Manipulation of gene expression using homologous recombination.

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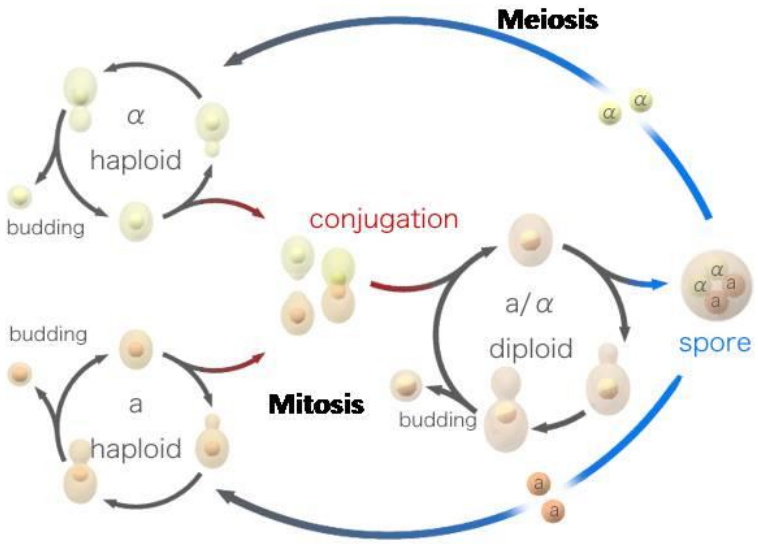
1.Introduction

Saccharomyces cerevisiae is an ideal model eukaryotic system for the systematic analysis of gene function due to the ease and precision with which its genome can be manipulated.The ability of budding yeast to undergo efficient homologous recombination with short stretches of sequence homology has led to an explosion of PCR-based methods to delete and mutate yeast genes and to create fusions to epitope tags and fluorescent proteins.

Budding yeast, an eukaryotic model system:

Budding yeast, *Saccharomyces cerevisiae* is commonly called baker's yeast. It is a single-celled eukaryote, thus it contains membrane-bound organelles, such as nucleus, endomembrane system, mitochondria etc. Yeast is a pertinent system for carrying out genetic manipulations such as transformation, gene deletion, tagging of ORF etc. Yeast cells divide as rapidly as once 90 min under optimal laboratory conditions, though a process of budding in which smaller daughter cells pinch or bud off the mother cell (**Miller et al,2015)**. *S.cerevisiae* cells in nature switch readily between two mating types: haploids a cells mate with haploid α cells to form diploids.Actively dividing eukaryote cells pass through a series of stages known collectively as the cell cycle.



Life cycle of budding yeast: a and α denote the two opposite mating types of haploid and produce a diploid (a/α) by mating, that upon nutrient starvation enters into meiosis sporulates whereas during nutrient rich conditions it undergoes mitosis and shows budding.

1.1. Homologous Recombination

Genomes of living organisms could be altered by gene targeting technology utilizing homologous recombination and producing a selectable and heritable genomic changes that pursues a specific scientific or technological puposes.Homologous recombination also termed as general recombination is the most important version of recombination in nature,being responsible for meiotic crossing-over in eukaryotes and the integration of acquired DNA by the process of conjugation,transduction and transformation into bacterial genomes.It involves a reciprocal exchange of sequences of DNA (Fig 1). Due to the fact that exogenous DNA recombines almost exclusively with its homologous counterpart in the chromosome this phenomenon is usually designated as gene targeting.

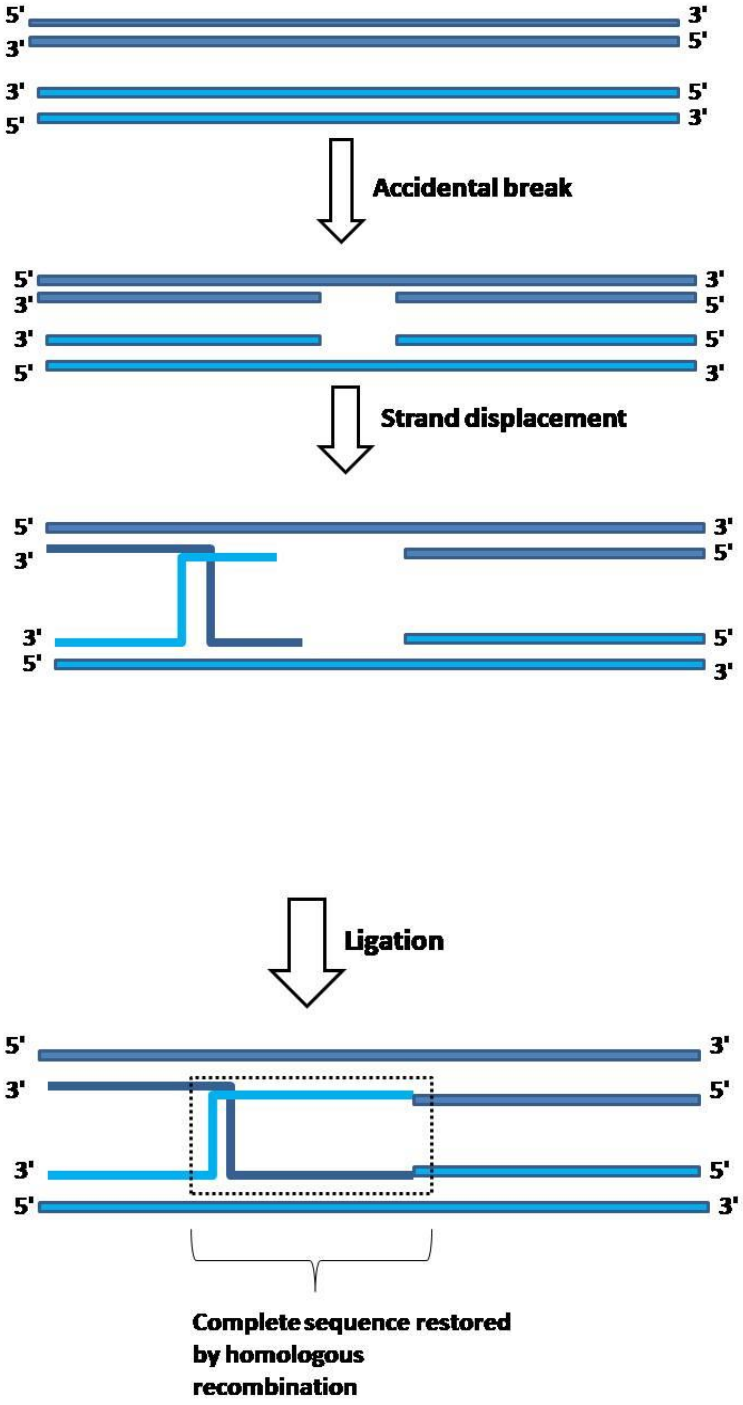


Figure 1: Homologous Recombination. The breaking and rejoining of two homologous DNA double helices causes genetic exchange between the two homologous DNA helices creating two DNA molecules.

