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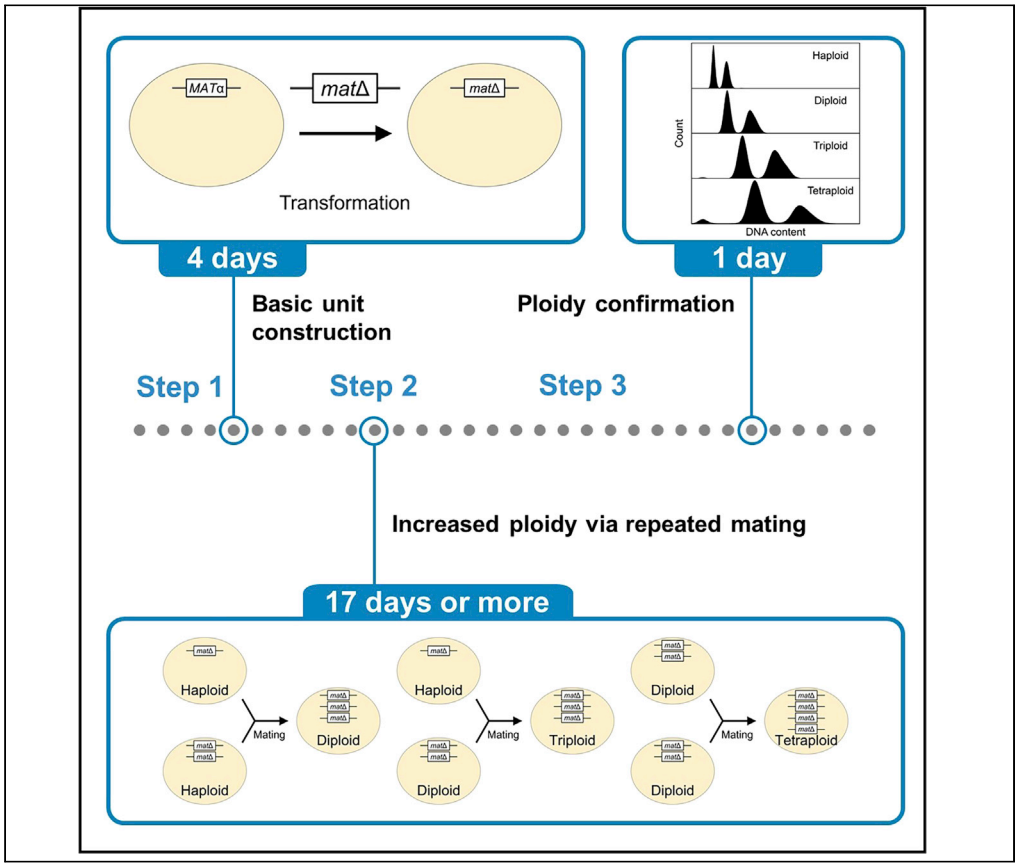
Protocol

Artificial control of mating type and repeated mating to produce polyploid cells in *Saccharomyces cerevisiae*



The budding yeast *Saccharomyces cerevisiae* is an excellent model for examining the effects of ploidy. Here, we provide a protocol for producing polyploid cells by creating a basic unit (*mat*Δ) and polyploidizing it via repeated mating. We describe steps for basic unit construction by one-step transformation, increased ploidy via repeated mating, and ploidy confirmation using flow cytometry. This protocol can be broadly applied to evaluate the physiology of polyploid cells.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Protocol for artificially controlling mating type and obtaining cells with desired ploidy

Plasmid-borne *MAT*a or α gene controls mating type of *MAT*-deficient (*mat*Δ) cell

Repeated mating of *mat*Δ strains with plasmids in and out increases ploidy

Ploidy is confirmed by propidium iodide staining and flow cytometry

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Artificial control of mating type and repeated mating to produce polyploid cells in *Saccharomyces cerevisiae*

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SUMMARY

The budding yeast *Saccharomyces cerevisiae* is an excellent model for examining the effects of ploidy. Here, we provide a protocol for producing polyploid cells by creating a basic unit (*mat*Δ) and polyploidizing it via repeated mating. We describe steps for basic unit construction by one-step transformation, increased ploidy via repeated mating, and ploidy confirmation using flow cytometry. This protocol can be broadly applied to evaluate the physiology of polyploid cells.

For complete details on the use and execution of this protocol, please refer to Oya and Matsuura (2022).[1]

BEFORE YOU BEGIN

We propose a simple method to artificially manipulate mating type in *Saccharomyces cerevisiae* to produce polyploid cells via repetitive mating. We prepared a mating type locus-deficient (*mat*Δ) strain and plasmids with *MAT*a or *MAT*α idiotypes (named YCpMATa and YCpMATα, respectively).[2]

Since the *MAT*α idiotype is responsible for mating type determination in *S. cerevisiae*, *mat*Δ cells fundamentally behave like *MAT*a cells. However, because *mat*Δ/*MAT*α diploids formed by mating *mat*Δ cells with *MAT*α cells do not have the *MAT*a idiotype, they behave as the α-mating type and are unable to sporulate. Because of these properties, *mat*Δ is called an a-like faker.[3] This protocol takes advantage of the fact that *mat*Δ with YCpMATa or YCpMATα shows the a- or α-mating type, respectively. Furthermore, when the plasmid is dropped, it becomes *mat*Δ again.

This protocol can be used to create cells with the desired ploidy or to investigate the copy number effect of the gene of interest on the polyploid phenotype. The latter is expected to provide insight into the nature and regulation of the gene of interest and the encoded protein.

Prepare mating type locus-deficient (*mat*Δ) strain

Timing: 4 days

The mating type (*MAT*) locus is recombined with a selective marker gene *kanMX* to generate the *mat*Δ strain. The *mat*Δ cell is the basic unit used to construct subsequent polyploid cells.

