 Reamp primer

Figure 11:Step 3 PCR

Lane 1: Step 2 PCR product (expect 1826bp)

Lane 2: Step 3 PCR product (expect 1958bp)

Lane 3: 1kb GeneRuler Ladder

(19/6/2012)

25ul 0.01uM LoxP-BC Cass-SacI F primer

25ul 0.01uM Lox2272-BC Cass-SacI R primer

25ul DMSO

55 μL of PCR quality H2O

250 μL High Fidelity Phusion Master Mix(NEB)

500 μL Total PCR mix

-Aliquot 25ul of this PCR mix to 10 PCR tubes

PCR program for Step 3 PCR:

98C for 30sec

98C for 15sec, 64C for 20sec, 72C for 65sec [26 cycles]

72C for 5min

4C forever

-Qiagen Qiaspin Purify the PCR products and Nanodrop(got yield of ~15ug)

-Run a 2% gel to confirm the products (Expected Size: 1958bp)

##### Confirming the Barcode Cassette:

To confirm that you have synthesized the correct product you can perform a PCR, Sanger sequence the product and carry out a small scale in yeast assembly.

**Barcode Cassette Confirmation PCRs:**

-Perform 2 PCRs: a SacI Reamp F **-** Us2 PCR and a Ds1- SacI Reamp RPCR

2ul 50ng/ul Step 3 PCR Barcode Cassette

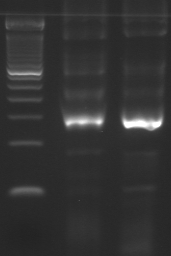
0.5 μL of 10uM SacI Reamp F or R primer

0.5 μL of 10uM Us2 primer or Ds1 Primer

2 μL of 5X HF Buffer (from NEB)

4.82 μL of PCR quality H2O

0.08 ul 25mM dNTP

0.1 μL Phusion Polymerase(Anna’s Stock)

10 μL Total PCR mix

PCR program for S3 confirm PCR:

98C for 30sec

98C for 10sec, 59C for 15sec, 72C for 30sec [25 cycles]

72C for 5min

4C forever

-Run a 4% Gel

Expected Bands:

Figure 12: S3 Barcode Cassette Confirmation PCR:

Lane 1:50bp ladder

Lane 2: SacI F-Us2 PCR(~132bp)

Lane 3: Ds1-SacI R PCR(~137bp)

132 bp for IT rgnt- HphMX4-LoxP Reamp-Us2 PCR

137 bp for Ds1-IT rgnt- HphMX4-Lox2272 Reamp PCR

### Step 2b:Transformation and In Yeast Assembly

After generating the fragments necessary for in yeast assembly (the SacI digested pSH47 and the Barcode Cassette(Step 3 PCR product) ) the pieces are transformed into yeast were they are assembled by yeast into a plasmid(Figure 12).

Figure 13: DNA pieces taken in by the transformants are assembled into a plasmid

##### Protocol:

-This protocol is based on the Gietz et al (Nature Protocols, 2007) transformation protocol

**Materials:**

-25ml 50% PEG 3350 solution

-5ml 1.0M LiAC solution

-5ml 2mg/ml Single-Stranded Carrier DNA or 1ml 10mg/ml Single-Stranded Carrier DNA(Invitrogen)

-Sterile Water(~1L)

-500ml 2xYPAD

-30ml SC-URA media

-6-8 large(245mmx245mm) YPG+HygromycinB Plates

**Day 1:**

1-Inoculate an HO:URALOX strain colony into 5ml SC-URA in a culture tube and culture in 30C incubator with rotation overnight

**Day2:**

1-Inoculate 20ul of the overnight HO:URALOX culture to 5 culture tubes each containing 5ml SC-URA and culture in 30C incubator with rotation overnight

**Day 3:**

1- First thing in the morning, combine the 5x5ml cultures of the HO:URALOX strain. Measure the OD600 of this mixture using a spectrophotometer (Make sure to dilute your sample to within the linear range of the spectrophotometer. OD600 ~0.1-0.5, Dilute ~ 1/10x)

2- Add 25 x 10^8 cells to 500 ml of pre-warmed 2XYPAD in a pre-warmed culture flask. The titer of this solution should be ~5 x10^6 cells/ml

3- Incubate the flask in the shaking incubator at 30C and 200 rpm until the cell titer is at least 2 x 10^7 cells/ml (OD600 ~ 2).This should take about 4 h.

4- Denature the Single Stranded Carrier DNA(5ml 2mg/ml or 1ml 10mg/ml) by placing in a beaker of boiling water on a hot plate. Boil the tube of Single Stranded DNA for 5 minutes and immediately place on ice till needed

5- Harvest the cells by centrifugation at 3,000g for 5 min and resuspend the pellet in 250 ml of sterile water and centrifuge at 3,000g for 5 min at 20C to pellet the cells. Repeat this wash with another 250 ml of sterile water by resuspending the cells and pelleting them again by centrifugation. Resuspend the cells in 10.0 ml of sterile water.

6- Transfer the cell suspension to a 50 ml Falcon tube, centrifuge for 5min at 3,000g and discard the supernatant.(**leave only a pellet)**

7- In another 50ml Falcon tube Prepare the following mix:

3.4 ml of Sterile Water containing the SacI digested pSH47 and Step 3 Barcode Cassette(use a ratio of 1:6 of pSH47:Step 3 Barcode Cassette , use ~12ug digested pSH47 and 15ug Step 3 Barcode Cassette)

5ml 2mg/ml Single-Stranded Carrier DNA

3.6ml 1.0M LiAc solution

24ml 50% PEG 3350

8- Vortex mix the PEG/LiAC/DNA mixture well and transfer all 36ml to the falcon tube with a pellet. Vortex vigorously to resuspend the pellet

9- Place the tube in a water bath at 42C and incubate for 60 min. Weigh the tube down to ensure it is fully immersed in the 42C water.**Important:** Every 15-20min vortex the tube to ensure that the cells remain suspended.

10- After the heat shock is done, pellet the cells by centrifuging at 3000g for 5 minutes. Remove the supernatant

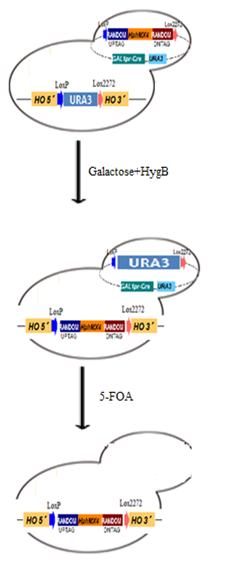
11- Resuspend the pellet in 100ml YPD and transfer to a 500ml flask. Incubate at 30C with 200rpm rotation for 2 hrs. (this step allows the cells to express the protein giving resistance to hygromycin)

Figure 14: Barcoder Strain Generation Process

12- After incubation, plate 10ul of the cell culture on small petri YPG+HygB plates to assess transformation efficiency

13- Transfer the remaining culture to two 50ml falcon tubes and spin at 3000g for 5min. Remove the supernatant and resuspend the pellet in each falcon tube in 1.5 ml sterile water

13- Plate ~600ul of the resuspended pellet on each of 6-8 large(245mm x 245mm) YPG+HygroB plates. Add ~20 beads to the plates and shake well to spread the cells evenly on the plate.

14- Incubate the plates at 30C for 3 days

### Step 2c:Induction and Selection Steps to Generate Barcoder Strain

1- Continuing from previous step: you now have **YPG+HygroB plates covered with Transformants** with the Cre plasmid carrying the HphMX4 Cassette Flanked with Barcodes.

2- After Incubating the strains on the YPG+HygroB plates for 3 days **scrape the cells** of the YPG+HygroB plates by using a pipette boy to add 20ml Sterile Water to a plate and resuspending the colonies using a plate spreader. Suck up the resuspended colonies from the plate using a pipet boy and transfer them to a sterile flask. Repeat this for all YPG+HygroB plates covered with transformants and combine the resuspended cells from those plates together

3- **Measure the OD600** of the Resuspended Strains from YPG+HygroB

4- Plate ~ 500 ul of OD600 = 160 of the resuspended cells from the YPG+HygroB **plate onto each of large 5-FOA plates**( ~6 large 5-FOA plates) and incubate at 30C for 2 days

5- Also take a small aliquot of the resuspended cells from the YPG+HygroB plates and dilute it enough so that ~500 cfus can be plated on Petri Dishes (ie :plate 20ul of ~25\* 10^3 cfus/ml dilution). **Plate an aliquot** of the dilution on regular YPG+HygB petri dish and 5-FOA dishes in order to assess what proportion of strains pass 5-FOA selection (ie: strains were the barcode cassette recombines into the HO locus and the Cre plasmid is lost). (Got around 4% pass 5-FOA selection)

6- After 2-3 days when the 5-FOA plates show a good lawn growth. **Scrape the cells** following the same procedure used for the YPG+HygB plates and **measure the OD600**

7- Make Glycerol stocks of the resuspended strains. This is the Barcoder Pool. Storing an excess barcoder pool in 4C

8- Take an aliquot of the Barcoder pool , dilute and **plate ~800cfus onto YPD+HygroB plates, 5-FOA plates and SC-URA plates.** Incubate at 30C for 3 days. Check the SC-Ura plates to confirm that 5-FOA selection was efficient (if 5-FOA selection was good you would expect no colonies on the SC-Ura plates .(Own Results: Got 960 colonies on the YPD+HygroB plates and 0 colonies on SC-Ura plates, so >99.8% 5-FOA efficiency.) Compare the YPD+HygroB and 5-FOA plates to check for discrepancies, you would expect similar colony counts on both plate if the barcoder selection process was successful

### Step 2d: Barcoder Strain Validation

1- Plate the Barcoder Strain Pool onto 5-FOA plates to get single colonies

2-Barcoder Strain Validation involves 3 steps : Step 1: PCR verification , Step 2: Sanger Sequencing Verification Step 3: Miseq run confirmation of barcode pool complexity