1.2. Gene Deletions

Deletion of an entire open-reading frame(ORF) of a gene deletion creates a null mutation,allowing for the analysis of loss-of-function phenotypes.To generate a deletion,the gene sequence from start to stop codon is removed and is generally replaced with a selectable marker.If the gene ORF is to be removed,the forward 5' PCR primer would contain 40-60 bp of DNA 5' to the ATG and the 3' reverse primer would contain 40-60 bp of DNA 3' to the stop codon.The marker is amplified from the cassette so both primers also contain sequences to recognize the deletion module.Following PCR amplification,the 40-60 bp of gene-specific homology are sufficient to direct gene replacement at the locus of interest (Fig 2). Integration into the target locus is verified by PCR of genomic DNA using primers that will amplify part of the integrated marker and an adjacent area of genomic DNA,confirming that the site of integration is correct.

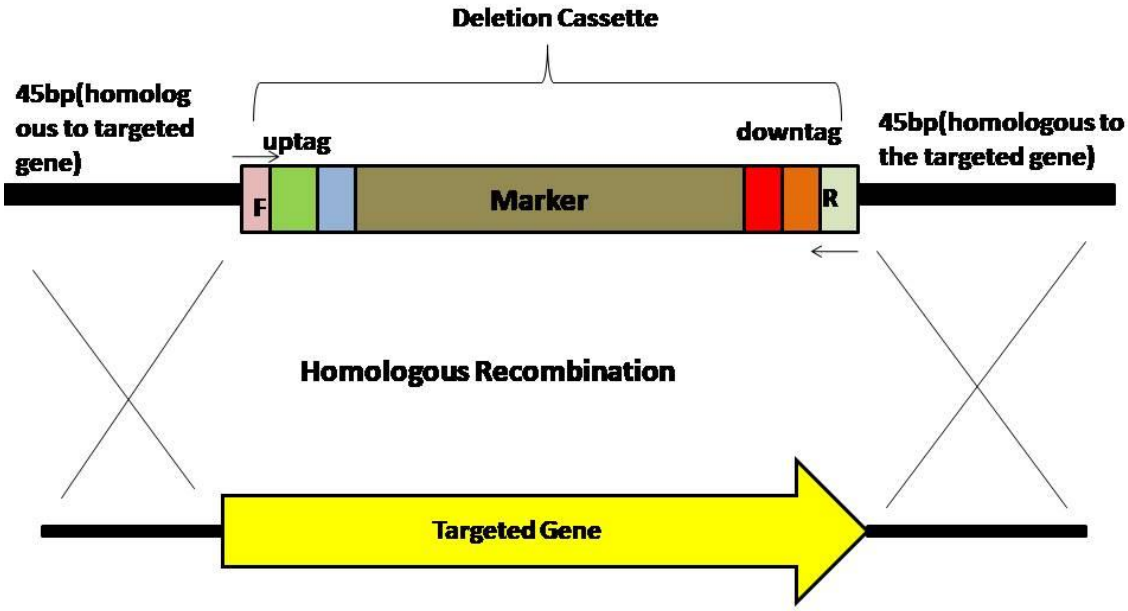


Figure 2.Strategy used for constructing mutants in the *S.cerevisiae* deletion collection (Andrusiak et al,2012).

1.3. Epitope Tagging and Construction of Fusion Proteins

The ability to epitope tag or create fusion proteinswith the endogenous wild-type or mutant copy of yeast genes makes yeast one of the most powerful systems for the study of multiple biological processes.Tagging with small peptide sequences such as HA (**Ling H-Y, Edwards AM, Gantier MP, DeBoer KD, Neale AD, et al. (2012**)), MYC (**Signal Transduction and Targeted Therapyvolume 3, Article number: 5 (2018))** are useful for immunochemistry,fusions with glutathione-S-transferase(GST),protein A (proA) or a tandem affinity tag(TAP) are useful for protein purification,and fusions to GFP or red fluorescent protein(RFP) or one of their variants (i.e.,BFP ,CFP ,YFP ,mCherry ,dsRed, RedStar, Venus, mKate2) are helpful for cell biological studies.

A plethora of C-terminal tagging modules have been developed for virtually every epitope and fluorescent protein variant.Using ~40-60 bp sequences immediately upstream and downstream of the stop codon as targeting sequences on the forward and reverse PCR primers,the resulting PCR fragments from these modules are integrated into the genome by one-step gene replacement.

N-terminal tagging is considered more complex because insertion of a marker in the 5' region of a gene will generally disrupt its expression.Therefore N-terminal tagging of essential genes must be done in diploids since gene function is disrupted during the tagging procedure.Furthermore, the marker must be excised in order to examine the expression of native versions of both essential and non-essential genes(**Jaspersen et al, 2014).**

1.4. Yeast Transformation

Success of PCR-mediated gene disruption or fusion ultimately involves efficient uptake of DNA and recombination into the target locus.It is imperative to use general transformation protocol. Transformations using many PCR products,especially drug resistance modules,cannot be directly plated to selection medium but rather need a period of outgrowth in which the drug resistance gene or nutritional marker can be expressed.

1.5. Promoter Shuffling

Genetic elements of interest can be introduced into the *Saccharomyces cerevisiae* genome via homologous recombination.

Promoter shuffling was done for construction of strains that can be arrested at metaphase I of meiosis. The endogenous promoter of a gene was replaced by another promoter of interest using recombination.

2.Materials and methods

2.1. Materials

- Culture
- Eppendorfs
- 10% SDS
- 10N NaOH
- Ethanol
- 50mM glucose
- 25mM Tris HCl
- 10mM EDTA
- Bromophenol blue dye
- Skimmed milk
- Primary antibody
- Secondary antibody
- BSA solution
- β-mercaptoethanol
- Triton X
- EtBr
- 5M potassium acetate
- Glacial acetic acid
- 10mg/ml Zymolase 20T
- 100% isopropanol
- 1M Sorbitol
- 0.1M Na_2EDTA
- Dilute HCl
- 1M Tris
- 0.5M EDTA
- dNTP
- Reverse Primer
- Forward Primer
- Taq polymerase
- 10*Lithium Acetate(10ml)
- ssDNA(10mg/ml)
- 50% PEG buffer
- 50X TAE buffer
- Buffer B
- Tango buffer

- NFH_2O
- 0.1M Phosphate buffer solution
- DAPI(1µg/ml)
- Distilled water

Reagents Preparation:

1.Alkaline Lysis Solution 1:

50mM glucose
25mM Tris HCl(pH=8)
10mM EDTA(pH=8)

2.Alkaline Lysis Solution 2:

0.2N NaOH(freshly diluted from 10N stock)
1%(w/v)SDS
Sterile water

3.Alkaline Lysis Solution 3:

5mM potassium acetate
Glacial acetic acid
Sterile water

4.Genomic DNA Solution 1:

9.108gm of 1M sorbitol

10ml of 0.5M Na_2EDTA(pH=7.5)