1. Amplification of PCR fragments.

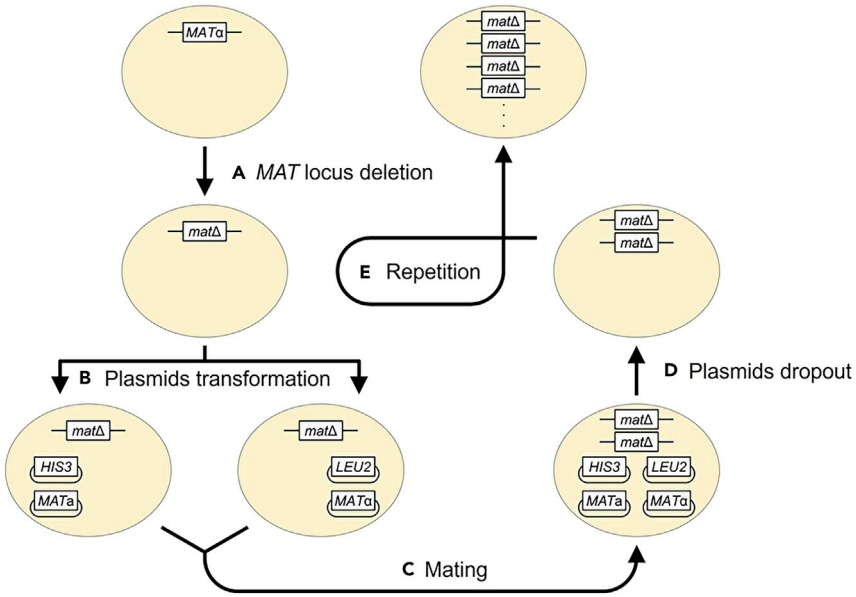

Figure 1. Scheme of *mat*Δ polyploid strain construction

(A) *mat*Δ cells were obtained by transforming the *mat*::*kanMX* PCR fragment into *MAT*α cells (BY4742).

(B) YCpMATa and pRS313 were successively introduced into *mat*Δ strain to obtain *mat*Δ YCpMATa pRS313 cells (left). In parallel, YCpMATα and pRS315 were successively introduced to obtain *mat*Δ YCpMATα pRS315 cells (right).

(C) *mat*Δ YCpMATa pRS313 cells and *mat*Δ YCpMATα pRS315 cells were mated to obtain *mat*Δ/*mat*Δ YCpMATa YCpMATα pRS313 pRS315 cells.

(D) Select cells dropped plasmids to obtain *mat*Δ/*mat*Δ (diploid) cells.

(E) By combining *mat*Δ cells with various ploidies and repeating steps (B) to (D), *mat*Δ polyploid cells with the desired ploidy can be produced.

PCR-mediated amplification of *mat*::*kanMX* from plasmid pFA6a-kanMX.[4] Amplify *mat*::*kanMX* cassette with primer pair MAT del F and MAT del R using KOD FX Neo polymerase. Successful amplification will yield PCR fragment of 1,570 bp.

> ***Note:*** KOD FX Neo enzyme solution has 3′→5′ exonuclease (proof-reading) activity and provides high fidelity. Other high-fidelity polymerases are possible alternatives.

2. Transformation of PCR fragments (Figure 1A).
   a. Inoculate BY4742 strain in 1 mL YPD liquid medium using a 5 mL culture tube and incubate at 30°C for 16 h with spinning at 45 rpm.
   b. Transplant 200 μL of overnight culture to 10 mL fresh YPD liquid medium in a 50 mL conical tube and incubate at 30°C for 4 h with shaking at 200 rpm.
   c. Pellet at 1,500 × *g*, 2 min, wash with 10 mL ddH_2O, repellet at 1,500 × *g*, 2 min and suspend the pellet in 1 mL LiAc and incubate at 30°C for 1 h.
   d. Transfer transformation mixture to 1.5 mL tube and pellet at 1,500 × *g*, 2 min and resuspend the pellet in 100 μL LiAc.
   e. Add 10 μL of 10 mg/mL denatured ssDNA and 10 μg of crude PCR product and incubate at 30°C for 30 min.
   f. Add 700 μL Li-PEG and incubate at 30°C for 30 min.
   g. Add 50 μL DMSO and heat shock at 42°C for 15 min.
   h. Pellet at 1,500 × *g*, 2 min, wash with 400 μL ddH_2O and repellet at 1,500 × *g*, 2 min.
   i. Suspend the pellet in 700 μL YPD and incubate at 30°C for 2 h with spinning at 45 rpm for curing.
   j. Pellet at 1,500 × *g*, 2 min and discard the supernatant.
   k. Suspend the pellet in 100 μL ddH_2O and plate onto a YPD plate containing 250 μg/mL G418.

l. Positive clones will appear after 2–3 days incubation at 30°C.
m. Successful integration of *mat*::*kanMX* will yield PCR fragment of 2,791 bp by amplifying the genomic DNA with primer pair MAT check F and MAT check R.

Pause point: The selected strain can be stored at −80°C as glycerol stocks until ready to proceed to the next steps.

⚠ CRITICAL: The *mat*::*kanMX* cassette amplified with primer pair MAT del F and MAT del R has short flanking sequences and is prone to mistargeting to loci other than *MAT*. We recommend checking more than 10 transformants (see troubleshooting section for additional information).

