

Single-step Marker Switching in *Schizosaccharomyces pombe* Using a Lithium Acetate Transformation Protocol

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[Abstract] The ability to utilize different selectable markers for tagging or mutating multiple genes in *Schizosaccharomyces pombe* is hampered by the historical use of only two selectable markers, *ura4⁺* and *kanMX6*; the latter conferring resistance to the antibiotic G418 (geneticin). More markers have been described recently, but introducing these into yeast cells often requires strain construction from scratch. To overcome this problem we and other groups have created transformation cassettes with flanking homologies to *ura4⁺* and *kanMX6* which enable an efficient and time-saving way to exchange markers in existing mutated or tagged fission yeast strains.

Here, we present a protocol for single-step marker switching by lithium acetate transformation in fission yeast, *Schizosaccharomyces pombe*. In the following we describe how to swap the *ura4⁺* marker to a *kanMX6*, *natMX4*, or *hphMX4* marker, which provide resistance against the antibiotics G418, nourseothricin (clonNAT) or hygromycin B, respectively. We also detail how to exchange any of the *MX* markers for nutritional markers, such as *arg3⁺*, *his3⁺*, *leu1⁺* and *ura4⁺*.

Keywords: *Schizosaccharomyces pombe*, Selectable marker, Marker switch, Li-Acetate transformation, Gene tagging, Gene deletion, Genetic manipulation

[Background] This single-step marker swap protocol for *Schizosaccharomyces pombe* allows for any tagged or mutated gene marked with an *MX*-type antibiotic marker to be swapped for a nutritional marker (cassettes containing the *arg3⁺*, *his3⁺*, *leu1⁺*, and *ura4⁺* have been constructed) and to exchange genetic *ura4⁺*-markers for any *MX*-type antibiotic resistance marker (*kanMX*, *natMX*, and *hphMX* constructs have been tested for this study) (Lorenz *et al.*, 2015a). Previously, this kind of approach was only feasible for *MX*-type antibiotic resistance markers (Sato *et al.*, 2005; Hentges *et al.*, 2005). Exchanging antibiotic resistance markers for each other already represented a basic set of useful genetic tools, the *ura4⁺*-to-*MX* as well as the *arg3MX4*, *his3MX4*, *leu1MX4*, and *ura4MX4* marker swap cassettes expand this genetic toolbox for tagging and mutating genes in fission yeast (Lorenz *et al.*, 2015a). The lithium acetate transformation protocol itself was described previously (Keeney and Boeke, 1994) and recently suggested modifications (<http://listserver.ebi.ac.uk/pipermail/pombelist/2014/004012.html>) were incorporated to provide a highly efficient procedure. Streamlining *Schizosaccharomyces pombe* strain construction in this way is time-saving and, therefore, will prove useful for fission yeast researchers.

