2-19 MAT/ste4 KO of strain #2 and 52

FRIDAY, 2019-05-10

The workflow assumes that the strains in question were derived from SEYa or SEYα, are haploid and do not carry HIS3 or TRP1 selection markers. The protocol can be modified to use other selection markers for knock-out if necessary. The protocol can also be adapted for other parental strains than SEYa or SEYα by optimizing the transformation protocol parameters.

First, a double knock-out of the strains of interest results in a MATΔ::His3 and ste4Δ::TRP1 genotype (ste4 is first knocked-out to render the strain sterile and prevent uncontrolled selfing of a MATα strain in the presence of a MATΔ::His3 strain during MAT knockout). Each knock-out strain is then selfed.

Repeating KO of line #2.

- [x] Enter batch of yeast strains being processed below [NOTE: these are new codes for this procedure, not MA line codes].

List of yeast strains:
2

ste4 knock-out

- [x] Patch each strain on grid on YPG plate [NOTE: used frozen strains from Aliquot 2 of MA lines].
- [x] Prepare STE4 KO PCR product (see 2-2 ste4 KO PCR product for double KO).

WEDNESDAY, 2019-05-15

- [x] Streak-out to single colonies on YPAD plate.

TUESDAY, 2019-05-21

- [x] Inoculate overnight culture (see ste4 KO Transformation protocol day 1). Linnea Sandell Matthew Stasiuk Christina Hsu
  Streak-out plate of #2 is in box on Stephan's lab bench.

WEDNESDAY, 2019-05-22

- [x] Prepare 1 mL competent cells (see ste4 KO Transformation protocol day 2).
- [x] Transform with 1 µg ste4 KO PCR product and plate on SC-Trp (see ste4 KO Transformation protocol day 2).

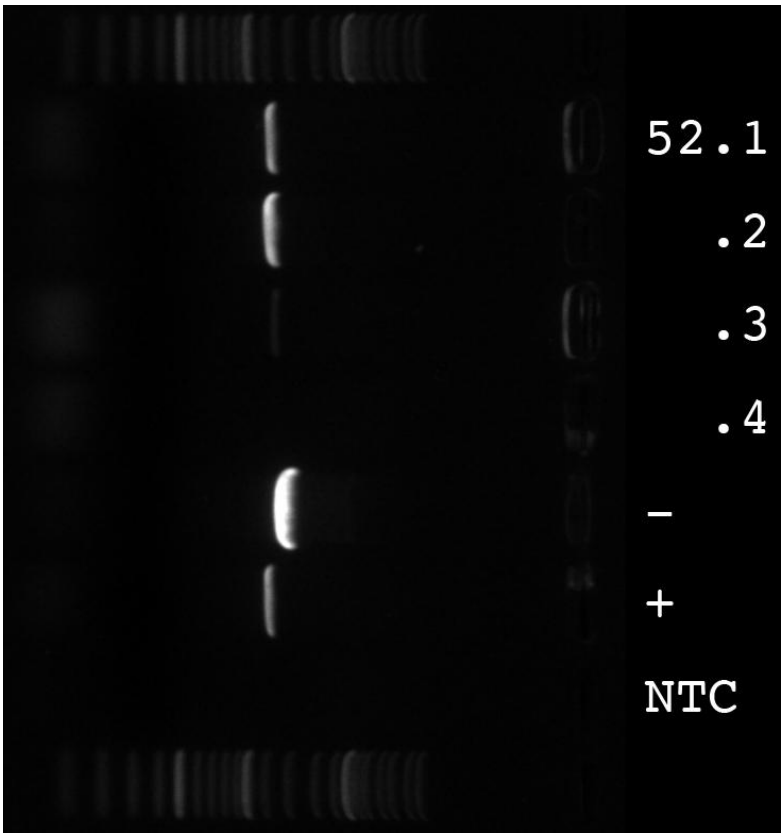
SATURDAY, 2019-05-25

- [x] Place plate at 4°C.

MONDAY, 2019-05-27

- [x] Streak-out a single transformant to single colonies on SC-Trp plate.
- [x] Add #52 from 2-4 MAT/ste4 KO of MA strains set 3 and confirm genotype of all 4 clones (see ste4 colony PCR protocol).