Note: Since the *mat*::*kanMX* strain from which the *MATα* idiotype has been removed will exhibit an a-mating type phenotype instead of α-mating type, successful integration of *mat*::*kanMX* can be confirmed by checking shmoo formation by incubating in YPD liquid medium containing 4 μg/mL α-factor at 30°C for 2 h.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Agar powder	Nacalai Tesque	01028-85
Bacto Yeast Extract	Gibco	212750
Hipolypepton	FUJIFILM Wako	392-02115
Difco Yeast Nitrogen Base w/o Amino Acids and Ammonium Sulfate (YNB w/o AA, AS)	Becton Dickinson	233520
Ammonium sulfate	Nacalai Tesque	02619-15
D-(+)-Glucose	Nacalai Tesque	16806-25
Bacto Casamino Acids	Becton Dickinson	223050
G418 Disulfate	Nacalai Tesque	16512-94
5-Fluoroorotic acid monohydrate 98%	Apollo Scientific	PC4054
L-Histidine	FUJIFILM Wako	084-00682
L-Leucine	Nacalai Tesque	20327-62
L-Lysine monohydrochloride	Sigma-Aldrich	L5626
Uracil	FUJIFILM Wako	212-00062
Glycerol	Nacalai Tesque	17018-25
α-Factor (WHWLQLKPGQPMY)	peptide synthesis	N/A
Dulbecco's Phosphate Buffered Saline (PBS)	Sigma-Aldrich	D5652-1L
KOD FX Neo	Toyobo	KFX-201
2× Buffer for KOD FX Neo	Toyobo	KFX-2B
2 mM dNTP	Toyobo	NTP-201
Lithium acetate dihydrate	Wako	120-01535
Tris(hydroxymethyl)aminomethane (Tris)	Nacalai Tesque	35406-91
Di-sodium dihydrogen ethylenediamine tetraacetate dihydrate (EDTA)	Nacalai Tesque	15111-45
Polyethylene glycol #4,000 (PEG)	Nacalai Tesque	11574-15
Deoxyribonucleic acid-cellulose single-stranded from calf thymus DNA (ssDNA)	Sigma-Aldrich	D8273-5G
Dimethyl sulfoxide (DMSO)	Nacalai Tesque	13407-45
Trisodium citrate dihydrate (sodium citrate)	FUJIFILM Wako	191-01785
Propidium iodide (PI)	FUJIFILM Wako	164-16721
Ribonuclease A from bovine pancreas (RNase)	Nacalai Tesque	30142-04
Proteinase K from *Tritirachium album* (Proteinase K)	Nacalai Tesque	29442-85
Hydrochloric acid (HCl)	FUJIFILM Wako	080-01066
Sodium hhydroxide	Nacalai Tesque	31311-05

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: Organisms/strains		
MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0	EUROSCARF	BY4741
MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0	EUROSCARF	BY4742
MATa/MATα his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 LYS2/lys2Δ0 met15Δ0/MET15 ura3Δ0/ura3Δ0 (Cross between BY4741 and BY74742)	This study	BY4743
mat::kanMX, BY4742 background	Oya and Matsuura[1]	OYA1
mat::kanMX/mat::kanMX, BY4742 background	Oya and Matsuura[1]	OYA28
mat::kanMX/mat::kanMX/mat::kanMX, BY4742 background	Oya and Matsuura[1]	OYA356
mat::kanMX/mat::kanMX/mat::kanMX/mat::kanMX, BY4742 background	Oya and Matsuura[1]	OYA228
mat::kanMX/mat::kanMX/mat::kanMX/mat::kanMX/mat::kanMX, BY4742 background	This study	OYA357
mat::kanMX/mat::kanMX/mat::kanMX/mat::kanMX/mat::kanMX/mat::kanMX, BY4742 background	This study	OYA358
mat::kanMX/mat::kanMX/mat::kanMX/MATα, BY4742 background	Oya and Matsuura[1]	OYA1332
Oligonucleotides		
ACTAATCTTACGGTTTTTGTTGGCCCTAGATAAGAATCCT AATATCGGATCCCCGGGTTAATTAA	Oya and Matsuura[1]	MAT del F
ACATTCAGTACTCGAAAGATAAACAACCTCCGCCACGA CCACACTGAATTCGAGCTCGTTTAAAC	Oya and Matsuura[1]	MAT del R
GAAGGACGTCTGTGATGTTG	Oya and Matsuura[1]	MAT check F
GTGGCGAAGATGAATAGTAATG	Oya and Matsuura[1]	MAT check R
Recombinant DNA		
pFA6a-kanMX6	Wach[4]	N/A
YCpMATa	Shin et al.[2]	N/A
YCpMATα	Shin et al.[2]	N/A
pRS313	Sikorski and Hieter[5]	N/A
pRS315	Sikorski and Hieter[5]	N/A
pRS317	Sikorski and Boeke[6]	N/A
Software and algorithms		
CytExpert software	Beckman Coulter	N/A

MATERIALS AND EQUIPMENT

Stock solutions

Reagent	Solvent	Final concentration
50 mg/mL G418, store at −20°C (Sterilize with filtration)	ddH_2O	50 mg/mL
L-Histidine stock solution (Sterilize with filtration)	ddH_2O	10 mg/mL
L-Leucine stock solution (Sterilize with filtration)	ddH_2O	10 mg/mL
L-Lysine monohydrochloride stock solution (Sterilize with filtration)	ddH_2O	10 mg/mL
Uracil stock solution (Sterilize with filtration)	ddH_2O	2 mg/mL
PI, store at 4°C	50 mM citrate buffer	1 mg/mL
RNase, store at −20°C	10 mM Tris-HCl (pH 7.5) 15 mM NaCl	10 mg/mL
Proteinase K, store at −20°C	ddH_2O	50 mg/mL
α-factor, store at 4°C	PBS	10 mg/mL
1 M Tris-HCl buffer, pH7.5	ddH_2O	1 M
0.5 M EDTA, pH8.0	ddH_2O	0.5 M
5 N NaOH	ddH_2O	5 N

YPD plate with or without G418

Reagent	Final concentration	Amount
Yeast extract	1%(w/v)	10 g
Hipolypepton	2%(w/v)	20 g

(*Continued on next page*)

Continued

Reagent	Final concentration	Amount
D-(+)-Glucose	2%(w/v)	20 g
Agar	2%(w/v)	20 g
50 mg/mL G418	250 μg/mL	5 mL
ddH_2O	–	Up to 995–1,000 mL

Adjust the amount of ddH_2O so that the total volume up to 1,000 mL.
Autoclave to sterilize.
50 mg/mL G418 are added after autoclave.
YPD plate with G418 are stored at 4°C for up to a month.
YPD plate without G418 are stored at 4°C for up to 6 months.

