PeerJ Preprints NOT PEER-REVIEWED

1

2 **Generation and Characterisation of Temperature Sensitive Mutants of Genes Encoding**
3 **the Fission Yeast Spindle Pole Body**

4

5 Ngang Heok Tang[±1], Chii Shyang Fong[*1], Hirohisa Masuda[#], Isabelle Jourdain[∨], Masashi
6 Yukawa[∧] and Takashi Toda[∧, ¶, ‡]

7

8 [±] Section of Neurobiology, Division of Biological Sciences, University of California, San
9 Diego, La Jolla, CA 92093, USA
10 [*] Cell Biology Program, Memorial Sloan Kettering Cancer Center, New York, NY 10065,
11 USA
12 [#] Telomere Biology Section, Laboratory of Biochemistry and Molecular Biology, National
13 Cancer Institute, National Institutes of Health, Bethesda, MD 20892-4255, USA
14 [∨]Biosciences, College of Life and Environmental Sciences, University of Exeter, Geoffrey
15 Pope Building, Stocker Road, Exeter EX4 4QD, United Kingdom
16 [∧] Hiroshima Research Center for Healthy Aging (HiHA), Department of Molecular
17 Biotechnology, Graduate School of Advanced Sciences of Matter, Hiroshima University, 1-3-
18 1 Kagamiyama, Higashi-Hiroshima, 739-8530, Japan
19 [¶] The Francis Crick Institute, Lincoln's Inn Fields Laboratory, 44 Lincoln's Inn Fields,
20 WC2A 3LY, London, United Kingdom

21

22 [1] Contributed equally
23 ‡ Corresponding author: E: takashi-toda@hiroshima-u.ac.jp; T: 81-(0)82-424-7868

24

25 **Key Words:** the spindle pole body; fission yeast; temperature sensitive mutants; the γ-tubulin
26 complex; PCR, drug resistance; replica plating

27

28 **Running Head:** Genetic study of the spindle pole body

29

30 **Abbreviations used:** γ-TuC, γ-tubulin complex; GOI, gene of interest; MT, microtubule;
31 MTOC, microtubule organising centre; PCR, polymerase chain reaction; RT, room
32 temperature; SPB, spindle pole body; ts, temperature sensitive

33

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.3377v1 | CC BY 4.0 Open Access | rec: 27 Oct 2017, publ: 27 Oct 2017

1 **Abstract**

2 The spindle pole body (SPB) in fungi is the equivalent of the animal centrosome. A number
3 of previous studies have identified many, if not all, components of the SPB. The SPB is the
4 structural platform for microtubule nucleation and plays important roles, both in mitosis and
5 meiosis. The SPB is absolutely essential for cell survival and its abnormalities give rise to
6 aberrant cell division and morphogenesis. Therefore, it is crucial to understand how the SPB
7 organises itself and how the functions of individual SPB components are regulated. We report
8 here a procedure to generate temperature sensitive mutants in the fission yeast,
9 *Schizosaccharomyces pombe*. The approach has proved useful to characterise functions of
10 individual SPB components. This original genetic manipulation is however not restricted to
11 analysis of SPB functions, and can be suited to investigate other cellular processes in *S.*
12 *pombe*.
13

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.3377v1 | CC BY 4.0 Open Access | rec: 27 Oct 2017, publ: 27 Oct 2017

1 **Introduction**

2 Microtubules (MTs) play essential roles in a variety of physiological processes, including
3 maintenance of cell polarity, intracellular trafficking and chromosome segregation. MTs are
4 composed of repeating αβ-tubulin heterodimers assembled in a head-to-tail configuration.
5 The α-tubulin faces the slow-growing minus end whereas the β-tubulin faces the fast-growing
6 plus end, giving rise to an intrinsic polarity of the MTs *(1)*. One of the key events in MT
7 assembly is its initial nucleation. The MTs are nucleated from a structural platform known as
8 the microtubule organising centre (MTOC) *(2)*. The major MTOC in animal cells is the
9 centrosome, which is made up of two perpendicular centrioles and a surrounding electron-
10 dense pericentriolar material *(3)*. One of the components enriched in the centrosome is a third
11 tubulin subunit, γ-tubulin, which forms the γ-tubulin complex (γ-TuC) and is essential for
12 initiation and regulation of microtubule assembly *(1, 4, 5)*.
13 The major MTOC in fungi is referred to as the spindle pole body (SPB), a disc-like structure
14 associated with or embedded in the nuclear envelope. In the fission yeast,
15 *Schizosaccharomyces pombe*, the SPB is observed using electron microscopy as an oblate
16 ellipsoid (90 nm thick and about 180 nm in diameter) *(6)*. The SPB resides on the
17 cytoplasmic surface of the nuclear envelope during interphase and mostly is inactive for
18 microtubule nucleation. Upon the G2-M transition, it undergoes structural alterations, thereby
19 being inserted into the nuclear envelope and activated to nucleate spindle microtubules. The *S.*
20 *pombe* SPB components have been identified by screening a bank of temperature sensitive
21 mutants for mitotic defects, studying intercellular protein localisation of GFP-fusion genomic
22 DNA library, and searching for genes encoding proteins homologous to centrosomal
23 components. Although the centrosome and the SPB are apparently different in their structures
24 and organisation, these two organelles in fact have many components in common due to their
25 functional similarity. These include integral components such as γ-TuC components (γ-

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.3377v1 | CC BY 4.0 Open Access | rec: 27 Oct 2017, publ: 27 Oct 2017

1 tubulin, GCP2~6, MOZART1), receptors of the γ-TuC (Pericentrin/Pcp1, etc), Centrin
2 (Cdc31) and its binding partner Sfi1 as well as transient passengers such as cell cycle-
3 regulating kinases (Cdk1, Polo, Wee1 etc) and spindle assembly checkpoint proteins (Mad1,
4 Mad2, etc) *(7)*.
5
6 Conditional mutants are a powerful tool for functional analysis of essential genes. The use of
7 temperature sensitive (ts) mutants in *S. pombe* has enabled detailed studies of many genes in
8 different cellular processes. A ts mutant is able to grow in complete medium at the permissive
9 temperature (27°C) as in wild type and display defective phenotypes only at the restrictive
10 temperature (36°C). Many methods, including random mutagenesis, have been successfully
11 used to generate ts mutants in *S. pombe*. This method involves the use of error-prone
12 polymerase chain reaction (PCR), which creates mutations in the amplified gene of interest
13 (GOI). Here, we describe the methods aiming for generating and screening of ts mutants
14 using random mutagenesis by error-prone PCR (schematically shown in Figure 1).
15
16 **Materials and Methods**
17 ***Media and S. pombe cells***
18 Prepare all solutions and media using MilliQ water (18.2 Mohm at 25°C). Unless indicated
19 otherwise, prepare and store all reagents at room temperature (RT). Diligently follow all
20 waste disposal regulations. The basic materials and procedures required to grow *S. pombe*
21 cells can be obtained from the Fission Yeast Handbook;
22 (http://research.stowers.org/baumannlab/documents/Nurselab_fissionyeasthandbook_000.pdf
23), and from literatures *(e.g. 8)*. There are community websites at the University of Southern
24 California that provides protocols, lists of plasmids and other information (www.pombe.net)
25 and at PomBase (http://www.pombase.org). *S. pombe* strains, plasmids, libraries, and cDNA