190527_2-19_STE4_Colony_PCR.jpg

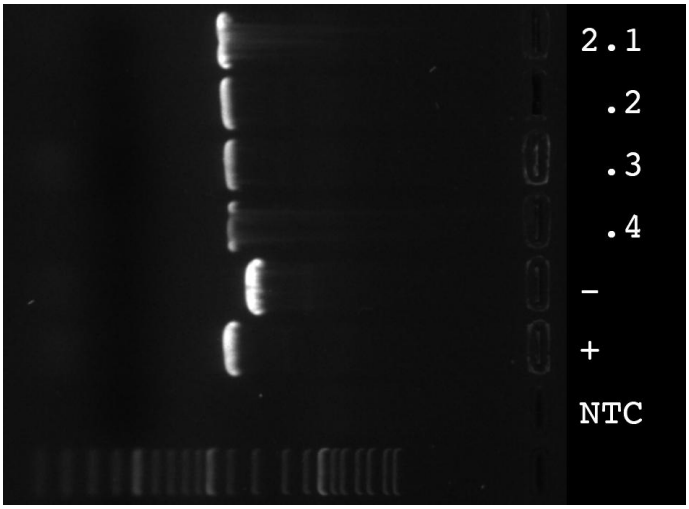


THURSDAY, 2019-05-30

- [x] Confirm genotype of all 4 clones of strain 2 (see ste4 colony PCR protocol). Christina Hsu Linnea Sandell

FRIDAY, 2019-05-31

190530_2-19_STE4_Colony_PCR.jpg



- [x] If ste4Δ::TRP1 genotype is confirmed, proceed with MAT knockout; otherwise repeat streak-out and colony PCR with another transformant.

ste4Δ::TRP1

	A	B	C	D	E	F
1	Strain #	# colonies	clone.1	clone.2	clone.3	clone.4
2	2	28	TRUE	TRUE	TRUE	TRUE
3	52	NA	TRUE	TRUE	TRUE	NA

MONDAY, 2019-06-03

MAT knock-out

- [x] Inoculate overnight culture (see MAT KO Transformation day 1).
- [x] Prepare MAT KO PCR product (see MAT KO PCR protocol).

TUESDAY, 2019-06-04

- [x] Prepare 1 mL competent cells (see MAT KO Transformation day 2).
- [x] Transform with 1 µg MAT KO PCR product and plate on SC-His-Trp (see MAT KO Transformation protocol day 2).

FRIDAY, 2019-06-07

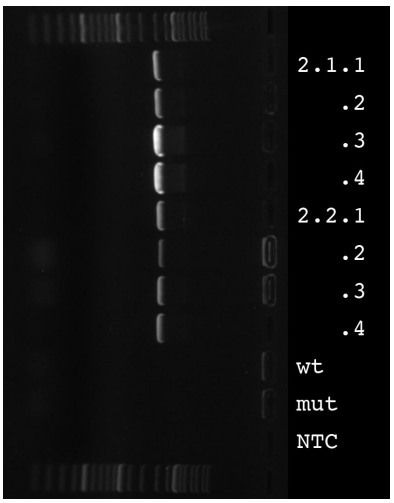
Did not receive any colonies for strain #52, will start again from beginning with ste4 KO.

- [x] Streak-out a single transformant to single colonies on SC-His-Trp plate.

MONDAY, 2019-06-10

- [x] Confirm genotype of single colony (see MAT Colony PCR).

190610_2-19_MAT_Colony_PCR.jpg



Controls failed (likely too many cells), but genotype of tested colonies could be determined based on product size (about 2 kb).
All tested colonies were MATΔ::His3.

MONDAY, 2019-06-17

- [x] Inoculate overnight culture of 2.1.1 in 2 mL YPAD.

TUESDAY, 2019-06-18

- [x] Prepare glycerol stock of 2.1.1 and store in -80°C freezer.

MATΔ::His3

	A	B	C	D	E	F
1	Strain #	# colonies	clone.1	clone.2	clone.3	clone.4
2	2.1	6	TRUE	TRUE	TRUE	TRUE
3	2.2	10	TRUE	TRUE	TRUE	TRUE
4	52.1	0				
5	52.2	0				
6	52.3	0				

ste4 KO Transformation

Introduction

Yeast strain derived from SEYa or SEYα are transformed with ste4 KO PCR product to replace ste4 with TRP1 and render the strain sterile. A heat shock time of 1 h increases transformation efficiency for SEY derived strains when compared to 20 min heat shock.