YPD liquid medium

Reagent	Final concentration	Amount
Yeast extract	1%(w/v)	10 g
Hipolypepton	2%(w/v)	20 g
D-(+)-Glucose	2%(w/v)	20 g
ddH_2O	–	Up to 1,000 mL

Autoclave to sterilize.
Store at 25°C for up to 6 months.

SD plate with or without His and/or Leu and/or Lys and/or Ura

Reagent	Final concentration	Amount
YNB w/o AA, AS	0.17% (w/v)	1.7 g
Ammonium Sulfate	0.5% (w/v)	5 g
D-(+)-Glucose	2%(w/v)	20 g
Agar	2%(w/v)	20 g
5 N NaOH	–	750 μL
L-Histidine stock solution	20 mg/mL	2 mL
L-Leucine stock solution	100 mg/mL	10 mL
L-Lysine monohydrochloride stock solution	30 mg/mL	3 mL
Uracil stock solution	20 mg/mL	10 mL
ddH_2O	–	Up to 975–1,000 mL

Adjust the amount of ddH_2O so that the total volume up to 1,000 mL.
Autoclave to sterilize.
NaOH, amino acids and Uracil are added after autoclave.
Store at 4°C for up to 6 months.

SDCA plate

Reagent	Final concentration	Amount
YNB w/o AA, AS	0.17% (w/v)	1.7 g
Ammonium Sulfate	0.5% (w/v)	5 g
D-(+)-Glucose	2%(w/v)	20 g
Casamino acids	0.5% (w/v)	5 g
Agar	2%(w/v)	20 g
ddH_2O	–	Up to 1,000 mL

Autoclave to sterilize.
Store at 4°C for up to 6 months.

SDCA liquid medium		
Reagent	Final concentration	Amount
YNB w/o AA, AS	0.17% (w/v)	1.7 g
Ammonium Sulfate	0.5% (w/v)	5 g
D-(+)-Glucose	2%(w/v)	20 g
Casamino acids	0.5% (w/v)	5 g
ddH_2O	–	Up to 1,000 mL
Autoclave to sterilize.		
Store at 25°C for up to 6 months.		

SDCA plate with 5-FOA and Ura		
Reagent	Final concentration	Amount
YNB w/o AA, AS	0.17% (w/v)	1.7 g
Ammonium Sulfate	0.5% (w/v)	5 g
D-(+)-Glucose	2%(w/v)	20 g
Casamino acids	0.5% (w/v)	5 g
Agar	2%(w/v)	20 g
5-FOA	0.1% (w/v)	1 g
Uracil stock solution	20 mg/mL	25 mL
ddH_2O	–	Up to 975 mL
Autoclave to sterilize.		
5-FOA and Uracil are added after autoclave.		
Store at 4°C for up to a month.		

LiAc		
Reagent	Final concentration	Amount
Lithium Acetate Dihydrate	100 mM	1 g
1 M Tris-HCl buffer, pH7.5	10 mM	1 mL
0.5 M EDTA, pH8.0	1 mM	200 μL
ddH_2O	–	Up to 100 mL
Autoclave to sterilize.		
Store at 25°C for up to a year.		

Li-PEG		
Reagent	Final concentration	Amount
Lithium Acetate Dihydrate	100 mM	1 g
1 M Tris-HCl buffer, pH7.5	10 mM	1 mL
0.5 M EDTA, pH8.0	1 mM	200 μL
PEG	50% (w/v)	50 g
ddH_2O	–	Up to 100 mL
Autoclave to sterilize.		
Store at 25°C for up to a year.		

1 M citrate buffer, pH7.4		
Reagent	Final concentration	Amount
Sodium Citrate	1 M	29.41 g
HCl	–	To pH 7.4
ddH_2O	–	Up to 100 mL
Autoclave to sterilize.		
Store at 25°C for up to a year.		

50 mM citrate buffer		
Reagent	Final concentration	Amount
1 M citrate buffer, pH7.4	50 mM	5 mL
ddH_2O	–	Up to 100 mL
Prepare before use.		

STEP-BY-STEP METHOD DETAILS

Production of *mat*Δ/*mat*Δ diploid (ΔΔ) strain

Timing: 17 days

Timing: 4 days (for step 1)

Timing: 4 days (for step 2)

Timing: 9 days (for step 3)

The *mat*Δ strain behaves as the a-mating type when it has YCpMATa and as the α-mating type when it has YCpMATα.

1. Control of yeast mating type by introduction of YCpMATa or YCpMATα to *mat*Δ strain (Figure 1B).
   a. Transformation of YCpMATa or YCpMATα plasmid (Table 1).
      Partial modification of "Transformation of PCR fragments" (see before you begin section, step 2).
      i. Inoculate *mat*::*kanMX* strain in 1 mL YPD liquid medium using a 5 mL culture tube and incubate at 30°C for 16 h with spinning at 45 rpm.
      ii. Add 0.2 μg of YCpMATa or YCpMATα plasmid instead of 10 μg of PCR product to transformation mixture of *mat*::*kanMX* strain.
      iii. Plate the heat shocked and washed samples onto SDCA plates.

