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Yeast protoplast fusion

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We are still developing and optimizing this protocol

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Abstract

This protocol was based on the original protocol by Ulschan Bathe and Kristen Van Gelder from the Hanson Lab at UF in 2023.

It details how to fuse 2 yeast protoplasts. It is focused on obtaining an evolution strain with the system OrthoRep, but should work for the fusion of any 2 yeast strains provided the selection scheme is functional.

Media and buffers

1 Make the following solutions ahead of time. They do not need to be sterilized.

Unless specified, all solutions are made in water

1.1 **CPB (Citrate Phosphate Buffer) solution**

Solution A

[M] 0.1 Molarity (M) Citric Acid (dihydrate)

Solution B

[M] 0.2 Molarity (M) Na2HPO4

Combine 34.8 mL A and 65.2 mL B and bring up final volume to 200 mL with MilliQ water

1.2 **3M KCl**

1.3 **0.5 M EDTA (pH = 8.0)**

1.4 **0.5 M CaCl2**

1.5 **Other media**

YPD

Autoclaved sterile water

2 **Solutions - made fresh the day of**

2.1 **BMEE solution**

3 mL [M] 0.5 Molarity (M) EDTA

50 µL beta-mercaptoethanol

21.95 mL MilliQ water

Filter sterilize

2.2 0.6 M KCl

20 mL [M] 3 Molarity (M) KCl
80 mL MilliQ water

Filter sterilize

2.3 Buffer 1

39 mL CPB Buffer
10 mL [M] 3 Molarity (M) KCl
1 mL [M] 0.5 Molarity (M) EDTA

Filter sterilize

2.4 Buffer 2

A - [M] 50 % w/v **PEG 3350**

Add 10 g PEG + 8 mL water, stir with high heat and adjust volume to 25 mL when done

B - Buffer 2

16.65 mL [M] 50 % w/v PEG 3350
5 mL [M] 3 Molarity (M) KCl
2.5 mL [M] 0.5 Molarity (M) CaCl2
850 µL MilliQ water

Filter sterilize

2.5 Buffer 3

5 mL [M] 3 Molarity (M) KCl
2.5 mL [M] 0.5 Molarity (M) CaCl2

17.5 mL MilliQ water

Filter sterilize

Plating media

3 Plating media (i.e., selective media) must be made in 100-150 mL batches. Each 25 mL supports a single plating, thus 100 mL supports 1 fusion with 2 platings at different concentrations

For 100 mL
93.5 mL MilliQ water
4.473 g **KCl**
0.667 g YNB with ammonium sulfate OR 0.5 g ammonium sulfate + 0.171 g YNB without ammonium sulfate (monosodium glutamate can also be used as an alternative nitrogen source if ammonium sulfate cannot be used due to interfering with antibiotics (G418 or Nat))
0.14 g g of appropriate dropout
Adjust pH to 6.1
3 g agar
Run on liquid 30 cycle, leave inside autoclave until later.

Yeast culture

4 2 or 3 days before the experiment, start 3 mL pre-cultures in appropriate selection media for the donor (**p1 carrying strain**) and recipient (**background + EP-DNAP**) strains. Grow at 30 °C with shaking 2d

5 The day before protoplast fusion, dilute pre-cultures 1:50 into 50 mL YPD culture and incubate shaking at 30 °C 16h

Be very mindful of the rate of growth of the cells - OD after outgrowth should not exceed 7 but should be above 2. Depending on your strain, you will need to figure out optimal time for starting the culture.

Protoplast fusion 1w 3d 3h 33m

6 The morning of protoplast fusion, make the solutions above and filter-sterilize them.

7 Take 100 µL of culture and add 900 µL YPD to it, then measure the OD600.

Multiply the OD by the dilution factor (x10)

Using the approximation that, at OD600 = 4.5, for 20 mL of culture, ~0.45 g of cells are yielded, calculate the volume of culture necessary to obtain 0.3 g of cells of each strain using the formula **c1.v1 = c2.v2**

8 Weigh your tubes before adding culture and make a note of it.

9 Centrifuge at 3000 x g, 00:05:00 , using blank tubes to balance the different culture volumes 5m

10 Weigh your tubes again, and adjust pellet weight by adding more culture or scooping out pellet.

Aim for a final weight of 0.3 g - preferably OVER

11 Resuspend in 10 mL sterile water and transfer to 15 mL falcon

12 Centrifuge at 3000 x g, 00:05:00 , and decant supernatant. Pipette out water. 5m

13 Resuspend in 1.8 mL in the same tube **BMEE solution TAPPING GENTLY to resuspend**

DO NOT VORTEX ANYTHING BEYOND THIS POINT

14 Incubate at 30 °C for 00:30:00 . 30m

At 15 min, invert a few times to mix gently.