**Step 1: PCR verification**

Perform a colony PCR on 20 colonies from the plated barcoder pool to verify that the barcoding procedure was successful(also include a negative control: a strain without barcodes ex: green monster GM512 strain)

Barcode Verification Colony PCR protocol:

1- For each of the Colonies perform a PCR on Mix(can prepare a master mix and aliquot)

2ul Sterile DNA Free Water

2ul 0.2M pH 7.4 Sodium Phosphate Buffer

0.5 ul 5U/ul zymoresearch zymolyase

2- Use a P20 pipette to inoculate ¼ of each colony to the zymo mix and mix well by pipetting

3- Place the colony-zymo reaction in a PCR machine programmed for 37C 25min , 95C 10 min

4- Add 125ul of sterile DNA-free Water to dilute the colony-zymo reactions when the incubation is done. These diluted zymo reactions are used as PCR templates

5-Barcoder Strain Validation PCRs:

1) 5’HO new seq primer - Us2 primer (checks for presence UPTAG in HO locus)

2) Ds1 primer - 3’HO new seq primer (checks for presence DNTAG in HO locus)

3) rtTA Fprimer – rtTA Rprimer (+ve control to confirm the zymo and PCR rxns work)

PCR Mix:

9ul diluted zymo rxn of a colony

1.5 μL of 10uM F primer (5’HO new sew or Ds1 or rtTA F primers)

1.5 μL of 10uM R primer (Us2, 3’ HO new seq or rtTA R primers)

6 μL of 5X HF Buffer (from NEB)

11.46 μL of PCR quality H2O

0.24 ul 25mM dNTP

0.3 μL Phusion Polymerase(Anna’s Stock)

30 μL Total PCR mix

PCR program for UPTAG, DNTAG verification:

98C for 30sec

98C for 10sec, 59C for 15sec, 72C for 30sec [25 cycles]

72C for 5min

4C forever

Run the samples on a 4% gel with a 100bp ladder

Expected Sizes: 5’HO new seq-Us2 PCR(263 bp)

Ds1 - 3’HO new seq PCR (251 bp)

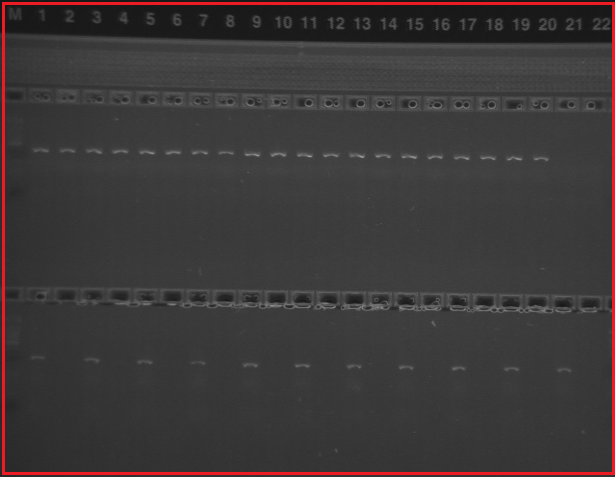
 rtTA F-rtTA R PCR(186bp)

Figure 15: Barcode Verification PCR

M: 50 bp ladder

UPPER Row:

1,3,5,..,19: 5’HO-Us2 PCR for 10 colonies on barcoder pool colonies

2,4,6,..,20: Ds1-3’HO PCR for 10 colonies on barcoder pool colonies

21,22: 5’HO-Us2 and Ds1-3’HO PCRs on –ve control(GM512)

BOTTOM Row:

1,3,5,..21: rtTA F-rtTA R PCRs on 10 barcoder pool colonies and GM512( +ve control for zymo rxn and PCR)

All 10 Barcoder colonies showed both UPTAG and DNTAG in the HO locus (**06/07/2012)**

**Step 2: Sanger Sequencing Verification**

1- Continue working with the PCR samples from the Barcode Strain PCR verification Step

2- EXOSAP purify the 20 5’HO-Us2 UPTAG PCR samples and the 20 Ds1-3’HO seq PCR samples

**EXOSAP purification protocol:**

1-For each PCR reaction to be purified prepare the following mix: (prep a master mix)

0.025ul ExoI (0.5U)

0.1ul Antarctic Phosphatase (0.5U)

3.5ul 10X Antarctic Phosphatase Buffer

6.375ul dH2O

10ul Total

2- Aliquot the 10ul of EXOSAP mix to 25ul of the PCR reaction

3- Incubate in a PCR machine at 37C 30min , 80C 20min

4-Dilute the samples with 35ul DNA-Free water

5- Aliquot 10ul of the diluted EXOSAP reactions to 96 well PCR plates and add 0.5ul 10uM 5’HO new seq primer to the 5’HO-Us2 PCRs and 0.5ul 3’HO new seq primer to the Ds1-3’HO new seq PCRs and send the plate to sanger sequencing

6- Use the sanger sequencing data to confirm barcode and Lox sites presence and to identify the UPTAG and DNTAG barcodes for these 20 colonies and form a rough estimate of the barcoder pool complexity. (See Engineered DNA pieces section below for expected templates for 5’HO-Us2 and Ds1-3’HO PCRs)

**Step 3: Miseq run confirmation of barcode pool complexity**

1- Take an aliquot from the Barcoder pool containing at least 1\* 10^8 cfus (ie 1ml OD600 10)

2- Genomic Prep the Barcoder Pool:

2- Spin the cells down at 13000rpm for 30 sec and remove the supernatant

3- Resuspend the cells in 300ul YD Digestion Buffer (check the zymoresearch genomic prep kit/protocol for recipes/details)

4- Add 12ul of 5U/ul zymoresearch zymolyase and 2.5ul of 20ng/ul RNase A

5- Incubate at 37C with rotation for 60 to 90 minutes

6- Add 200ul MPC Protein Precipitation Reagent and vortex sample

7- Pellet the debris at 13,000rpm for 10 minutes.

8-Transfer the supernatant to a new epitube and add 625 ul isopropanol to precipitate the DNA. Mix by inverting

9- Spin the tube at 13,000rpm for 10 minutes

10- Remove the supernatant and wash the DNA pellet with 800ul 70% EtOH. Remove the EtOH. Spin down and remove any remaining EtOh.

11- Dry the pellet in a Speedvac

12- Resuspend the DNA in 100ul EB Buffer

3-Picogreen the genomic prepped barcoder pool

4- Perform a PCR to add the Illumina Sequencing Adaptors to the Barcoder pool:

PCR Mix:

5ul 100ng/ul Genomic Prepped Barcoder Pool DNA

1.25ul 10uM P01\_Us1 Primer

1.25ul 10uM P01\_Us2 Primer

1.25ul 10uM P01\_Ds1 Primer

1.25ul 10uM P01\_Ds2 Primer

15ul water

25ul Phusion HF Master Mix

50ul Total

PCR program for adding Tag and Sequencing Adaptor to Barcodes

98C for 30sec

98C for 10sec, 59C for 15sec, 72C for 30sec [15 cycles]

72C for 5min

4C forever

5- Run the PCR rxn on a 4% gel(use multiple lanes). The expected product size is ~215bp

6- Gel Cut the desired band

7- qPCR the Gel Cut Band and Run on Miseq

8- Use the data to assess barcoder pool complexity

## Step 3: Mating and Sporulation of Barcoder Pool and Green Monster

The Barcoder Pool generated (Mating Type ‘alpha’) is mated to GM512(Green Monster strain Mating Type ‘a’) and grown in the appropriate selective media to generate the TWAS children. The TWAS children pool consists of haploid strains carrying strain specific barcodes at the HO locus and having different genotypes at the 16 ABC transporter genes.

**Mating and Sporulation Protocol** (basedon Yo’s Mating Protocol**):**

**NOTE 1:** if possible start the mating as soon as the barcoder pool is done ie: when scrape cells from 5-FOA plates in the barcode generation protocol, immediately use those cells in the mating protocol while the cells are fresh. If this is not possible then grow the barcoder pool from glycerol stocks on YPD plates or in YPD liquid culture.

**NOTE 2:** The barcoder pool generated and used in TWAS version 2 is the LS2 barcoder pool (Large Scale Plate Based Barcoder Pool 2). Glycerol stocks of this barcoder pool can be found in Freezer Box 1.

**NOTE 3:**you want to carry out the mating and sporulation at a large enough scale so after sporulation you get at least 100,000 unique sporulants. Verify this by checking your mating and sporulation efficiencies and estimating the number of unique sporulants you got,

Mating (Diploid Generation):

1- Inoculate a single GM512 colony(Green Monster MAT type ‘a’) into YPD and grow overnight. In the morning inoculate to several culture tubes to a start OD of 0.5 and culture for a few hours till OD600 ~ 1.0. You need ~30 to 50 ml of OD 1.0 GM512 culture

2- Pellet 50ml of OD1.0 GM512 culture at 3000g for 5min and resuspend in 50ml sterile water

3- Pellet Xml of Barcoder Pool and resuspend in enough Sterile Water to get an OD600 of 1.0 and ~50ml

4- Leave the GM512 and Barcoder pools resuspended in sterile water on the bench for 2 hours . This allows the cell cycles of the pools to synchronize and increases mating efficiency

5- Combine 25ml of the OD1.0 Barcoder Pool and 25ml of the OD 1.0 GM512 pools that are in water and vortex this mix well

6- Pellet the cells at 3000g for 5minutes.