Pause point: The selected strain can be stored at −80°C as glycerol stocks until ready to proceed to the next steps.

Note: Since casamino acids is an acid hydrolysate of casein and is a mixture of amino acids except tryptophan, we use it to select yeast cells carrying *URA3*. SDCA medium can be substituted with synthetic complete (SC) medium not containing uracil.

Note: When the *mat*::*kanMX* YCpMATα strain drops the plasmid, a-mating type cells may appear in the α-mating type population, and mating may result in *mat*::*kanMX*/*mat*::*kanMX* YCp*MATα* diploid cells. To prevent this, we recommend that selection in medium lacking uracil is continually applied to the *mat*::*kanMX* YCpMATα strain.

Note: Microscopic observation of the *mat*::*kanMX* YCpMATα strain reveals cells showing aberrant morphology, which we call Barbapapa-like (Figure 2).[1]

Table 1. Selection marker information

Plasmid name	Selection marker
YCpMATa	*URA3*
YCpMATα	*URA3*
pRS313	*HIS3*
pRS315	*LEU2*
pRS317	*LYS2*

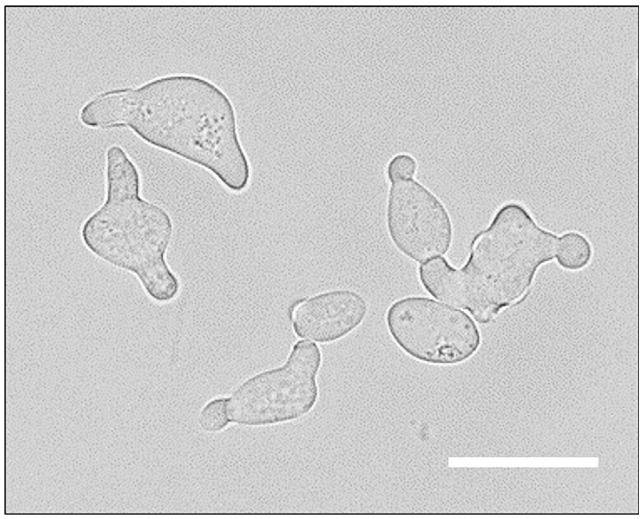

Figure 2. The morphology of "Barbapapa-like" cells

A representative image of the Barbapapa-like morphology shown by tetraploid cells with only one copy of *MATα* (ΔΔΔα). Scale bar = 20 μm.

2. Introduce selection markers to select mating products (Figure 1B).
   a. Transformation of pRS313 or pRS315 plasmid (Table 1).[5]
      Partial modification of "Transformation of PCR fragments" (see before you begin section, step 2).
      i. Inoculate *mat*::*kanMX* YCpMATa and *mat*::*kanMX* YCpMATα strains each in 1 mL SDCA liquid medium using a 5 mL culture tube and incubate at 30°C for 16 h with spinning at 45 rpm.
      ii. Add 0.2 μg of pRS313 and pRS315 plasmid instead of 10 μg of PCR product to transformation mixture of *mat*::*kanMX* YCpMATa and *mat*::*kanMX* YCpMATα strains, respectively.
      iii. Plate the heat shocked and washed samples onto SD +Leu +Lys and SD +His +Lys plates, respectively.

Pause point: The selected strain can be stored at −80°C as glycerol stocks until ready to proceed to the next steps.

3. Mating, diploid selection and plasmids dropout (Figures 1C and 1D).
   a. Mating.
      i. Using a sterile toothpick, patch part of a colony of *mat*::*kanMX* YCpMATa pRS313 strain on a YPD plate.
      ii. Similarly, patch part of a colony of *mat*::*kanMX* YCpMATα pRS315 strain and mix it well with *mat*::*kanMX* YCpMATa pRS313 strain already on the plate.
      iii. Incubate mating patch consisting of the mixture at 30°C for 16 h.
   b. Diploid selection.
      i. Using a plate that is selective for mated cells only (SD +Lys), streak the mating patch onto the plate for single colonies.
      ii. Incubate at 30°C for 2 days.
   c. YCpMATa and YCpMATα plasmids dropout.
      i. Using a plate to select cells that dropped YCpMATa and YCpMATα (SDCA +Ura +5-FOA), streak the diploid colony onto the plate for single colonies.
      ii. Incubate at 30°C for 3 days.
   d. Selective marker plasmids dropout.

i. Streak the diploid colony with dropped YCpMATa and YCpMATα on YPD plate for single colonies.
ii. Incubate at 30°C for 2 days.
iii. Patch colonies to SD +Leu +Lys +Ura, SD +His +Lys +Ura, SDCA and YPD plates, maintaining the correspondence between each patch.
iv. Incubate at 30°C for 1 day.
v. Negative clones in SD +Leu +Lys +Ura, SD +His +Lys +Ura and SDCA plates are selected from YPD plate.

Pause point: The selected strain can be stored at −80°C as glycerol stocks until ready to proceed to the next steps.

Note: We recommend picking up 10–20 colonies. When continuously cultured in SDCA + Ura +5-FOA and YPD media without selection for His+ and Leu+, the pRS313 and pRS315 plasmids would be expected to be lost in about half of the patches.

Note: For identifying colonies that have lost the plasmids, the patches process can be replaced by replica plating.

Production of a series of *mat*Δ polyploid strains

Timing: 17 days or more

Similar to the *mat*Δ haploid (Δ) strain, the mating type of *mat*Δ polyploid strains can be controlled by YCpMATa and YCpMATα.