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.3377v1 | CC BY 4.0 Open Access | rec: 27 Oct 2017, publ: 27 Oct 2017

1 and genomic DNA clones can be purchased from the National BioResource Project – Yeast
2 (http://yeast.lab.nig.ac.jp/nig/index_en.html).
3 1. Yeast Extract 5 Supplements (YE5S): 0.5% yeast extract, 3% D-glucose, 250 mg/L of
4 each supplement (Adenine, Leucine, Uracil, Histidine, Lysine). Adjust the pH to 5.6
5 with hydrochloric acid (HCl) (note that *S. pombe* cells cannot grow at a neutral pH).
6 Autoclave at 120°C for 15 min. YE5S-broth can be kept for several months at RT.
7 2. YE5S-agar: add 20 g agar to YE5S media prior to adjusting the pH. Poured plates can
8 be kept for several weeks in the fridge. Polymerised YE5S-agar can also be kept for
9 several months at RT, microwaved until homogenous, and poured at a later date.
10 If preferred, YE5S powder, and YE5S-agar powder are commercially available
11 (Sunrise Science Products).
12 3. Mating/Sporulation media (ME4S): 3% Bacto-malt extract. Supplements added as for
13 YE5S except lysine. Adjust to pH 5.5 with NaOH.
14 4. Antibiotics: geneticin (G418, aka kan) at a final concentration of 100 µg/mL,
15 hygromycin B (hph) at 300 µg/mL, Nourseothricin-dihydrogen (nat) at 100 µg/mL.
16 Antibiotics are added to YE5S-agar only, not to YE5S-broth. Plates with antibiotics
17 can be kept for several weeks at 4°C.
18 5. Strains are kept in a -80°C freezer in Yellow Freezing Mix: 1% yeast extract, 1%
19 bacto tryptone, 2% glucose, 25% glycerol.
20 6. Wild type strains 972 h^-, 975 h^+ or auxotrophic derivatives.
21 7. Benchtop Bunsen burner or access to a laminar flow hood.
22 8. Spectrophotometer (595 nm), haemocytometer or automatic cell counter (type
23 Sysmex or Beckman coulter counter) to count cells.
24 9. Benchtop optical microscope, slides and coverslips.

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.3377v1 | CC BY 4.0 Open Access | rec: 27 Oct 2017, publ: 27 Oct 2017

1 10. Erlenmeyer conical borosilicate, narrow neck 250 mL flasks. Cover flasks with
2 aluminium foil paper and sterilise.
3 11. Sterile plastic ware: pipette tips, 1.5 mL microcentrifuge tubes, 50 mL tubes, etc.
4 12. Sterile Petri dishes: 3xVent 9 cm.
5 13. Sterile wooden toothpicks or inoculating loops.
6 14. A shaking incubator set at 27°C.
7 15. Non-shaking incubators set at different temperatures, ranging from 27-36°C.

8 ***Replica-plating***

9 1. Cylindrical wooden block of the diameter of Petri dishes used, or replica plater from
10 Sigma-Aldrich (#Z363391-1EA).
11 2. Cling film.
12 3. Tissue paper.
13 4. Rubber band.
14 5. Whatman filter paper. Alternatively, velvet squares Sigma-Aldrich) can be used.
15 6. 70% v/v ethanol.

16 ***PCR Amplification of a drug-resistant cassette***

17 1. Plasmids pFA6-kanMX6 (#39296, Addgene), pFA6a-5FLAG-hphMX6 (#19342,
18 Addgene) or pFA6a-natMX6-Purg1 (#39350, Addgene) *(9, 10)*.
19 2. 100 mer-long, PAGE-purified oligonucleotides (we use Sigma-Aldrich
20 oligonucleotide synthesis service).
21 3. PCR machine.
22 4. PrimeSTAR polymerase PCR kit (Takara).
23 5. dNTP mix.
24 6. 1% agarose gel.
25 7. PCR purification kit.

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.3377v1 | CC BY 4.0 Open Access | rec: 27 Oct 2017, publ: 27 Oct 2017

1 ***Colony PCR***

2 1. PCR machine.

3 2. PCR tubes.

4 3. Heat block (95°C).

5 4. Z taq DNA polymerase (Takara).

6 5. dNTP mix.

7 6. 1% agarose gel.

8 7. 1% Sarcosyl (N-lauroylsarcosine sodium salt).

9 8. 10 M NaOH: 40 g NaOH and top up to 100 mL with ddH_2O.

10 ***Random mutagenesis using error-prone PCR***

11 1. MasterPure Yeast DNA purification kit (Cambio Ltd.).

12 2. PCR purification kit.

13 3. PCR machine.

14 4. Vent DNA polymerase (New England Biolabs).

15 5. dNTP set: all 4 dNTP provided separately.

16 6. Two standard 20-30 mer primers that correspond to the 500 bp 5'- and 3'-flanking

17 regions of the gene of interest.

18 ***Transformation of PCR fragments into S. pombe cells***

19 1. Incubator-shaker (27˚C).

20 2. Cell counter (type Sysmex or Beckman coulter counter), haemocytometer or

21 spectrometer.

22 3. Rotary shaker.

23 4. Centrifuges: bench top for Eppendorf tube, large for 50 mL tubes. All rotor types are

24 suitable.

25 5. Waterbath at 42˚C.

