HALOFERAX MATING

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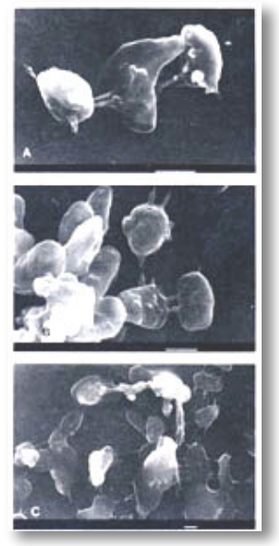


DAY 1

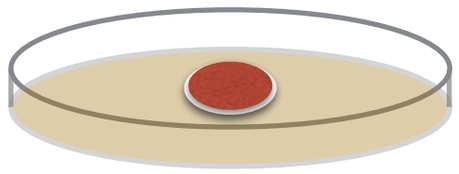
- Set up 5ml O/Ns of strains to be mated in Hv-broth (YPC or Ca, +Thy if needed)

DAY 2

- Dilute O/Ns in fresh broth, use 10 µl (1/500) for 5ml O/N
- Prepare Millipore Swinnex 25 filter units with Millipore 0.45 um type MCE 25 mm filters, wrap in aluminium foil and autoclave

DAY 3

- When OD = 0.8 (~4x10^8 cells) for both strains, mix equal volumes (1 ml each) of O/Ns
  - Also use 2 ml of unmixed parental O/Ns as negative controls
  - For different OD values, see this table
- Use 5 ml syringe to gently filter cell suspension through the sterile Swinnex filter unit
  - Repeat with 2 ml of unmixed parental O/Ns as negative controls
- Disassemble filter unit and use sterile forceps to remove filter disc. Place filter cell-side uppermost on Hv-YPC (+Thy) plate)



- Incubate overnight at 45°C

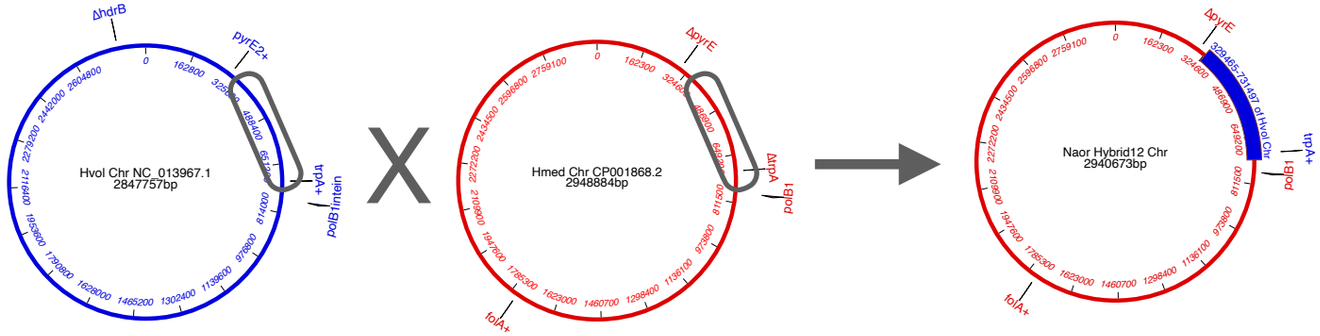
Rosenshine et al (1989); Naor et al (2012); Naor et al (2013)

HALOFERAX MATING CONTINUED…

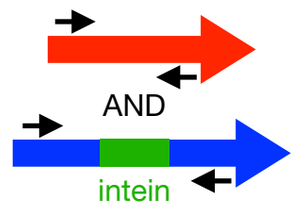
DAY 4

- Use sterile forceps to remove filter disc, transfer to 5 ml eppendorf with 1 ml 18% SW
  - Leave in rotator at 45°C ~1 hour to gently resuspend cells
- Dilute cells in 18% SW, plate 100 µl at 10^0, 10^{-1} and 10^{-2} on selective (e.g. Hv-Ca) plates
  - Optional: spot 20 µl at 10^{-4}, 10^{-6} and 10^{-8} on Hv-YPC for viable count
  - Incubate at 45°C for 5-6 days; expect recombinants at frequency of 10^{-4}