4. Production of *mat*Δ/*mat*Δ/*mat*Δ triploid (ΔΔΔ) and *mat*Δ/*mat*Δ/*mat*Δ/*mat*Δ tetraploid (ΔΔΔΔ) strain (Figure 1E).
   a. Produce the ΔΔ strain by mating Δ strains (step-by-step method details section, steps 1, 2 and 3).
   b. Produce the ΔΔΔ and ΔΔΔΔ strain by mating ΔΔ with a Δ strain and ΔΔ strain, respectively.
   c. Production of further polyploid strains in the same way.

Note: We have successively obtained hexaploid cells using this method (Figure 3). As the ploidy increases, the colonies tend to be non-uniform in size (Figure 4A). This is presumably due to aneuploid formation caused by genomic instability of polyploid cells.[7] In addition, longer incubation times result in more frequent appearance of small colonies (Figure 4B).

Alternative method for creation of polyploid cells starting from *MAT*α strain

Timing: 13 days or more

Timing: 4 days (for step 5a)

Timing: 9 days (for step 5b)

A *mat*Δ YCpMATa strain and a *MAT*α strain mate to form a non-mater *mat*Δ/*MAT*α YCpMATa strain. Dropping the YCpMATa plasmid from the non-mater diploid produces *mat*Δ/*MAT*α diploid (Δα) that behaves as the α-mating type. Δα can mate with *mat*Δ YCpMATa again (Figure 5A).

⚠ CRITICAL: *mat*Δ YCpMATa must be used instead of *mat*Δ. This is because *mat*Δ can cause uncontrolled changes in ploidy after repeated mating in a single mating stage (Figure 5B).

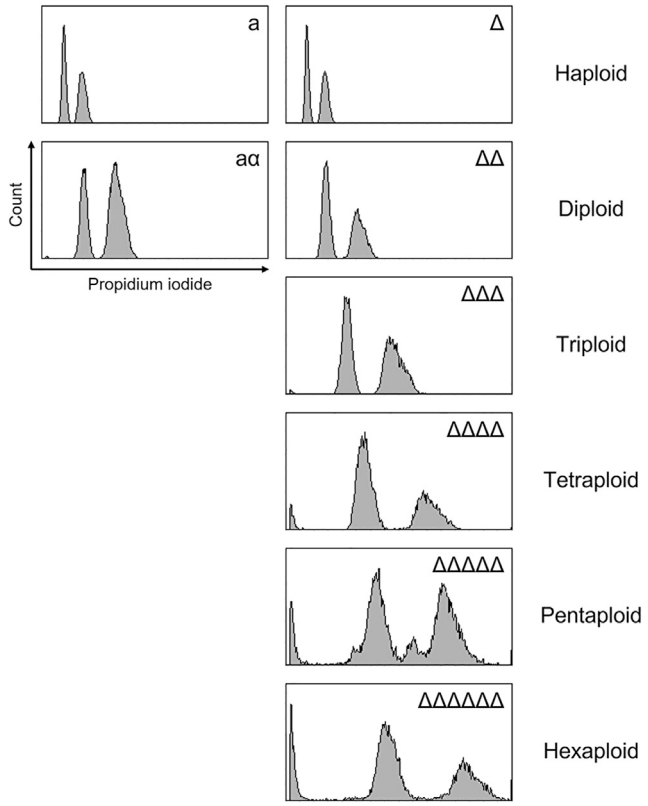

Figure 3. Confirmation of ploidy by DNA content measurement

Histograms showing DNA content represent ploidy. Low fluorescence peaks prominent in tetraploid, pentaploid, and hexaploid correspond to dead cells, which indicates instability of the genome due to increased ploidy.

5. Sequential mating of *mat*Δ cells based on *MAT*α cells can successively increase ploidy.
   a. Introduce selection marker to select mating products.
      Partial modification of "Transformation of PCR fragments" (see before you begin section, step 2).
      i. Add 0.2 μg of pRS315 plasmid instead of 10 μg of PCR product to transformation mixture of BY4742 strain.
      ii. Plate the heat shocked and washed samples onto SD +His +Lys +Ura plate.

   Pause point: The selected strain can be stored at −80°C as glycerol stocks until ready to proceed to the next steps.

   b. Mating, diploid selection and plasmids dropout.
      Partial modification of "Mating, diploid selection and plasmids dropout" (see step-by-step method details section, step 3).
      i. Mating *mat*::*kanMX* YCpMATa strain and *MAT*α pRS315 strain.
      ii. Use SD +His +Lys plate for diploid selection.
      iii. Use SD +His +Lys +Ura, SDCA and YPD plates for plasmids dropout check.

   Note: In a similar way, the mating of Δα and Δ produces *mat*Δ/*mat*Δ/*MAT*α (ΔΔα) triploid strain, and furthermore, the mating of ΔΔα and Δ produces *mat*Δ/*mat*Δ/*mat*Δ/*MAT*α (ΔΔΔα) tetraploid strain.

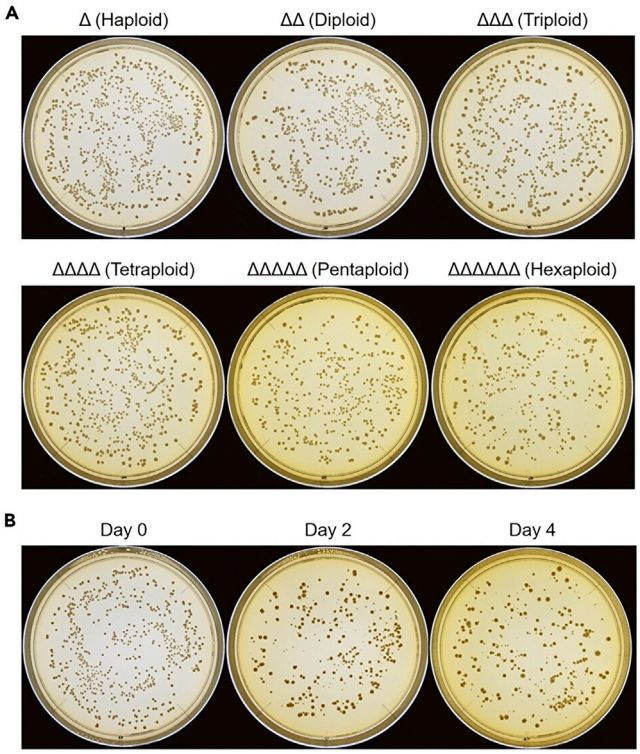

Figure 4. Higher-order ploidy strains are genetically unstable

(A) Strains were cultured for 16 h, and cells corresponding to 10^{-4} ODU were spread on YPD plates and incubated at 30°C. Colonies were photographed after 2 days.