7- Discard the supernatant and resuspend the cells in 500ul water

8- Transfer the resuspended cells to an epitube and pellet them again at 13,000rpm for 30 sec

9- Remove the supernatant

10- Using a sterile pipette tip or sterilized metal spatula , remove the pellet from the epitube and spread it as a patch on a YPD plate

11- Incubate the YPD plate with the patched GM512\_Barcoder pool on your bench at room temp overnight

12-After at least 24 hrs, scrape and resupend the patch of GM512+Barcoder pool and resuspend in water

13- take a small aliquot and dilute and plate onto YPD and YPD+G418+ClonNat plates to assess mating efficiency( mating/diploid formation efficiency = # colonies on YPD+G418+ClonNat/# colonies on YPD , got ~40% diploid formation eff)

14- Inoculate X ul of the resuspended patch in 30ml GNA+ClonNat+G418 so that the starting OD is 0.1 and incubate at 30C Overnight. (This step selects for diploids)

15- Measure OD600 of GNA+G418+ClonNat culture of GM512+BC pool patch

16- Inoculate Xul of this culture to 50ml YPD+G418+ClonNAt culture for starting OD600 of 0.3-0.4 and culture for 4-5 hours until it reaches OD600 of 1.0

17- When the diplod GM512+BC pool reaches OD600 1.0, pellet the cells at 3000g for 5 minutes and resuspend in 50ml sterile water

18- Repeat this process and wash the pellet with sterile water 3 more times

19- After the final wash resuspend the pellet in 50ml of minimal sporulation media(supplemented with 7.5 ug/mL lysine,7.5 ug/mL leusine, 5 ug/mL histidine, 5 ug/mL methionine , 1.25 ug/mL uracil , check Yo’s sporulation protocol for more details)

20- Aliquot the cells suspended in sporulation mix to a few culture tubes so 5-7ml per tube

21-Incubate the sporulating cells at room temp in a rotator for 5 days

Spore Dissection and TWAS children selection:

22- After 5-days, confirm the cells have sporulated by looking at them under a microscope and looking for tetrads

23- if sporulation was successful , pool the sporulation mix from all the culture tubes into a 50ml falcon tube

24-Pellet the cells at 3000g , 5min

25- Resuspend cells in 12ml mix of : 100mM Sodium Phosphate Buffer pH 7.4

1m Sorbitol

96ul 5U/ul zymoresearch zymo (480 units)

26- Incubate at 30C for 1hr , mix every 20min to resuspend cells

27-Add 12ml 0.02% NP-40 to the spore dissection zymo mix and vortex mix and leave at room temp for 5 minutes

28- Stop the reaction by adding 120ml water to the spore dissection mix and pace it on ice. Split it into 4 50ml Falcon tubes

29- Sonicate the Spore mix in falcon tubes with a sonicator set to Setting 1, Duty Cycle 50 and on ice

30- After all tubes have been sonicated Take a 40ul aliquot of dissected spores and plate 10ul onto SC-His, SC-Leu and YPD plates in order to assess sporulation efficiency. (For TWAS ver 2 got YPD: ~3360 colonies, SC-His:~600 colonies, SC-Leu:~840 colonies. So a sporulation efficiency of ~18% , so about 3\* 10^ 7 different spores inoculated into the SC-His and SC-Leu cultures and since only 1/4th of spores carry appropriate mating type and barcode, about 8\*10^5 unique spores

31- Pellet the remaining spore mix at 3000g for 5 minutes. For 2 of the 4 falcon tubes of dissected spore mix resuspend in 110ml SC-His liquid cultures to select for Mating Type ‘a’ spore/TWAS children. For the other 2 falcon tubes of dissected spore mix resuspend in 110ml SC-Leu liquid cultures to select for Mating Type ‘alpha’ spore/TWAS children.

32- Culture the SC-His and SC-Leu cultures of dissected spores in a shaking incubator at 30C, 200rpm overnight.

33- The following day measure the OD600 of the SC-His and SC-Leu cultured spores and aliquot an appropriate amount to 100ml YPD+HygroB for a starting OD600 of 0.1 ( a separate culture for each of the SC-His and SC-Leu selected spores). Culture overnight at 30C,200rpm in a shaking incubator.

34- The strains from YPD+HygroB cultures are the TWAS children which will be genotypes and used in drug assays. The strains cultured in SC-His then YPD+HygroB are the TWAS Mating Type ‘a’ children and the strains cultured in SC-Leu then YPD+HygroB are the TWAS Mating Type ‘alpha’ children. Make glycerol stocks of both pools. (TWAS ver 2 barcoded sporulants pools are in Freezer Box 1 labelled as LS2+GM512 TWAS ‘a’ or ‘alpha’ spores , check Freezer Box documents)

35- Assess the strains by plating the strains from both pools on SC-His, SC-Leu, YPD+G418+ClonNat plates in order to 1- assess the plating density on SC-His and SC-Leu plates 1- to check for any diploid presence, only diploids have both mating type ‘a’ and ‘alpha’ cassettes and grow on YPD+G418+ClonNat (TWAS ver 2 gave 1 colony on YPD+G418+ClonNat plate and 440 colonies on SC-His and 775 colonies on SC-Leu for same plating densities ie: diploid percent in population is <0.25% )

36 – The barcode complexity of the sporulated strains can also be assessed following the same procedures used to assess barcoder pool complexity (sanger sequencing the UPTAG and DNTAGs from many colonies or running a Miseq run of the barcodes) (For TWAS ver 2 picked 48 colonies and sanger sequenced the UPTAGs and out of 48 colonies , only 2 colonies shared a barcode : Refer to ‘Sanger UPTAG Seq 48 colony TWAS2 spores’ Excel File

37- After the plating density of the TWAS children Mating Type ‘a’ and Type ‘alpha’ is assessed plate the TWAS Mating Type ‘a’ barcoded spores on 5 large SC-His plates(245mmx245mm plates) for a plating density of ~ 1500 to 2000 strains per large SC-His plate

38- Plate the TWAS Mating Type ‘alpha’ barcoded spores on 5 large SC-Leu plates(245mmx245mm plates) for a plating density of ~ 1500 to 2000 strains per large SC-Leu plate

39- These Large SC-His and SC-Leu spores plated with the barcoded GM512+barcoder pool sporulants and the plates used for colony picking of strains

## Step 4: Colony Picking the Plated TWAS Children using Qpix Robot:

Use the Qpix robot to pick single colonies from the large SC-His and SC-Leu plates to 384 well plates containing SC-His or SC-Leu liquid media. The SC-His plates contain the TWAS Mating Type ‘a’ strains, while the SC-Leu plates contain the TWAS Mating Type ‘alpha’ strains. 15X384 well plates are picked for each of the Type ‘a’ and Type ‘alpha’ strains , ie: ~5460 Mating Type ‘a’ and 5460 Mating Type ‘alpha’ strains for a total of ~10,920 strains.

**Colony Picking Protocol:**

**1-** **Sterilize the Qpix robot**: unscrew the 96 pin picking head.Rinse the wash basins with distilled water. Place the picking head, the screw and all the wash basins face up in the Qpix robot and start the UV sterilization procedure(30 minutes)

**2-Prepare the 384 deep well destination plates:**Use the liquidator to fill 20 Genetix 384 deep well plates(X7007) with 55ul SC-His(for type ‘a’ sporulants) or SC-Leu(for type ‘alpha’ sporulants. Remove the 4 corner pipette tips so the 4 quadrants at each corner are empty.

Use the Random Wells list(see below) to remove the SC-His or SC-Leu from 4 wells in a randomly selected quadrant(Label the plate with the wells removed)

**3-** **Spin down** the plates to ensure there are no air bubbles

**4-** **Prepare** 800ml fresh 1% bleach solution and 800ml 80% ethanol solution and 1L of sterile autoclaved water.

**5-** **Take** the Large 245mmx245mm plates containing the plated TWAS children ,the sterilization solutions ,gloves, a large beaker for waster and the filled 384 well plates to the colony picker

**6-** Press the ‘Change Head’ icon and install the 96 pin picking head, make sure to only use the head’s handle and not to touch any pins

**7-** Fill the wash basins with the correct solutions and place in the correct locations in the robot

**8-** Place 2 large plates to be picked in the robot and uncover them

**9-** **Set pin depth**: Select the ‘Ruler’ command , use the x and y axis commands to move the picking head until it is over the large plates.Use the z axis command to slowly lower the A1 pin until it penetrates the agar and two of its notches are covered. Check this happens for both large plates and make sure the pin does hit the bottom of the plate

**10-** **Check Alignment:** press on the ‘Target’ icon and Align, make sure the crossmarks and the hole are aligned

**11-** **Ensure the plates are loaded in the proper orientation so that the A1 well position lines up** with the A1 pin. The A1should be at the bottom right( Confirm this by firing the A1 pin and making sure it is the bottom right pin)

**12-** Check the head,source and destination options and select the proper settings.Deselect Stack use and load plates by hand(stacker tends to fail)

**13-** Select ‘Full’ is using two plates to pick colonies or ‘Partial’ if only 1 plate is picked

**14-** **Display image data** and adjust the diameter and distance parameters to maximize the number of colonies picked and minimize the chances of picking two colonies simultaneously. Scan through the images to make sure all is fine and no undesired colonies are picked. Deselect, any ambiguous colonies or colonies that are too close.