Sterile water(quantum sufficient for 50ml)

Adjust pH to 7.5 with dil.HCl

5.Genomic DNA Solution 2:

2.5ml of TRIS-HCl from 1M stock(pH=8)

2ml of Na_2EDTA from 0.5M stock

Sterile water(quantum sufficient for 50ml)

Adjust the pH to 7.5 with dil.HCl

6.Genomic DNA Solution 3:

4.907gm of potassium acetate in sterile water(quantum sufficient for 10ml)

7.Solution 1:Li-TE Buffer(Lithium Acetate-TRIS EDTA buffer)

10×TE(10ml):1ml of 1M TRIS+0.2ml of 0.5M EDTA+8.8ml of Distilled water

10×Li-Ac(10ml):1.01gm of Li-Ac in 10ml distilled water

1×Li-TE buffer(100ml):10ml of 10×TE+10ml of 10×Li-Ac+80ml of DW(pH=7.5)

8.Solution 2: 40%PEG4000

50% PEG(25ml):17.85ml of 70%PEG+7.15ml distilled water

40%PEG buffer(30ml):24ml of 50% PEG+3ml of 10×TE+3ml of 10×Li-Ac(8:1:1 proportion);pH=7.5

9.Skimmed milk:

For 2ml of skimmed milk, 200µl of PBS (Phosphate buffer solution)+ BSA (Bovine serum albumin) was added. Volume makeup was done by PBS solution upto 2ml.

10.Preparing primary antibody (α-tubulin):

Preparation of primary antibody must be done in 1:5 ratio. For 250µl, 50µ of α-tubulin and 200µl of PBS+BSA solution were mixed.

Growth and Transformation medium

YPD media(broth):

For 100ml,Yeast Extract=1g

Peptone=2g

Dextrose=2g

YPD media(plates):

For 100ml,Yeast Extract=1g

Peptone=2g

Dextrose=2g

Agar=2g

Drop out media:

For 100ml,Nitrogen base without NH_4SO_4=0.17g

Monosodium Glutamate=0.1g

Agar=2g

Dropout media=0.2g

Dextrose=2g

G418 plate:

For 100ml,Yeast Extract=1g

Peptone=2g

Dextrose=2g

Agar=2g

G418 Antibiotic=100µl

YPA Media:

For 100ml,Yeast Extract=1g

Peptone=2g

Potassium Acetate=1g

SPM Media:

For 100ml,Potassium Acetate=0.3g

Raffinose=0.011g

2.2.Plasmid Isolation

- Take 1ml of sample in eppendorf tubes.
- Centrifuge at 13500 rpm for 1min;remove the supernatant.
- Add ALS-1(Alkaline Lysis Solution) 200µl and vortex.
- Add freshly prepared ALS-2 400µl (Composition of ALS-2:0.1% SDS from 10%SDS +0.2N NaOH from 10N NaOH+water for volume makeup)
- Add ALS-3 300µl.
- Keep in ice for 30min.
- Then centrifuge at 13500rpm for 5 min.
- Collect the supernatant in fresh tubes.
- Add 1ml ethanol.
- Keep in room temperature for 5min.
- Centrifuge at 13500rpm for 2min.
- Discard the supernatant.
- Dry the pellet.
- Add 20µl of water(nuclease free water);dissolve the pellet in water.
- Load the gel.

2.3. Yeast DNA Isolation

Methods of isolation:

- Grow the cells overnight at 30°C in 5ml YPD medium.
- Collect the cells by centrifugation at 5000rpm for 5min.Discard the supernatant.
- Resuspend the cells in 0.5ml of Genomic DNA solution 1 and transfer it to 1.5ml tube.
- Add 5µl of 10mg/ml solution of Zymolase 20T and incubate for 1hr at 37°C.(Note that switch on 65°C waterbath)
- Centrifuge at 10000rpm for 1min.
- Discard the supernatant;resuspend the cells in 0.5ml of Genomic DNA solution 2.
- Add 50µl of 10% SDS and vortex for 1min.
- Incubate the mixture for 30min at 65°C.
- Allow the mixture to cool down to room temperature and add 0.2ml of Genomic DNA solution 3.

- Place the microfuge tubes in ice for 1hr.
- Centrifuge at 13500rpm for 5min.
- Transfer the supernatant to a fresh tube and add 750µl of 100% isopropanol at room temperature.
- Mix by inverting the tubes several times and allow it to sit at room temperature for 5min.
- Centrifuge at 13500rpm for 2min.
- Pour off the supernatant and air dry the pellet.
- Dissolve the pellet in 25µ water.
- Load the gel.

2.4. Yeast Transformation

Method of transformation:

- Grow the cells overnight in YPD medium(5ml).
- Inoculate it in 50ml of YPD medium in 250ml flask at OD_{600}=0.2.
- Grow the cells to an OD_{600} of 0.8-1.0 at 30°C and 200rpm for approximately 4 to 5hrs.
- Harvest the cells by centrifugation at 5000rpm for 4min at temperature 23°C.
- Discard the supernatant and resuspend the pellet in 40ml sterile distilled water.
- Harvest the cells by centrifugation at 5000rpm for 4min at temperature.
- Discard the supernatant.
- Add 10×Li-Ac(Lithium-Acetate),10×TE(Tris-EDTA) buffer and sterile distilled water in the ratio of 1:1:8.
- Then it is kept in shaking condition for at least 1hr at 30°C.
- Switch on and set the dry bath at 100°C and water bath at 42°C and 30°C.
- Keep the ssDNA for 10min in 100°C dry bath.
- Take 100µl of the sample in eppendorfs.One is for positive and the other is for control.

- Add 5µl of ssDNA from the stock given 10mg/ml ssDNA to all the eppendorfs.
- Make 40% PEG(Polyethylene Glycerol)buffer from 50% PEG(Polyethylene Glycerol)buffer.
- Add PCR product of isolated plasmid equally to all the positive eppendorfs.
- Add 300µl of PEG buffer to all the eppendorfs.
- Incubate the samples for 30min in 30°C and for 25min in 42°C.
- Add 800µl sterile water.
- Centrifuge it at 3000rpm for 2min.
- Discard the supernatant.
- Add 150µl of sterile distilled water(no vortexing).
- Plate the cells on appropriate drop out plate(Ura^- plates).
- Incubate it at 30°C until colony appears(~24 to 48 hours).