(B) ΔΔΔα were repeatedly subcultured every day, and cells corresponding to 10^{-4} ODU were spread on YPD plates on Day 0, 2, and 4, incubated at 30°C. Colonies were photographed after 2 days.

DNA content measurement

Timing: 1 day

6. Confirmation that the producing polyploid cells exhibit the desired ploidy by measuring DNA content.
   a. Fix sample strains.
      i. Inoculate sample strains in 1 mL YPD liquid medium using a 5 mL culture tube and incubate at 30°C for 16 h with spinning at 45 rpm.
      ii. Transfer 1 OD_{600} units (1 ODU = 1 mL cell culture of an OD_{600} = 1) of overnight culture to 1.5 mL tube.
      iii. Pellet at 1,500 × *g*, 2 min and discard the supernatant.
      iv. Fix pellets with 1 mL 70% ethanol at 25°C for 30 min.
   b. Prepare sample.
      i. Pellet fixed sample at 20,000 × *g*, 5 min and wash with 1 mL 50 mM citrate buffer.
      ii. Pellet at 20,000 × *g*, 5 min and discard the supernatant.
      iii. Pellet at 20,000 × *g*, 2 min and discard the supernatant completely.
      iv. Suspend the pellet in 1 mL 50 mM citrate buffer containing 0.25 mg/mL RNase and incubate at 50°C for 1 h.
      v. Add 10 μL 50 mg/mL Proteinase K and incubate at 50°C for 1 h.
      vi. Pellet at 20,000 × *g*, 5 min and discard the supernatant.
      vii. Suspend the pellet in 1 mL 50 mM citrate buffer containing 16 μg/mL Propidium iodide and incubate at 25°C for 30 min in the dark.

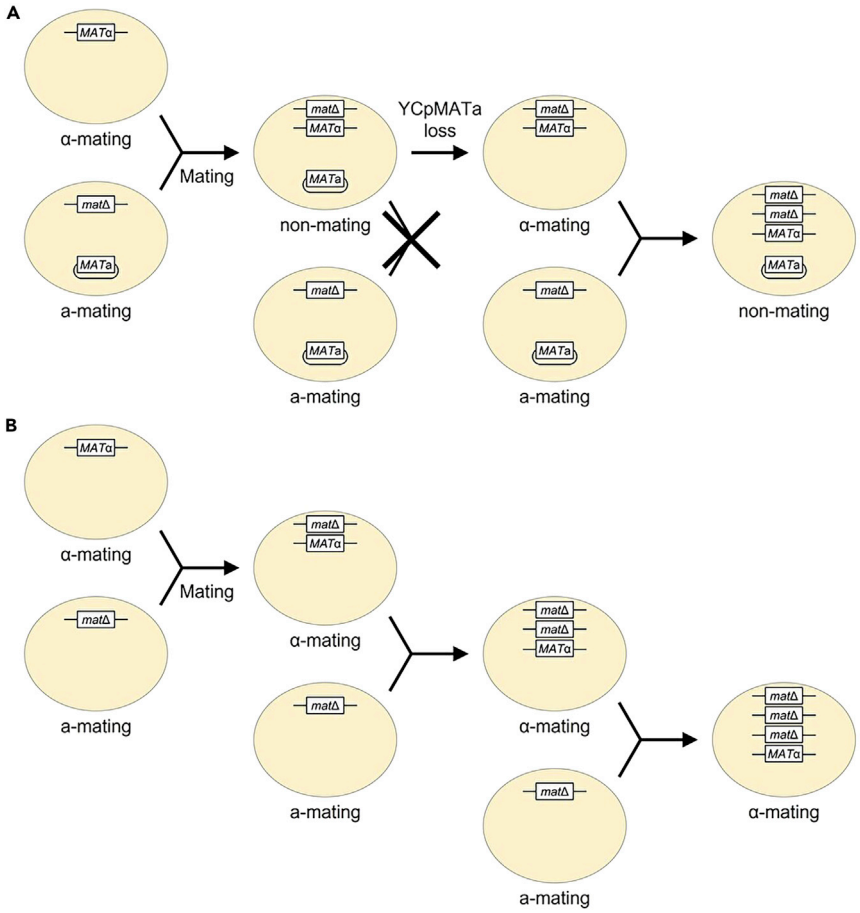

Figure 5. Difference between *mat*Δ and *mat*Δ YCpMATa in repeated mating with *MAT*α

(A) Since *mat*Δ/*MAT*α YCpMATa diploids generated by mating between *mat*Δ YCpMATa and *MAT*α is non-mater, they never enter the mating stage again. However, when they have lost YCpMATa, they behave as the α-mating type and can mate with *mat*Δ YCpMATa again to increase the ploidy.

