HERP: Haploid Engineering and Replacement Protocol for Saccharomyces

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Abstract

This protocol is from:
Alexander WG, Doering DT,and Hittinger CT (2014) High-Efficiency Genome Editing and Allele Replacement in Prototrophic and Wild Strains of Saccharomyces. *Genetics* 198:859-866; doi:10.1534/genetics.114.170118

Please see the full manuscript for additional details.

The purpose of this document is to provide you with an easy-to-follow guide to using the HERP cassettes. We will go through the preparation of the selection and counterselection media, the culturing and transformation for insertion of the HERP cassettes, and counterseletive replacement of the HERP cassettes.

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Guidelines

C. Inserting the HERP cassettes

1) Design primers with overhangs that target the cassette to your desired locus.

a) The 5' overhangs dictate where the cassette will be integrated, and the length needed depends on the
species you're manipulating (40 bp for *S. cerevisiae, S. paradoxus, S. uvarum*, & *S. eubayanus*, 50 bp for *S. mikatae*,
and 70 for *S. kudriavzevii; S. arboricola*'s length requirement is unknown). The longer these overhangs are the more
efficient integration will be, although longer overhangs usually mean longer oligonucleotides, which are expensive
and sometimes difficult to use.

b) The 3' ends amplify the cassette from either the primer or yeast genomic DNA. While we have constructed both plasmids and stably-integrated yeast strains with all three HERP cassettes, the yeast strains provide
an advantage over the plasmid constructs. HERP cassettes with an adjacent I-SceI recognition site are unstable in
bacteria, resulting in the plasmid never being recovered. In yeast, SCE1 is actively repressed while growing on glucose,

which prevents leaky nuclease expression. Because of this active repression, the yeast strains tolerate an I-SceI site
adjacent to the HERP cassettes, which in turn reduces the length of oligonucleotide needed to provide both a priming
site and a targeting overhang. The authors **strongly** recommend using the yeast strains as PCR templates for HERP
cassette amplification. If the plasmids are used, then the 18-bp I-SceI sequence must be included on the oligo
between the 3' amplification sequence and the 5' targeting overhang (Table S3).

Protocol

Preparing Media: Yeast Extract-Peptone-Glycerol +Antifolates (YPGly+ AF)

Step 1.
Add the following components to a 2-L Erlenmeyer flask:

PROTOCOL

. Yeast Extract-Peptone-Glycerol +Antifolates (YPGly+ AF) Media

CONTACT: Tracey DePellegrin

Step 1.1.
10 g yeast extract

Step 1.2.
20 g peptone

Step 1.3.
5 g sulfanilamide

Step 1.4.
50 mg hypoxanthine

Step 1.5.
18 g agar

Step 1.6.
900 mL ddH2O

Preparing Media: Yeast Extract-Peptone-Glycerol +Antifolates (YPGly+ AF)

Step 2.
Mix to dissolve as much as possible (agar and sulfanilamide won't dissolve until heated).

Preparing Media: Yeast Extract-Peptone-Glycerol +Antifolates (YPGly+ AF)

Step 3.
Autoclave for no more than 20 minutes on a liquid cycle.

DURATION
00:20:00

Preparing Media: Yeast Extract-Peptone-Glycerol +Antifolates (YPGly+ AF)

Step 4.
Once autoclaved, cool to 50° in a water bath, then add the following and mix:
-5 g thymidine
-200 mg methotrexate

-100 mL 50% (v/v) glycerol, sterilized

NOTES

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NOTA BENE: the standard operating procedure for adding compounds after autoclaving is to dissolve them in a solvent, filter, then add to the media; this generally is difficult or impossible for methotrexate and thymidine due to the amount required. For the last two years, I've been adding the solid chemicals directly to the cooled media, and I've never had contamination. I suspect that the extreme conditions prevent microbial growth. Also, both methotrexate and thymidine are sensitive to heat, so take care to not add them early.

Preparing Media: Yeast Extract-Peptone-Glycerol +Antifolates (YPGly+ AF)

Step 5.

Pour 20 mL into plastic petri dishes and allow to set. You have now made YPGly +AF media.

Preparing Media: Synthetic Complete +5-fluorodeoxyuridine (SC +FUdR)

Step 6.

In a 2-L Erlenmeyer flask, make 1 L of Synthetic Complete agar using your favorite provider's formulation.

Preparing Media: Synthetic Complete +5-fluorodeoxyuridine (SC +FUdR)

Step 7.

Autoclave then cool to 50° in a water bath.

Preparing Media: Synthetic Complete +5-fluorodeoxyuridine (SC +FUdR)

Step 8.

Dissolve 55 mg of FUdR into 1.1 mL of ddH2O and filter sterilize

Preparing Media: Synthetic Complete +5-fluorodeoxyuridine (SC +FUdR)

Step 9.

Add 1 mL of FUdR solution to cooled SC agar and mix.

Preparing Media: Synthetic Complete +5-fluorodeoxyuridine (SC +FUdR)

Step 10.

Pour 20 mL into plastic petri dishes and allow to set. You have now made SC +FUdR agar.

NOTES

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NB: an alternate method to make SC +FUdR plates is to make a 1000x stock solution of 50 mg/mL FUdR in water, filter, then spread enough concentrate onto the surface of a premade SC plate to bring the final concentration to 50 µg/mL (20 µL of concentrate mixed with 80 µL of water, then spread onto the surface of a plate containing 20 mL of SC agar)

Inserting the HERP cassettes

Step 11.