2.5. Restriction digestion of plasmid(PB1764) :

Reagents used	**25µl**
Buffer B	2.5
Enzyme	1
Template	10
NFH_2O	12

2.6. Polymerase Chain Reaction(PCR)

The general PCR reaction mixture has the following components:

Components	For 50µl
• 10mM dNTP mixture	5µl
• PCR buffer	5µl
• Forward primer	2µl

- Reverse primer 2µl
- DNA template 1.5µl
- Taq polymerase 0.6µl
- Nuclease free water 34µl

The PCR reaction was performed with the following cycling parameters:

- Initial denaturation temperature: 95°C for 5min
- Denaturation temperature: 95°C for 30 sec
- Primer annealing temperature: 52°C(may vary) for 30 sec
- Annealing temperature: 57°C for 30sec
- Primer extension temperature: 72°C for 3min 15 sec(may vary)
- Extension temperature: 72°C for 5min
- Holding temperature: 4°C
- No.of PCR cycles:30 cycles
- Quantity in each vile: 50µl

2.7. Agarose gel electrophoresis:

100ml agarose gel was prepared by dissolving 0.8g agarose in 99ml of distilled water, 1ml of 50X TAE buffer and also supplemented with ethidium bromide(1µg/ml).Gels were generally run at 120 V in 1X TAE buffer and the DNA was visualized on a UV trans-illuminator.

2.8. DAPI Staining:

DAPI or 4',6-diaminido-2-phenylindole is a fluorescent stain that binds strongly to adenine-thymine rich regions in DNA.It is used in fluorescence microscopy.It is done by the following method:

- Take the culture in 1.5µl eppendorf tubes.
- Centrifuge it at 13500 rpm for 1 min.
- Discard the supernatant.
- Add 0.1M phosphate buffer solution for washing(add equal amount of the initial volume).
- Centrifuge it at 13500 rpm for 1 min.
- Discard the supernatant.
- Again add 0.1M phosphate buffer solution for washing.
- Add 500µl of 50% ethanol.
- Centrifuge it immediately at 5000 rpm for 1 min.

- Wash with 200µl of phosphate buffer solution(0.1M).
- Centrifuge it at 5000 rpm for 1 min.
- Add 40µl of DAPI (1µg/ml).

2.9. Fluorescence microscopy

All fluorescence images were acquired with a Zeiss Axio Observer.Z1(100*objectives,NA=1.40;Carl Zeiss MicroImaging Inc.)equipped with Axiocam camera controlled by Axiovision software controlled by Axiovision software.Images were processed by image J for brightness and contrast adjustment and for merging of the images.Spindle lengths were measured from at least 100 cells unless mentioned otherwise using Zeiss Axiovision software.

2.10. Immunofluorescence:

- Grow the culture in 5ml YPD tubes overnight.
- Inoculate in YPA media with OD 0.2.Keep it to 1.6-1.8 OD.
- Inoculate in 50ml SPM media. This is called meiotic induction.
- To arrest the cells in metaphase, we can certain time points like 3 hours, 5 hours, 7 hours etc,after we will collect the culture.
- Centrifuge at 13500 rpm for 1 min.
- Add 135 µl formaldehyde and keep it for 5 min.
- Centrifuge it at 10000 rpm for 1 min.
- Discard the supernatant.
- Add 1 ml Phosphate buffer solution and centrifuge at 10000 rpm for 1 min.
- Discard the supernatant.
- Add 100µl of PBS.
- Add 5µl zymolase and 3.5µl of β-mercaptoethanol to the cells.
- Incubation at 30°C for 15 min. Observe the spheroplastic cells with phase microscopy.
- Centrifuge the sample at 6000 rpm for 1 min.
- Discard the supernatant.
- Add 50µl PBS in the pellet. Mix it gently.
- Prepare polylysine wells in a wax coated slide. Amount of polylysine given per well is 20 µl . Keep it for 45 min.
- Wash the polylysine with the help of PBS by vaccum pump twice.
- Add 20µl culture within the wells. Keep it for 30 min.

- Wash with PBS by vaccum pump.
- Add 20µl of Triton X in each well.
- Keep it for 2 min.
- Wash with PBS .
- Add 20µl of skimmed milk to each well.
- Keep it for half an hour.
- Wash with PBS with vaccum pump thrice.
- Add 20µl primary antibody (α-tubulin) to each well.
- Keep it for one hour.
- Wash the wells 2 times with PBS by vaccum pump.
- Add 20µl of 2^{nd} antibody (Alexaflour) in each well.
- Keep it for one hour.
- Wash it with PBS.
- Add DAPI and keep it for 30 min.
- Wash with PBS by vaccum pump.

2.11.Meiotic Induction

- Grow the culture in YPD media overnight.
- Inoculate in YPA media in 0.2 OD.
- Keep in 30°C for 16 hours.
- Then inoculate in 50µl SPM media and keep it in 30°C under shaking condition.
- To arrest the cells in metaphase, we can certain time points like 3 hours, 5 hours, 7 hours etc,after we will collect the culture.
- Centrifuge at 13500 rpm for 1 min.
- Add 135 µl formaldehyde and keep it for 5 min.
- Centrifuge it at 10000 rpm for 1 min.
- Discard the supernatant.
- Add 1 ml Phosphate buffer solution and centrifuge at 10000 rpm for 1 min.
- Discard the supernatant.
- Add 100µl of PBS.
- Add DAPI

Results

3.1.Shuffling of the endogenous promoter of gene to regulate its expression.

In this work, promoter of CDC20 was replaced with mitosis specific promoter pCLB2 using pFA6a vector. At the metaphase–anaphase transition, Securin/Pds1 is targeted for proteosomal degradation by an ubiquitin ligase known as the Anaphase-Promoting Complex/Cyclosome (APC/C) and its specificity factor Cdc20 (APC/C–Cdc20). APC/C–Cdc20 is regulated at multiple levels. Surveillance mechanisms such as the DNA damage and spindle assembly checkpoints negatively regulate APC/C–Cdc20 activity. In budding yeast, the DNA damage checkpoint arrests the cell cycle in metaphase by stabilizing Securin/Pds1 (**Cohen-Fix and Koshland 1997; Sanchez et al. 1999; Tinker-Kulberg and Morgan 1999; Wang et al. 2001; Agarwal et al. 2003; Searle et al. 2004**). In response to unattached kinetochores, the spindle assembly checkpoint prevents the metaphase–anaphase transition by inhibiting the interaction between Cdc20 and APC/C (**Musacchio and Salmon 2007**). However, these two checkpoints are not the only mechanisms that restrict APC/C–Cdc20 activity to the metaphase–anaphase transition because their combined inactivation does not cause precocious anaphase entry **(Lee et al. 2002**)(Fig 4).

Oligonucleotides were used in which 5' end of forward primer (MA23) contains 50 bps sequences upstream to start codon of gene and reverse primer's(MA24) 5' end contains 50 bps sequences just downstream to start codon (including start codon). A vector, carrying promoter sequences of interest, was used as a template in PCR amplification and amplified cassette was transformed in haploid SK1 wild type strains and selected on YPD+G418 plate

For the confirmation of transformants diagnostic PCR was done using diagnostic primers. Forward primer (MA25) consist of sequence upstream to start codon of CDC20, whereas reverse primer(MA26) within the CDC20 gene. Diagnostic primers gives 799 kb band in absence of shuffling, whereas upon shuffling it gives 3.15 kb fragment (Fig 5). The positive transformants were allowed to mate to construct a diploid homozygous for *pCLB2-CDC20*.