Materials and Reagents

1. Conical tubes:
 - 15 ml (Greiner Bio One, catalog number: 188261)
 - 50 ml (Greiner Bio One, catalog number: 227261)
2. 1.5 ml centrifuge tubes (Greiner Bio One, catalog number: 616201)
3. 0.2 ml flat-cap PCR tubes (Corning, Axygen®, catalog number: PCR-02-C)
4. Petri dishes (Greiner Bio One, catalog number: 633185)
5. BD Plastipak 10 ml syringes (BD, catalog number: 302188)
6. Millex-GP 33 mm diameter sterile syringe filter units (Polyethersulfone [PES] membrane, pore size: 0.22 µm) (EMD Millipore, catalog number: SLGPO33RS)
7. Appropriate *Schizosaccharomyces pombe* strains: for a marker swap the strain must already carry a mutant or tagged gene marked with either an *ura4⁺* or *MX*-type marker, such as *kanMX*, *natMX*, or *hphMX* (Bähler *et al.*, 1998; Goldstein and McCusker, 1999). When introducing *arg3MX4*, *his3MX4*, *leu1MX4*, or *ura4MX4* into a strain, this strain needs to be mutated for the respective gene at its original locus, *e.g.*, *arg3-D4* (Waddell and Jenkins, 1995), *his3-D1* (Burke and Gould, 1994), *leu1-32* (Keeney and Boeke, 1994), or *ura4-D18* (Grimm *et al.*, 1988).
8. Plasmids: pALo120 (FYP2884), pALo121 (FYP2885), pALo122 (FYP2886), pFA6a-*arg3MX4* (FYP2890), pFA6a-*his3MX4* (FYP2891), pFA6a-*leu1MX4* (FYP2892), and pFA6a-*ura4MX4* (FYP2893) (Lorenz, 2015a and 2015b); plasmid can be obtained from the National BioResource Project (NRBP) of MEXT, Japan (please refer to FYP numbers when ordering).
9. Sonicated salmon sperm DNA (Sigma-Aldrich, catalog number: 31149)
10. Agarose, molecular grade (Bioline, catalog number: BIO-41026)
11. Q5 high fidelity DNA polymerase (New England Biolabs, catalog number: M0491)
12. 5x Q5 reaction buffer (accessory part of Q5 high fidelity polymerase) (New England Biolabs, catalog number: M0491)
13. 10 mM dNTPs (Thermo Fisher Scientific, Thermo Scientific™, catalog number: R0191)
14. 10 µM oligonucleotides (to be used as primers in PCR reactions) (Sigma-Aldrich, USA)
15. MilliQ H₂O drawn from a MilliQ Advantage A10 system (EMD Millipore, catalog number: Z00Q0V0WW)
16. CutSmart buffer (accessory part of restriction enzymes, can also be ordered separately under New England Biolabs, catalog number: B2704)
17. Restriction enzymes:
 - Xba*I restriction enzyme (New England Biolabs, catalog number: R0145)
 - Bam*HI-HF restriction enzyme (New England Biolabs, catalog number: R3136)
 - Eco*RI-HF restriction enzyme (New England Biolabs, catalog number: R3101)
 - Pvu*II-HF restriction enzyme (New England Biolabs, catalog number: R3151)
 - Sac*I-HF restriction enzyme (New England Biolabs, catalog number: R3156)
18. Di-methyl sulfoxide (DMSO) (Sigma-Aldrich, catalog number: D8418)

19. Ethylenediaminetetraacetic acid (EDTA) (Thermo Fisher Scientific, Fisher Scientific, catalog number: 10526383)
20. Sodium hydroxide (NaOH) pellets (anhydrous) (Sigma-Aldrich, catalog number: S8045)
21. Tris(hydroxymethyl)aminomethane (VWR, catalog number: 103156X)
22. Glacial (100%) acetic acid (VWR, catalog number: 20104.334)
23. Yeast extract, microgranulated (ForMedium™, catalog number: YEM02)
24. Glucose anhydrous (Thermo Fisher Scientific, Fisher Scientific, catalog number: 10373242)
25. Adenine (Sigma-Aldrich, catalog number: A5665)
26. Uracil (ForMedium™, catalog number: DOCO212)
27. Leucine (ForMedium™, catalog number: DOCO156)
28. Lysine (Sigma-Aldrich, catalog number: L8662)
29. Histidine (Sigma-Aldrich, catalog number: H5659)
30. Arginine (Sigma-Aldrich, catalog number: A6969)
31. Agar granulated (ForMedium™, catalog number: AGR10)
32. G418 disulfate (ForMedium™, product number: G4181)
33. Nourseothricin-dihydrogen sulfate (clonNAT) (Werner BioAgents, catalog number: clonNAT)
34. Hygromycin B (ForMedium™, catalog number: HYG1000)
35. Yeast nitrogen base without amino acids and without ammonium sulfate (ForMedium™, catalog number: CYN0502)
36. L-glutamic acid monosodium salt hydrate (sodium glutamate) (Sigma-Aldrich, catalog number: G5889)
37. 5 N hydrochloric acid (HCl) (VWR, catalog number: 30018.320)
38. Lithium acetate (Sigma-Aldrich, catalog number: L4158)
39. Polyethylene glycol (PEG) MW 3,350 (Sigma-Aldrich, catalog number: P4338)
40. DNA molecular weight marker, HyperLadder™ 1 kb (Bioline, catalog number: BIO-33025)
41. 0.5 M EDTA (pH 8.0) (see Recipes)
42. 50x TAE gel electrophoresis buffer (see Recipes)
43. YES (Yeast extract supplemented) broth (see Recipes)
44. YES plates (see Recipes)
45. Concentration of antibiotics in YES media (see Recipes)
46. YNG (Yeast nitrogen base glutamate) plates (see Recipes)
47. 1 M Tris/HCl (pH 7.5) (see Recipes)
48. 1 M LiAc (pH 7.5) (see Recipes)
49. 10x TE (pH 7.5) (see Recipes)
50. LiAc/TE (see Recipes)
51. 40% PEG (see Recipes)