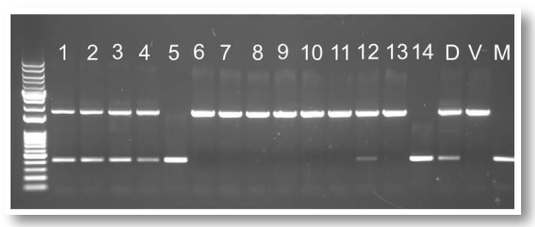
DAY 10 ONWARDS



- Set up 5ml O/Ns of heterodiploid cells in non-selective broth (e.g. Hv-YPC, +Thy)
  - Optional: use colony PCR to verify heterodiploid cells
    - e.g. PCR for polB, differs between Hvol and Hmed (intein in former), expect both bands in diploid



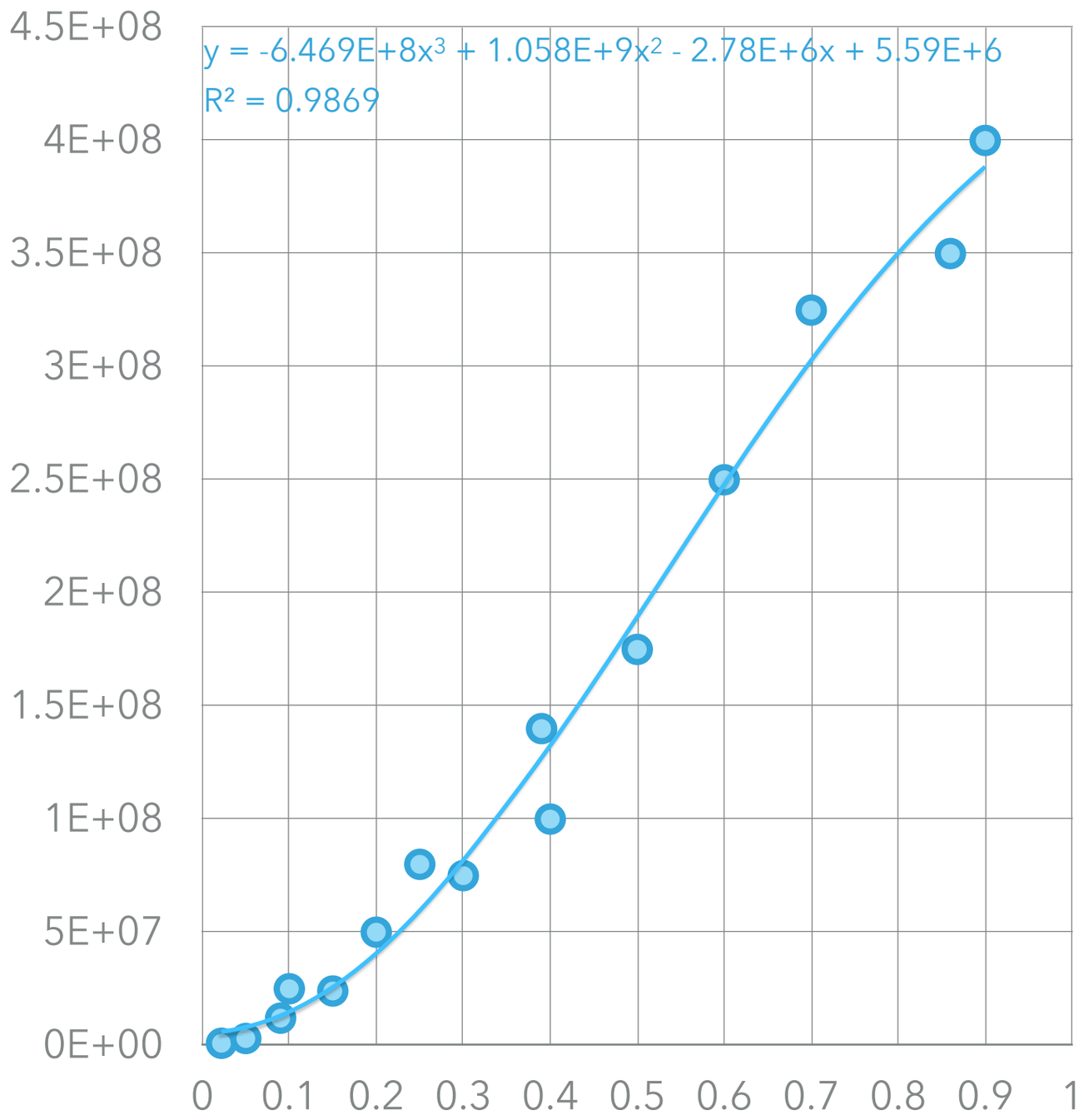
- Next day, dilute cells in 18% SW, plate 100 µl at 10^{-1}, 10^{-2},10^{-3} and 10^{-4} on selective plates
  - e.g. Hv-Ca+5FOA, incubate at 45°C for 5-6 days