Diploid was inoculated in YPD media and meiotic induction was done as described in material and methods. It was kept for 8-9 hours. Then immunofluorecence was done of the culture (Figure 8).

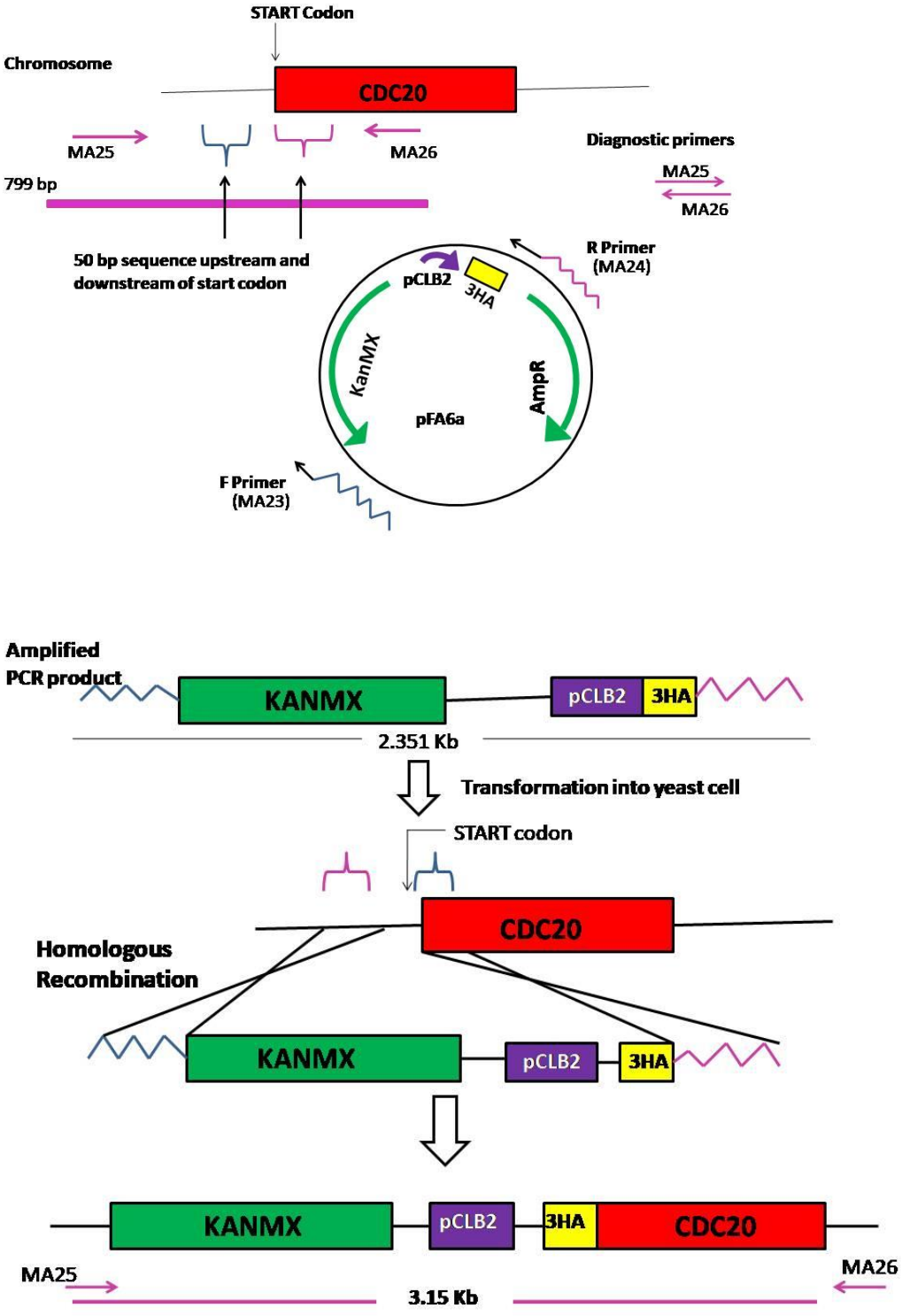
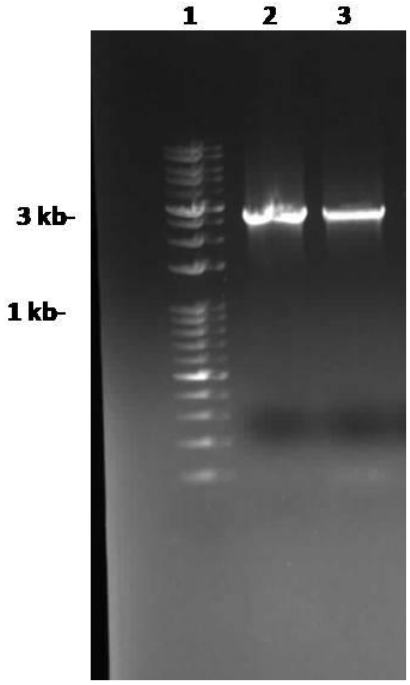


Figure 4: Promoter shuffling of CDC20. After PCR amplification, transformation was done. After transformation the gene integrates within the genome in the N-terminal.



1. DNA Ladder
2 and 3.Transformants

Figure 5: The transformation is positive.Both the transformants i.e. pCLB2-CDC20 are nearly 3 kb in size.

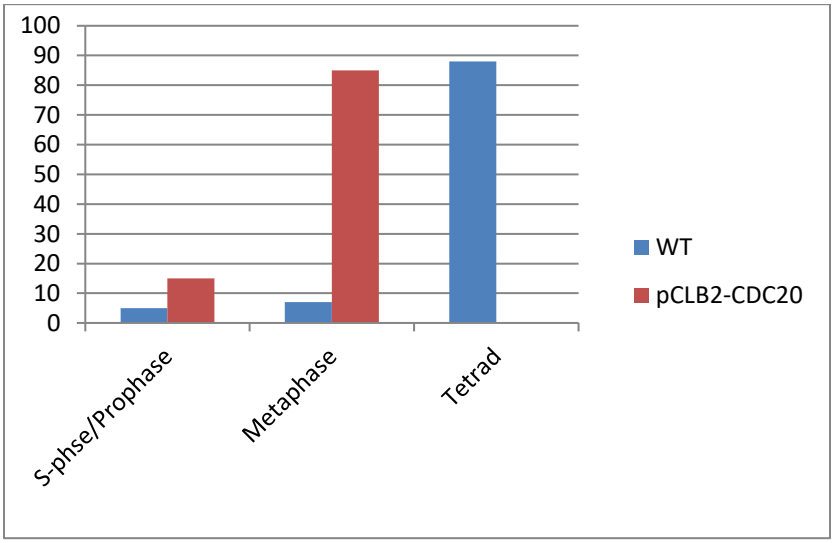
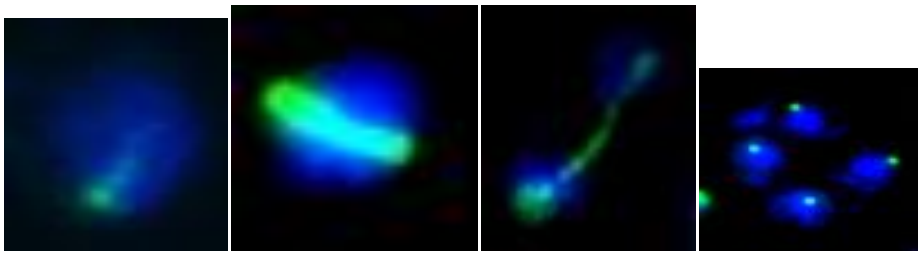


Figure 6: Bar graph showing percentage of cells in different meiotic phases in WT (wild type) and Mutant (pCLB2-CDC20). For WT, tetrads is of 88% whereas S-phase/Prophase are of 5% and metaphase is of 7%. For pCLB2-CDC20, S-phase/Prophase is of 15% and metaphase is of 85% but Tetrads is zero percentage.



