RTR_1v2

High-throughput crossing of the *S. pombe* deletion library by Synthetic Genetic Array (SGA) using the Singer RoToR HDA

(updated SGA protocol based on RTR_1v1, by **R. R. Barrales** as of December 8th 2014)

***S. pombe* library:** The original *Bioneer* deletion library comes in a 96-well format (total 36 plates). For the large-scale crosses, the library has been reassembled into 384 plates (total 9 plates or—after reconfiguration—8 plates, which is the newer version). The library has the mating type *h+,* and the deletions can be selected by Geneticin (G418).

Query strain: Any query strain that is *h-* (or *h90*) and contains another selectable marker (e.g. gene *y*Δ*::natMX*) can be used for crossing. For selecting haploid spores of only one specific mating type (e.g. *h+*), the Cycloheximide (Cyh) Method by Krogan and co-worker (Roguev et al. 2007) can be applied. In this case, the gene deletion of the query strain needs to be introduced into the original strain (or a derivate) that harbors a cyh-resistant allele of the ribosomal RNA gene *rpl42* at its original locus and an additional wild-type Cyh-sensitive allele next to the active mating type locus (*h-*). After the cross, haploid spores that contain only the the Cyh-resistant allele and are thus *h+* are selected in presence of Cyh. For performing silencing assays based on *ura4+* expression/FOA counter-selection, the query strain has additionally a reporter strain with *ura4+* expressed from a heterochromatin locus (i.e. pericentromeric *imr* or *otr*; silent *mat3-EcoRV*). To select for the silent reporter, a selectable marker (usually *hygMX*) is inserted next to the heterochromatin region into a neutral euchromatic locus. In case of the *imrL::ura4+* reporter, *ura4+* is leaky expressed and allows also for selection on EMM plates lacking uracil.

Selection of haploid spores can be done using either using the ***Temperature Method*** (Dixon et al., PNAS 2008) or the ***Cycloheximide Method*** (Roguev et al., Nat Methods 2007)

General note for RoToR HDA station settings: On the RoToR touchscreen is a left side tab where several options can be switched on/off (*recycle*, *offset*, *dry mix* or *wet mix*, etc). When loading a profile, these options are not always selected as needed — check first the settings before starting the robot.

Step 1: Generating query strain array and copy of the deletion library

(2 days before mating)

1.1 Create Query Array (384 format): From an over-night culture of the query strain pour 30mL into an empty square plate. Use this plate as source plate for the Singer RoToR HDA station. Several copies (minimum 3 plates) are generated.

*RoToR settings:

- **Select:** Source-Wet 384 bath, Target-Dry 384 plate.
  - Long Pin Pads.
  - Spot Many

- **Load Profile: Query Array**
- **General**
  - **Quantity** Select number of query plates
  - **Pairs** 2 times each (if culture is not saturated, 4 times is recommended)
  - **Recycle** Until repeat (revisit *ON*)
- **Source**
  - **Pinning**
    - **Speed** 19 mm/sec
    - **Backoff** 0.5 mm
    - **Repeat Pin** 1 time
  - **Wet Mix**
  - **NO WET MIX**
- **Target**
  - **Pinning**
    - **Pinning Pressure** 32%
    - **Speed** 19 mm/sec
    - **Overshoot** 2 mm
    - **Repeat Pin** 1 time
  - **Dry Mix**
    - **NO DRY MIX**

1.2 Create Fresh Library Copy (384 format): replicate all plates of the library (384 format, either assembled in 1-8 or 1-9 plates) onto new YES plates.

*RoToR settings:

- **Select**: Source-Dry 384 plate, Target-Dry 384 plate.
  - Short Pin Pads
  - Replicate Many (in case more than one copy is required)
- **Load Profile**: **SOLID2SOLID SGA**
- **General**
  - **Quantity** 2 (or number of target plates)
  - **Pairs** 1 time each
  - **Recycle** Until repeat (revisit *ON*)
- **Source**
  - **Pinning**
    - **Pinning Pressure** 32%
    - **Speed** 8 mm/sec
    - **Overshoot** 2 mm
    - **Repeat Pin** 1 time
  - **Dry Mix**
    - **Clearance** 1 mm
    - **Diameter** 0,5 mm
    - **Cycles** 1 rotations
- **Target**
  - **Pinning**
    - **Pinning Pressure** 32%
    - **Speed** 19 mm/sec
    - **Overshoot** 2 mm
    - **Repeat Pin** 1 time
  - **Dry Mix**
    - **Clearance** 0,5 mm
    - **Diameter** 1 mm
    - **Cycles** 1 rotations

Step 2: Mating

2.1 Mix: For mating, replicate each one copy of the query strain and one plate of the deletion library (1, 2…8) onto the *same* SPAS plate (one query strain plate can be used several times, e.g. for up to three library plates).