Materials

- YPAD
- 50% PEG
- 1 M LiAc
- 2mg/ml ssDNA
- ste4 KO PCR product (at least 30 ng/µL)
- Sterile H20
- Water bath or heating block at 42°C
- Disposable 17x100 mm culture tubes with closures (VWR Catalog # 60818-725)
- SC-Trp plates

Procedure

Competent cells

Day 1

- [x] 1. Overnight culture (ONC): Inoculate 2 mL of YPAD with a single yeast colony and incubate culture on the shaker at 30°C/200 rpm/overnight.

Day 2

- [x] 2. Inoculate 2.5 mL of YPAD in disposable 10 mL culture tubes with 200 µL of ONC and grow it on the shaker at 30°C/200 rpm/4 h.

04:00:00

- [x] 3. Set water bath or heating block to 42°C.
- [x] 4. Indicate number of transformations and PCR product concentration:

Settings

	# of Transformations	ste4 KO PCR product [ng/µL]
1	-1	100

- [x] 5. If the carrier DNA has not been denatured yet, denature the carrier DNA in heating block at 95°C for 5 min and chill immediately on ice before use. If carrier DNA has already been denatured then thaw it on ice.

- [x] 6. Prepare 0.1 M LiAc, freshly diluted from 1 M LiAc:

0.1 M Li Ac

	Materials	mL
1		1 X [mL]
2	1 M LiAc	0.1
3	ddH2O	0.9
4	**Total**	**1**

- [x] 7. Centrifuge 3,000 g/5 min; discard supernatant and resuspend cell pellet in 1 mL sterile H_2O.

- [x] 8. Centrifuge 3,000 g/5 min; discard supernatant and resuspend cells in 1 mL of 0.1 M LiAc.

- [x] 9. Centrifuge 3,000 g/5 min; discard supernatant.

Transformation

- [x] 10. Prepare transformation master mix by adding reagents in order (pipetting 50% PEG slowly), two additional reactions are included to account for pipetting discrepancies:

Transformation master mix

	A	B
1		1 X [µL]
2	50% PEG	240
3	1 M LiAc	36
4	ssDNA (2 mg/ml)	50
5	ste4 KO PCR product	10
6	ddH2O	24
7	**Total**	**360**

- [x] 11. Add 360 µL of transformation master mix to each tube and immediately vortex until the cells are smoothly suspended.

- [x] 12. Heat shock cells in water-bath or heat-block: 42°C/1 h.

01:00:00

- [x] 13. Centrifuge 3,000 g/5 min; discard supernatant.
- [x] 14. Resuspend each cell pellet in 100 µL sterile H_2O, then plate cells on the SC-Trp.

Additional resources

PCR product amou...

	KO PCR product [ng]
1	1000

Transformation single reaction

	A	B
1		[µL]
2	50% PEG	240
3	1 M LiAc	36
4	2mg/ml ssDNA	50
5	PCR product	10
6	dHd2O	24
7	**Total**	**360**

ste4 Colony PCR

Introduction

For genotyping of ste4 locus: the primers bind upstream (F) and downstream (R) of ste4, and PCR is used to determine if TRP1 has replaced ste4 by homologous recombination.

Materials

- TaKaRa LA Taq® DNA Polymerase (Mg2+ free buffer) RR002A
- 10 µM Primer F: OLPr022 STE4 -133 F
- 10 µM Primer R: OLPr023 STE4 1402 R
- SC-Trp plates
- Template: yeast colonies transformed with ste4 KO PCR product

Procedure

PCR

1. Chose the number of 10 µL reactions (include 3 additional reactions to account for wild-type, mutant, and no-template control):

Settings

	# of reactions
1	7

2. Combine the following reagents to prepare the master mix (contains 0.5 additional reaction to account for pipetting discrepancies), and aliquot 10 µL master mix/pcr tube:

Master mix

	A	B
1		7.5 X [µL]
2	ddH2O	44.25
3	10X LA PCR Buffer	7.5
4	25 mM MgCl2	7.5
5	dNTP Mix (2.5 mM each)	12
6	10 µM OLPr022 STE4 -133 F	1.5
7	10 µM OLPr023 STE4 1402 R	1.5
8	LA Taq Polymerase (5 U/µL)	0.75
9	**Total**	**75**

- ✓ 3. In sterile work environement (e.g. laminar flow), gently touch a yeast colony with a 10 µL pipette tip so a small number of cells stick to it, touch the tip to a fresh reference SC-Trp plate, and resuspend cells in PCR reaction.