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.3377v1 | CC BY 4.0 Open Access | rec: 27 Oct 2017, publ: 27 Oct 2017

1 6. Dimethyl sulfoxide (DMSO).
2 7. Salmon Sperm DNA (Invitrogen).
3 8. Sterilised glass balls (VWR).
4 9. At least 3L of YE5S broth, 150 plates of YE5S and 100 plates of YE5S+G418. Plates
5 do not have to be all poured in advance.
6 10. 1 M (10x) Lithium acetate (LiOAc), pH 7.5. Adjust the pH with 0.05 M acetic acid,
7 autoclave, and store at room temperature.
8 11. 1 M Tris-HCl, pH 7.5.
9 12. 10 M sodium hydroxide (NaOH).
10 13. 0.1 M EDTA, pH 8.0.
11 14. 10x TE buffer, pH 7.5: 100 mM Tris-HCl pH 7.5, 10 mM EDTA pH 8.0. Autoclave
12 and store at room temperature.
13 15. LiOAc/TE solution: 0.1 M LiOAc in 1x TE. Make fresh immediately prior to use and
14 syringe-sterilise (0.2 µm pore size).
15 16. 40% PEG/LiOAc/TE solution: for 20 mL use 8g PEG3640, 2 mL 10x LiOAc
16 solution, 2 mL 10x TE, in ddH2O. Microwave briefly to solubilise. Syringe-sterilise
17 (0.2 µm pore size) and cool down before use. Do not store for longer than 1 month.
18 ***Isolation and backcross check of ts mutants***
19 1. Phloxin B stock 5 mg/mL (Sigma-Aldrich).
20 2. YE5S+Phloxin B plates: add Phloxin B to YE5S at 5.5 µg/mL.
21 3. Glusulase (PerkinElmer).
22 4. 95% Ethanol.
23 ***Assessment of temperature sensitivity by spot test***
24 1. Sterile, flat bottom, 96-well plates.
25 2. 20-200 µL multi-channel pipette.

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.3377v1 | CC BY 4.0 Open Access | rec: 27 Oct 2017, publ: 27 Oct 2017

3. 48-pin replicator (type #R2383-1EA, Sigma-Aldrich)

3. Results

S. pombe cells are usually plated on rich agar media (i.e. YE5S) for growth and maintenance. However, the cells can be replica-plated into different selective plates (e.g. drug selection plates or minimal agar plate) to select for the colonies with the genotype of interest. In brief, all colonies from the original plates are transferred to a filter paper, and then from the filter paper to the final plates.

1. Assemble the replica plating kit as shown in Figure 2A. In summary, a piece of cling film is secured on top of a wooden block by a rubber band. This forms the base of a replica kit. (*See* **Note 1**).
2. Then, assemble a piece of Whatman paper on top of a piece of tissue paper. Secure them onto the wooden block using another rubber band. This forms the filter paper used for replica plating. (*See* **Note 2**).
3. The following steps are summarised in Figure 2B.
4. Apply the plate to be replicated onto the Whatman paper. Tap gently with the palm of the hand, to ensure all the colonies on plate are transferred to the Whatman paper.
5. Remove the plate from the Whatman paper. (*See* **Note 3**).
6. Apply selection plates onto the replica kit containing colonies. Gently tap to ensure even distribution of the agar-paper surface.
7. Remove the plate from the Whatman paper, as in step 5. (*See* **Note 4**).
8. Step 6 & 7 can be repeated for multiple times with other selection plates, if desired.
9. Incubate replica-plated plates at desired temperature (e.g. 27°C). Colonies take 2-3 days to appear after replica-plating at 27°C.

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.3377v1 | CC BY 4.0 Open Access | rec: 27 Oct 2017, publ: 27 Oct 2017

10. When a set of replica plating is done, untie the second rubber band and discard the tissue + Whatman papers.
11. Clean the cling film with 70% ethanol. Ensure the cling film is still securely attached on the wooden block.
12. The apparatus can be re-used for next replica plating experiment.

PCR amplification of a drug-resistant cassette

In order to make the selection process easier, we first construct a wild-type strain that contain a drug resistance marker in the 3' end of the gene of interest (GOI). The procedure presented here is for the insertion of a kan^R cassette. (*See* **Note 5**).

1. Design a set of 100-mer primers in which 80 bases target the GOI and 20 bases target the resistance cassette (Table 1). Primers can be designed using PPPP (Pombe PCR Primer Programs; http://www.bahlerlab.info/resources/). Select the C-terminal tagging option and follow the steps. Be aware that this function will delete the STOP codon between the GOI and insertion cassette. For insertion of drug resistant cassette, it is very important that the STOP of GOI is re-included in the 20-mer GOI sequence. For gene targetting (e.g. GFP), the 80-mer should include 80 bases immediately upstream of the stop codon. (*See* **Note 6**).
2. Set up a PCR reaction and run it as indicated in Figure 3.
3. Run 1-2 µL of the amplified PCR fragment on 1% agarose gel. For the kan^R cassette, the correct PCR fragment should be of ~1.6 kb long. (*See* **Note 7**).
4. Concentrate the remaining PCR fragments into 10 µL of double-distilled water. (*See* **Note 8**).
5. Transform the rest of the PCR fragment into WT h^- *S. pombe* cells (see below for transformation protocol). (*See* **Note 9**).

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.3377v1 | CC BY 4.0 Open Access | rec: 27 Oct 2017, publ: 27 Oct 2017

1 6. Once the kan^R strain is obtained, streak it along with the WT on YE5S at several
2 temperatures, to check that the presence of the cassette does not affect growth. (*See*
3 **Note 10**).

4 **Transformation of PCR fragments into S. pombe cells**

5 Transformation of *S. pombe* cells is a method used to introduce genetic modification in the
6 cell by incorporation of exogenous DNA fragments. The basic principle of transformation is
7 to facilitate delivery of DNA fragments through the cell wall and cell membrane into the
8 cytosol to reach the nucleus. Once the exogenous DNA fragments are delivered into the
9 nucleus, homologous recombination takes place. Several methods have been reported for
10 yeast cell transformation, including the lithium acetate method, electroporation and
11 spheroplasting. Here, we describe the lithium acetate method *(9, 11)*. The procedure is carried
12 out at room temperature, unless otherwise specified.

13 1. Before starting, boil the salmon sperm DNA for 5 min, then leave on ice (2 µL of a 10
14 mg/mL stock will be needed per transformation). Prepare the PEG/LiOAc/TE if it is
15 older than 1 month old, or microwave it if it has crystalised to return the components
16 into solution.
17 2. Grow cells in 50 mL YE5S liquid culture overnight at 27˚C, with shaking at 200 rpm.
18 (*See* **Note 11**).
19 3. Make sure the cells are in exponentially growing phase before transformation. (*See*
20 **Note 12**).
21 4. Harvest exponentially growing cells into 50 mL tubes by centrifugation at 3000 rpm
22 for 3-5 minutes. (*See* **Note 13**).
23 5. Discard supernatant and wash the cells with 10 mL of Lithium Acetate with TE
24 (LiOAc/TE), for 5 minutes with gentle rotation. Spin them down as before. Repeat
25 this step once (wash twice in total).