Equipment

1. Erlenmeyer glass flasks for culturing yeast (DURAN Group, catalog number: 2177144)
2. Infors HT multitron standard shaking incubator (Infors, model: Multitron Standard)
3. Static incubator (Gallenkamp forced air incubator) (Weiss Technik UK)
4. Water baths (Grant Instruments, catalog number: JBN5)
5. Eppendorf microcentrifuge (Eppendorf, model: 5424R)
6. MJ Research PTC-100 programmable thermal cycler
7. Nucleic acid gel electrophoresis system consisting of a gel electrophoresis chamber with a 7 x 10 cm tray (Bio-Rad Laboratories, model: Mini-Sub[®] Cell GT) and a power supply (Bio-Rad Laboratories, model: PowerPac[™] Basic Power Supply)
8. NanoDrop 2000c UV/Vis-spectrophotometer (Thermo Fisher Scientific, Thermo Scientific[™], model: ND-2000C)
9. Sigma 4-16K centrifuge (Sigma Laborzentrifugen, model: Sigma 4-16K)
10. Haemocytometer, type Neubauer improved (Marienfeld-Superior, catalog number: 0630010)
11. Leica DM500 microscope (Leica Microsystems, model: Leica DM500)
12. Classic Media 12 L autoclave (Prestige Medical, model: 210048)

Procedure

A. Generation of marker swap cassettes for transformation

Marker swap cassettes to be used in the lithium acetate protocol below can be generated by two alternative means; by PCR amplification from or by restriction endonuclease digestion of the plasmids previously described in Lorenz (2015a and 2015b) (see below). Using appropriate modifications, this protocol can also be applied to constructs described elsewhere (Sato *et al.*, 2005; Hentges *et al.*, 2005; Gadaleta *et al.*, 2013; Chen *et al.*, 2015). After the PCR or restriction digest run 1/20 volume of each reaction on a 0.8% agarose gel in 1x TAE at 80 V for 45 min to verify that the reaction has worked properly (band sizes to be expected for each reaction are detailed below). The remainder of the PCR reaction or restriction is stopped by the addition of 2 μ l of 0.5 M EDTA (pH 8.0). DNA concentration is measured on a NanoDrop 2000c UV/Vis-spectrophotometer, and an appropriate volume (maximum 20 μ l) to result in 1-5 μ g of cassette DNA is used for each transformation.

1. *ura4⁺* marker swap cassette amplification
 - a. A 50 μ l PCR reaction contains:
 - 100 ng of DNA from plasmid pALo120, pALo121, or pALo122 (Lorenz, 2015a and 2015b) to create cassettes by PCR for swapping an *ura4⁺* marker to a *kanMX6*, *natMX4*, or *hphMX4* marker, respectively.
 - 10 μ l 5x Q5 reaction buffer
 - 200 μ M dNTPs