  Do not use too many cells as they might inhibit the PCR reaction. The PCR reaction should only look slightly opaque from the resuspended cells. If the reaction looks milky then you might have used too many cells.
  The reference plate is incubated 30°C/2–3 days.

- ✓ 4. PCR program "STE4COLO" in folder "STEPHAN":

  94°C/5 min—[94°C/0.5 min—61°C/0.5 min—68°C/1.5 min]x35—68°C/5 min—10°C/∞

Gel

- ✓ 5. Gel: 10 µL/0.5 µg O'GeneRuler DNA Ladder Mix/1% TAE/120 V/35 min

00:35:00

Expected product sizes:

ste4 1535 bp

ste4Δ::TRP1 1232 bp

Stop processing any yeast strains with the wrong genotype (i.e. discard any corresponding overnight cultures and patches on reference plate).

Additional resources

Primers and template

	A	B
1	Forward Primer	OLPr022 STE4 -133 F
2	Reverse Primer	OLPr023 STE4 1402 R
3	Template	yeast colonies

PCR Volume and reagent concentrations

	A	B
1	PCR Volume [µL]	10
2	Starting buffer conc [X]	10
3	Final buffer conc [X]	1
4	Starting Mg2+ conc [mM]	25
5	Final Mg2+ conc [mM]	2.5
6	Starting dNTP conc each [mM]	2.5
7	Final dNTP conc each [mM]	0.4
8	Starting Primer F conc [µM]	10
9	Final Primer F conc [µM]	0.2
10	Starting Primer R conc [µM]	10
11	Final Primer R conc [µM]	0.2
12	Starting Polymerase conc [U/µL]	5
13	Final Polymerase conc [U/µL]	0.05
14	Starting Template conc [ng/µL]	5
15	Final Template conc [ng/µL]	0

Single PCR reaction

	A	B
1		[µL]
2	ddH2O	5.9
3	10X PCR Buffer	1
4	25 mM MgCl2	1
5	dNTP Mix (2.5 mM each)	1.6
6	10 µM Primer F	0.2
7	10 µM Primer R	0.2
8	LA Taq Polymerase (5 U/µL)	0.1
9	Template (5 ng/µL)	0
10	Total	10

ste4 Colony PCR.2

Introduction

For genotyping of ste4 locus: the primers bind upstream (F) and downstream (R) of ste4, and PCR is used to determine if TRP1 has replaced ste4 by homologous recombination.

Materials

- TaKaRa LA Taq® DNA Polymerase (Mg2+ free buffer) RR002A
- 10 µM Primer F: OLPr022 STE4 -133 F
- 10 µM Primer R: OLPr023 STE4 1402 R
- SC-Trp plates
- Template: yeast colonies transformed with ste4 KO PCR product

Procedure

PCR

1. Chose the number of 10 µL reactions (include 3 additional reactions to account for wild-type, mutant, and no-template control):

Settings

	# of reactions
1	7.5

2. Combine the following reagents to prepare the master mix (contains 0.5 additional reaction to account for pipetting discrepancies), and aliquot 10 µL master mix/pcr tube:

Master mix

	A	B
1		**8 X [µL]**
2	ddH2O	47.2
3	10X LA PCR Buffer	8
4	25 mM MgCl2	8
5	dNTP Mix (2.5 mM each)	12.8
6	10 µM OLPr022 STE4 -133 F	1.6
7	10 µM OLPr023 STE4 1402 R	1.6
8	LA Taq Polymerase (5 U/µL)	0.8
9	**Total**	**80**

3. In sterile work environement (e.g. laminar flow), gently touch a yeast colony with a 10 µL pipette tip so a small number of cells stick to it, touch the tip to a fresh reference SC-Trp plate, and resuspend cells in PCR reaction.

   Do not use too many cells as they might inhibit the PCR reaction. The PCR reaction should only look slightly opaque from the resuspended cells. If the reaction looks milky then you might have used too many cells.
   The reference plate is incubated 30°C/2–3 days.