**15-** **Record** the colony parameters used (Used Diameter min 9 max 23. Roundness 0.30, Axis Ratio 0.73 , Proximity 4 for TWAS ver 2)

**16- Pick enough colonies to fit one 384 well plate**

**17-** **Repeat** until the 2 large plates are fully picked

**18-** When the two plates are fully picked, **remove the plates.** Check the plates to confirm colonies where picked correctly

**19-** Remove the wash basins and dump the solutions and **replace with fresh** ones and repeat the protocol from Step 8 for the new plates

**20-** **When you are done, empty and rinse** the basins, place the picking head in its container and switch off the machine

**21-** When done **label** each plate with the appropriate number and record the wells which were left empty (ex: His ‘a’ TWAS2 plate : Corners+C3 quad empty , etc)

**22-** After you are done picking all the plates place the 384 deep well plates in a Styrofoam box lined with wet paper towels(to reduce evaporation) and **Incubate** them in 30C incubator overnight

**Random Wells Left Empty in each 384 well Plate List**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Plate | Rows (A->H) | Col (1->12) |  | Corresponding 384 plate quadrant | |
| His 1 | A | 5 |  | A | 9 |
| His 2 | F | 2 |  | K | 3 |
| His 3 | D | 5 |  | G | 9 |
| His 4 | E | 4 |  | I | 7 |
| His 5 | E | 1 |  | I | 1 |
| His 6 | D | 3 |  | G | 5 |
| His 7 | B | 8 |  | C | 15 |
| His 8 | D | 6 |  | G | 11 |
| His 9 | A | 3 |  | A | 5 |
| His 10 | B | 2 |  | C | 3 |
| His 11 | F | 5 |  | K | 9 |
| His 12 | C | 5 |  | E | 9 |
| His 13 | D | 3 |  | G | 5 |
| His 14 | G | 11 |  | M | 21 |
| His 15 | B | 4 |  | C | 7 |
| Leu 1 | G | 8 |  | M | 15 |
| Leu 2 | G | 3 |  | M | 5 |
| Leu 3 | E | 2 |  | I | 3 |
| Leu 4 | B | 9 |  | C | 17 |
| Leu 5 | F | 8 |  | K | 15 |
| Leu 6 | A | 8 |  | A | 15 |
| Leu 7 | E | 6 |  | I | 11 |
| Leu 8 | B | 6 |  | C | 11 |
| Leu 9 | B | 1 |  | C | 1 |
| Leu 10 | D | 10 |  | G | 19 |
| Leu 11 | B | 7 |  | C | 13 |
| Leu 12 | F | 8 |  | K | 15 |
| Leu 13 | D | 6 |  | G | 11 |
| Leu 14 | C | 1 |  | E | 1 |
| Leu 15 | H | 8 |  | O | 15 |

This Table shows the wells and quadrants left empty for the TWAS ver 2 384 well plates picked with the robot. The 4 corner quadrants of all the 384 well plates (Quad 1: wells A1,A2,B1,B2 Quad 2: wells A23,A24,B23,B24 , Quad3: wells O1,O2,P1,P2 Quad4: wells O23,O24,P23,P24) where left empty for all 384 well colony picked plates. This was done by removing the pipette tips at A1 , A12, H1 and H12 on the 96 tip liquidator pipette tip boxes. In addition 1 random quadrant was left empty for each plate. This table shows the additional random quadrants left empty for each 384 well plate. The first two columns show the coordinates of the pipette tips removed in the 96 pipette tip liquidator boxes and the second set of coordinates shows the corresponding quadrant of the 384 well plates that is left empty because of this. For example: removing the pipette tip at A5 in the 96 tip boxes causes the well at A9,A10,B9 and B10 in the corresponding 384 well plate to be empty.

## Step 5: Perform Zymolyase Digests on the TWAS Strains:

After Colony Picking Individual TWAS strains and arraying them to 384 well plates and culturing them overnight. Now you need to prepare a lysed aliquot of cells which can be used as the template for Row-Column PCRs

In addition to lysing the picked TWAS strains, during this step the controls are also add to the zymolyase plates. This includes Green Monster(GM512), URABCv2( a barcoder strain colony having no deletions) and Gold Standard Strains(GS), these are previously genotyped TWAS children whose exact genotype and strain-specific barcode is known.

**Lysing Cells for Row-Column PCR Protocol:**

1-Prepare liquidator tips boxes.

Open 60X20ul Liquidator Tip Boxes and remove the 4 corner tips and the appropriate random tip and label the boxes

Open 60x200ul Liquidator Tip Boxes

2- Make zymolyase-master mix for 15x 384 plates( do the 30 plates in 2 batches of 15 plates over 2 days):

( 1 rxn mix:

8ul 0.2M Sodium Phosphate Buffer pH 7.4

4 ul dH2O sterile DNA free water

0.05 ul 5u/ul zymoresearch zymolyase

4ul overnight yeast culture )

5 x 384 plate master mix: (Make 3 tubes of 5x384 plate master mix so have enough for 15x384 plates)

16.8ml 0.2M Sodium Phosphate Buffer pH 7.4

8.4ml dH2O sterile DNA free water

105 ul 5u/ul zymoresearch zymolyase

3- Vortex the zymolyase master mix well and keep on ice

4- Fill a sterile 96 well plate lid with zymo master mix

5- Use the Liquidator and 20ul pipette tips(1 box) (CALIBRATE THE LIQUIDATOR FIRST) to add 12ul zymo master mix to 15x384 well plates(Corning Assay Plate #3702)

6- spin the plates down on short cycle so all the liquid is at the bottom of the well.

7-Set 5 zymo 384 well plates aside and place rest in 4C

8- Label 5 of the zymo rxn-384 plates with the corresponding colony picked plates you will use (**and the corresponding random wells left out: CHECK ‘Gold Standards Arrayed TWAS v2’ Excel file, ‘Random Quadrants Tab’ )**

9- Use the 20ul tip boxes with the corners and random pipette tip location (identical location removed as zymo plates, check Table above) removed and aliquot 4 ul of cell culture from the overnight grown yeast cultures. When pipette , first pump up and down to resusupend the settled cells and also pump once or twice in the zymo plate to mix

10- When all 5 are plates ready **ADD the GM512, BC strain and Gold Standard Controls according to the** ‘**Gold Standards Arrayed TWAS v2’ Excel file to each of the wells in the zymo plate corresponding to the 20 emptied wells**

11-Place the 5x384 zymo plates in the 37C incubator for 35 minutes

12-Meanwhile prepare another 5plate batch of zymo plates

13- When the 5 plates incubation in 37C is done. Remove those plates and add 64ul sterile DNA free H2O to each well (use the 200ul tip boxes prepared earlier)

14- **Take a 10ul Aliquot from 2 wells of the 5th plate of each zymo batch:** 1 random well and 1 of the control wells(NOTE DOWN which wells and which plate aliquots come from).These aliquots will be used to confirm the zymo rxns worked before performing large scale Row-Column PCR on all plates

15- When done, seal the 384 well zymo rxn plates with an aluminum foil and place in the -20C fridge for Row-Column PCR. After the plates are used for Row-Colum PCR, store them in the -80C freezer

## Step 6: Making Glycerol Stocks of the TWAS strains and Pooled Plate Samples

Glycerol stocks of the individual strains/384 well plates are made and glycerol stocks containing the strains pooled by plate are made as well ex: His 1 glycerol stock contains all 364 strains from His plate 1 pooled together

**Preparing Glycerol Stocks Protocol:**

1- Take the cell culture plates used to prepare the zymo rxn . Open # plates X4 200ul liquidator tip boxes. REMOVE the corner tips and the proper random tip corresponding to each plate and label the box(Follow same table used in zymo and colony picking protocol, see above)

2- **Take 3ul from 1 random well** on each plate(a non-control well) and plate onto SC-His(for mating type ‘a’ haploids) or SC-Leu plates(for mating type ‘alpha’ haploids) ( **see random pick Table Below).** These are randomly picked TWAS strains that can be genotyped in the future if the gold standards inoculated weren’t enough.

3-Use 200ul Liquidator tips to add 40 ul 50% glycerol to each well ( 4 times for each 384 well plate)

4- Pump up and down to mix well and then transfer 40ul of this glycerol-cell culture mix to a sterile lid.

5-When 1 384 well plate is done. Seal that plate with an aluminum foil seal and label. Use a pipetboy pipette to suck up the glycerol stock in the lids and transfer to a 15ml culture tube and label with corresponding plate(this is a mix of all strain from one 384 well plate)

6- Repeat this for all the cell culture plates

7- When all the plates are done, aliquot 200ul from the plate mix tubes to 300ul 96 well sterile round bottom plates in the following pattern

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** |
| **A** | His1 | His1 | His1 | His2 | His2 | His2 | His3 | His3 | His3 | His4 | His4 | His4 |
| **B** | His5 | His5 | His5 | His6 | His6 | His6 | His7 | His7 | His7 | His8 | His8 | His8 |
| **C** | His9 | His9 | His9 | His10 | His10 | His10 | His11 | His11 | His11 | His12 | His12 | His12 |
| **D** | His13 | His13 | His13 | His14 | His14 | His14 | His15 | His15 | His15 |  |  |  |
| **E** | Leu1 | Leu1 | Leu1 | Leu2 | Leu2 | Leu2 | Leu3 | Leu3 | Leu3 | Leu4 | Leu4 | Leu4 |
| **F** | Leu5 | Leu5 | Leu5 | Leu6 | Leu6 | Leu6 | Leu7 | Leu7 | Leu7 | Leu8 | Leu8 | Leu8 |
| **G** | Leu9 | Leu9 | Leu9 | Leu10 | Leu10 | Leu10 | Leu11 | Leu11 | Leu11 | Leu12 | Leu12 | Leu12 |
| **H** | Leu13 | Leu13 | Leu13 | Leu14 | Leu14 | Leu14 | Leu15 | Leu15 | Leu15 |  |  |  |

8- Label,Seal and place the plates in -80C storage

**Random Singles Picked from each plate:**

|  |  |  |
| --- | --- | --- |
| His Plates | Row | Column |
| 1 | 5 | 1 |
| 2 | 15 | 5 |
| 3 | 2 | 22 |
| 4 | 16 | 16 |
| 5 | 8 | 21 |
| 6 | 4 | 7 |
| 7 | 8 | 12 |
| 8 | 9 | 12 |
| 9 | 3 | 8 |
| 10 | 8 | 5 |
| 11 | 7 | 18 |
| 12 | 5 | 17 |
| 13 | 16 | 3 |
| 14 | 9 | 11 |
| 15 | 13 | 21 |

|  |  |  |
| --- | --- | --- |
| Leu Plates | Row | Column |
| 1 | 7 | 6 |
| 2 | 16 | 17 |
| 3 | 15 | 9 |
| 4 | 5 | 24 |
| 5 | 7 | 11 |
| 6 | 15 | 22 |
| 7 | 5 | 22 |
| 8 | 13 | 1 |
| 9 | 15 | 10 |
| 10 | 12 | 19 |
| 11 | 11 | 21 |
| 12 | 10 | 10 |
| 13 | 3 | 19 |
| 14 | 9 | 4 |
| 15 | 8 | 17 |