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.3377v1 | CC BY 4.0 Open Access | rec: 27 Oct 2017, publ: 27 Oct 2017

6. Resuspend the cells in 100 μL of LiOAc/TE and transfer the cell solution into a 1.5 mL microcentrifuge tube.
7. Add 7.5 μL of carrier DNA and 10 μL DNA fragments into the cell solution. (*See* **Note 14**).
8. Gently mix the solution by vortex for 3 seconds, followed by 10 minutes on a rotating wheel mixer.
9. Add 260 μL of 40% PEG/LiOAc/TE and vortex briefly for 3 seconds. (*See* **Note 15**).
10. Incubate the solution at 27˚C for 2 hours, shaking at 220 rpm.
11. Add 43 μL of DMSO and vortex briefly for 3 seconds.
12. Heat shock at 42˚C for 5 minutes. (*See* **Note 16**).
13. Wash the cells with 500 μL of YE5S. Spin down at 5000 rpm for 10 seconds.
14. Discard supernatant and resuspend the cells in 500 μL of YE5S.
15. Incubate the cells at 27˚C for 1.5 hours, shaking at 220 rpm. (*See* **Note 17**).
16. Spin down the cells at 5000 rpm for 10 seconds. Discard 200 μL of the supernatant and resuspend the cells in the remaining 300 μL of YE5S. (*See* **Note 18**).
17. Plate 150 μL of cell solution onto YE5S plate, using glass balls. Shake gently to evenly spread the solution on the plate. Throw the glass balls into a beaker containing a bit of ethanol 70%. (*See* **Note 19**).
18. Incubate the plates upside-down at 27˚C for 18-24 hours.
19. Replica-duplicate the colonies from these YE5S plates into YE5S+G418 plates. Incubate plates at 27˚C for 4-5 days. (*See* **Note 20**).
20. Cells that have grown on G418 plates should have kan^R cassette inserted into the GOI. In addition, check correct insertion of the kan^R cassette by colony PCR (*See* **Note 21**).
21. Pick the grown colonies, streak on a fresh YE5S plate and freeze in YFM as lab stock for future use.

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.3377v1 | CC BY 4.0 Open Access | rec: 27 Oct 2017, publ: 27 Oct 2017

Colony PCR

1. Use a sterilised toothpick or pipette tip to pick a small amount of cells from a single colony and transfer to a PCR tube containing solution as in step 2.
2. Suspend the cells in 12 µL of 40 mM NaOH, 0.01% sarcosyl (freshly made from 10 M NaOH and 1% sarcosyl).
3. Boil the sample at 95°C for 15 minutes.
4. Place the tube on ice to cool for 3 minutes.
5. Vortex for 3-5 seconds to mix the solution.
6. Spin down the cells at 5000 rpm for 10 seconds.
7. Use 1.5 µL of the supernatant and 18.5 µL of reaction mixture for PCR (Figure 4).

Random mutagenesis by error-prone PCR

In order to create point mutations in the GOI, random mutagenesis by error-prone PCR can be performed. This method has proved very useful to identify novel functions of several genes *(12-15)*. In brief, the GOI is amplified by PCR using unbalanced dNTPs, where one of the nucleotides is ten times more concentrated than the three others, thereby making the PCR amplification error-prone. Such PCRs are less efficient than PCRs that use a standard dNTP mix, and conditions of amplification may need to be tested and optimised (e.g. type of polymerase). Thus far, the best results we have obtained were by using 10× dGTP and Vent DNA polymerase. This protocol describes methods involved in creating random mutations anywhere in the gene, without preference. (*See* **Note 22**).

1. Purify genomic DNA from a strain containing GOI-kan^R using the commercial MasterPure Yeast DNA purification kit (Cambio Ltd.), following the manufacturer's instructions.

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.3377v1 | CC BY 4.0 Open Access | rec: 27 Oct 2017, publ: 27 Oct 2017

1 2. Design a set of primers (20-30 mer) that anneal to 500 bp upstream (forward primer;
2 500 bp before the START codon) and 500 bp downstream (reverse primer in reverse
3 complimentary form; 500 bp after the STOP codon) of the GOI-kan^R. (*See* **Note 23**).
4 3. Amplify the GOI-kan^R fragment with error-prone PCR using Vent DNA polymerase
5 supplemented with 10× dGTP (100 mM for dGTP; 10 mM for dATP, dTTP and
6 dCTP). Follow manufacturer's instructions for PCR conditions.
7 4. Run a portion (1-2 μL is sufficient) of the amplified PCR fragment on 1% agarose gel.
8 5. If a product was amplified at the right size and in sufficient amount (a total of approx.
9 400 μg are needed for each PCR), re-do another 40 PCRs using the same conditions.
10 6. Using a commercial kit or by ethanol precipitation, concentrate each PCR reaction
11 individually into 10 μL of water.
12 7. Transform each concentrated PCR reaction twice into WT *h*$^-$ *S. pombe* cells (i.e. do 80
13 transformations). (*See* **Note 24**).
14 8. Allow cells to grow at 27°C overnight for endogenous gene replacement to occur.
15 9. Replica the lawn of *S. pombe* cells on YE5S+G418 plates and incubate them at 27°C
16 for 3 to 4 days. Ensure that there are at least a total of 5000 colonies (~200 colonies
17 per plate is ideal) at the end of the incubation.
18 **Isolation of temperature sensitive (ts) mutants**
19 1. Replica each plate onto two YE5S and two YE5S+Phloxin B plates.
20 2. Incubate one YE5S and one YE5S+Phloxin B plate at 27°C for 2 days. Incubate the
21 other YE5S and YE5S+Phloxin B plates at 36°C for 2 days.
22 3. Visually identify candidate ts mutants by their ability to grow and form a colony at
23 27°C, but not at 36°C. Positive clones should not only grow less at 36°C than 27°C, but
24 should also be stained dark pink by Phloxin B, which stains dead cells.