- 500 nM each of AL1forw (5'-agctacaaatcccactgg-3') and AL1rev (5'-gtgatattgacgaaactttttg-3') oligonucleotides as primers
1 U Q5 high-fidelity DNA polymerase
Make up to 50 µl by adding the appropriate amount of sterile MilliQ water (see Note 1).
- b. Use the following PCR programme:
 - 30 sec at 98 °C
 - 35 x (10 sec at 98 °C, 20 sec at 55 °C, 85 sec at 72 °C)
 - 120 sec at 72 °C
 - c. Expected band sizes for transformation cassettes:
 - pALo120 (*ura4⁺*-to-*kanMX6* swapping cassette): 1.97 kb
 - pALo121 (*ura4⁺*-to-*natMX4* swapping cassette): 1.74 kb
 - pALo122 (*ura4⁺*-to-*hphMX4* swapping cassette): 2.2 kb
2. Restriction digest to liberate *ura4⁺* marker swap cassettes
 - a. A 20 µl reaction contains:
 - 12 µg of DNA from plasmid pALo120, pALo121, or pALo122 (this will result in ~5 µg of marker swap cassette DNA) (Lorenz, 2015a and 2015b).
 - 2 µl CutSmart buffer
 - 2 µl *Xba*I restriction enzyme
 Make up to 20 µl by adding the appropriate amount of sterile MilliQ water (see Note 1).
 - b. Incubate reaction at 37 °C for 1 h.
 - c. Expected band sizes after restriction digest:
 - pALo120: 1.98 kb (cassette) and 2.64 kb (vector backbone)
 - pALo121: 1.75 kb (cassette) and 2.64 kb (vector backbone)
 - pALo122: 2.2 kb (cassette) and 2.64 kb (vector backbone)
 3. *MX* marker swap cassette amplification
 - a. A 50 µl PCR reaction contains:
 - 100 ng of DNA from plasmid pFA6a-*arg3MX4*, pFA6a-*his3MX4*, pFA6a-*leu1MX4*, or pFA6a-*ura4MX4* (Lorenz, 2015a and 2015b) to create cassettes by PCR for swapping any *MX*-type marker to a *arg3⁺*, *his3⁺*, *leu1⁺*, or *ura4⁺* marker, respectively.
 - 10 µl 5x Q5 reaction buffer
 - 200 µM dNTPs
 - 500 nM each of AL2forw (5'-gtttagcttgctcgtccc-3') and AL2rev (5'-gatggcggcggttagtatcg-3') oligonucleotides as primers
 - 1 U Q5 high-fidelity DNA polymerase
 Make up to 50 µl by adding the appropriate amount of sterile MilliQ water (see Note 1).
 - b. Use the following PCR programme:
 - 30 sec at 98 °C
 - 35 x (10 sec at 98 °C, 20 sec at 64 °C, 90 sec at 72 °C)
 - 120 sec at 72 °C

- c. Expected band sizes for transformation cassettes:
 - pFA6a-*arg3MX4*: 2.54 kb
 - pFA6a-*his3MX4*: 2.65 kb
 - pFA6a-*leu1MX4*: 2.19 kb
 - pFA6a-*ura4MX4*: 2.39 kb
 4. Restriction digest to liberate *arg3MX4*, *leu1MX4*, and *ura4MX4* marker swap cassettes
 - a. A 20 µl reaction contains:
 - 10 µg of DNA from plasmid pFA6a-*arg3MX4*, pFA6a-*leu1MX4*, or pFA6a-*ura4MX4* (this will result in ~5 µg of marker swap cassette DNA) (Lorenz, 2015a and 2015b).
 - 2 µl CutSmart buffer
 - 1 µl *Bam*HI-HF restriction enzyme
 - 1 µl *Eco*RI-HF restriction enzyme
 - Make up to 20 µl by adding the appropriate amount of sterile MilliQ water (see Note 1).
 - b. Incubate reaction at 37 °C for 1 h.
 - c. Expected band sizes after restriction digest:
 - pFA6a-*arg3MX4*: 2.6 kb (cassette) and 2.47 kb (vector backbone)
 - pFA6a-*leu1MX4*: 2.24 kb (cassette) and 2.47 kb (vector backbone)
 - pFA6a-*ura4MX4*: 2.44 kb (cassette) and 2.47 kb (vector backbone)
 5. Restriction digest to liberate the *his3MX4* marker swap cassette
 - a. A 20 µl reaction contains:
 - 10 µg of DNA from plasmid pFA6a-*his3MX4* (this will result in ~5 µg of marker swap cassette DNA) (Lorenz, 2015a and 2015b).
 - 2 µl CutSmart buffer
 - 1 µl *Pvu*II-HF restriction enzyme
 - 1 µl *Sac*I-HF restriction enzyme
 - Make up to 20 µl by adding the appropriate amount of sterile MilliQ water (see Note 1).
 - b. Incubate reaction at 37 °C for 1 h.
 - c. Expected band sizes after restriction digest:
 - pFA6a-*his3MX4*: 2.72 kb (cassette) and 2.44 kb (vector backbone)
- B. Lithium acetate transformation
1. Grow 100 ml of yeast cells from the strain(s) to be transformed in YES broth in a shaking incubator at 30 °C to a density of ~1 x 10⁷ cells/ml (count with haemocytometer to establish density) (see Notes 2 and 3).
 2. Harvest by centrifugation (in two 50 ml conical tubes) at 1,900 x g for 3 min at 20 °C.
 3. Resuspend cells in 25 ml of sterile MilliQ water, and combine into one 50 ml conical tube.
 4. Harvest by centrifugation at 1,900 x g for 3 min at 20 °C.
 5. Resuspend cells in 5 ml LiAc/TE (see Recipes), centrifuge at 1,900 x g for 3 min at 20 °C.
 6. Resuspend cells in 1 ml LiAc/TE and transfer to 1.5 ml centrifuge tube.