These tables show the wells from which random single were picked from each well

## Step 7: Perform the Row-Column PCRs:

After diluted zymo reactions are ready, you can perform the Row-Column PCRs.A single row-column PCR is performed for each 384 well plate and the primers for identifying gene deletion barcodes and strain specific barcodes at the HO locus are multiplexed together. The first step is add row and column tags to the samples from each individual plate. Following that a unique set of plate tag is added to each plate in order to be able to identify which plate a certain row or column tag corresponds to. Following that the samples from the multiple plates are pooled together and are enriched for strain specific barcodes whose representation in the Row-Column Pool can be low. They are then sequenced on a flow cell and the genotypes and strain specific barcodes of each colony can be identified

**TWAS Row-Colum PCR Protocol:**

**1- Thaw the primer stocks and colony-zymo rxns and HF buffers used**

**2- Prepare Row-Column Primer Plate:**

a- Prepare 96 well Row primer mixes and Column Primer mixes:

96 well Row Primer plate (In each well of Columns 1 and 2) :

Mix

50ul of 100uM GT-UPTAG U1 Row primer,

50ul of 100uM GT-DNTAG D1 Row Primer

50ul of 100uM BC-UPTAG Us1 Row Primer

50ul of 100uM BC-DNTAG Ds1 Row Primer

In following Pattern:

|  |  |
| --- | --- |
| Column 1 | Column2 |
| Row 1 Primers | Row 2 |
| Row 3 | Row 4 |
| R5 | R6 |
| R7 | R8 |
| R9 | R10 |
| R11 | R12 |
| R13 | R14 |
| R15 | R16 |

This yields a 96 well plate with each well have 25uM of each Row primer

96 well Column Primer plate (In each well of Rows 1 and 2) :

Mix

50ul of 100uM GT-UPTAG U2 Column primer,

50ul of 100uM GT-DNTAG D2 Column Primer

50ul of 100uM BC-UPTAG Us2 Column Primer

50ul of 100uM BC-DNTAG Ds2 Column Primer

In following Pattern:

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Row 1 | Col 1 primers | C3 | C5 | C7 | C9 | C11 | C13 | C15 | C17 | C19 | C21 | C23 |
| Row 2 | Col 2 primer | C4 | C6 | C8 | C10 | C12 | C14 | C16 | C18 | C20 | C22 | C24 |

This yields a 96 well plate with each well have 25uM of each Column primer

b-Array the row column primers to a 384 well PCR plate(max capacity wells is 30ul)

Using a multichannel pipette aliquot 7ul of the 25uM each primer mixes in the 96 well plates to the corresponding rows in the 384 well plates. Repeat the process for the column primers.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| 384 plate | 1 | 2 | 4 | … |
| A | R1 row primers / C1 Col primers | R1 row primers / C2 Col primers | R1 row primers / C3 Col primers | … |
| B | R2 row primers / C1 Col primers | R2 row primers / C2 Col primers | R2 row primers / C3 Col primers | … |
| C | R3 row primers / C1 Col primers | R3 row primers / C2 Col primers | R3 row primers / C3 Col primers | … |
| D | R4 row primers / C1 Col primers | R4 row primers / C2 Col primers | R4 row primers / C3 Col primers | … |
| … | … | … | … | … |

And each well will have 14ul 12.5uM of **each** GT-UPTAG,GT-DNTAG,BC-UPTAG-BC-DNTAG row and column primer

**3- Start thawing the 25mM stocks of dNTPs**

**4- PCR mix for single well PCR rxn:**

2ul 5xHF Buffer

0.1ul Anna’s Phusion

0.08 25mM dNTP

4.22 ul dH2O

2ul zymo-colony mix

0.2 ul 12.5uM GT-UPTAG U1 Row Primer

0.2 ul 12.5uM GT-DNTAG D1 Row Primer

0.2 ul 12.5uM BC-UPTAG Us1 Row Primer

0.2 ul 12.5uM BC-DNTAG Ds1 Row Primer

0.2 ul 12.5uM GT-UPTAG U2 Column Primer

0.2 ul 12.5uM GT-DNTAG D2 Column Primer

0.2 ul 12.5uM BC-UPTAG Us2 Column Primer

0.2 ul 12.5uM BC-DNTAG Ds2 Column Primer

10ul Total

MAKE PCR Master Mix for 10 plates

In 50ml Falcon tube Mix:

11,520 ul 5xHF Buffer

576ul Anna’s Phusion Polymerase

460.8ul 25mM dNTP

24,307.2ul dH2O (Sterile DNA free Sigma Water)

~36.8 ml

-Place the Phusion master mix in a sterile reservoir and use a multichannel pipette to aliquot 250 ul of Phusion Master mix to each well of a 96 well plate

-Use a Liquidator to Aliquot 91.2ul of Homemade Phusion Master Mix to each well of a 384 well assay plate (enough for 9 Row-Column PCRs)

-CALIBRATE liquidator to add 2.85ul use P20 tips

- Use a liquidator to aliquot 2.85ul of the 12.5uM Row-Column Primer mixes prepared earlier to the corresponding well in the 384 well assay plate( ie: use 4 P20 boxes to aliquot primers to proper Phusion master mix wells). Pump up and down to mix

**5- Setting up the Row-Colum PCRs:**

1-Label 8 384 well BioRad PCR plates with corresponding plates performing Row-Column PCR on ( use a waterproof permanent marker)

2- Calibrate the liquidator to add 8ul with P20 tips so can add appropriate volume by only pressing to the first stop

3- Use the liquidator to aliquot 8ul of the Phusion-Primer Master Mix made earlier. (Add the Phusion-Primer mix to the top left of all PCR plates using one liquidator box, then add to Top Right of all plates with a different box,etc)

4- When all plates ready,spin zymo plates down and use the liquidator to add 2ul of the diluted zymolyase rxns on the picked colonies.**(SAVE a liquidator lid for each plate perform a RC PCR on)**

5- Seal the RC-PCR plates using a ThermoBond Aluminum Seal(Kbioscience) . Place the seal on the plate carefully so it is aligned with the plate and use the plate sealer at 165C,3 sec program to seal the plate. Quicly use a seal roller to press the seal and make sure it is bond to the wells.

6- Spin the PCR Plates Down

7- Use the Hydrocycler and run the following PCR(**LOAD a maximum of 8 plates only in the Hydrocycler**):

95C 5min

95C 1min,57C 35sec, 72C 45sec (23 cycles)

72C 2 min

8- Pick up the plates as soon as done. If not much time left, can store overnight in the -20C.**Double check the plates to make sure no leaks or changes in volume or cross contamination among wells.**

Also confirm the row-column PCR and zymo worked by running 2ul of a a few randomly selected wells on a 4% gel( select at least one sample from BC and GM512, GS standard controls)(Expected Size is ~120-130bp)

|  |  |
| --- | --- |
| Plate | Conc (ng/ul) |
| Leu1 | 31.11 |
| Leu2 | 42.31 |
| Leu3 | 49.61 |
| Leu4 | 48.11 |
| Leu5 | 45.31 |
| Leu6 | 62.91 |
| Leu7 | 66.51 |
| Leu8 | 21.41 |
| Leu9 | 23.41 |
| Leu10 | 25.71 |
| Leu11 | 36.01 |
| Leu12 | 30.01 |
| Leu13 | 30.41 |
| Leu14 | 34.41 |
| Leu15 | 39.31 |

9- Spin plates down and Carefully remove the seal of each RC-CPR plate and Place a sterile P20 liquidator tip box lid over the 384 well PCR plate and seal them together with parafilm.Invert the plates so the plate is upside down on the top and the lid is on the bottom where it can receive the PCR samples.

10- Place those lid-plate combinations in the centrifuge and spin for a short time(5sec)

11-Carefully remove the plates so as little sloshing as possible. Use a pipette to mix and aliquot the Row-Column PCR mixes to 2x2ml tubes

12- Vortex the RC-PCR tubes and use one to Qiagen Qiaspin Purify , aliquot 260ul of RC-PCR and purify each plate separately(use same column twice for each plate for the PBI spin step)

13- Quantify the amount of DNA from each Row-Column PCR ,Run a gel (Expected Size is ~120-130bp) and perform a Different Plate Tag PCR on each Row-Column PCR sample(see below)

TWAS ver 2 RC-PCR Nanoquant Results(18/7/2012):

|  |  |
| --- | --- |
| Plate | Conc (ng/ul) |
| His1 | 71.71 |
| His2 | 26.41 |
| His3 | 11.21 |
| His4 | 19.21 |
| His5 | 62.51 |
| His6 | 72.41 |
| His7 | 84.01 |
| His8 | 64.81 |
| His9 | 17.91 |
| His10 | 24.11 |
| His11 | 32.01 |
| His12 | 43.41 |
| His13 | 48.11 |
| His14 | 54.71 |
| His15 | 60.41 |

**Plate Tag PCRs Protocol( TWAS ver 2):**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Plate Tag Primers** | **P01**\_PE 1.0 | **P02**\_PE 1.0 | **P03**\_PE 1.0 | **P04**\_PE 1.0 | **P05**\_PE 1.0 | **P06**\_PE 1.0 |
| **P01**\_PE 2.0 | **His 1** RC PCR | **His 2** RC PCR | **His 3** RC PCR | **His 4** RC PCR | **His 5** RC PCR | **His 6** RC PCR |
| **P02**\_PE 2.0 | **His 7** RC PCR | **His 8** RC PCR | **His 9** RC PCR | **His 10** RC PCR | **His 11** RC PCR | **His 12** RC PCR |
| **P03**\_PE 2.0 | **His 13** RC PCR | **His 14** RC PCR | **His 15** RC PCR | **Leu 1** RC PCR | **Leu 2** RC PCR | **Leu 3** RC PCR |
| **P04**\_PE 2.0 | **Leu 4** RC PCR | **Leu 5** RC PCR | **Leu 6** RC PCR | **Leu 7** RC PCR | **Leu 8** RC PCR | **Leu 9** RC PCR |
| **P05**\_PE 2.0 | **Leu 10** RC PCR | **Leu 11** RC PCR | **Leu 12** RC PCR | **Leu 13** RC PCR | **Leu 14** RC PCR | **Leu 15** RC PCR |

Figure 16: Arraying of Plate Tag Primers for TWAS ver 2

Each plate PCR is done using a different Plate Tag Primer Combination( see table above).