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.3377v1 | CC BY 4.0 Open Access | rec: 27 Oct 2017, publ: 27 Oct 2017

1 4. Pick some cells from each candidate colony, transfer on a microscope slide with a bit
2 (~2 µL) of water, cover with a coverslip and observe under a benchtop microscope to
3 rapidly assess the phenotype. Select clones that show a phenotype of interest, clones
4 that have different phenotypes, and clones that have a range of temperature sensitivity
5 (variations of pinkness on Phloxin B plates).
6 5. Re-streak candidate ts mutants from the YE5S plates incubated at 27°C, onto fresh
7 YE5S plates. Incubate at the permissive temperature 27°C.
8 6. Freeze all candidates in YFM.
9 **Backcross check of ts mutants**
10 1. Cross each ts candidate (having been made in a WT h^- strain, they are h^-) with a WT
11 strain of the opposite mating type (WT h^+) on ME4S to induce conjugation and
12 sporulation. Incubate plates at 27°C for 2 to 3 days. (*See* **Note 25**).
13 2. Confirm asci formation under the microscope. Expect at least 5% asci formation
14 efficiency.
15 3. Inoculate a loopful of asci mix (about one colony size) into 100 µL of 0.5~1%
16 glusulase to breakdown the ascus wall. Incubate tubes at 27°C for 2 hours.
17 4. Add 43 µL of ethanol 95% to the tube to kill any non-sporulating cells. Incubate tubes
18 at room temperature for 30 minutes.
19 5. Pellet the spores by centrifugation (2000 rpm, 1 minute) and resuspend them in 1 mL
20 of H_2O.
21 6. Plate onto YE5S plates (2 µL, 10 µL or 50 µL depending on the efficiency of
22 sporulation) and incubate at 27°C for 3 days.
23 7. Replica each plate onto 1× YE5S, 1× YE5S+G418 and 2× YE5S+Phloxin B plates.
24 8. Incubate the YE5S, YE5S+G418 and 1× YE5S+Phloxin B at 27°C; and the other
25 YE5S+Phloxin B at 36°C.

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.3377v1 | CC BY 4.0 Open Access | rec: 27 Oct 2017, publ: 27 Oct 2017

9. Check the plates after 2 days. A correct ts mutant should always show co-segregation of temperature sensitivity and geneticin resistance.
10. Freeze confirmed ts mutants in YFM. (*See* **Note 26**).

Assessment of temperature sensitivity by spot test

A spot test is a semi-quantitative growth assay where the same number of cells from different strains are "spotted" on plates, which, in this instance, are incubated at various temperatures. This assay can be used to indicate for each mutant, which temperature(s) are permissive, semi-permissive or restrictive. It is also used to assess the extent of the growth defect at a given temperature.

1. Propagate exponentially growing cultures of wild type and ts mutants in YE5S broth.
2. Determine cell density with a cell counter.
3. Dilute cultures to 2×10^7 cells/mL and transfer 100 μL of the diluted culture to a 96-well plate.
4. Prepare four ten-fold serial dilutions in the 96-well plate.
5. Spot cells onto YE5S plates with a 48-pin replicator.
6. Incubate plates at temperatures ranging from 19°C to 36°C, including one at 27°C, for 3 days. See Figure 5 for an example of spot test of ts mutants.

Ts mutants of interest may later be sequenced and utilised in a range of techniques- cell biology, biochemistry, genetics, to study the function of the gene.

Discussion

Several important points for the isolation of temperature sensitive mutants are described below.

1. Avoid bubble formation between the wooden block and cling film. Bubbles will make the pressure applied onto the surface uneven and results in uneven replica plates.

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.3377v1 | CC BY 4.0 Open Access | rec: 27 Oct 2017, publ: 27 Oct 2017

This base (wooden block + cling film) does not have to be changed for every replica plating.

2. Avoid touching the surface of Whatman paper to avoid potential contamination. The papers will contain colonies in the following steps. The papers have to be changed when a set of replica plating is done.
3. Perform this step fast. Slow plate removal may result in uneven colony transfer (colonies still stick onto plate and not transferred onto Whatman paper).
4. Colonies will be transferred from the Whatman paper to the plate. A "colony shape" may be seen on the plate, which contain many cells. However, no colony will be visible on the plate at this point.
5. Available markers include G418 (kan^R), hygromycin B (hph^R) and nourseothricin ($ClonNat^R$). All plasmids containing these drug resistant cassettes have the same backbone (pFA6a) and the region of the primers designed to amplify the cassette is the same between plasmids. Thus, the primers that we present below can suitably be used to insert any of the three drug resistant cassettes.
6. Although it is sometimes non-avoidable, the presence of predicted strong secondary structure or primer dimer (can be predicted by Sigma-Aldrich ordering website) may affect efficiency of PCR amplification.
7. A general protocol for agarose gel electrophoresis can be found at https://www.addgene.org/plasmid-protocols/gel-electrophoresis/.
8. We use QIAquick PCR purification kit for this. Follow the manufacturer's instruction and elute the PCR fragments using 10 µL of double-distilled water.
9. PCR fragment should be transformed into WT cells, so only the correct transformant can grow on kan^R selection plates in the subsequent steps. Avoid transforming GOI-

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.3377v1 | CC BY 4.0 Open Access | rec: 27 Oct 2017, publ: 27 Oct 2017

1 kan^R fragment into other strains containing kan^R or other selection markers, to avoid
2 selection of false positive cells.
3 10. Marker switch can be performed to create hph^R or nat^R strain. Relevant information
4 for marker switch has been reported *(10)*.
5 11. Larger culture could be used to obtain more cells.
6 12. Exponentially growing cells give the best transformation efficiency. Several methods
7 including Beckman Coulter's cell counter and spectrometer can be used to measure
8 cell density. In cell counter, exponentially growing cells give a reading of 5-10 × 10^6
9 cells/mL; in spectrometer, OD_{595} = 0.2 - 0.5
10 13. We use 2 × 10^8 exponentially growing cells for a single transformation. Total cells to
11 be used can be calculated from the cell density reading in step 2.
12 14. Carrier DNA increases the transformation efficiency. We use Salmon sperm DNA
13 (Invitrogen). 300 ng of DNA fragments are usually sufficient to create more than 20
14 kan^R colonies.
15 15. The use of PEG is indispensable for this protocol. Together with carrier DNA, this
16 will enhance the delivery of DNA fragments through the cell wall.
17 16. Although not indispensable, heat shock increases the transformation efficiency,
18 possibly by increasing permeability of the membrane.
19 17. This step will enhance recovery of the cells.
20 18. This will shorten the time to dry the plates in the following steps.
21 19. We use 2 plates for each transformation, to avoid cells over-growing in a single plate.
22 This also creates a back-up in the case of contamination. Direct plating could be
23 performed onto YE5S+G418 plates. However, plating onto YE5S plates will enhance
24 recovery of the cells. Glass balls can be recycled by washed them in ethanol, then
25 water, and autoclaving.