- Recombinant haploids will have novel combination of selectable markers
  - Optional: use colony PCR to verify haploid recombinants, e.g. *polB*, expect one band only

CELLS PER ML @ OD650 VALUE

OD650	Cells/ml
0.022	9.00E+05
0.05	3.00E+06
0.09	1.20E+07
0.15	2.40E+07
0.25	8.00E+07
0.39	1.40E+08
0.86	3.50E+08
0.1	2.50E+07
0.2	5.00E+07
0.3	7.50E+07
0.4	1.00E+08
0.5	1.75E+08
0.6	2.50E+08
0.7	3.25E+08
0.9	4.00E+08



COLONY PCR: ONETAQ

DNA prep:

- Touch DNA with yellow tip (the less cells the better)
- Pipette cells up and down in 100µl SDW
- Boil at 100°C for 10mins
- Leave on ice for 10mins

PCR mix per Reaction	
DNA	1
Forward primer	0.4
Reverse primer	0.4
dNTPs	4
GC buffer (5X)	4
OneTaq	0.1
SDW	10.1
	20µl

PCR cycles	Temperature /°C	Time	
Initial D	94	30s	
D	94	30s	x 30
A	x	30s	
E	68	y (1min per kb)	
Final E	68	5mins	

Can use Q5 hotstart polymerase instead, but costs more

Q5 PCR

PCR mix per Reaction	
DNA*	1
Forward primer (50 µM)	1
Reverse primer (50 µM)	1
dNTPs (1 mM)	10
GC enhancer (5X)	10
Q5 buffer (5X)	10
Q5 hotstart polymerase	0.5
SDW	16.5
	50µl

PCR cycles	Temperature /°C	Time	
Initial Denature	98	30s	
Denature	98	10s	x 30
Anneal	x**	20s	
Extension	72	y† (secs per kb)	
Final Extension	72	10mins	

- DNA* Use ~50 ng plasmid DNA; if using genomic DNA, dilute 1/10 in dH_2O
- x** = annealing temperature

  = 81.5 + 0.41%GC - 600/Length + $16.6\text{Log}_{10}[Na^+]$
  Assuming Na^+ concentration = 50 mM
  Subtract 1°C for each 1% mismatch

- y† = extension time, 20 sec/kb for plasmid DNA, 30 sec/kb for genomic DNA,

TOUCHDOWN PCR

TD PCR cycles	Temperature /°C	Time	
Initial Denature	98	30s	
Denature	98	10s	x 10
Anneal	Low - High temp**	20s	
Extension	72	y† (secs per kb)	
Denature	98	10s	x 20
Anneal	High temp**	20s	
Extension	72	y† (secs per kb)	
Final Extension	72	10mins	