7. Centrifuge at 2,300 x g for 1 min at room temperature. Discard supernatant and resuspend cells at $\sim 3 \times 10^9$ cells/ml in LiAc/TE (normally 300 μ l, if starter culture was 100 ml of 1×10^7 cells/ml).
8. To 100 μ l of cells add 2 μ l of sonicated salmon sperm DNA (10 mg/ml) and the DNA to transform (1-5 μ g in a maximum volume of 20 μ l). Mix well by carefully pipetting up and down.
9. Incubate at room temperature for 10 min.
10. Add 260 μ l 40% PEG (see Recipes and Note 4) and incubate for 2-6 h at 30 °C. Mix well by carefully pipetting up and down.
11. Add 43 μ l DMSO (dimethyl sulfoxide), mix well by inverting the tube five times, and heat shock for 5 min at 42 °C (see Note 5).
12. Plate suspension by evenly spreading onto 3 YES plates (~ 135 μ l on each plate) without selection (see Recipes).
13. After 24-48 h incubation at 30 °C, replica-plate these plates onto selective plates; either onto YES containing the corresponding antibiotic, or onto YNG lacking the corresponding supplement.
14. Allow the selective plates to grow at 30 °C for several days until single colonies reach a diameter of 3-4 mm.
15. Patch at least 20 colonies onto a fresh selective plate and incubate 24-48 h.
16. Replica-plate onto a fresh selective plate and onto a plate selecting for the original marker, to ensure that the marker swap is correct (e.g., if the original strain was *ura4⁺* and pALo120 was used to swap to *kanMX6*, the resulting strain must be resistant to G418 and unable to grow on media lacking uracil).

Data analysis

The critical step of the single-step marker swap procedure is to confirm that the markers have been truly exchanged in a transformant, *i.e.*, that the new marker is correctly integrated at the target site, thereby removing the original marker. It is known that marker integration at its intended target site is not perfectly efficient in *Schizosaccharomyces pombe*, and a wide range of correct integration frequencies have been reported (Bähler *et al.*, 1998; Sato *et al.*, 2005). Correct integration is influenced by several parameters, including the chromatin status of the genomic site and length of sequence homologies flanking the marker cassettes. In the presented single-step marker swap cassettes flanking sequence homologies are between 200 and 400 bps, which is longer than the required minimum 80 bps (Bähler *et al.*, 1998), but not long enough to enable 100% correct targeting in all cases. Swapping the *ura4⁺* marker to any of the antibiotic *MX* markers occurred at frequencies between 22.9-100%. The correct integration efficiency of swapping an *MX* marker to a nutritional marker was tested at the meiotic gene *hop1*; this is more challenging due to the closed chromatin state at meiotic open reading frames (Bähler *et al.*, 1998). Therefore, the frequency of exchanging a *kanMX6*-marker deleting *hop1* with a nutritional marker was lower and varied between 15.3-36.6% (Lorenz, 2015a).