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.3377v1 | CC BY 4.0 Open Access | rec: 27 Oct 2017, publ: 27 Oct 2017

1 20. Incubation time varies with different gene tagging. On average, 4-5 days are
2 sufficient. Replica plate when the colony size is easily visible.
3 21. Design a set of primers: A 20-mer forward primer ~200 bp before the STOP codon of
4 GOI and another 20-mer reverse primer (in reverse complement form) ~ 200 bp after
5 the STOP codon. Cells without kan^R cassette integration have a band size of ~ 400
6 bp, whereas correct integrants with kan^R cassette have a band size of ~2 kb.
7 22. This protocol can be modified to introduce mutations in a specific region of the GOI
8 *(16)*. For example, for a gene of 1000 bp length, the first 500 bp were amplified
9 using error-prone PCR whereas the last 500 bp were amplified using high-fidelity
10 PCR. The first 500 bp fragment should contain several mutations whereas the second
11 500 bp fragment should be indifferent from the wild type. Fusion PCR of these two
12 fragments using a subsequent high-fidelity PCR should produce a 1000 bp DNA
13 fragment that contains mutations only in the first 500 bp fragment. This PCR product
14 can then be transformed into the cells as described previously.
15 23. Primers need not to be long as the amplified, mutated fragments will itself recombine
16 with the endogenous WT gene. The amplified fragment should contain 500bp-GOI-
17 kan^R cassette-500bp. Since the kan^R cassette was inserted immediately downstream
18 of the STOP codon. The presence of 500 bp in each direction facilitates homologous
19 recombination for incorporation of the fragment into the genomic locus after
20 transformation. It is not a problem to subject the kan^R fragment for error-prone PCR.
21 If the cassette is mutated in error-prone PCR, it should not grow in YE5S+G418
22 plate during the selection process.
23 24. Each PCR tube may have produced and amplified different point mutations. In order
24 not to select several times the same mutant, it is preferable not to pull all PCR
25 products together but to transform them individually.

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.3377v1 | CC BY 4.0 Open Access | rec: 27 Oct 2017, publ: 27 Oct 2017

25. Further information on mating can be found in www.pombe.net.
26. Note that this is the backcrossed version. Future experiments should be conducted using the backcrossed strains (non-backcrossed strain may contain excessive background mutations).

Conclusions

The procedure provided in this study is applicable not only for the creation of temperature sensitive mutants for the SPB components but also for more general mutant isolation of any genes of interest. The strategy is based upon a simple principle and experimentation is straightforward, by which everybody working on molecular genetics and cell biology in the fission yeast system can follow. We hope that this methodology will be useful for the fission yeast community.

Acknowledgments

We thank Yoshinori Watanabe (Tokyo University) for initially implementing this method and introducing it to us. This work was supported by Cancer Research UK/the Francis Crick Institute, the Japan Society for the Promotion of Science KAKENHI Scientific Research (A) (16H02503), Challenging Exploratory Research (16K14672) (T.T.) and KAKENHI Scientific Research (C) (16K07694) (M.Y.).

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.3377v1 | CC BY 4.0 Open Access | rec: 27 Oct 2017, publ: 27 Oct 2017

1 **References**

2 1. Nogales, E. (2000). Structural insights into microtubule function. *Annu Rev Biochem*
3 69, 277-302.
4 2. Luders, J. and Stearns, T. (2007). Microtubule-organizing centres: a re-evaluation. *Nat*
5 *Rev Mol Cell Biol* 8, 161-167.
6 3. Bettencourt-Dias, M. and Glover, D.M. (2007). Centrosome biogenesis and function:
7 centrosomics brings new understanding. *Nat Rev Mol Cell Biol* 8, 451-463.
8 4. Petry, S. and Vale, R.D. (2015). Microtubule nucleation at the centrosome and beyond.
9 *Nat Cell Biol* 17, 1089-1093.
10 5. Oakley, B.R., Paolillo, V. and Zheng, Y. (2015). γ-Tubulin complexes in microtubule
11 nucleation and beyond. *Mol Biol Cell* 26, 2957-2962.
12 6. Ding, R., West, R.R., Morphew, M., Oakley, B.R. and McIntosh, J.R. (1997). The
13 spindle pole body of *Schizosaccharomyces pombe* enters and leaves the nuclear
14 envelope as the cell cycle proceeds. *Mol Biol Cell* 8, 1461-1479.
15 7. Fu, J., Hagan, I.M. and Glover, D.M. (2015). The centrosome and its duplication cycle.
16 *Cold Spring Harb Perspect Biol* 7, a015800.
17 8. Forsburg, S.L. and Rhind, N. (2006). Basic methods for fission yeast. *Yeast* 23, 173-
18 183.
19 9. Bähler, J., Wu, J., Longtine, M.S., Shah, N.G., McKenzie III, A., Steever, A.B., Wach,
20 A., Philippsen, P. and Pringle, J.R. (1998). Heterologous modules for efficient and
21 versatile PCR-based gene targeting in *Schizosaccharomyces pombe*. *Yeast* 14, 943-951.
22 10. Sato, M., Dhut, S. and Toda, T. (2005). New drug-resistant cassettes for gene
23 disruption and epitope tagging in *Schizosaccharomyces pombe*. *Yeast* 22, 583-591.
24 11. Okazaki, K., Okazaki, N., Kume, K., Jinno, S., Tanaka, K. and Okayama, H. (1990).
25 High-frequency transformation method and library transducing vectors for cloning

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.3377v1 | CC BY 4.0 Open Access | rec: 27 Oct 2017, publ: 27 Oct 2017