15 Centrifuge at 3000 x g, 00:05:00 , decant supernatant. 5m

16 Add 3 mL **0.6 M KCl** and resuspend with serological pipette.

Note: avoid the creation of bubbles

17 Centrifuge at 3000 x g, 00:05:00, decant supernatant. 5m

18 Add 4.8 mL **Buffer I** and resuspend with serological pipette.

19 Add Zymolyase solution at a total concentration of [M] 6 U/mL

Note: Hanson Lab storage buffer for Zymolyase is as follows. If using this recipe make at a concentration of 5 U/uL, then add 5.8 uL :

i. 100 mg (2000 U) of zymoylase from amsbio (Cat# = 120491-1)

ii. 0.1 M Na2PO4 (pH 7.5, adjusted with NaOH)

iii. 50% glycerol

iv. 3 mM β-ME

To make 400 uL of solution
1. 100 mg zymolyase
2. 200 uL glycerol
3. 192 uL 0.1 M Na2PO4 (pH 7.5, adjusted with NaOH)
4. 8 uL of a 1:100 beta-mercaptoethanol dilution in 0.1 M Na2PO4

20 Incubate at 30 °C statically for 01:00:00 and rotate every 00:15:00 by hand. 1h 15m

Use these incubations to make the plating media and autoclave it.

Additionally, set up your 42 °C water bath next to the fume hood. Use a thermometer to make sure the temperature is right.

21 Centrifuge at 700 x g, 0°C, 00:10:00, decant supernatant. 30m

Add 3 mL **0.6 M KCl**. Resuspend gently by adding the buffer along the side of the tube.

Centrifuge at 700 x g, 0°C, 00:10:00, decant supernatant.

Add 3 mL **0.6 M KCl**. Resuspend gently by adding the buffer along the side of the tube.

Centrifuge at 700 x g, 0°C, 00:10:00, decant supernatant.

22 Resuspend in 3 mL **Buffer 1**

23 In new 15 mL tubes, mix 1.5 mL of donor strain with 1.5 mL of recipient strain.

	A	B	C
		Donor strain	Donor strain
	Recipient strain	Evolution strain	Evolution strain
	NA	Control 1	-

Use appropriate EP-DNAPs

The remaining volumes can be carried forward as negative controls.

Mix by inversion gently a few times.

24 Centrifuge at 700 x g, 0°C, 00:10:00, decant supernatant. Resuspend in 5 mL of Buffer 2. 10m

The PEG will make resuspension difficult, so try your best with the serological pipette. Tiny, visible clumps of cells are OK, but should be avoided as they can make identification of emerging colonies difficult later on - avoid using the small pipettes.

25 Incubate at 30 °C for 00:30:00 with occasional inverting. 30m

26 Centrifuge at 700 x g, 0°C, 00:10:00, decant supernatant. 15m

The media should be done autoclaving at this point. Take out, add glucose and other required auxotrophic additives, and return back to the autoclave to maintain liquidity for at least

00:05:00

BE VERY CAREFUL NOT TO ADD ADDITIVES WHICH LOSE YOUR SELECTION SCHEME!!!! - PAY ATTENTION TO SELECTION MARKER IN P1 (LEU2) AND SELECTION MARKER IN EP-DNAP CONSTRUCT (In our case, this is URA3, but other plasmids have HIS3)

27 Resuspend in 5 mL **Buffer 3** with EXTREME GENTLENESS - the cells are protoplasted at this point.

28 Take media in the autoclave and place in 42 °C water bath, for a minimum of 3m

00:03:00 . **TIME IT!!!!!!**

You should be able to touch the flask with your wrist's bare skin and not feel pain, only warmth.

29 In a 50 mL falcon tube, add cells 1w 3d

Make 2-3 tubes per fusion:

- One with 250 µL of cells
- One with 500 µL of cells
- One with 1 mL of cells

Gently, add 25 mL of plating media, mix gently, and then pour in labelled plates. Allow plates to solidify and incubate at 30 °C for up to 240:00:00

30 On the day after, tape the lid with parafilm.