PCR Mix:

3->6 ul (~150ng) Row-Column PCR purified product

2.25 ul 10uM P0X\_PE 1.0 Primer

2.25 ul 10uM P0X\_PE 2.0 Primer

0.45ul Anna’s Phusion Polymerase

0.36 ul 25mM dNTPs

9ul 5xHF buffer

27.7->24.7ul water

45 ul total

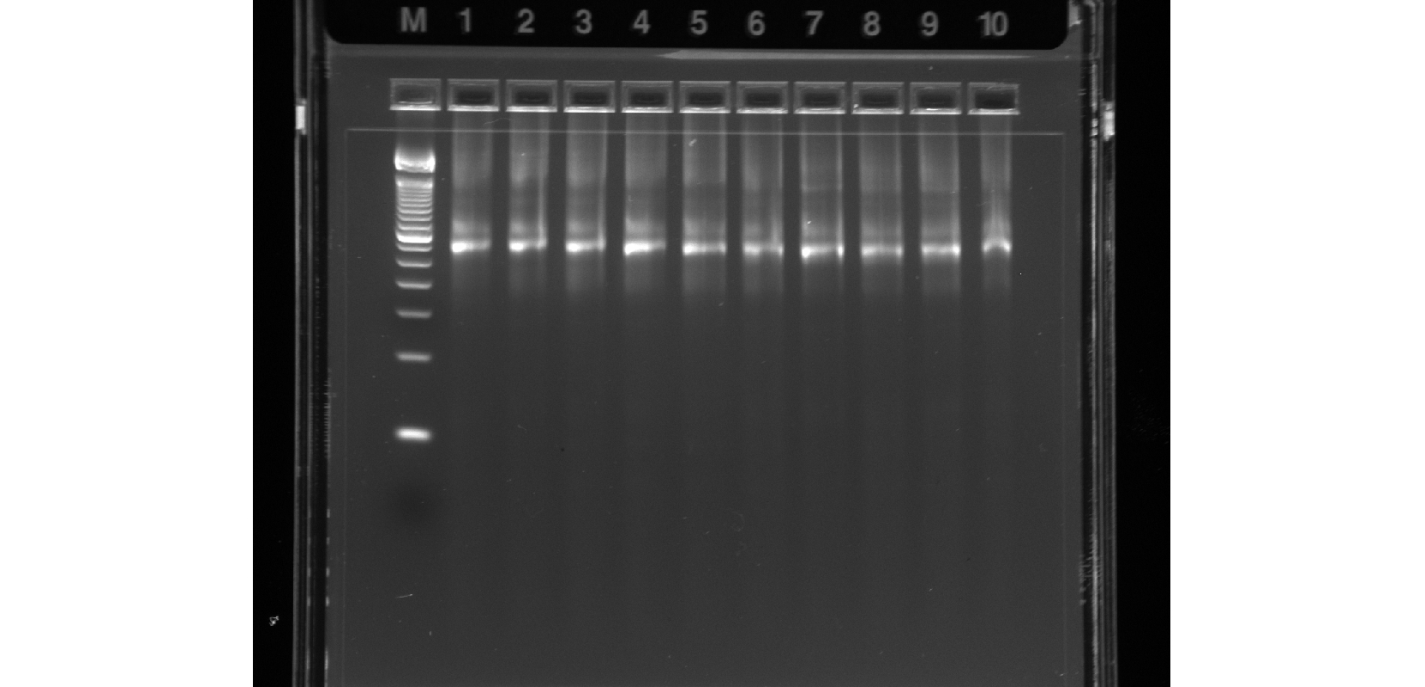
- prepare 2x45 ul for each plate and setup in PCR tubes, spread tubes in PCR machine to minimize Plate Effects

Figure 17: Plate Tag PCR results

M:50 bp ladder

Lane 1->10: Plate Tag PCRs of 10 randomly chosen plates from TWAS ver2.Expected Size is 269->286 bp

**18/7/2012**

PCR Program:

98C for 30sec

98C for 10sec, 59C for 15sec, 72C for 40sec [15 cycles]

72C for 5min

4C forever

Ran a 4% Gel to Confirm the Plate Tag PCR Products:

Expected Size: 269->286 bp

Multiplexing Plate Tag PCRs:

1-If the Plate Tag PCRs were successful Qiagen Qiaquik Spin purify all 30 Plate Tag PCRs separately

2- qPCR all 30 Plate Tag PCRs using the light cycler and the KAPA illumine sequencing quantification kit (need to use qPCR rather than nanoquant because the plate tag PCR has some nonspecific products which can give inaccurate results for the amount of desired plate tag products whereas the qPCR only gives the amount of desired Plate Tag Product)

3- Use the qPCR results to multiplex all 30 Plate Tag samples so that an equal amount of Plate Tag PCRs are in each pool

4- Ran 100 ul of this multiplexed Plate Tag PCR sample on multiple lanes of a 4% gel

5- Gel extract the band of the desired size (260-290bp)

6- Qiagen gel purify and run a qPCR to quantify this sample. Run the sample on a Miseq to confirm that you have a uniform Plate Tag Distribution and to check the strain specific Row-Column PCR percentage of pool. You can also run 1 plate tag PCR on the Miseq to confirm the strain specific barcode complexity

**TWAS ver 2 Plate Tag PCR qPCR Results:**

|  |  |  |
| --- | --- | --- |
|  | Plate Tag PCR concentrations | |
| Plate | **nM** | **ng/ul** |
| His1 | 87.31 | 16.42 |
| His2 | 126.16 | 23.73 |
| His3 | 127.19 | 23.93 |
| His4 | 130.29 | 24.51 |
| His5 | 106.81 | 20.09 |
| His6 | 91.03 | 17.12 |
| His7 | 79.46 | 14.95 |
| His8 | 97.46 | 18.33 |
| His9 | 96.98 | 18.24 |
| His10 | 84.45 | 15.89 |
| His11 | 104.44 | 19.64 |
| His12 | 92.46 | 17.39 |
| His13 | 86.28 | 16.23 |
| His14 | 94.92 | 17.85 |
| His15 | 75.17 | 14.14 |
| Leu1 | 78.19 | 14.71 |
| Leu2 | 81.92 | 15.41 |
| Leu3 | 84.06 | 15.81 |
| Leu4 | 62.33 | 11.72 |
| Leu5 | 81.60 | 15.35 |
| Leu6 | 79.85 | 15.02 |
| Leu7 | 74.06 | 13.93 |
| Leu8 | 77.63 | 14.60 |
| Leu9 | 68.36 | 12.86 |
| Leu10 | 64.47 | 12.13 |
| Leu11 | 73.03 | 13.74 |
| Leu12 | 48.21 | 9.07 |
| Leu13 | 75.57 | 14.21 |
| Leu14 | 75.81 | 14.26 |
| Leu15 | 81.84 | 15.39 |

**Strain Specific Barcode Plate Tag PCR Product Enrichment:**

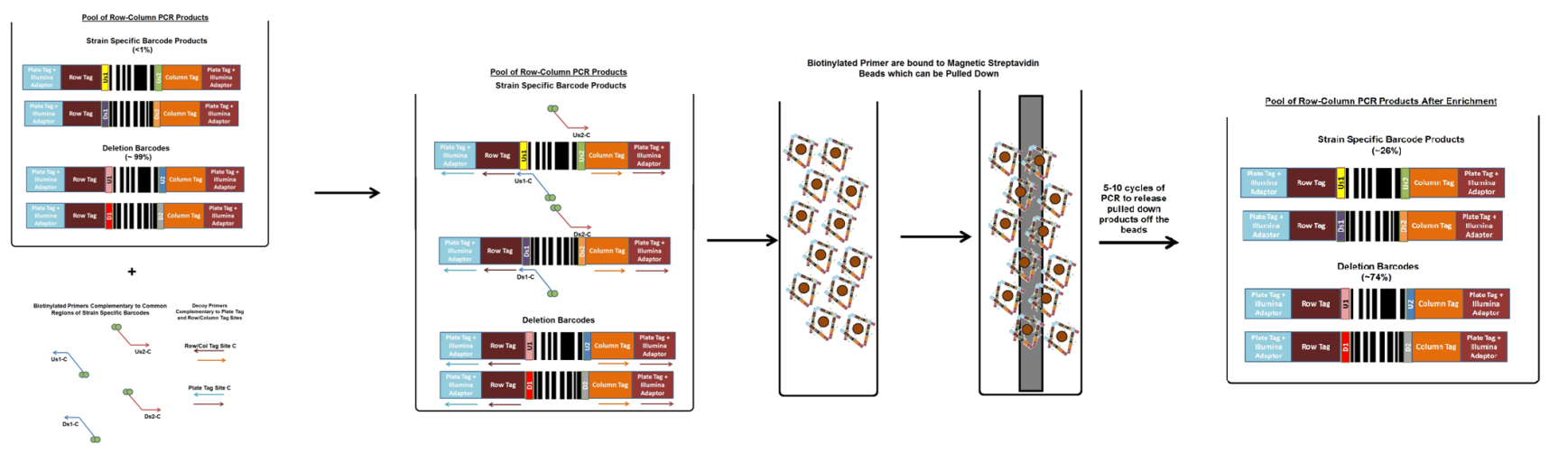
 On our previous run we found the proportion row-column PCR products from the strain specific barcodes was much less than expected( predict 1 in 9, since on average 1 strain specific barcode for every 8 gene deletion barcodes, but the number was much lower than that). Therefore we developed a method to enrich the proportion of the plate tag PCR pool which comes from the strain specific barcodes. This protocol relies on using primers that hybridize to the Us and Ds sites present only in the strain specific barcodes and using magnetic beads to pull down those products.