*RoToR settings:

- **Select:** Source-Dry 384 plate, Target-Dry 384 plate.
  - Short Pin Pads.
  - Mate
- **Load Profile: Mating**
- **General**
  - **Quantity** 0
  - **Pairs** 1 time
  - **Recycle** NO
- **Source**
  - **Pinning**
    - **Pinning Pressure** 32%
    - **Speed** 19 mm/sec
    - **Overshoot** 2 mm
    - **Repeat Pin** 1 time
  - **Dry Mix**
    - **Clearance** 0,5 mm
    - **Diameter** 0,5 mm
    - **Cycles** 1 rotation

- **Target**
  - **Pinning**
    - **Pinning Pressure** 32%
    - **Speed** 19 mm/sec
    - **Overshoot** 2 mm
    - **Repeat Pin** 1 time
  - **Dry Mix**
    - **Clearance** 0,5 mm
    - **Diameter** 1 mm
    - **Cycles** 1 rotation

2.2 After completing the *Mating* program, adding **a water droplet** on top of cells increases the mating efficiency (Dixon et al. PNAS, 2008). To do this, fill an empty plate with sterile water (20 mL approx.) and run the *H2O Mating* program on the RoToR:

*RoToR settings:

- **Select:** Source-Wet 384 bath, Target-Dry 384 plate.
  - Long Pin Pads.
  - Spot Many

- **Load Profile: H2O Mating**
- **General**
  - **Pairs** 1 time
  - **Recycle** NO
- **Source**
  - **Pinning**
    - **Backoff** 0.5 mm
    - **Repeat Pin** time
  - **Wet Mix**
  - **NO WET MIX**
- **Target**
  - **Pinning**
    - **Pinning Pressure** 32%
    - **Speed** 19 mm/sec
    - **Overshoot** 2 mm
    - **Repeat Pin** 1 time
  - **Dry Mix**
    - **Clearance** 0,5 mm
    - **Diameter** 0,2 mm
    - **Cycles** 1 rotation

2.3. Sporulation. Put plates with mated cells in plastic bags and seal them. Incubate **3 to 4 days at room temperature** (25 to 28°C).

Step 3: Haploid Selection

3.1. For **selection of spores** using the ***Temperature Method***, put the plates in bags in a **42°C** incubator with several containers filled with water (1-2 liters) for **4 days**. Prolonged high-temperature-exposure will kill all cells but spores (i.e. non-mated parental haploid cells, non-sporulated diploid cells).
For selection with the ***Cycloheximide (cyh) Method***, continue directly with step 3.2.

3.2. Germination of Spores: For ***Temperature Method*** replicate spores from SPAS onto YES with 100mg/L of Hyg. For ***Cycloheximide (cyh) Method***, replicated spores onto YES with 100 mg/L Cyh). Incubate for 2-3 days at 30°C. During this step, all clones are duplicated changing from 384 to 768 format.

*RoToR settings:

- **Select:** Source-Dry 384 plate Target-Dry 768 plate.
  - Short Pin Pads.
  - 1:2 Array single source.
- **Load Profile: SOLID2SOLID SGA**
- **General**
  - **Quantity** 2 (or number of target plates)
  - **Pairs** 1 time each
  - **Recycle** NO
- **Source**
  - **Pinning**
    - **Pinning Pressure** 32%
    - **Speed** 8 mm/sec
    - **Overshoot** 2 mm
    - **Repeat Pi** 1 time
  - **Dry Mix**
    - **Clearance** 1 mm
    - **Diameter** 0,5 mm
    - **Cycles** 1 rotations
- **Target**
  - **Pinning**
    - **Pinning Pressure** 32%
    - **Speed** 19 mm/sec
    - **Overshoot** 2 mm
    - **Repeat Pin** 1 time
  - **Dry Mix**
    - **Clearance** 0,5 mm
    - **Diameter** 0,3 mm
    - **Cycles** 1 rotations

Step 4: Selection of haploid double mutants

For ***Cycloheximide (cyh) Method,*** use YES plates supplemented with Cyh (mating type selection), G418 (deletion mutants from library), Hyg (selection marker next to heterochromatin *ura4+* reporter) and another antibiotic (usually Nat for selecting for an additional mutation in the query strain); all antibiotics are at 100 mg/L. For ***Temperature Method*** *(cyh)* ***Method***, use YES plates supplemented with G418, Hyg and Nat. **Optional**: When *ura4+* is used as reporter gene within the *imrL* region, an additional EMM-Ura selection step *before* the antibiotics selection step can improve the reproducibility of results.

*RoToR settings:

- **Select:** Source-Dry 768 plate Target-Dry 768 plate.
  - Short Pin Pads.
  - Replicate 768 New2
    Note: The option for replicating in the 768 format (*Replicate 768 New2)* is not provided by the RoToR settings but this can be manually programmed and saved to the presets.
- **Load Profile: SOLID2SOLID 768**
- **General**
  - **Quantity** 1
  - **Pairs** 1 time each
  - **Recycle** NO

- **Source**
  - **Pinning**
    - **Pinning Pressure** 10%
    - **Speed** 19 mm/sec
    - **Overshoot** 2 mm
    - **Repeat Pin** 1 time
  - **Dry Mix**
    - **NO DRY MIX**
- **Target**
  - **Pinning**
    - **Pinning Pressure** 10%
    - **Speed** 19 mm/sec
    - **Overshoot** 2 mm
    - **Repeat Pin** 1 time
  - **Dry Mix**
    - **Clearance** 0,5 mm
    - **Diameter** 0,3 mm
    - **Cycles** 1 rotations

IMPORTANT: If cells have been grown on **EMM-Ura**, it is necessary to use **32% of pinning pressure** and **Dry Mix (0,3 mm diameter)** in *Source* for replica plating to the next selection plate.