4. PCR program "STE4COLO" in folder "STEPHAN":

   94°C/5 min—[94°C/0.5 min—61°C/0.5 min—68°C/1.5 min]x35—68°C/5 min—10°C/∞

Gel

5. Gel: 10 µL/0.5 µg O'GeneRuler DNA Ladder Mix/1% TAE/120 V/35 min

00:35:00

Expected product sizes:

ste4 1535 bp

ste4Δ::TRP1 1232 bp

Stop processing any yeast strains with the wrong genotype (i.e. discard any corresponding overnight cultures and patches on reference plate).

Additional resources

Primers and template

	A	B
1	Forward Primer	OLPr022 STE4 -133 F
2	Reverse Primer	OLPr023 STE4 1402 R
3	Template	yeast colonies

PCR Volume and reagent concentrations

	A	B
1	PCR Volume [µL]	10
2	Starting buffer conc [X]	10
3	Final buffer conc [X]	1
4	Starting Mg2+ conc [mM]	25
5	Final Mg2+ conc [mM]	2.5
6	Starting dNTP conc each [mM]	2.5
7	Final dNTP conc each [mM]	0.4
8	Starting Primer F conc [µM]	10
9	Final Primer F conc [µM]	0.2
10	Starting Primer R conc [µM]	10
11	Final Primer R conc [µM]	0.2
12	Starting Polymerase conc [U/µL]	5
13	Final Polymerase conc [U/µL]	0.05
14	Starting Template conc [ng/µL]	5
15	Final Template conc [ng/µL]	0

Single PCR reaction

	A	B
1		[µL]
2	ddH2O	5.9
3	10X PCR Buffer	1
4	25 mM MgCl2	1
5	dNTP Mix (2.5 mM each)	1.6
6	10 µM Primer F	0.2
7	10 µM Primer R	0.2
8	LA Taq Polymerase (5 U/µL)	0.1
9	Template (5 ng/µL)	0
10	Total	10

MAT KO Transformation

Introduction

ste4Δ::TRP1 yeast strain derived from SEYa or SEYα are transformed with MAT KO PCR product to replace MATa or MATα with His3. A heat shock time of 1 h increases transformation efficiency for SEY derived strains when compared to 20 min heat shock.

Materials

- YPAD
- 50% PEG
- 1 M LiAc
- 2mg/ml ssDNA
- MAT KO PCR product (at least 30 ng/µL)
- Sterile H20
- Water bath or heating block at 42°C
- Disposable 17x100 mm culture tubes with closures (VWR Catalog # 60818-725)
- SC-His-Trp plates

Procedure

Competent Cells

Day 1

- [x] 1. Overnight culture (ONC): Inoculate 2 mL of YPAD with a single yeast colony and incubate culture on the shaker at 30°C/200 rpm/overnight.

Day 2

- [x] 2. Inoculate 2.5 mL of YPAD in disposable 10 mL culture tubes with 200 µL of ONC and grow it on the shaker at 30°C/200 rpm/4 h.

04:00:00

- [x] 3. Set water bath or heating block to 42°C.
- [x] 4. Indicate number of transformations and PCR product concentration:

Settings

	# of Transformations	MAT KO PCR product [ng/µL]
1	3.5	100

- [x] 5. If the carrier DNA has not been denatured yet, denature the carrier DNA in heating block at 95°C for 5 min and chill immediately on ice before use. If carrier DNA has already been denatured then thaw it on ice.

- [x] 6. Prepare 0.1 M LiAc, freshly diluted from 1 M LiAc:

0.1 M Li Ac

	Materials	mL
1		5.5 X [mL]
2	1 M LiAc	0.55
3	ddH2O	4.95
4	**Total**	**5.5**

- [x] 7. Centrifuge 3,000 g/5 min; discard supernatant and resuspend cell pellet in 1 mL sterile H_2O.

- [x] 8. Centrifuge 3,000 g/5 min; discard supernatant and resuspend cells in 1 mL of 0.1 M LiAc.

- [x] 9. Centrifuge 3,000 g/5 min; discard supernatant.

Transformation

- [x] 10. Prepare transformation master mix by adding reagents in order (pipetting 50% PEG slowly), two additional reactions are included to account for pipetting discrepancies:

Transformation master mix

	A	B
1		5.5 X [µL]
2	50% PEG	1320
3	1 M LiAc	198
4	ssDNA (2 mg/ml)	275
5	MAT KO PCR product	55
6	ddH2O	132
7	**Total**	**1980**

- [x] 11. Add 360 µL of transformation master mix to each tube and immediately vortex until the cells are smoothly suspended.