1 mammalian cDNAs by trans-complementation of *Schizosaccharomyces pombe*. *Nucleic*
2 *Acids Res* 18, 6485-6489.
3 12. Fong, C.S., Sato, M. and Toda, T. (2010). Fission yeast Pcp1 links polo kinase-
4 mediated mitotic entry to γ-tubulin-dependent spindle formation. *EMBO J* 29, 120-130.
5 13. Hsu, K.S. and Toda, T. (2011). Ndc80 internal loop interacts with Dis1/TOG to ensure
6 proper kinetochore-spindle attachment in fission yeast. *Curr Biol* 21, 214-220.
7 14. Masuda, H., Mori, R., Yukawa, M. and Toda, T. (2013). Fission yeast
8 MOZART1/Mzt1 is an essential γ-tubulin complex component required for complex
9 recruitment to the MTOC, but not its assembly. *Mol Biol Cell* 24, 2894-2906.
10 15. Tang, N.H., Takada, H., Hsu, K.S. and Toda, T. (2013). The internal loop of fission
11 yeast Ndc80 binds Alp7/TACC-Alp14/TOG and ensures proper chromosome
12 attachment. *Mol Biol Cell* 24, 1122-1133.
13 16. Chmielewska A.M., Tang, N.H. and Toda T. (2016). The hairpin region of Ndc80 is
14 important for the kinetochore recruitment of Mph1/MPS1 in fission yeast. *Cell Cycle*
15 15, 740-747.
16 17 Zhang, J. and Megraw, T. L. (2007) Proper recruitment of γ-tubulin and D-TACC/Msps
17 to embryonic *Drosophila* centrosomes requires centrosomin motif 1. *Mol Biol Cell* 10,
18 4037-404.
19 18 Flory, M. R., Morphew, M., Joseph, J. D., Means, A. R. and Davis, T. N. (2002) Pcp1p,
20 an Spc110p-related calmodulin target at the centrosome of the fission yeast
21 *Schizosaccharomyces pombe*. *Cell Growth Differ* 13, 47-58.
22 19 Gillingham, A. K. and Munro, S. (2000) The PACT domain, a conserved centrosomal
23 targeting motif in the coiled- coil proteins AKAP450 and pericentrin. *EMBO Rep* 1,
24 524-529.

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.3377v1 | CC BY 4.0 Open Access | rec: 27 Oct 2017, publ: 27 Oct 2017

1 20 Lin, T. C., Neuner, A., Schlosser, Y. T., Scharf, A. N., Weber, L. and Schiebel, E.
2 (2014) Cell-cycle dependent phosphorylation of yeast pericentrin regulates γ-TuSC-
3 mediated microtubule nucleation. *eLife* 3, e02208
4

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.3377v1 | CC BY 4.0 Open Access | rec: 27 Oct 2017, publ: 27 Oct 2017

1 **Table 1. Oligonucleotides used for insertion of drug-resistant cassette.** Forward and
2 reverse primers are long oligonucleotides used for PCR-based gene targeting method (Bähler
3 et al., 1998).
4

Primer	**Sequence 5' to 3'** 5
Forward	(80-mer GOI sequence)- CGGATCCCCGGGTTAATTAA
Reverse	(80-mer GOI sequence)- GAATTCGAGCTCGTTTAAAC

6
7
8

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.3377v1 | CC BY 4.0 Open Access | rec: 27 Oct 2017, publ: 27 Oct 2017

1 **Figure Legends**

2 **Figure 1. Schematic diagram of the temperature sensitive mutant isolation strategy**

3 Randomly mutagenised PCR fragments of GOI are transformed into a wild type fission yeast
4 strain. Cells are allowed to grow on non-selective medium overnight for endogenous gene
5 replacement to occur. Transformants are then selected on plates containing the selection drug
6 at 27°C for 3 to 4 days. Replicated colonies are incubated at 27°C and 36°C. Temperature
7 sensitive mutants are those that do not grow at 36°C, but grow normally at 27°C. Phloxin B is
8 used to confirm dead cells.

9

10 **Figure 2. Replica plating**

11 A) Assembly of the replica plating kit. B) Replica plating of plates.
12 See Method 3.1 for the detailed procedures.

13

14 **Figure 3. Composition of PCR reaction mixture for amplification of a drug-resistant**
15 **cassette**

16 A) Components of PCR reaction mixture, as indicated by manufacturer's instructions
17 (#R006A, Takara). B) Conditions used for PCR reaction.

18

19 **Figure 4. Composition of PCR reaction mixture for colony PCR**

20 A) Components of PCR reaction mixture, as indicated by manufacturer's instructions
21 (#R006A, Takara). B) Conditions used for PCR reaction.

22

23 **Figure 5. Example of spot test and identified alleles of temperature-sensitive mutants**

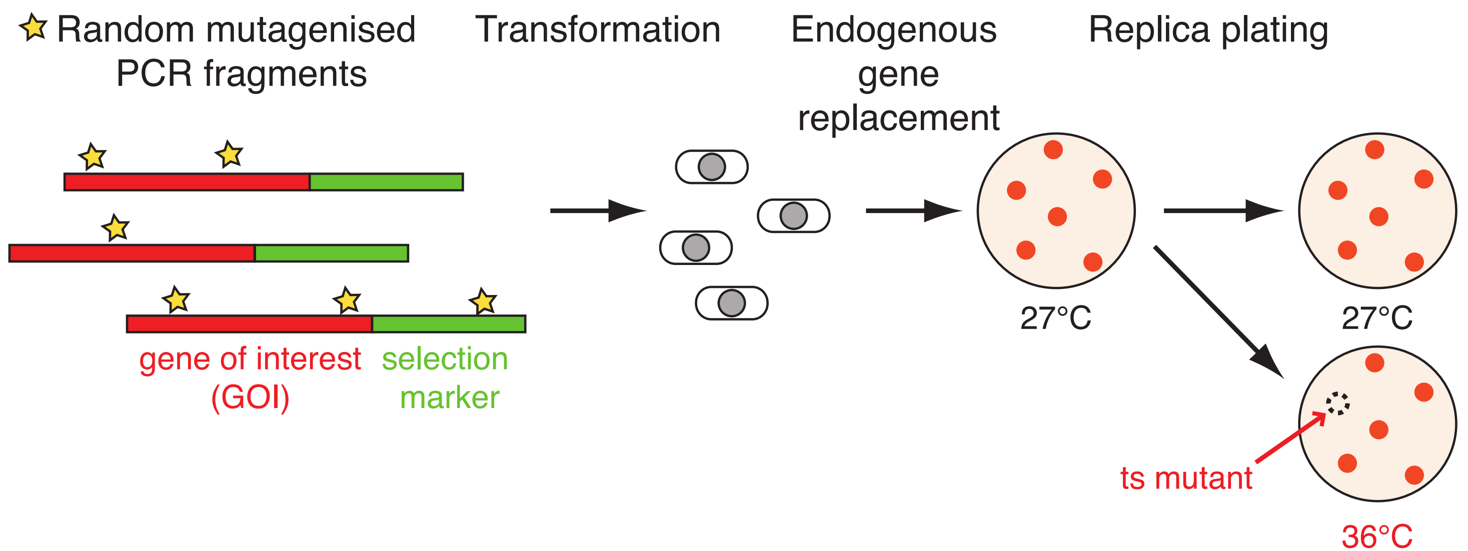
24 A) Shown here are *pcp1* ts mutants. Pcp1 plays a role in spindle microtubule organisation as
25 a receptor/platform for the γ-TuC at the SPB *(12)*. Ten-fold serial dilution assays on YE5S