(B) Since *mat*Δ/*MAT*α diploids generated by the mating between *mat*Δ and *MAT*α behave as the α-mating type, they can mate with *mat*Δ again to produce *mat*Δ/*mat*Δ/*MAT*α triploids. Therefore, the use of *mat*Δ for mating results in uncontrolled ploidy. For simplicity, plasmids used to select mated cells are omitted.

viii. Sonicate 5 times (1 s. ON and 1 s. OFF) at 30% power.

c. Measure the DNA content of sample.

Measure on a CytoFLEX S flow cytometer with 561-nm laser.

Pause point: Prepared samples can be stored at 4°C for 1 week.

Note: The purpose of sonication is to separate cells. The optimal power and duration for each sonicator should be adjusted while checking with microscopic observation.

⚠ CRITICAL: If the measurement sample is too concentrated, dilute all samples with 50 mM citrate buffer at the same ratio to equalize the fluorescence intensity of the background.

EXPECTED OUTCOMES

This protocol can be used to create cells with the desired ploidy (Figure 3).

To evaluate the defective phenotype of a gene of interest in polyploid cells, all copies of the gene must be deleted. Our method of creating a polyploid series with the *mat*Δ strain as the basic unit can create polyploid cells with mutations in all gene copies by introducing mutations in the *mat*Δ strain. Furthermore, by combining basic units with and without the gene of interest, the effect of copy number variation of the gene on the polyploid phenotype can be examined. The phenotype would represent the nature and regulation of the gene and the protein it encodes. Using this method, we created a ΔΔΔα strain and found that the *MAT*α idiotype is composed of haploinsufficient genes.[1] We also created a *STE4* quadruple-deficient ΔΔΔα strain and showed that aberrant morphology and high mortality in ΔΔΔα were dependent on activation of the mating pheromone response pathway (mating pathway).[1]

LIMITATIONS

Because this polyploid creation protocol relies on mating, it is not possible to create polyploid cells with multiple deficiencies in the genes involved in mating. However, this problem can be solved by cloning the gene of interest into pRS317 (Table 1).[6] We used pRS317-*STE4* to produce the *STE4* quadruple-deficient ΔΔΔα strain.[1]

We assume that the sex of the *mat*Δ YCpMATα strain is unstable, since the Barbapapa-like morphology is observed, suggesting abnormal activation of the mating pathway. Therefore, it is not possible to introduce deletion of a gene such as *SST2*, which acts as a repressor of the mating pathway, into the *mat*Δ YCpMATα strain. The gene of interest must be cloned into pRS317 and the gene deleted in the genome while carrying the plasmid.

TROUBLESHOOTING

Problem 1

mat::*kanMX* transformants were not obtained. PCR fragments amplified with MAT check F and MAT check R which specifically amplify the *MAT* locus yielded one band of 2,261 bp or two bands of 2,261 and 2,791 bp (before you begin section, step 2m).

Potential solution

PCR fragments of 2,261 and 2,791 bp represent *MAT*α and *mat*::*kanMX* at *MAT* locus, respectively. The former is due to mistargeting of the introduced PCR product that possesses short flanking sequences derived from the primers MAT del F and MAT del R. The latter is due to *mat*::*kanMX*/*MAT*α diploid, caused by mating between a transformant behaving as the a-mating type and a surrounding *MAT*α cell.

Genomic DNA of *mat*::*kanMX*/*MAT*α diploid is useful for a template of PCR with primer pair MAT check F and MAT check R. The PCR product possesses 500 bp flanking sequences, longer than the plasmid-based PCR fragment, and is expected to increase the efficiency of correct targeting. Consequently, the use of this fragment improves the efficiency of obtaining *mat*::*kanMX* strain. However, we still recommend checking at least 5 transformants, since this method cannot avoid the contamination of diploids via mating.

Problem 2

Delayed colony formation in the processes of Mating, polyploid selection and plasmids dropout (step-by-step method details section, steps 3, 4 and 5).

Potential solution

Since the growth of the polyploids decreases slightly as the ploidy increases, the duration of each step of colony formation should be extended as necessary. In addition, as the ploidy increases, the frequency of chromosome loss increases.[7] It is therefore recommended that genetically unstable strains with higher-order ploidy should not experience repeated culture.

Problem 3

No single colonies formed on a SDCA +Ura +5-FOA plate in YCpMATa and YCpMATα plasmid dropouts (step-by-step method details section, steps 3c and 5b).

Potential solution

Increase the number of cells inoculated from the diploid or polyploid colony and spread on SDCA + Ura +5-FOA plate. Alternatively, a single colony formed on a diploid or polyploid selection plate is spread on a YPD plate and incubated at 30°C for 16 h. Using a SDCA +Ura +5-FOA plate, streak the grown patch onto the plate to form single cell-derived colonies.

Problem 4

Addition of 70% ethanol caused the cells to aggregate, and thus they could not be suspended well (step-by-step method details section, step 6a).

Potential solution

Once the pellet is suspended in 300 μL ddH_2O, add 700 μL 100% ethanol and resuspend again.

Problem 5

Sample concentration for measuring DNA content was too low (step-by-step method details section, step 6c).

Potential solution

The amount of cells to be fixed is increased to 5 ODU, and the final measurement sample is diluted appropriately with 50 mM citrate buffer before measurement. If diluted, all samples must be diluted at the same ratio.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Akira Matsuura (amatsuur@faculty.chiba-u.jp).

Materials availability

OYA1 (*mat*::*kanMX*, BY4742 background) strain and YCpMATa and YCpMATα plasmids will be deposited to National BioResource Project-Yeast.

Data and code availability

This study did not generate nor analyze datasets.

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AUTHOR CONTRIBUTIONS

Conceptualization, K.O., A.M.; methodology, K.O., A.M.; investigation, K.O.; resources, K.O., A.M.; writing - original draft, K.O.; writing - review and editing, A.M.; funding acquisition, K.O., A.M.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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