✓ 12. Heat shock cells in water-bath or heat-block: 42°C/1 h.

01:00:00

✓ 13. Centrifuge 3,000 g/5 min; discard supernatant and resuspend each cell pellet in 100 µL sterile H_2O, then plate cells on the SC-His-Trp.

Additional resources

PCR product amou...

	KO PCR product [ng]
1	1000

Transformation single reaction

	A	B
1		[µL]
2	50% PEG	240
3	1 M LiAc	36
4	2mg/ml ssDNA	50
5	PCR product	10.0
6	dHd2O	24.0
7	**Total**	**360**

MAT Colony PCR

Introduction

For genotyping of MAT locus: the primers bind upstream (F) and downstream (R) of MAT locus, and PCR is used to determine if His3 has replaced MAT by homologous recombination.

Materials

- TaKaRa LA Taq® DNA Polymerase (Mg2+ free buffer) RR002A
- 10 µM Primer F: OLPr014 MAT -283 F
- 10 µM Primer R: OLPr015 MAT +295 R
- SC-His-Trp plates
- Template: yeast colonies from transformation of STE4Δ::TRP1 strains with MAT KO PCR product

Procedure

PCR

1. Chose the number of 10 µL reactions (include 3 additional reactions to account for wild-type, mutant, and no-template control):

Settings

	# of reactions
1	11

2. Combine the following reagents to prepare the master mix (contains 0.5 additional reaction to account for pipetting discrepancies), and aliquot 10 µL master mix/pcr tube:

Master mix

	A	B
1		**11.5 X [µL]**
2	ddH2O	67.85
3	10X LA PCR Buffer	11.5
4	25 mM MgCl2	11.5
5	dNTP Mix (2.5 mM each)	18.4
6	10 µM OLPr014 MAT -283 F	2.3
7	10 µM OLPr015 MAT +295 R	2.3
8	LA Taq Polymerase (5 U/µL)	1.15
9	**Total**	**115**

- 3. In sterile work environement (e.g. laminar flow), gently touch a yeast colony with a 10 µL pipette tip so a small number of cells stick to it, touch the tip to a fresh reference SC-His-Trp plate, and resuspend cells in PCR reaction.

  Do not use too many cells as they might inhibit the PCR reaction. The PCR reaction should only look slightly opaque from the resuspended cells. If the reaction looks milky then you might have used too many cells.
  The reference plate is incubated 30°C/2–3 days.

- 4. PCR program "MATCOLON" in folder "STEPHAN":

  94°C/5 min—[94°C/0.5 min—61°C/0.5 min—68°C/3 min]x35—68°C/5 min—10°C/∞

Gel

- 5. Gel: 10 µL/0.5 µg O'GeneRuler DNA Ladder Mix/1% TAE/120 V/35 min

00:35:00

Expected product sizes:
MATa 2974 bp
MATα 3085 bp
MATΔ::His3 1958 bp
Stop processing any yeast strains with the wrong genotype (i.e. discard any corresponding overnight cultures and patches on reference plate).

Additional resources

Primers and template

	A	B
1	Forward Primer	OLPr014 MAT -283 F
2	Reverse Primer	OLPr015 MAT +295 R
3	Template	yeast colonies

PCR Volume and reagent concentrations

	A	B
1	PCR Volume [µL]	10
2	Starting buffer conc [X]	10
3	Final buffer conc [X]	1
4	Starting Mg2+ conc [mM]	25
5	Final Mg2+ conc [mM]	2.5
6	Starting dNTP conc each [mM]	2.5
7	Final dNTP conc each [mM]	0.4
8	Starting Primer F conc [µM]	10
9	Final Primer F conc [µM]	0.2
10	Starting Primer R conc [µM]	10
11	Final Primer R conc [µM]	0.2
12	Starting Polymerase conc [U/µL]	5
13	Final Polymerase conc [U/µL]	0.05
14	Starting Template conc [ng/µL]	5
15	Final Template conc [ng/µL]	0

Single PCR reaction

	A	B
1		[µL]
2	ddH2O	5.9
3	10X PCR Buffer	1
4	25 mM MgCl2	1
5	dNTP Mix (2.5 mM each)	1.6
6	10 µM Primer F	0.2
7	10 µM Primer R	0.2
8	LA Taq Polymerase (5 U/µL)	0.1
9	Template (5 ng/µL)	0
10	Total	10