Figure 18: Beads Enrichment of Strain Specific Barcodes

**Beads Based Barcode Enrichment Protocol:**

1- Mix

0.125ul 100uM Us1-Biotin Primer

0.125ul 100uM Us2-Biotin Primer

0.125ul 100uM Ds1-Biotin Primer

0.125ul 100uM Ds2-Biotin Primer (Total of ~50pmol of biotinalyated primers,keep in mind for beads binding capacity)

45ul 4M NaCl

1ul 50mM EDTA

44ul DNA-free H2O

50ul of ~15ng/ul Multiplxed Plate Tag PCRs sample(use the multiplexed sample from BEFORE gel cutting)

180ul Total

2-Hybridization of primers to product: Aliquot 36ul of this mix to 5 PCR tubes and incubate in PCR machine at:

100C 3min

60C to 35C ,reduce temperature by 1C every 7 minutes

4C (Place on ice as soon as PCR done to avoid unspecific hybridization at low temps)

3-

# Primers List:

**5’HO-loxP-URA F primer:**

5’-CCATATCCTCATAAGCAGCAATCAATTCTATCTATACTTTAAA ATAACTTCGTATAGCATACATTATACGAAGTTATCGGTGCCTGACTGCGTTAGC **-3’**

**URA-Lox2272-3’HO R primer:**

5’-TTACTTTTATTACATACAACTTTTTAAACTAATATACACATT ATAACTTCGTATAAAGTATCCTATACGAAGTTATCGACCGAGATTCCCGGGTAATAAC **– 3’**

**midURA-w5’ primer:**

5’ CGCTCTTCGCAATGTCAACAG -3’

**midURA-w3’:**

5’ GGAACCTAGAGGCCTTTTGATG -3’

**5’HO new seq primer:**

5’- AGCATGATGAAGCGTTCTAAACGCA -3’

**3’ HO new seq primer:**

5’- TCAGTGCCGGTAACGCTTTTTGT -3’

**sBC-STEP1F primer:**

5’- CCTCAGAACCTCGGCTCACACTGGATCCCCGGGTTAATTAAGG -3’

**sBC-STEP1R primer:**

5’- CTAACCACGGCAGTCGGGATTCTTTTCGACACTGGATGGCGG -3’

**STEP2F N ver primer:**

5’ATGCCTTTGACGACCCGAGATGNNNNNNNNNNNNNNNNNNNNNNNNNCCTCAGAACCTCGGCTCACACT-3’

**STEP2R N ver primer:**

5’GCGTATGGTGCGAGTATCAGCGNNNNNNNNNNNNNNNNNNNNNNNNNCTAACCACGGCAGTCGGGATTC-3’

**IT rgnt- HphMX4-LoxP Reamp primer(SacI Reamp F primer):**

5’- GCTCGGCGGCTTCTAATCCGTACTAGAGC -3’

**IT rgnt- HphMX4-Lox2272 Reamp primer(SacI Reamp R primer):**

5’- TTAACCCTCACTAAAGGGAACAAAAGCTGGAGCT -3’

**LoxP-BC Cass-SacI F primer:**

5-GCTCGGCGGCTTCTAATCCGTACTAGAGCTATAACTTCGTATAGCATACATTATACGAAGTTATATGCCTTTGACGACCCGAGATG-3’

**Lox2272-BC Cass-SacI R primer:**

5’ - TTAACCCTCACTAAAGGGAACAAAAGCTGGAGCTATAACTTCGTATAAAGTATCCTATACGAAGTTATGCGTATGGTGCGAGTATCAGCG 3’

**Us1 primer:**

5’- ATGCCTTTGACGACCCGAGATG -3’

**Us2 primer:**

5’- AGTGTGAGCCGAGGTTCTGAGG -3’

**Ds1 primer:**

5’- GAATCCCGACTGCCGTGGTTAG -3’

**Ds2 primer:**

5’- GCGTATGGTGCGAGTATCAGCG -3’

**P01\_Us1 primer:**

AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNN  CAAGTGTTC ATGCCTTTGACGACCCGAGATG -3’

**P01\_Us2 primer:**

CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCTNNNNN  CAAGTGTTC AGTGTGAGCCGAGGTTCTGAGG -3’

**P01\_Ds1 primer:**

AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNN  CAAGTGTTC GAATCCCGACTGCCGTGGTTAG-3’

**P01\_Ds2 primer:**

CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCTNNNNN  CAAGTGTTC GCGTATGGTGCGAGTATCAGCG-3’

**FOR ROW,COLUMN and PLATE Tag Primer sequences , refer to the TWAS Design folder or ask Nozomu**

# Engineered DNA Pieces:

**Cassette transformed into the HO Locus:**

**5’HO overlap–LoxP-URA3-Lox2272-3’HO Overlap**

5’-CCATATCCTCATAAGCAGCAATCAATTCTATCTATACTTTAAAATAACTTCGTATAGCATACATTATACGAAGTTATCGGTGCCTGACTGCGTTAGCAATTTAACTGTGATAAACTACCGCATTAAAGCTTTTCAATTCATCATTTTTTTTTTATTCTTTTTTTTGATTTCGGTTTCCTTGAAATTTTTTTGATTCGGTAATCTCCGAACAGAAGGAAGAACGAAGGAAGGAGCACAGACTTAGATTGGTATATATACGCATATGTAGTGTTGAAGAAACATGAAATTGCCCAGTATTCTTAACCCAACTGCACAGAACAAAAACCTGCAGGAAACGAAGATAAATCATGTCGAAAGCTACATATAAGGAACGTGCTGCTACTCATCCTAGTCCTGTTGCTGCCAAGCTATTTAATATCATGCACGAAAAGCAAACAAACTTGTGTGCTTCATTGGATGTTCGTACCACCAAGGAATTACTGGAGTTAGTTGAAGCATTAGGTCCCAAAATTTGTTTACTAAAAACACATGTGGATATCTTGACTGATTTTTCCATGGAGGGCACAGTTAAGCCGCTAAAGGCATTATCCGCCAAGTACAATTTTTTACTCTTCGAAGACAGAAAATTTGCTGACATTGGTAATACAGTCAAATTGCAGTACTCTGCGGGTGTATACAGAATAGCAGAATGGGCAGACATTACGAATGCACACGGTGTGGTGGGCCCAGGTATTGTTAGCGGTTTGAAGCAGGCGGCGGAAGAAGTAACAAAGGAACCTAGAGGCCTTTTGATGTTAGCAGAATTGTCATGCAAGGGCTCCCTAGCTACTGGAGAATATACTAAGGGTACTGTTGACATTGCGAAGAGCGACAAAGATTTTGTTATCGGCTTTATTGCTCAAAGAGACATGGGTGGAAGAGATGAAGGTTACGATTGGTTGATTATGACACCCGGTGTGGGTTTAGATGACAAGGGAGACGCATTGGGTCAACAGTATAGAACCGTGGATGATGTGGTCTCTACAGGATCTGACATTATTATTGTTGGAAGAGGACTATTTGCAAAGGGAAGGGATGCTAAGGTAGAGGGTGAACGTTACAGAAAAGCAGGCTGGGAAGCATATTTGAGAAGATGCGGCCAGCAAAACTAAAAAACTGTATTATAAGTAAATGCATGTATACTAAACTCACAAATTAGAGCTTCAATTTAATTATATCAGTTATTACCCGGGAATCTCGGTCGGTTATTACCCGGGAATCTCGGTCGATAACTTCGTATAGGATACTTTATACGAAGTTATAATGTGTATATTAGTTTAAAAAGTTGTATGTAATAAAAGTAA -3’

**Barcode Cassette used for In Yeast Assembly:**

**5’pSH47(SacI)homology–LoxP-Us1-Random UP barcode-Us2-HphMX4-Ds1-Random Down Barcode-Ds2-Lox2272-3’pSH47(SacI)homology**