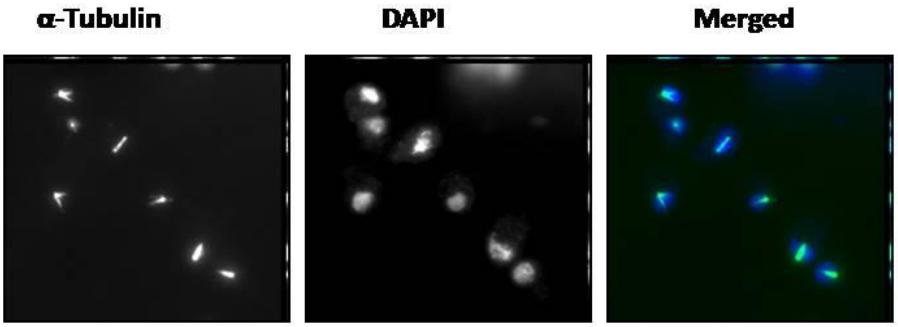


Figure 8: Metaphase I arrested cell after doing Immunofluorescence

3.2.In frame fusion of ORFs to study protein localization.

We first isolated the plasmid (pB1764). The plasmid we choose contains an CFP-TUB1 fusion under the control of the HIS3 promoter (HIS3p) and a URA3 selectable marker. Upon digestion with Stu1, which cuts within the URA3 , the exposed ends of the linearized plasmid would theoretically target the construct for integration into the URA3 locus. After digesting with Stu1 and we transformed digestion product into SK1 diploid strain.

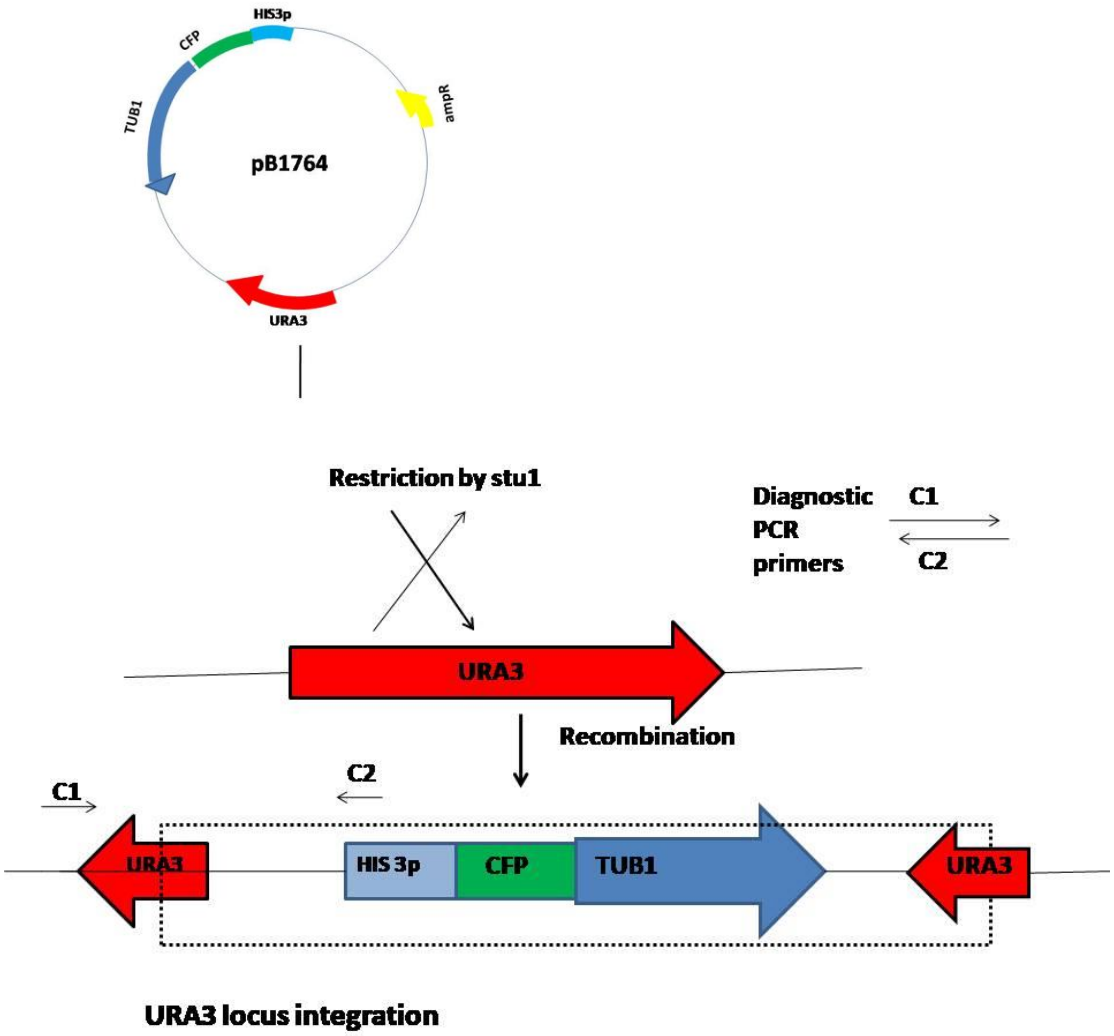
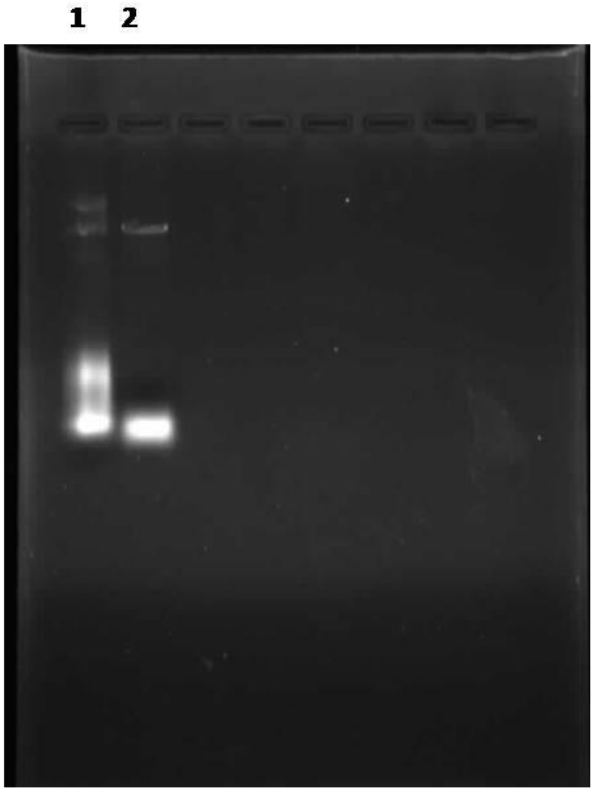