Design primers with overhangs that target the cassette to your desired locus. (See the guidelines for details.)

Inserting the HERP cassettes

Step 12.

Amplify the HERP cassette of choice using your targeting primers and a high-fidelity polymerase such as New England Biolab's Phusion system. If your reaction makes use of DMSO or other harsh chemicals, clean your PCR product with a column before proceeding.

REAGENTS

Phusion High-Fidelity DNA Polymerase - 100 units M0530S by New England Biolabs

Inserting the HERP cassettes

Step 13.
Culture your strain of choice by inoculating 50 mL of YPD media with enough overnight culture of your strain to bring the OD600 to 0.2-0.25. Shake at the optimal temperature for your strain or species until the culture's OD600 reaches 0.85-1.0.

Inserting the HERP cassettes

Step 14.
Shake at the optimal temperature for your strain or species until the culture's OD600 reaches 0.85-1.0.

Inserting the HERP cassettes

Step 15.
Harvest the cells by centrifugation in a 50-mL conical vial at 3000 RPM for 5 minutes.

DURATION
00:05:00

Inserting the HERP cassettes

Step 16.
Remove supernatant, wash with 25 mL water, and spin at 3000 RPM for 5 minutes.

DURATION
00:05:00

Inserting the HERP cassettes

Step 17.
Remove supernatant and suspend cells in 1 mL of water.

Inserting the HERP cassettes

Step 18.
Aliquot 100 µL cell suspension to microcentrifuge tubes, spin for 30 seconds at max speed in a microcentrifuge, and remove supernatant.

DURATION
00:00:30

Inserting the HERP cassettes

Step 19.
Add the following reagents to each cell pellet IN ORDER:

PROTOCOL

. HERP Insertion Mixture

CONTACT: Tracey DePellegrin

Step 19.1.
240 µL 50% polyethylene glycol, average MW 4000, filter sterilized

Step 19.2.
36 µL 1 M lithium acetate, filter sterilized

Step 19.3.
5 µL 20 mg/mL boiled sonicated salmon sperm DNA

Step 19.4.
79 µL HERP cassette PCR product or water (for control)

Inserting the HERP cassettes

Step 20.

Suspend cell pellet in transformation mixture and heat shock.

NOTES

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NB: for optimal transformation efficiency, you must empirically determine what time, temperature, and/or additive conditions give the most transformants for your species or strain. Our suggestion is to use a yeast replicating plasmid with a dominant drug marker and evaluate a number of conditions as in Gietz & Woods, 2002. In general, 30 minute heat shocks at 42° works well for S. cerevisiae, while the psychrophillic species generally only tolerate heat shocks of 37° (S. kudriavzevii only tolerate 34°. S. mikatae doesn't tolerate the transformation reaction conditions well, and requires a room temperature incubation of 10 minutes followed by a 37° shock for 5 minutes.

Inserting the HERP cassettes

Step 21.

Once heat-shocking has been completed, spin the reactions for 30 seconds at max speed, remove the supernatant, and suspend the cells in 600 µL of YPD.

DURATION

00:00:30

Inserting the HERP cassettes

Step 22.

Transfer to glass culture tubes and spin in a culture wheel for 3 hours at the strain's or species' optimal temperature.

DURATION

03:00:00

Inserting the HERP cassettes

Step 23.

Spread 200 µL of recovered cells to each of three YPGly +AF plates. Only one 200 µL volume of control reaction, however, needs to be plated. Once all the liquid has been absorbed, store agar up at the optimal temperature. Colonies will appear in 3-10 days.

Inserting the HERP cassettes

Step 24.

Streak colonies out to fresh YPGly +AF plates. Analyze by amplifying target locus via PCR and/or sequencing across the insertion junction.

Counterselective Replacement of the HERP Cassette

Step 25.

Once you have molecularly confirmed the insertion of the HERP cassette, phenotypically confirm its sensitivity to FUdR by spotting 1,000 cells onto SC +FUdR plates multiple times. Sensitive strains should exhibit no growth, while insensitive strains will rapidly grow.

Counterselective Replacement of the HERP Cassette

Step 26.

Once your HERP insertion is confirmed and you have established FUdR sensitivity, begin by inoculating the strain in 50 mL of 2X YPA100 +4% galactose (see main text) to an OD600 of 0.2-0.25 and culture at the optimal temperature.

Counterselective Replacement of the HERP Cassette

Step 27.

Once an OD600 of 0.85-1.0 is reached, repeat steps C4 to C6, except replace the HERP cassette PCR product in C6 with your desired replacement PCR product.

Counterselective Replacement of the HERP Cassette

Step 28.

Once the heat shock is completed, remove the supernatant and suspend in 600 µL water.

Counterselective Replacement of the HERP Cassette

Step 29.

Spread 200 µL onto each of three SC plates.

Counterselective Replacement of the HERP Cassette

Step 30.

Incubate at optimal temperature for 24 hours.

DURATION

24:00:00

Counterselective Replacement of the HERP Cassette

Step 31.

After 24 hours, incubate plates at 4° for one hour.

DURATION

01:00:00

Counterselective Replacement of the HERP Cassette

Step 32.

Lightly replicate plates to SC +FUdR plates.

Counterselective Replacement of the HERP Cassette

Step 33.

Re-replicate to fresh FUdR plates no more than once a day to reduce background growth. Colonies will appear in 2-5 days, longer if glucose is replaced by glycerol.