5’-GCTCGGCGGCTTCTAATCCGTACTAGAGCTATAACTTCGTATAGCATACATTATACGAAGTTAT ATGCCTTTGACGACCCGAGATGNNNNNNNNNNNNNNNNNNNNNNNNNCCTCAGAACCTCGGCTCACACT ggatccccgggttaattaaggcgcgccagatctgtttagcttgcctcgtccccgccgggtcacccggccagcgacatggaggcccagaataccctccttgacagtcttgacgtgcgcagctcaggggcatgatgtgactgtcgcccgtacatttagcccatacatccccatgtataatcatttgcatccatacattttgatggccgcacggcgcgaagcaaaaattacggctcctcgctgcagacctgcgagcagggaaacgctcccctcacagacgcgttgaattgtccccacgccgcgcccctgtagagaaatataaaaggttaggatttgccactgaggttcttctttcatatacttccttttaaaatcttgctaggatacagttctcacatcacatccgaacataaacaaccATGGGTAAAAAGCCTGAACTCACCGCGACGTCTGTCGAGAAGTTTCTGATCGAAAAGTTCGACAGCGTCTCCGACCTGATGCAGCTCTCGGAGGGCGAAGAATCTCGTGCTTTCAGCTTCGATGTAGGAGGGCGTGGATATGTCCTGCGGGTAAATAGCTGCGCCGATGGTTTCTACAAAGATCGTTATGTTTATCGGCACTTTGCATCGGCCGCGCTCCCGATTCCGGAAGTGCTTGACATTGGGGAATTCAGCGAGAGCCTGACCTATTGCATCTCCCGCCGTGCACAGGGTGTCACGTTGCAAGACCTGCCTGAAACCGAACTGCCCGCTGTTCTGCAGCCGGTCGCGGAGGCCATGGATGCGATCGCTGCGGCCGATCTTAGCCAGACGAGCGGGTTCGGCCCATTCGGACCGCAAGGAATCGGTCAATACACTACATGGCGTGATTTCATATGCGCGATTGCTGATCCCCATGTGTATCACTGGCAAACTGTGATGGACGACACCGTCAGTGCGTCCGTCGCGCAGGCTCTCGATGAGCTGATGCTTTGGGCCGAGGACTGCCCCGAAGTCCGGCACCTCGTGCACGCGGATTTCGGCTCCAACAATGTCCTGACGGACAATGGCCGCATAACAGCGGTCATTGACTGGAGCGAGGCGATGTTCGGGGATTCCCAATACGAGGTCGCCAACATCTTCTTCTGGAGGCCGTGGTTGGCTTGTATGGAGCAGCAGACGCGCTACTTCGAGCGGAGGCATCCGGAGCTTGCAGGATCGCCGCGGCTCCGGGCGTATATGCTCCGCATTGGTCTTGACCAACTCTATCAGAGCTTGGTTGACGGCAATTTCGATGATGCAGCTTGGGCGCAGGGTCGATGCGACGCAATCGTCCGATCCGGAGCCGGGACTGTCGGGCGTACACAAATCGCCCGCAGAAGCGCGGCCGTCTGGACCGATGGCTGTGTAGAAGTACTCGCCGATAGTGGAAACCGACGCCCCAGCACTCGTCCGAGGGCAAAGGAATAAtcagtactgacaataaaaagattcttgttttcaagaacttgtcatttgtatagtttttttatattgtagttgttctattttaatcaaatgttag**c**gtgatttatattttttttcgcctcgacatcatctgcccagatgcgaagttaagtgcgcagaaagtaatatcatgcgtcaatcgtatgtgaatgctggtcgctatactgctgtcgattcgatactaacgccgccatccagtgtcgaaaa GAATCCCGACTGCCGTGGTTAGNNNNNNNNNNNNNNNNNNNNNNNNNCGCTGATACTCGCACCATACGCATAACTTCGTATAGGATACTTTATACGAAGTTATAGCTCCAGCTTTTGTTCCCTTTAGTGAGGGTTAA -3’

**5’HO new seq - Us2 primers PCR expected product(NNNN is UPTAG barcode sequence):**

AGCATGATGAAGCGTTCTAAACGCACTATTCATCATTAAATATTTAAAGCTCATAAAATTGTATTCAATTCCTATTCTAAATGGCTTTTATTTCTATTACAACTATTAGCTCTAAATCCATATCCTCATAAGCAGCAATCAATTCTATCTATACTTTAAAATAACTTCGTATAGCATACATTATACGAAGTTATATGCCTTTGACGACCCGAGATGNNNNNNNNNNNNNNNNNNNNNNNNNCCTCAGAACCTCGGCTCACACT

**Ds1-3’HO new seq primers PCR expected product(NNNN is DNTAG barcode sequence):**

GAATCCCGACTGCCGTGGTTAGNNNNNNNNNNNNNNNNNNNNNNNNNCGCTGATACTCGCACCATACGCATAACTTCGTATAGGATACTTTATACGAAGTTATAATGTGTATATTAGTTTAAAAAGTTGTATGTAATAAAAGTAAAATTTAATATTTTGGATGAAAAAAACCATTTTTAGACTTTTTCTTAACTAGAATGCTGGAGTAGAAATACGCCATCTCAAGATACAAAAAGCGTTACCGGCACTGA

**Expected Sequence at HO Locus after Barcode-HpHMX4 recombination into HO Locus:**

AGCATGATGAAGCGTTCTAAACGCACTATTCATCATTAAATATTTAAAGCTCATAAAATTGTATTCAATTCCTATTCTAAATGGCTTTTATTTCTATTACAACTATTAGCTCTAAATCCATATCCTCATAAGCAGCAATCAATTCTATCTATACTTTAAAATAACTTCGTATAGCATACATTATACGAAGTTATATGCCTTTGACGACCCGAGATGNNNNNNNNNNNNNNNNNNNNNNNNNCCTCAGAACCTCGGCTCACACTggatccccgggttaattaaggcgcgccagatctgtttagcttgcctcgtccccgccgggtcacccggccagcgacatggaggcccagaataccctccttgacagtcttgacgtgcgcagctcaggggcatgatgtgactgtcgcccgtacatttagcccatacatccccatgtataatcatttgcatccatacattttgatggccgcacggcgcgaagcaaaaattacggctcctcgctgcagacctgcgagcagggaaacgctcccctcacagacgcgttgaattgtccccacgccgcgcccctgtagagaaatataaaaggttaggatttgccactgaggttcttctttcatatacttccttttaaaatcttgctaggatacagttctcacatcacatccgaacataaacaaccATGGGTAAAAAGCCTGAACTCACCGCGACGTCTGTCGAGAAGTTTCTGATCGAAAAGTTCGACAGCGTCTCCGACCTGATGCAGCTCTCGGAGGGCGAAGAATCTCGTGCTTTCAGCTTCGATGTAGGAGGGCGTGGATATGTCCTGCGGGTAAATAGCTGCGCCGATGGTTTCTACAAAGATCGTTATGTTTATCGGCACTTTGCATCGGCCGCGCTCCCGATTCCGGAAGTGCTTGACATTGGGGAATTCAGCGAGAGCCTGACCTATTGCATCTCCCGCCGTGCACAGGGTGTCACGTTGCAAGACCTGCCTGAAACCGAACTGCCCGCTGTTCTGCAGCCGGTCGCGGAGGCCATGGATGCGATCGCTGCGGCCGATCTTAGCCAGACGAGCGGGTTCGGCCCATTCGGACCGCAAGGAATCGGTCAATACACTACATGGCGTGATTTCATATGCGCGATTGCTGATCCCCATGTGTATCACTGGCAAACTGTGATGGACGACACCGTCAGTGCGTCCGTCGCGCAGGCTCTCGATGAGCTGATGCTTTGGGCCGAGGACTGCCCCGAAGTCCGGCACCTCGTGCACGCGGATTTCGGCTCCAACAATGTCCTGACGGACAATGGCCGCATAACAGCGGTCATTGACTGGAGCGAGGCGATGTTCGGGGATTCCCAATACGAGGTCGCCAACATCTTCTTCTGGAGGCCGTGGTTGGCTTGTATGGAGCAGCAGACGCGCTACTTCGAGCGGAGGCATCCGGAGCTTGCAGGATCGCCGCGGCTCCGGGCGTATATGCTCCGCATTGGTCTTGACCAACTCTATCAGAGCTTGGTTGACGGCAATTTCGATGATGCAGCTTGGGCGCAGGGTCGATGCGACGCAATCGTCCGATCCGGAGCCGGGACTGTCGGGCGTACACAAATCGCCCGCAGAAGCGCGGCCGTCTGGACCGATGGCTGTGTAGAAGTACTCGCCGATAGTGGAAACCGACGCCCCAGCACTCGTCCGAGGGCAAAGGAATAAtcagtactgacaataaaaagattcttgttttcaagaacttgtcatttgtatagtttttttatattgtagttgttctattttaatcaaatgttagcgtgatttatattttttttcgcctcgacatcatctgcccagatgcgaagttaagtgcgcagaaagtaatatcatgcgtcaatcgtatgtgaatgctggtcgctatactgctgtcgattcgatactaacgccgccatccagtgtcgaaaaGAATCCCGACTGCCGTGGTTAGNNNNNNNNNNNNNNNNNNNNNNNNNCGCTGATACTCGCACCATACGCATAACTTCGTATAGGATACTTTATACGAAGTTATAATGTGTATATTAGTTTAAAAAGTTGTATGTAATAAAAGTAAAATTTAATATTTTGGATGAAAAAAACCATTTTTAGACTTTTTCTTAACTAGAATGCTGGAGTAGAAATACGCCATCTCAAGATACAAAAAGCGTTACCGGCACTGA

**Expected Plate Tag,Row-Col PCR final product:**

5’-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNNNNNCAAGTGTTCTAACTTACGGAGTCGCTCTACGCAAGTGTTCATGCCTTTGACGACCCGAGATGNNNNNNNNNNNNNNNNNNNNNNNNNCCTCAGAACCTCGGCTCACACTGAACACTTGCAGGACCTAAAGAATCCCATCCGAACACTTGNNNNNNNNNAGATCGGAAGAGCGGTTCAGCAGGAATGCCGAGACCGATCTCGTATGCCGTCTTCTGCTTG -3’

Illumina Adaptors

Plate Tags( up to 6 different tags:Upstream Plate Tag CAAGTGTTC / AGGACATTC/ CACTAATGG / AGCCTGATG / TTACGCTAA / ACTCTCCGT

Downstream Plate Tag: GAACACTTG / GAATGTCCT/ CCATTAGTG / CATCAGGCT / TTAGCGTAA / ACGGAGAGT)

Ps1.0 , Ps2.0 regions( Constant regions)

Row and Column Tags : 16 different Row Tags , 24 different Column Tags (see TWAS design folder for primers)

Us1/U1/Ds1/D1 or Us2/U2/Ds2/D2 sequences

U1: GATGTCCACGAGGTCTCT U2: CGTACGCTGCAGGTCGAC

D1: CGGTGTCGGTCTCGTAG D2:ATCGATGAATTCGAGCTCG

Us1:ATGCCTTTGACGACCCGAGATG Us2:CCTCAGAACCTCGGCTCACACT

Ds1:GAATCCCGACTGCCGTGGTTAG Ds2:CGCTGATACTCGCACCATACGC