Figure 8: Schematic depicting TUB1-tagging plasmid Pb1764:HIS3p:CFP-TUB1 and chromosomal locus URA3 with open reading frame and direction of coding sequence indicated.



1.Uncut plasmid
2.Stu1 cut pB1764

Figure 9: Plasmid pB1764 is linearised by stu1

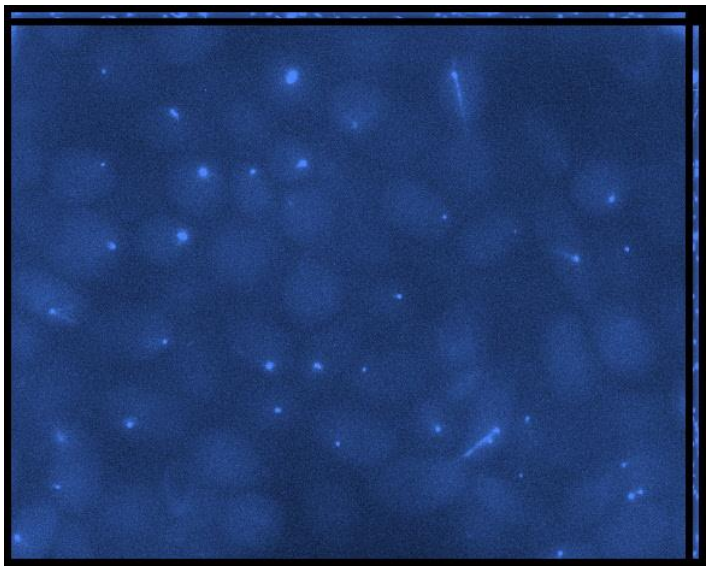
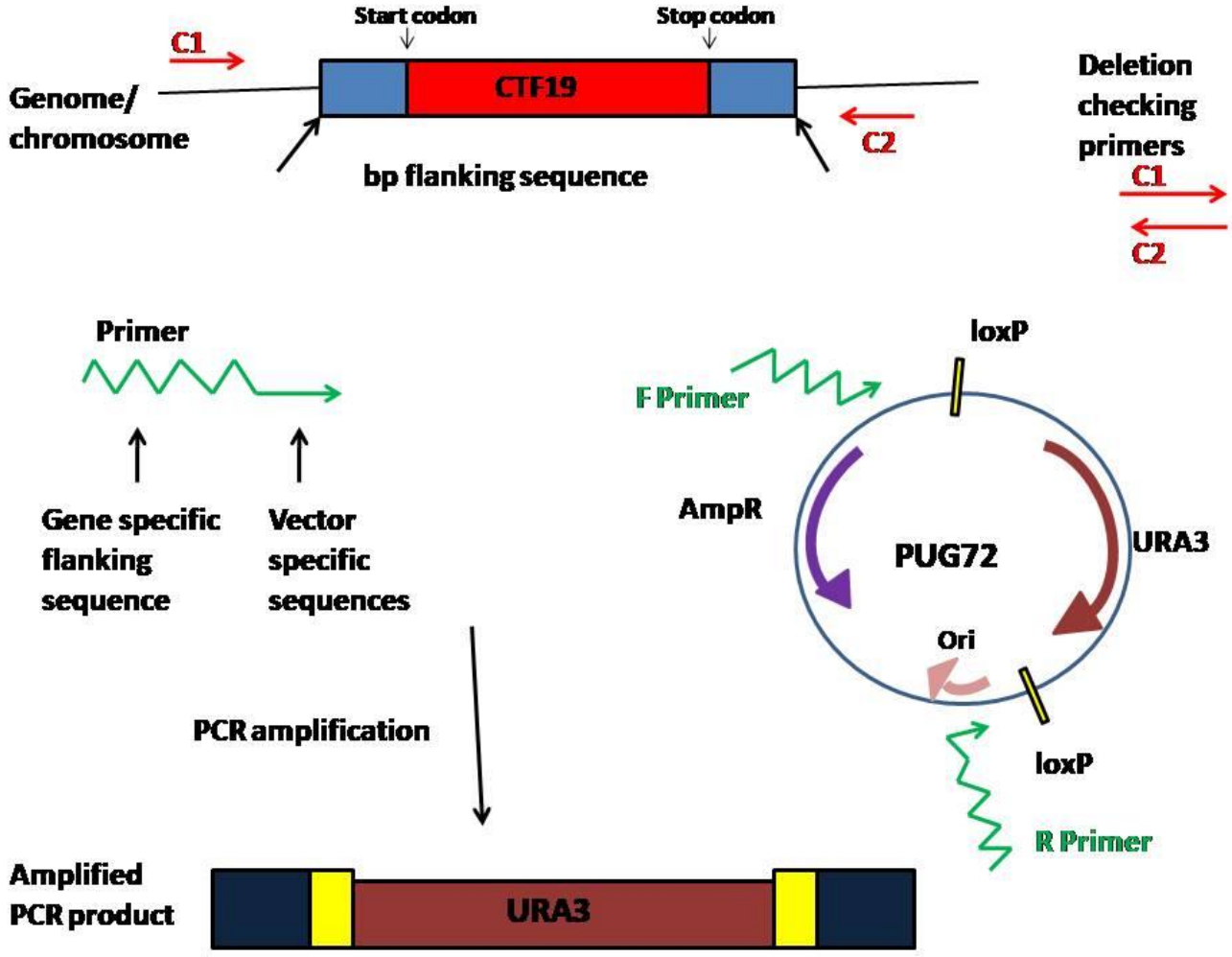


Figure 10: Immunofluorescence detection of CFP-TUB1

3.3.Construction of Gene deletion strains:

A PCR based gene deletion strategy was adopted for deletion of gene from with marker cassette in haploids of opposite mating type.Primers containing a region of similarity to the flanking region of target gene and constant priming region were synthesized.As shown in the figure 10, it was expected that the integration of such a PCR amplified cassette would lead to deletion of the functional target gene by homologous recombination.

We have isolated plasmid PUG72 and amplification of deletion cassette from plasmid was done by PCR. Then transformation was done. The desired plasmid was integrated into the strain by homologous recombination and the gene CTF19 was deleted from the strain.