Notes

1. When pipetting a PCR reaction or restriction digest, always start with the MilliQ water, add the buffer, then all other components, and finally the enzyme(s). Working from these protocols, it is important to calculate the amount of water required before one sets up the reaction.
2. For the transformation protocol use yeast cells streaked onto the appropriate plate not longer than 7 days beforehand. Always check the genotype of yeast strains carefully before usage!
3. It is crucial to grow the fission yeast cells to late logarithmic phase ($\sim 1 \times 10^7$ cells/ml) to achieve the most efficient transformation frequency. Growing cells to higher density will result in drastically reduced numbers of transformants, because cells enter stationary phase and develop a thicker cell wall (*i.e.*, do not grow cells to higher than $\sim 1 \times 10^7$ cells/ml and dilute).
4. 40% PEG (see Recipes) needs to be prepared freshly on the day when the transformation is performed.
5. The duration and temperature of the heat shock (step B11) is critical!

Recipes

1. 0.5 M EDTA (pH 8.0) (1 L)
 - 186.1 g ethylenediaminetetraacetic acid (EDTA)
 - Add 800 ml MilliQ water
 - Adjust pH to 8.0 by adding NaOH pellets (solution will only become clear when pH > 7)
 - Make up with MilliQ water to volume
 - Sterilize by autoclaving
2. 50x TAE gel electrophoresis buffer (1 L)
 - 242 g Tris(hydroxymethyl)aminomethane
 - 57.1 ml glacial acetic acid
 - 100 ml 0.5 M EDTA (pH 8.0)
 - Make up with MilliQ water to volume
 - Sterilize by autoclaving
 - Use as 1x TAE by diluting 1:50 in MilliQ water
3. YES (Yeast extract supplemented) broth (1 L)
 - 5 g yeast extract, microgranulated
 - 30 g glucose anhydrous
 - 250 mg adenine
 - 250 mg uracil
 - 250 mg leucine
 - 250 mg lysine
 - 250 mg histidine
 - 250 mg arginine

- Make up with MilliQ water to volume
 Sterilize by autoclaving
4. YES plates (1 L, makes about 40 plates)
 - As YES broth
 - 20 g agar granulated
 - Sterilize by autoclaving
 5. Concentration of antibiotics in YES media (per 1 L)
 - Note: Antibiotics are added to YES agar cooled down to 55 °C after autoclaving!*
 - 200 mg G418 disulfate
 - 200 mg nourseothricin-dihydrogen sulfate (clonNAT)
 - 400 mg hygromycin B
 6. YNG (Yeast nitrogen base glutamate) plates (1 L, makes about 40 plates)
 - 1.9 g yeast nitrogen base (without amino acids and without ammonium sulfate)
 - 3.7 g L-glutamic acid monosodium salt hydrate (sodium glutamate)
 - 30 g glucose anhydrous
 - 20 g agar granulated
 - 75 mg adenine
 - 75 mg uracil
 - 75 mg leucine
 - 75 mg lysine
 - 75 mg histidine
 - 75 mg arginine
 - Make up with MilliQ water to volume
 - Sterilize by autoclaving
 7. 1 M Tris/HCl (pH 7.5) (1 L)
 - 121.1 g Tris(hydroxymethyl)aminomethane
 - Add 800 ml MilliQ water
 - Adjust pH to 7.5 by adding 5 N HCl
 - Make up with MilliQ water to volume
 - Sterilize by autoclaving
 8. 1 M LiAc (pH 7.5; 100 ml)
 - 20.4 g lithium acetate
 - Add 70 ml MilliQ water
 - Adjust pH to 7.5 with acetic acid, if necessary
 - Make up with MilliQ water to volume
 - Sterilize by autoclaving
 9. 10x TE (pH 7.5; 100 ml)
 - 2 ml 0.5 M EDTA (pH 8.0) (final concentration 0.01 M)
 - 10 ml 1 M Tris/HCl (pH 7.5) (final concentration 0.1 M)

Make up with MilliQ water to volume

Sterilize by autoclaving

10. LiAc/TE (10 ml)

1 ml 1 M LiAc (pH 7.5)

1 ml 10x TE (pH 7.5) (0.1 M Tris, 0.01 M EDTA)

8 ml sterile MilliQ water

11. 40% PEG (10 ml)

4 g PEG (polyethylene glycol) MW 3,350

1 ml 1 M LiAc (pH 7.5)

1 ml 10x TE (0.1 M Tris, 0.01 M EDTA)

4 ml sterile MilliQ water

Dissolve PEG completely (incubate at 37 °C for a few minutes, if necessary).

Make up to 10 ml

Filter sterilize

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