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.3377v1 | CC BY 4.0 Open Access | rec: 27 Oct 2017, publ: 27 Oct 2017

1 media. 5×10^4 cells were applied in the first spot and plates were incubated at 27°C or 36°C
2 for 3 days. B) Summary of mutation sites in *pcp1* ts mutants. Functional domains within Pcp1
3 (e.g. SPM, CM 1 and PACT) *(12, 17-20)* are indicated together with mutated amino acid
4 residues in individual ts mutants.
5

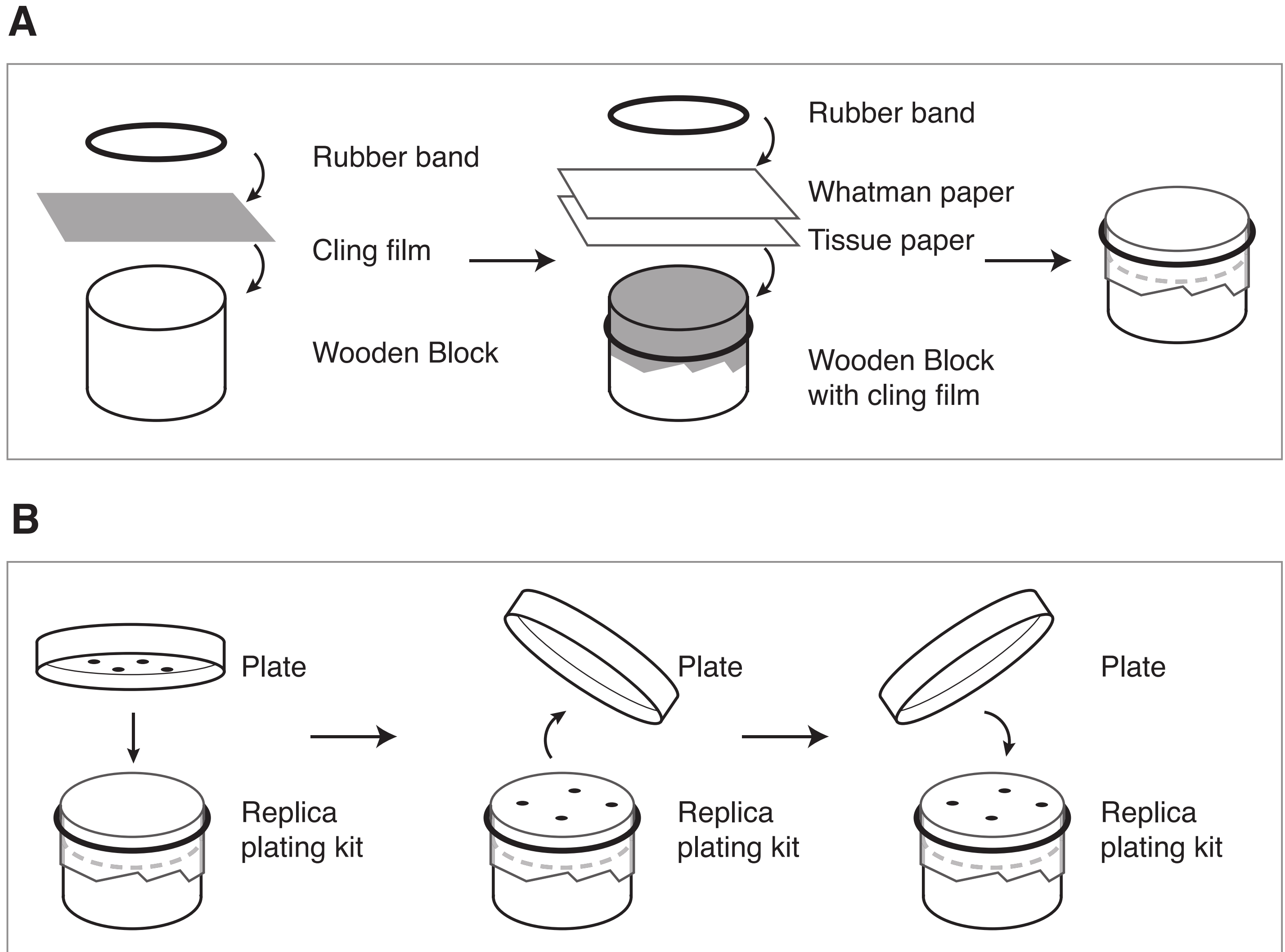
PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.3377v1 | CC BY 4.0 Open Access | rec: 27 Oct 2017, publ: 27 Oct 2017

Figure 1. Tang et al.



PeerJ Preprints NOT PEER-REVIEWED

Figure 2. Tang et al.



PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.3377v1 | CC BY 4.0 Open Access | rec: 27 Oct 2017, publ: 27 Oct 2017

Figure 3. Tang et al.

A

Component	Amount
5X PrimeSTAR Buffer (Mg^{2+} Plus)	10 μl
dNTP Mixture (2.5 mM each)	4 μl
Forward Primer	50 pmol
Reverse Primer	50 pmol
Template (pFA6a-kanMX6)	5-10 ng
PrimeSTAR HS DNA Polymerase (2.5 U/μl)	0.5 μl
Double-Distilled Water	up to 50 μl

B

Temperature	Time	
94˚C	1 min	
98˚C	10 sec	35 cycles
52˚C	15 sec	35 cycles
72˚C	2 min	35 cycles
72˚C	2 min	
4˚C	storage	

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.3377v1 | CC BY 4.0 Open Access | rec: 27 Oct 2017, publ: 27 Oct 2017

Figure 4. Tang et al.

A

10X Z buffer	10 μl
dNTP Mixture (2.5 mM each)	4 μl
Forward Primer	10 pmol
Reverse Primer	10 pmol
Template (supernatant)	1.5 μl
Z taq	0.5 μl
Double-Distilled Water	up to 20 μl

B

95˚C	- 5 min	
98˚C	- 5 sec	40 cycles
55˚C	- 10 sec	40 cycles
72˚C	- 10 sec/kb	40 cycles
72˚C	- 5 min	
4˚C	- storage	

Figure 5. Tang et al.