Step 5: Silencing assay (with *ura4+* reporter)

Replica-plate from last selection plate onto EMM (control plate), EMM+FOA (1 mg/ml) and EMM-Ura. During this step, the format is changed back from 784 to to 384 format (i.e. splitting each plate with the duplicated colonies from step 3.2. into two plates)

*RoToR settings:

- **Select:** Source-Dry 768 plate Target-Dry 384 plate.
  - Short Pin Pads.
  - Breakdown

- **Load Profile: Silencing Assay From YES**
- **General**
  - **Quantity** 1
  - **Pairs** 1 time each
  - **Recycle** NO
- **Source**
  - **NO Offset**
  - **Pinning**
    - **Pinning Pressure** 10%
    - **Speed** 19 mm/sec
    - **Overshoot** 2 mm
    - **Repeat Pin** 1 time
  - **Dry Mix**
    - **NO DRY MIX**
- **Target**
  - **Pinning**
    - **Pinning Pressure** 10%
    - **Speed** 19 mm/sec
    - **Overshoot** 2 mm
    - **Repeat Pin** 1 time
  - **Dry Mix**
    - **NO DRY MIX**

Note: Using these settings, colonies are sometimes not transferred properly from the center of the plate. In this case **increase the pinning pressure to 32 %** and **reduce the spinning speed to 8 mm/sec** for both source and target plate.

Pictures are taking from day 2 to day 4 with a digital camera. Colony sizes are quantified using the software *ht-colony-grid-analyzer-1.1.7* (freeware: http://ht-col-measurer.sourceforge.net/)

Required materials:

All quantities (amount of plates) are calculated for one round of large-scale cross (i.e. one query strain with 2 duplicates). Yeast extracts is obtained from Dicfo, EMM is obtained from ForMedium (EMM without dextrose, #PMD0410). Agar is obtained from Difco (high quality) or Serva (low quality) — we noticed strong variation with respect to growth when using low-quality agar and use for the final steps, in which colony size is quantitatively assessed, only high-quality agar. All antibiotics are used at 100 µg/mL (G418 (geneticin), Invitrogen #1013027; clonNAT (nourseothricin), Werner Bioreagents; hygromycine B, Invitrogen #10687-010; cycloheximide, Singer #C7698). FOA (Thermo Scientific, *#R0812*) is used at 1 mg/mL.

Media Plates

Plate type	Amount	Step	Comment
YES	3-4	Query strain array (1.1)	Can also be prepared with Nat (or Hyg) to reduce contamination risk
YES	8-10	Library copy (1.2)	Can also be prepared with Gen (G418) to reduce contamination risk
SPAS	8-10	Mating (2.1)	
YES+Hyg	16-20	Germination (3.2)	Temperature method-only
YES+Cyh	16-20	Germination (3.2)	Cycloheximide method-only
EMM-Ura	16-20	Reporter selection (4)	Optional (only for *imr::ura4+* reporter)
YES+Hyg+Gen+Nat	16-20	Double mutant select (4)	Temperature method-only
YES+Cyh+Hyg+Gen+Nat	16-20	Double mutant select (4)	Cycloheximide method-only
EMM	16-20	Silencing assay (5)	Non-selective conditions
EMM-Ura	16-20	Silencing assay (5)	Ura-positive selection
EMM+FOA	16-20	Silencing assay (5)	Ura-counter selection

Singer RoToR HDA Consumables

dishes and lids (plates): *PlusPlates #PLU-001*
384-pinning pads: *RePads 384 short #RP-MP-384* (quantity 1000);
RePads 384 long #RP-MP-3L (quantity 200);

Pinning pads	Amount	Step	Comment
384 long	3-4	Query strain array (1.1)	For pinning from liquid cultures, long pins are required
384 short	8	Library copy (1.2)	calculated for 8 plates/library
384 short	16	Mating (2.1)	
384 long	8	Mating (2.2)	Adding water droplet
384 short	16	Germination (3.2)	Temperature method-only
384 short	16	Germination (3.2)	Cycloheximide method-only
384 short	16	Reporter selection (4)	Optional (only for *imr::ura4+* reporter)
384 short	16	Double mutant select (4)	Temperature method-only
384 short	16	Double mutant select (4)	Cycloheximide method-only
384 short	16	Silencing assay (5)	
384 short	16	Silencing assay (5)	
384 short	16	Silencing assay (5)	
Total number 384 short	**120**		either temperature or cycloheximide method
Total number 384 long	**12**		
Total number plates **)	**144**		

Scheme of screening procedure