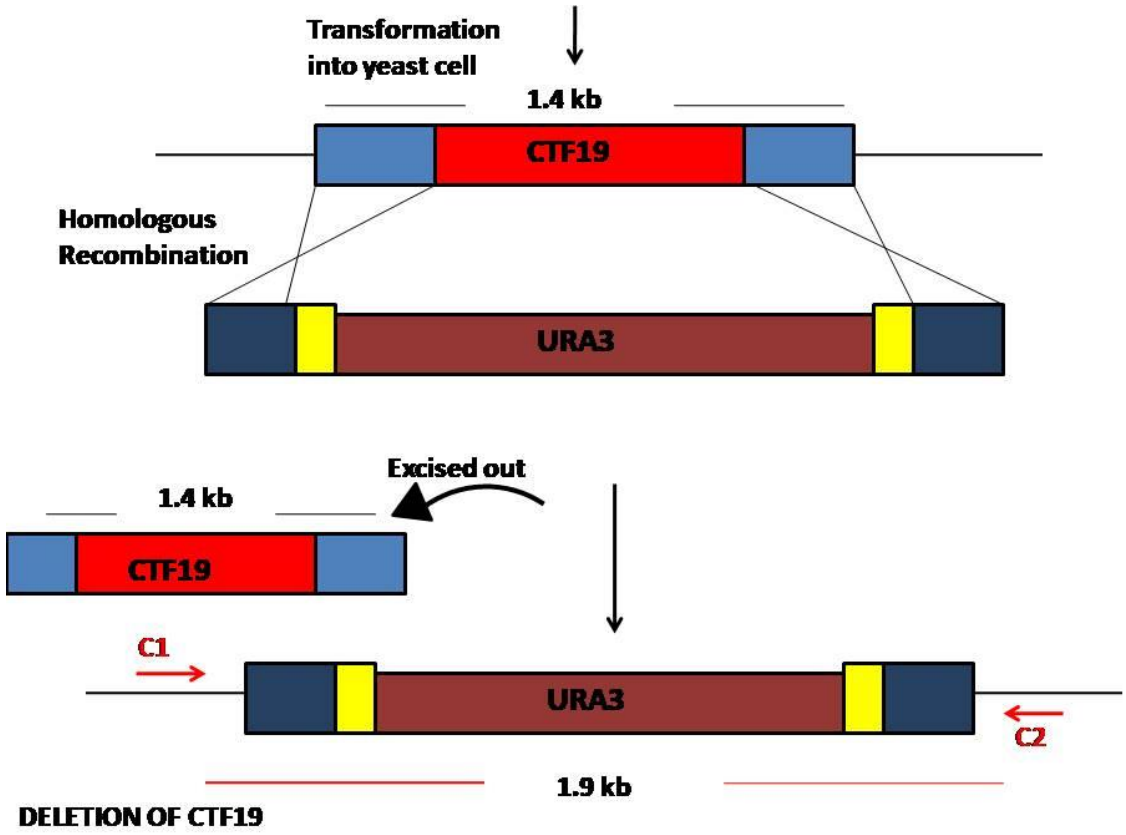


Figure 11: PCR based strategy for gene deletion using URA3 marker (Agarwal el al,2015).

Confirmation deletion mutant:

Genomic DNA was isolated from transformants. PCR confirmation was done by using another set of forward and reverse primers. Primers were located 50 nucleotide upstream of the start codon and downstream of the stop codon respectively, of the disrupted gene,CTF19.

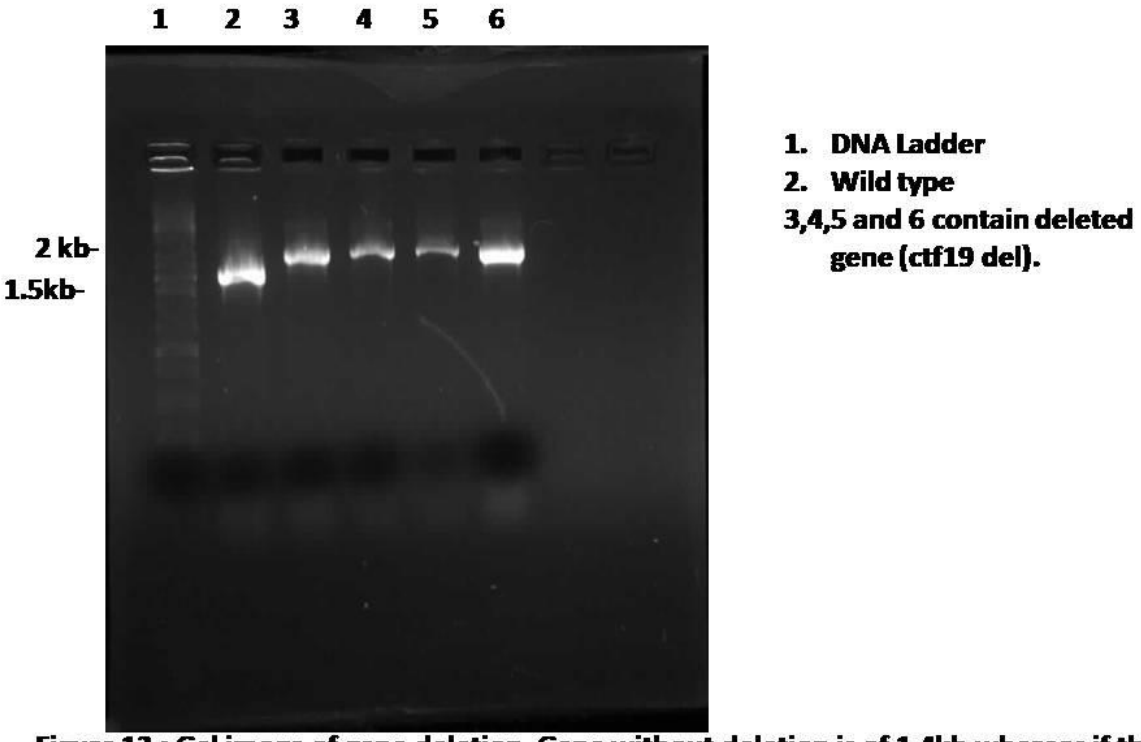


Figure 12 : Gel image of gene deletion. Gene without deletion is of 1.4kb whereas if the gene is deleted then the amplified product of the gene is of 1.9kb.

4. Strains constructed:

Strain	Genotype
1.	MATa/α, cdc20::pCLB2-CDC20::KANMX6/cdc20::pCLB2-CDC20::KANMX6
2.	MATa/α, cdc20::pCLB2-CDC20::KANMX6/cdc20::pCLB2-CDC20::KANMX6, ctf19Δ::KANMX6/ctf19Δ::KANMX6,REC8-6HA::HIS3?REC8-6HA::HIS3
3.	MATa/α, SPC42-EGFP::TRP1/SPC42-EGFP::TRP1

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